

HPV CHIMAERIC PARTICLE

BACKGROUND OF THE INVENTION

This invention relates to a chimaeric human papillomavirus (HPV) virus like particle (VLP) having a diameter of about 30nm and a method of treatment and/or prophylaxis of HPV infection and/or cervical cancer by administration of the chimaeric HPV VLP of the invention.

Cervical cancer is primarily caused by HPV infection and is the third most common cancer among women worldwide (Ferlay *et al.*, 2010). As a result, HPV vaccine development is a priority for preventative cancer research. The L1 major capsid protein is the antigen of choice for prophylactic vaccines, as it is immunodominant and self-assembles into VLPs which are structurally and immunologically similar to authentic virions. Vaccination with VLPs elicits high titres of neutralisation antibodies (NAbs) in both animals and humans and two multivalent HPV L1 VLP-based prophylactic vaccines have been licensed and are highly effective in the prevention of vaccine-type HPV-16 and 18 infections and associated disease (Schiller *et al.*, 2008).

Despite the high efficacy of current L1 VLP-based HPV vaccines, the type-specificity (Brown *et al.*, 2009; Wheeler *et al.*, 2009), the lack of therapeutic efficacy (FUTURE II Study Group, 2007; Hildersheim *et al.*, 2007) and the high

cost of vaccines (Schiller *et al.*, 2008) have limited their widespread application, particularly in developing countries with >80% of the cervical cancer burden (Parkin and Bray, 2006). Therefore, there is an urgent need for affordable second generation HPV vaccines, which broaden protection to include multiple oncogenic HPV types, and improve the therapeutic efficacy to clear established HPV infections and cancerous lesions.

Broad-spectrum prophylactic HPV vaccines can be developed using cross neutralising L2 epitopes. The L2 epitopes can be incorporated into surface regions of L1 to create L1/L2 chimaeras displaying the L2 peptide on the surface of assembled L1 (WO 03/097673; Kawana *et al.*, 1999, 2003; Slupetzky *et al.*, 2007; Kondo *et al.*, 2007, 2008).

The use of plant expression systems for the large-scale production of foreign antigens has been proposed as a cost-effective alternative for vaccine production (Fischer *et al.*, 2004), with a definitive trend toward the use of transient expression for high-level protein expression and optimisation (Rybicki, 2009). Several groups have expressed HPV-16 L1 in plants (Biemelt *et al.*, 2003; WO 2006/119516; Maclean *et al.*, 2007).

A practical limitation of plant systems is low yields of recombinant protein, potentially a result of protein instability or low-level expression (Fischer *et al.*, 2004; Obembe *et al.*, 2011). It is estimated that plant-expressed recombinant protein yields need to be greater than 1% of the total soluble protein (TSP) to be economically viable (Fischer *et al.*, 2004). This is particularly problematic for the expression of recombinant proteins using nuclear-transformed transgenic plants, as these systems are often associated with low yields of recombinant protein (Rybicki, 2009).

HPV-16 L1 has been expressed transgenically in nuclear-transformed potato and tobacco plants, but low expression levels of HPV-16 L1 (<1% TSP) have consistently reported and the elicited immune responses were relatively weak (Biemelt *et al.*, 2003; Varsani *et al.*, 2003b; Varsani *et al.*, 2006a).

However, human codon-optimisation of the L1 gene and targeting to the chloroplast have significantly improved HPV-16 L1 expression in both transgenic

and *Agrobacterium*-mediated transient tobacco expression systems to up to about 17% TSP (Maclean *et al.*, 2007).

A recent development in plant-derived HPV vaccines was the expression of the first HPV-16 L1 chimaera in plants. The L1/E6/E7 chimaera consisted of HPV-16 L1 C-terminally fused to several E6 and E7 epitopes and it was expressed in transgenic tomatoes (Paz De la Rosa *et al.*, 2009). However, yields were low (0.05 – 0.1% TSP) and therefore not commercially viable.

WO 2011/077371 describes a method for producing chimaeric HPV L1 polypeptides with increased expression levels relative to HPV L1 protein in an insect, plant or yeast expression system. Although human codon-optimised L1/L2 chimaeras produced from HPV L1 and BPV L2 (amino acids 1-88) in plants formed VLPs of about 55 nm, the other HPV L1/L2 chimaeras were only able to form capsomeres of approximately 17 nm in diameter.

Although capsomeres are stable at room temperature, they are only able to induce 20 to 40-fold lower humoral immune responses in comparison to VLPs (Thönes *et al.*, 2008). It would therefore be beneficial to develop a chimaeric VLP comprising L1 and L2 which is expressed at commercially viable levels in an expression system. Such a chimaeric VLP would be easier to purify and is likely to be more immunogenic than a chimaeric capsomere.

SUMMARY OF THE INVENTION

According to a first aspect of the invention there is provided a chimaeric human papillomavirus (HPV) virus like particle (VLP) having a size of about 30 nm in diameter, the chimaeric HPV VLP comprising a chimaeric HPV 16 L1/L2 polypeptide encoded by a human codon-optimised nucleotide sequence, the chimaeric HPV 16 L1/L2 polypeptide further comprising an HPV L1 polypeptide that includes an HPV L2 peptide of between about 13 amino acids to about 26 amino acids inserted from residue 414 of the HPV 16 L1 polypeptide, and wherein the amino acids of the inserted HPV L2 peptide replace the corresponding amino acids of the HPV 16 L1 polypeptide.

For example, the inserted HPV L2 peptide may be a 13 amino acid LVEETSFIDAGAP peptide (SEQ ID NO: 3) encoded by a human codon-optimised nucleotide sequence as set forth in SEQ ID NO: 7, or a 20 amino acid QLYKTCKQAGTCCPDIIIPKV peptide (SEQ ID NO: 5) encoded by a human codon-optimised nucleotide sequence as set forth in SEQ ID NO: 9, or a 26 amino acid GGLGIGTGSGTGGRTGYIPLGTRPPT peptide (SEQ ID NO: 4) encoded by a human codon-optimised nucleotide sequence as set forth in SEQ ID NO: 8.

Preferably the inserted HPV L2 peptide is the 13 amino acid LVEETSFIDAGAP peptide (SEQ ID NO: 3) encoded by a human codon-optimised nucleotide sequence as set forth in SEQ ID NO: 7.

The HPV type 16 L1 protein may further be encoded by a nucleotide sequence modified to be nuclear localisation signal deficient.

Preferably, the HPV-16 L1/L2 polypeptide comprises an amino acid sequence as set out in SEQ ID NO: 22, SEQ ID NO: 23 or SEQ ID NO: 24, or a variant or derivative thereof.

Preferably, the HPV-16 L1 polypeptide is as set out in SEQ ID NO: 1 and the HPV-16 L1 polypeptide is encoded by a human-codon optimised HPV-16 L1 polynucleotide sequence as set out in SEQ ID NO: 2.

The approximately 30 nm diameter, chimaeric HPV VLP may be a plant expressed chimaeric HPV VLP purified from a plant expression system. Preferably, the expressed chimaeric VLP may be targeted to the chloroplast of the plant.

According to a further aspect of the invention, there is provided a pharmaceutical composition comprising a 30 nm diameter chimaeric HPV VLP according to the invention and a pharmaceutically acceptable carrier.

The composition may also comprise an adjuvant.

According to a further aspect of the invention, there is provided a method of producing a chimaeric HPV VLP having a size of about 30 nm in diameter, the method comprising the steps of:

- (i) providing a chimaeric human codon-optimised nucleotide sequence encoding a chimaeric HPV 16 L1/L2 polypeptide, the chimaeric HPV 16 L1/L2 polypeptide comprising an HPV 16 L1 polypeptide having an HPV L2 peptide of between about 13 amino acids to about 26 amino acids inserted from residue 414 of the chimaeric HPV 16 L1/L2 polypeptide, wherein the amino acids of the inserted HPV L2 peptide replace the corresponding amino acids of the HPV 16 L1 polypeptide;
- (ii) cloning the chimaeric human codon-optimised nucleotide sequence into an expression vector adapted to express a polypeptide in a plant;
- (iii) transforming or infiltrating a plant cell with the expression vector of step (ii);
- (iv) expressing the chimaeric HPV 16 L1/L2 polypeptide in the plant cell of step (iii) such that the expressed chimaeric HPV 16 L1/L2 polypeptide assembles into a chimaeric HPV VLP having a uniform shape and a diameter of about 30nm; and
- (v) recovering the chimaeric HPV VLP from the plant cell.

The expression vector preferably includes promoters and other regulatory sequences, or the like, that are operably linked to the coding sequence of the expression vector.

Preferably, the expression vector of step (ii) is adapted to target a chloroplast of a plant cell and in step (iv) the expressed chimaeric HPV protein is targeted to the plant chloroplast.

Step (iii) may further include introducing into a plant cell a suppressor protein adapted to inhibit post-transcriptional gene silencing in a plant. Preferably, the suppressor protein is the NSs protein of the tomato spotted wilt virus or the p19 of tomato bushy stunt virus.

For example, the inserted HPV L2 peptide may be a 13 amino acid LVEETSFIDAGAP peptide (SEQ ID NO: 3) encoded by a human codon-optimised nucleotide sequence as set forth in SEQ ID NO: 7, or a 20 amino acid QLYKTCKQAGTCPPDIIPKV peptide (SEQ ID NO: 5) encoded by a human codon-optimised nucleotide sequence as set forth in SEQ ID NO: 9, or a 26 amino acid GGLGIGTGSGTGGRTGYIPLGTRPPT peptide (SEQ ID NO: 4) encoded by a human codon-optimised nucleotide sequence as set forth in SEQ ID NO: 8.

Preferably the inserted HPV L2 peptide is the 3 amino acid LVEETSFIDAGAP peptide (SEQ ID NO: 3) encoded by a human codon-optimised nucleotide sequence as set forth in SEQ ID NO: 7.

According to a further aspect of the invention, there is provided an approximately 30 nm diameter, chimaeric HPV VLP according to the invention for use in a method of preventing and/or treating HPV infection and/or cervical cancer in a subject.

More specifically, the chimaeric HPV VLP may be for use in a method of eliciting an immune response in the subject, such as a neutralising antibody and/or CTL response. Preferably, the chimaeric HPV VLP is for use in eliciting a cross-protective immune response to multiple HPV types in the subject.

According to a further aspect of the invention, there is provided a use of a regularly shaped, approximately 30 nm diameter, chimaeric HPV VLP according to the invention in the manufacture of a medicament for use in a method of preventing and/or treating HPV infection and/or cervical cancer in a subject.

More specifically, the medicament may be for use in a method of eliciting an immune response in the subject, such as a neutralising antibody and/or CTL response. Preferably, the medicament is for use in eliciting a cross-protective immune response to multiple HPV types in the subject.

According to a further aspect of the invention, there is provided a method of preventing and/or treating HPV infection and/or cervical cancer in a subject, the method comprising a step of administering a prophylactically or therapeutically effective amount of a uniformly shaped, approximately 30 nm diameter, chimaeric HPV VLP according to the invention to the subject.

More specifically, the method may comprise eliciting an immune response in the subject, such as a neutralising antibody and/or CTL response. Preferably, the method comprises eliciting a cross-protective immune response to multiple HPV types in the subject.

The subject is preferably a human.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows plasmids used to create the HPV chimaera plant expression constructs. C) HPV chimaera genes from pGA4 constructs were directionally subcloned into the *Agrobacterium* plant expression vectors: A) pTRAc-rbcs1-cTP, B) pTRAc and D) pRIC3. The vector elements necessary for plant expression are shown in the figure. P35SS: CaMV 35S promoter containing duplicated transcriptional enhancer, CHS: chalcone synthase 5' untranslated region, pA35S: CaMV 35S polyadenylation signal for foreign gene expression, ColE1ori: *E. coli* origin of replication, RK2ori: *Agrobacterium* origin of replication, *bla*: ampicillin / carbenicillin-resistance gene, and LB/RB: left and right borders for T-DNA integration. The pTRAc vector contains SAR: tobacco Rb7 scaffold attachment regions flanking the expression cassette. In addition, the pTRAc-rbcs1-cTP vector contains *npt II*: the kanamycin-resistant gene, Pnos/pAnos: promoter / polyadenylation signal of the nopaline synthase gene and rbcs1-cTP: *Solanum tuberosum* chloroplast-transit peptide sequence of the Rubisco small-subunit gene *rbcs1*. The pRIC3 vector contains LIR: BeYDV long intergenic region, SIR: BeYDV short intergenic region, and Rep/RepA: BeYDV rep gene.

Figure 2 shows chloroplast-targeted L1/L2 chimaera expression time trial 1-9 days post-infiltration (dpi) in *N. benthamiana*, either with (+) or without (-) the NSs silencing suppressor. The L1/L2 chimaeras A) L1/L2(108-120), B) L1/L2(56-81), C) L1/L2(17-36) and D) L1/L2 BPV (1-88) in crude leaf extracts were detected by CamVir1 western blot analysis. M = protein marker with the size in kDa indicated on the left. NSs negative control = pBIN-NSs infiltrated crude plant extract (5 dpi). Positive controls: *N. benthamiana* (+) = plant-derived HPV-16 L1.

The black arrows indicate the position of the L1/L2 chimaeras (~56 kDa) and the grey arrow indicates degraded protein.

Figure 3 a) shows a Western blot of the L1/L2 chimaeras expressed using 3 plant expression vectors: pTRAc, pTRAc-rbcs1-cTP and pRIC3. Chimaeras were co-expressed with NSs, extracted 5 dpi and detected with CamVir1. HPV-16 L1 was expressed as a positive expression control for pTRAc and pTRAc-rbcs1-cTP (pRIC3 construct not available) and the negative expression control was NSs-infiltrated plants. M = protein marker with the size of the protein indicated in kDa on the left. The black arrows indicate the the L1/L2 chimaeras or HPV-16 L1 (~56 kDa); and b) shows comparison of the L1/L2 chimaeras expressed using 3 plant expression vectors: pTRAc, pTRAc-rbcs1-cTP and pRIC3. The error bars indicate the standard deviation.

Figure 4 shows assembly of L1/L2 chimaeras expressed using 3 different plant expression vectors: pTRAc, pTRAc-rbcs1-cTP and pRIC3. Proteins were co-expressed with the NSs silencing suppressor and extracted 5 dpi. Chimaeras assembled into higher-ordered structures such as capsomeres or VLPs (detected by conformational-specific H16.V5 MAb) is expressed as a percentage of the total chimaera protein (detected by the linear-specific H16.J4 MAb). HPV-16 L1 was expressed as a positive expression control and the negative expression control was NSs-infiltrated plants. The error bars indicate the standard deviation.

Figure 5 shows purity of the plant-produced vaccine antigens. A) Coomassie-stained protein gel. B) Western blot detection of HPV antigens. M = Protein marker with size in kDa indicated on the left. C = clarified crude plant extract. P = purified antigen. V1 = L1/L2(108-120), V2 = L1/L2(56-81), V3 = L1/L2(17-36), V4 (+) = HPV-16 L1 and V5 (-) = NSs-infiltrated plant extract. The black arrows indicate the HPV antigens and the white arrows indicate the plant protein Rubisco.

Figure 6 shows total soluble protein (TSP) and total HPV protein in the crude and purified samples. TSP was determined using the Lowry assay and HPV protein was detected with H16.J4 (linear epitope-specific). V1: L1/L2(108-120), V2: L1/L2(56-81), V3: L1/L2(17-36), V4: HPV-16 L1 (positive control), V5:

NSs plant extract (negative control). The error bars indicate the standard deviation.

Figure 7 shows transmission electron micrographs of CamVir1-immunotrapped crude and purified vaccine antigens A) V1: L1/L2(108-120), B) V2: L1/L2(56-81), C) V3: L1/L2(17-36), D) V4: HPV-16 L1 (positive control), E) V5: NSs plant extract (negative control). Grids were viewed on a Zeiss 912 Omega Cryo EFTEM. Left scale bar = 50 nm, right scale bar = 200 nm. Light grey arrows indicate HPV-16 capsomeres (~10 nm), white arrows represent capsomere aggregates or small VLPs (~30 nm) and dark grey arrows indicate full-sized VLPs (~55 nm).

Figure 8 shows a transmission electron micrograph of CamVir1-immunotrapped crude vaccine antigen L1/L2(56-81). Grids were viewed on a Zeiss 912 Omega Cryo EFTEM. Scale bar = 100 nm.

Figure 9 shows a direct ELISA of mouse sera using insect cell-produced HPV-16 L1 as the coating antigen. V1 = L1/L2(108-120), V2 = L1/L2(56-81), V3 = L1/L2(17-36), V4 = HPV L1 (+ vaccine control), V5 = plant extract (- vaccine control). A) Titration of the mouse antisera for all the vaccines. B) Graph showing the values obtained for the ELISA positive control MAbs H16.V5 and CamVir1. C) Vaccine pre-bleed absorbance values at 1:50 dilution. Markers represent the mean value of triplicate samples and error bars indicate the standard deviation.

Figure 10 shows a western blot detection of *E. coli*-expressed His-tagged HPV-16 L2 by mouse sera at a dilution of 1:100. M = protein marker with the protein size in kDa. V1 = L1/L2(108-120), V2 = L1/L2(56-81), V3 = L1/L2(17-36), V4 = HPV L1 (+ vaccine control), V5 = plant extract (- vaccine control). PB = pre-bleed sera. FB = final bleed sera. For the western blot controls: +ve = mouse anti-His (1:2000; Serotec), -ve = no primary antibody. The black arrow indicates L2 (~80 kDa).

Figure 11 shows a HPV-16 PsV neutralisation assay. Pooled sera from mice vaccinated with V1-V5 were tested for their ability to neutralise HPV-16 PsVs. A) V1 = L1/L2(108-120), B) V2 = L1/L2(56-81), C) V3 = L1/L2(17-36), D) V4 = HPV-16 L1 (+ve vaccine control), E) V5 = NSs-infiltrated plant extract (-ve vaccine

control). F) H16.V5 = +ve neutralisation control. Cell control = -ve infection / SEAP expression control. PsV control = +ve infection / SEAP expression control. Samples were assayed in triplicate and error bars indicate the standard deviation.

Figure 12 shows a HPV-18 PsV neutralisation assay. A) V1 = L1/L2(108-120), B) V2 = L1/L2(56-81), C) V3 = L1/L2(17-36), D) V4 = HPV-16 L1, E) V5 = NSs-infiltrated plant extract (-ve vaccine control). F) Rabbit anti-Cervarix sera = +ve neutralisation control.

Figure 13 shows a HPV-45 PsV neutralisation assay. A) V1 = L1/L2(108-120), B) V2 = L1/L2(56-81), C) V3 = L1/L2(17-36), D) V4 = HPV-16 L1, E) V5 = NSs-infiltrated plant extract (-ve vaccine control). F) H45.N5 = +ve neutralisation control.

Figure 14 shows a HPV-52 PsV neutralisation assay. A) V1 = L1/L2(108-120), B) V2 = L1/L2(56-81), C) V3 = L1/L2(17-36), D) V4 = HPV-16 L1, E) V5 = NSs-infiltrated plant extract (-ve vaccine control). F) H52.C1 = +ve neutralisation control.

Figure 15 shows the amino acid (SEQ ID NO: 1) of HPV-16 L1.

Figure 16 shows the human-codon optimised nucleotide sequences (SEQ ID NO: 2) of HPV-16 L1.

Figure 17 shows the amino acid sequence (SEQ ID NO: 3) of the L2 (108-120) epitope which was inserted into the HPV L1 sequence.

Figure 18 shows the amino acid sequence (SEQ ID NO: 4) of the L2 (56-81) epitope which was inserted into the HPV L1 sequence.

Figure 19 shows the amino acid sequence (SEQ ID NO: 5) of the L2 (17-36) epitope which was inserted into the HPV L1 sequence.

Figure 20 shows the amino acid sequence (SEQ ID NO: 6) of the L2 BPV (1-88) epitope which was inserted into the HPV L1 sequence.

Figure 21 shows the human-codon optimised DNA nucleotide sequence (SEQ ID NO: 7) of L2 (108-120) which was inserted into the HPV L1 sequence.

Figure 22 shows the human-codon optimised DNA nucleotide sequence (SEQ ID NO: 8) of L2 (56-81) which was inserted into the HPV L1 sequence.

Figure 23 shows the human-codon optimised DNA nucleotide sequence (SEQ ID NO: 9) of L2 (17-36) which was inserted into the HPV L1 sequence.

Figure 24 shows the human-codon optimised DNA nucleotide sequence (SEQ ID NO: 10) of L2 BPV (1-88) which was inserted into the HPV L1 sequence.

Figure 25 shows the amino acid sequence (SEQ ID NO: 22) of the HPV 16 L1/L2(108-120) chimaeric polypeptide.

Figure 26 shows the amino acid sequence (SEQ ID NO: 23) of the HPV 16 L1/L2(56-81) chimaeric polypeptide.

Figure 27 shows the amino acid sequence (SEQ ID NO: 24) of the HPV 16 L1/L2(17-36) chimaeric polypeptide.

Figure 28 shows the amino acid sequence (SEQ ID NO: 25) of the HPV 16 L1/L2 BPV(1-88) chimaeric polypeptide.

Figure 29 shows the human-codon optimised DNA nucleotide sequence (SEQ ID NO: 26) encoding the HPV 16 L1/L2(108-120) chimaeric polypeptide.

Figure 30 shows the human-codon optimised DNA nucleotide sequence (SEQ ID NO: 27) encoding the HPV 16 L1/L2(56-81) chimaeric polypeptide.

Figure 31 shows the human-codon optimised DNA nucleotide sequence (SEQ ID NO: 28) encoding the HPV 16 L1/L2(17-36) chimaeric polypeptide.

Figure 32 shows the human-codon optimised DNA nucleotide sequence (SEQ ID NO: 29) encoding the HPV L1/L2 BPV(1-88) chimaeric polypeptide.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be described more fully hereinafter with reference to the accompanying figures, in which some, but not all embodiments of the invention are shown.

The invention as described should not to be limited to the specific embodiments disclosed and modifications and other embodiments are intended to be included within the scope of the invention. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

Terms used herein have their meaning recognised in the art unless otherwise indicated.

The current invention provides a chimaeric human papillomavirus (HPV) virus like particle (VLP) having a regular shape and a size of about 30 nm in diameter and a method of treatment and/or prophylaxis of HPV infection and/or cervical cancer by administration of the chimaeric HPV VLP of the invention. In particular, the regularly shaped and 30 nm diameter chimaeric HPV VLP comprises a HPV type 16 L1 protein into which a HPV L2 peptide of between about 13 amino acids and about 26 amino acids encoded by a human codon-optimised nucleotide sequence has been inserted at amino acid residue 414, thereby replacing the corresponding HPV L1 amino acids.

The L1 major capsid protein spontaneously self-assembles into virus-like particles (VLPs), which form the basis of the current prophylactic HPV vaccines (Schiller *et al.*, 2008). Recombinant VLPs have been expressed in several diverse host systems including mammalian, insect, yeast, bacteria and plants.

The HPV-16 L1 C-terminal helix 4 (h4) plays a role in VLP assembly and is located between residues 414-426 (Varsani *et al.*, 2003a). The removal of these motifs results in capsomere formation and prevents further self-assembly into VLPs (Bishop *et al.*, 2007). Furthermore, there are disulphide bonds between highly conserved cysteine residues 175 and 428, and mutations of these cysteines results in the formation of capsomeres rather than VLPs (Li *et al.*, 1998; McCarthy *et al.*, 1998; Sapp *et al.*, 1998; Fligge *et al.*, 2001; Varsani *et al.*, 2006b). However,

in this study, it was shown that insertion of a HPV L2 peptide of between about 13 amino acids and about 26 amino acids encoded by a human codon-optimised nucleotide sequence, when inserted at amino acid residue 414, thereby replacing the corresponding HPV L1 amino acids, was able to successfully assemble into small, regularly shaped chimaeric VLPs of about 30nm in diameter.

Commercial HPV vaccines (currently expressed in yeast or insect cells) are expensive (Schiller *et al.*, 2008), partially as a result of costly production and purification protocols. In addition, complicated purification methods and extensive pre-treatment can affect the stability and recovery of assembled L1 protein and denatured L1 does not induce neutralising antibodies. As a result, the production of vaccine antigens using low-cost expression systems and simple production and purification processes remain high priorities in any commercial protein production system.

The invention will be described by way of the following examples which are not to be construed as limiting in any way the scope of the invention.

EXAMPLES

EXAMPLE 1: TRANSIENT PLANT EXPRESSION OF L1 CHIMAERAS

METHODS AND MATERIALS

Plant expression vectors

Three binary *Agrobacterium* plant expression vectors were used to optimize HPV chimaera expression: pTRAc and pTRAc-rbcs1-cTP (provided by Prof. Rainer Fischer; Fraunhofer Institute for Molecular Biology and Applied Ecology, Germany) and the Bean yellow dwarf geminivirus (BeYDV) vector pRIC3 (created by Richard Halley-Stott). Two are non-replicative vectors which target the expressed protein to either the cytoplasm (pTRAc) or chloroplast (pTRAc-rbcs1-cTP) (Maclean *et al.*, 2007), and the third is a self-replicating cytoplasm-targeting vector (pRIC3). The pRIC3 vector is a third-generation pRIC vector (Regnard *et*

al., 2010), which has been reduced in size and has shown similar amplification of transgene expression *in planta*.

The vectors contain a number of elements necessary for protein expression in plants (Figure 1). The pTRAc-rbcs1-cTP vector (Figure 1A) is a derivative of pTRAc (Figure 1B), and contains the chloroplast-transit peptide sequence of the potato *rbcs1* gene. The pRIC3 (Figure 1D) contains the BeYDV replication-associated proteins necessary for self-replication (Regnard *et al.*, 2010).

Synthesis of the L1 chimaeras

The four HPV-16 L1/L2 chimaeras used in this study are described in Table 1. The chimaeras consist of a South African HPV-16 L1 isolate gene sequence (SALI: GenBank accession no. AY177679) with an L2 epitope located in the h4 helix at aa 414 (denoted the "F-position"). These chimaeric genes were designed by Dr Inga Hitzeroth (Plant Vaccine Group, UCT), human codon-optimised and synthesized *in silico* by GENEART AG (Regensburg, Germany) using high throughput gene assembly. Synthesized L2 epitope sequences replaced the L1 sequence in the F-position and were not simply inserted into the L1 protein.

Table 1: Summary of the HPV-16 L1 chimaeric constructs

Construct	Inserted epitope	L1 position of epitope		Sequence substitution (aa)
L1/L2(108-120)	HPV-16 L2 aa 108-120	F-position	aa 414-426	13
L1/L2(56-81)	HPV-16 L2 aa 56-81		aa 414-439	26
L1/L2(17-36)	HPV-16 L2 aa 17-36		aa 414-433	20
L1/L2 BPV(1-88)	BPV-1 L2 aa 1-88		aa 414-505	88

Subcloning of the L1 chimaera genes

The HPV-16 L1/L2, chimaera sequences were excised from pGA4 vectors using 3' *XhoI* and either 5' *BspHI*, *MluI* or *HindIII* restriction enzyme (RE) sites that flank the chimaeric genes (Figure 1C). The HPV genes were directionally subcloned into the plant expression vectors, using *AflIII* and *XhoI* for pTRAc (Figure 1B), *MluI* and *XhoI* for pTRAc-rbcs1-cTP (Figure 1A), and *HindIII* and *XhoI* for pRIC3 (Figure 1D). DH5- α chemically competent *E. coli* cells (E.cloni™, Lucigen) were transformed with the chimaera plasmid constructs and

recombinants were selected using ampicillin resistance (100 µg/ml). The pTRAc HPV-16 L1/L2 chimaera constructs L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36) were provided by Mark Whitehead (Plant Vaccine Group, UCT). The plasmid constructs used in this study are summarized in Table 2.

Table 2: *Agrobacterium* expression constructs used in this study

Plant Expression Vector	Chimaeras tested	Plasmid replication	Subcellular localisation	Source
pTRAc	L1/L2	Non-replicative	Cytoplasm	M. Whitehead
pTRAc-rbcs1-cTP	L1/L2	Non-replicative	Chloroplast	This study
pRIC3	L1/L2	Self-replicative	Cytoplasm	This study

Identification of recombinant L1 chimaeras

L1 chimaera recombinant clones were screened by colony PCR, using pTRAc vector-specific primers and chimaera-specific primers binding to different L2 epitopes (Table 3). PCR was performed using GoTaq Flexi DNA Polymerase kit (Promega) as per the manufacturer's instructions using 1 µM of each primer in a final MgCl₂ concentration of 3 mM.

Table 3: Primers used in PCR and sequencing of the HPV chimaeras

Primer target	Chimaera detected	Primer name	Primer sequence	PCR product (kb)
pTRAc vector	All chimaeras	pTRAc Fwd	5'-CATTTTCATTGGAGAGGACACG-3' (SEQ ID NO:11)	~1.7
		pTRAc Rvs	5'-GAAGTACTCACACATTATTCTGG-3' (SEQ ID NO:12)	
L1/L2 chimaeras	All chimaeras	ModNew Fwd	5'-CGACGACCTGTACATCAAGG-3' (SEQ ID NO:13)	-
	L1/L2(108-120)	VEET Rvs	5'-GATGAAGCTGGTCTCCTCC-3' (SEQ ID NO:14)	0.41
	L1/L2(56-81)	SAF2 Rvs	5'-GGATGTAGCCGGTCCTGC-3' (SEQ ID NO:15)	0.44
	L1/L2(17-36)	QLYK Rvs	5'-ACCTTGGGGATGATGTCAGG-3' (SEQ ID NO:16)	0.44
	L1/L2 BPV(1-88)	SALIBPV Rvs	5'-TATCTAGGGCTTCCTCCAGC-3' (SEQ ID NO:17)	0.56

Colony PCR using vector-specific primers

The pTRAc vector-specific primers (designed by Mark Whitehead) bind upstream and downstream of the multiple cloning site (MCS) to detect the gene

insertions. The PCR profile consisted of an initial denaturation step at 95°C for 3 min, followed by 25 cycles at 95°C for 30s, 59°C for 30s and 72°C for 3 min, and a final elongation step at 72°C for 3 min. PCR products were separated on a 0.8% TBE agarose gel and detected using ethidium bromide.

Colony PCR using epitope-specific primers

HPV L2 epitope-specific primers (designed by Marieta Burger) were used to verify the correct chimaera insert in recombinant pTRAc-rbcs1-cTP and pRIC3 clones. The PCR profile consisted of an initial denaturation step at 95°C for 2 min, followed by 25 cycles at 95°C for 30s, 55°C (L1/L2 chimaeras) for 20s and 72°C for 30s, and a final elongation step at 72°C for 3 min. PCR products were separated on a 1.2% TBE agarose gel and detected using ethidium bromide.

Restriction enzyme digestion

Recombinants were verified by restriction enzyme digestion using RE sites which flank the 1.5 kb chimaera gene insert (*EcoRI* / *XhoI* for pTRAc-rbcs1-cTP clones, or *HindIII* / *XhoI* for pRIC3 clones). Recombinant DNA (~500 µg) was digested for 1-2 hrs at 37°C, using 1U enzyme per reaction as per manufacturer's instructions (Roche/Fermentas). Digested DNA was separated on a 0.8% TBE agarose gel and stained with ethidium bromide.

Sequencing of L1 chimaeras

The HPV chimaera gene insert in pTRAc-rbcs1-cTP recombinants were sequenced using the pTRAc vector-specific primers. Sequences were aligned with the HPV chimaera sequences using DNAMAN multiple alignment software.

Agrobacterium transformation

Agrobacterium tumefaciens GV3101::pMP90RK cells were made electrocompetent using the method described by Shen and Forde (1989). Transformation of *Agrobacterium* was performed as described by Maclean *et al.* (2007) and recombinant clones were screened by antibiotic selection (50 µg/ml Carbenicillin, 50 µg/ml Rifampicin and 30 µg/ml Kanamycin). Successful

transformation was confirmed by colony PCR and restriction enzyme digestion (as described above).

Agroinfiltration of *N. benthamiana*

A. tumefaciens recombinant chimaera cultures, as well as *A. tumefaciens* LBA4404 cultures containing the pBIN-NSs plasmid encoding the tomato spotted wilt virus (TSWV) NSs silencing suppressor (Takeda *et al.*, 2002), were prepared for infiltration as described by Maclean *et al.* (2007). The *Agrobacterium* cells were diluted in infiltration media (10 mM MgCl₂, 10 mM MES, 3% sucrose and 150 µM acetosyringone in water, pH 5.6) to give a final OD₆₀₀ of 0.25 for individual *Agrobacterium* chimaera strains and a combined OD₆₀₀ of 0.5 for the constructs co-infiltrated with *A. tumefaciens* LBA4404 (pBIN-NSs). The strains were incubated at 22°C for 2 hrs to allow for expression of the *vir* genes prior to infiltration.

Six-week old *N. benthamiana* leaves were agroinfiltrated by injecting the bacterial suspension into the abaxial air spaces from the ventral side of the leaf (Maclean *et al.*, 2007). The plants were grown under conditions of 16 hr light, 8 hr dark at 22°C for the desired time period. Chimaera expression time trials were conducted 1-9 days post-infiltration (dpi), and chimaeras were either co-expressed with or without the NSs silencing suppressor. Separate plants were used for each chimaera, and separate leaves on the same plant were infiltrated with either pTRAc, pTRAc-rbcs1-CTP or pRIC3 chimaera constructs for the comparative vector expression.

Protein extraction from plants

Leaf discs, cut using the cap of an eppendorf tube, were harvested from agroinfiltrated leaves (~10 mg per disc, 3 discs per construct) and ground in liquid nitrogen. Leaf material was resuspended in 250µl per disc of 1.5M NaCl high salt PBS (HS PBS) extraction buffer containing protease inhibitor (EDTA-free Complete Protease Inhibitor; Roche). The crude plant extract was clarified twice by centrifugation at 13,000 rpm for 5 min and the supernatant was stored at -20°C.

Western blot detection of plant-expressed L1 chimaeras

The plant extracts were incubated at 95°C for 5 min in loading buffer (Sambrook *et al.*, 1989), separated by a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane by semi-dry electroblotting. The membrane was blocked in blocking buffer for 30 min at room temperature (5% skim milk, 0.1% Tween-20 in 1x PBS, pH 7.6) and incubated overnight at 4°C in anti-L1 primary antibody, diluted in blocking buffer. HPV-16 L1 protein was detected with either mouse monoclonal (MAb) CamVir1 (1:10000; Abcam, UK), which binds to the L1 linear epitope GFGAMDF located at aa 230-236 (McLean *et al.*, 1990), or H16.J4 (1:2500) which binds a linear epitope located at aa 261-280 within the FG loop of the L1 protein (Christensen *et al.*, 1996). Both binding sites are not destroyed by the L2 epitope insertions.

Membranes were washed with blocking buffer for 4x 15 min, and incubated in secondary goat-anti-mouse-alkaline phosphatase conjugate (1:10000; Sigma) diluted in blocking buffer for 2 hrs at room temperature. Membranes were finally washed with wash buffer (0.1% Tween-20 in 1x PBS, pH 7.6) for 4x 15 min and developed with Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl phosphate substrate (NBT/BCIP substrate; Roche). Chimaera expression was compared by measuring the density of the bands detected on anti-L1 western blots using GeneTools (SYNGENE).

Chimaera quantification by capture ELISA

The L1 chimaeras extracted from *N. benthamiana* were quantified by capture ELISA using a modified polyvinyl alcohol (PVA)-blocking ELISA method (Studentsov *et al.*, 2002). Briefly, a 96-well Maxisorp microtitre plate was coated with 1:2000 mouse anti HPV-16 L1 MAb (either CamVir1 or H16.J4) overnight at 4°C and blocked with PVA. Plant extract was added to the wells and incubated for 1 hr at 37°C. This was followed by a washing step and the addition of rabbit anti-HPV-16 polyclonal serum (1:1000). The plate was incubated overnight at 4°C and HPV-16 L1 protein was detected with swine anti-rabbit horseradish peroxidase (HRP) conjugate (1:5000; DAKO) and 1,2-phenylenediamine dihydrochloride substrate (OPD; DAKO; Denmark).

The commercial HPV vaccine Cervarix was used as a positive ELISA control and as a HPV-16 L1 VLP standard. Each sample was analysed in triplicate and quantified using the Cervarix standard curve. The amount of chimaera protein present in each sample (mg) was expressed as chimaera per kilogram of plant tissue (mg/kg).

Total soluble protein (TSP) for each crude leaf extract was determined using the Lowry protein assay (Biorad DC Protein Assay; Microplate Assay Protocol) as per the manufacturer's instructions using a Bovine plasma gamma globin IgG protein standard (Bio-Rad). The relative chimaera yield was calculated where the ELISA-quantified chimaera protein (mg) was expressed as a percentage of TSP, in order to account for differences in leaf tissue mass and protein extraction efficiency.

Statistical analysis of chimaera expression yields

Statistical differences in chimaera expression using the different plant expression vectors were determined using ANOVA and the Fischer LSD Post Hoc test. Differences were reported as statistically significant at $p < 0.01$.

Chimaera assembly

Assembly of the HPV proteins into higher-order immunogenic structures was assessed using a H16.J4 and H16.V5 capture ELISA as described above. The H16.J4 MAb binds to a L1 linear epitope comprising of aa 261-280 (Christensen *et al.*, 1996) and thus gives the total HPV protein present in the plant extract. H16.V5 binds to a conformational L1 epitope (Christensen *et al.*, 1996, 2001) containing essential aa 260-290 and specifically binding L1 residues Phe-50, Ala-266, and Ser-282 (White *et al.*, 1999), thus it was used for the detection of assembled HPV protein. In order to compare the assembly of chimaeras expressed using different vectors, the amount of assembled HPV protein was expressed as a percentage of the total HPV protein.

RESULTS

Verification of L1 chimaera clones

The L1 chimaeras (Table 1) were successfully cloned into the pTRAc-rbcs1-cTP and pRIC3 plant expression vectors and transformed into *E. coli* and *Agrobacterium* GV3101.

The pTRAc-rbcs1-cTP recombinant clones were screened by colony PCR using pTRAc-specific primers binding upstream and downstream of the MCS, or chimaera-specific primers binding to different L2 epitopes. All chimaeras produced fragments of the expected size as predicted in Table 3.

Clones were further verified by restriction enzyme (RE) digestion using *EcoRI* and *XhoI* RE sites which flank the chimaera gene insert. As expected, all chimaeras contained a 1.5 kb gene insert. Clones were sequenced and individual chimaeras were confirmed using DNAMAN multiple sequence alignment software.

The pRIC3 recombinant clones were similarly verified by colony PCR using the chimaera epitope-specific primers and *HindIII* / *XhoI* restriction enzyme digestion. All chimaeras produced the 0.2–0.6 kb chimaera-specific PCR bands described in Table 3 and the 1.5 kb gene fragment in the RE digests. Thus all the HPV chimaeras were successfully subcloned into the pTRAc-rbcs1-cTP and pRIC3 plant expression vectors.

Optimisation of L1 chimaera expression in *N. benthamiana*

Co-expression with the NSs silencing suppressor

Chloroplast-targeted HPV-16 L1/L2 expression in *N. benthamiana* was examined in a 1-9 day post-infiltration (dpi) time trial. Chimaeras were expressed either with (+) or without (-) the NSs silencing suppressor protein to examine its effects on chimaera expression. Expression was analysed by western blotting using the anti-L1 MAb CamVir1. All the L1/L2 chimaeras were detected, with the predicted ~56 kDa L1 band (Figure 2), although L1/L2 (108-120) runs higher than the other chimaeras.

All chimaeras showed a prolonged increase in expression when co-infiltrated with the silencing suppressor protein NSs (Figure 2 A-D), suggesting it was effective in preventing post-translational gene silencing and enhancing protein accumulation in plants. ELISA detection using the linear-epitope specific MAb H16.J4 confirmed the results, with up to a 16-fold increase in L1/L2 yields (data not shown). Chimaera expression without NSs was detected 1-3 dpi and peaks 3-5 dpi, while chimaeras co-expressed with NSs was detected at 3 dpi and expression peaked at 5-7 dpi. There was a small decrease in expression between 5-9 dpi, suggesting there is a slow decline in expression levels (ELISA results, data not shown). As a result, all chimaeras were co-expressed with NSs in further experiments.

Several high molecular bands were detected for the L1/L2 (17-36) chimaera, suggesting the chloroplast signal sequence (cTP) may not have been cleaved or the chimaera may have been glycosylated. However, L1/L2 (17-36) analysed on subsequent western blots did not display these high molecular weight bands, suggesting the protein was partially denatured in Figure 2C.

The L1/L2 chimaera containing the BPV L2 aa 1-88 epitope had low expression levels in comparison to the chimaeras containing HPV-16 L2 epitopes. The bands on the L1/L2 BPV (1-88) western blots were only visible after 16 hours of development (Figure 2D), in comparison to the 15 min development time required for the other chimaeras (Figure 2A-C). ELISA quantification estimated L1/L2 BPV(1-88) achieved maximum yields of 40 mg/kg plant tissue, while high expression yields of 1000 - 4600 mg/kg were estimated for the other L1/L2 chimaeras (data not shown). In addition, the L1/L2 BPV(1-88) plant extract contained a characteristic ~45 kDa band (Figure 2D) associated with L1 degradation, suggesting L1/L2 BPV(1-88) is unstable in this expression system. These results were confirmed by several L1/L2 BPV(1-88) western blots from different time trials.

Effect of chloroplast targeting on L1/L2 chimaera yield

Targeting of HPV proteins to the chloroplast can significantly improve plant expression yields (Maclean *et al.*, 2007). To determine the importance of chloroplast-targeting, the pTRAc (cytoplasmic-targeting) and the pTRAc-rcs1-

cTP (chloroplast-targeting) L1/L2 chimaera constructs were co-infiltrated with pBIN-NSs in *N. benthamiana* in a 3-9 dpi time trial. The L1/L2 BPV(1-88) chimaera was not included in this study, as it shows very low expression in *N. benthamiana* when compared to the other L1/L2 chimaeras.

Western blots and ELISA data consistently demonstrated low expression for the cytoplasm-targeted L1/L2 chimaeras, with maximum expression of chimaeras 3 dpi and yields of 20-45 mg/kg plant tissue (data not shown). Expression of cytoplasm-targeted L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36) was weakly detected in comparison to the chloroplast-targeted L1/L2(108-120) chimaera diluted 3x prior to loading and included as a positive control. Comparison of chimaera yields indicates that L1/L2 chimaera expression was increased 40 - 80 fold when targeted to the chloroplast. Taking these results into consideration, further chimaera expression studies were done using the pTRAc-rbcs1-cTP vector.

Optimisation using the self-replicative pRIC3 plant expression vector

In an attempt to improve chimaera yields, particularly for the low-expressing L1/L2 BPV(1-88), the plant expression vector pRIC3 (self-replicative, cytoplasm-targeting vector) was compared to pTRAc-rbcs1-cTP (non-replicative chloroplast-targeting vector) in a 3-9 dpi time trial in the presence of NSs.

Maximum chimaera yields for both vectors were obtained 3-5 dpi. The three L1/L2 chimaeras containing the HPV-16 L2 epitopes aa 108-120, 56-81 and 17-36 were better expressed using the chloroplast-targeting pTRAc-rbcs1-cTP vector compared to the self-replicative pRIC3 vector. L1/L2 BPV(1-88) was not highly expressed for either vector and degradation was visible for both constructs.

ELISA quantification shows the self-replicative pRIC3 vector did not improve expression yields for the majority of the chimaeras. Yields were up to 3-fold higher using the pTRAc-rbcs1-cTP vector, suggesting that chloroplast-targeting is more effective in the high-expression of chimaeras than the pRIC3 vector, which ultimately targets the expressed protein to the cytoplasm. The L1/L2 BPV(1-88) expression levels were similar to the NSs negative control, suggesting

plants are not a viable system for the production of L1/L2 BPV(1-88) and the expression of L1/L2 BPV(1-88) was not pursued further.

The results from the expression optimization using the pTRAc-rbcs1-cTP and pRIC3 vectors are summarised in Table 4. The L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36) were highly-expressed. The parameters shown to maximise expression in the preliminary time trials are: co-expression with NSs, extraction 5 dpi and use of the pTRAc-rbcs1-cTP vector to target the expressed L1/L2 protein to the chloroplast.

Table 4: Summary of L1 chimaera expression and optimization

Chimaera	Maximum chimaera expression				
	Extraction (dpi)	Vector	Yield (mg/kg)	Yield (%TSP)	Fold increase (pTRAc-rbcs1-cTP vs. pRIC3)
L1/L2(108-120)	5	pTRAc-rbcs1-cTP	600	3.7	1.8
L1/L2(56-81)	5	pTRAc-rbcs1-cTP	280	1.7	2.4
L1/L2(17-36)	5	pTRAc-rbcs1-cTP	440	2.9	1.8
L1/L2 BPV(1-88)	5	pTRAc-rbcs1-cTP	-	-	-

Comparative vector expression of L1/L2 chimaeras

Three high-expressing L1/L2 chimaeras were chosen as vaccine antigens for the mouse immunogenicity studies: L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36). A final expression study including three biological repeats was performed to confirm the L1/L2 results and obtain statistically valid data. All three vectors (pTRAc, pTRAc-rbcs1-cTP and pRIC3) were directly compared for expression of each of the L1/L2 vaccine antigens, HPV-16 L1 was included as a positive control (pTRAc and pTRAc-rbcs1-cTP constructs were available) and NSs-infiltrated plants served as the negative control. Chimaeras were co-expressed with NSs and extracted 5 dpi.

Effect of expression on plants

Examination of the infiltrated leaves prior to extraction 5 dpi suggested that the self-replicative pRIC3 vector had adverse effects on the health of the plant. Leaves infiltrated with pRIC3 were yellow/brown in colour and necrosis of the leaf tissue was visible in the infiltrated areas. This was observed to a lesser degree in

the pTRAc leaves, which also targeted chimaeras to the plant cytoplasm. The pTRAc-rbcs-cTP leaves appeared to be the healthiest, resembling the leaves of the NSs-infiltrated negative control and the uninfiltrated leaves, suggesting accumulation of the chimaras in the chloroplast has less of an impact on plant health (results not shown). Infiltration appears to have no observable effect on plant health, as the NSs-infiltrated leaf looked similar to the uninfiltrated leaf (excluding the syringe injection markings). These results were consistently observed for all the time trials.

Western blot detection of the HPV proteins

HPV protein was detected by anti-L1 western blotting (Figure 3a). The NSs-infiltrated plant extract (negative control) was not detected and plant-derived HPV-16 L1 (positive control) was detected using the chloroplast-targeting vector. Expression using the different vectors was directly compared with pTRAc-rbcs1-cTP consistently giving the highest expression yields, followed by pRIC3, and then pTRAc.

ELISA quantification of the HPV proteins

Capture ELISA was used to quantify the HPV chimaeras using CamVir1. The L1/L2 chimaera and HPV-16 L1 yields are shown in Figure 3b. Statistical differences in chimaera expression using the 3 plant expression vectors were determined using ANOVA and the Fischer LSD Post Hoc test. Differences were reported at statistically significant at $p < 0.01$.

Chloroplast-targeted expression of the L1/L2 chimaeras and HPV-16 L1 using pTRAc-rbcs1-cTP gave significantly higher yields than the NSs-infiltrated negative control ($p = 0.000 - 0.002$), and the cytoplasm-targeting pTRAc vector ($p = 0.000 - 0.004$). In addition, pTRAc-rbcs1-cTP significantly improved L1/L2(56-81) expression compared to pRIC3 ($p = 0.006$). The pRIC3 vector did not statistically improve expression of any of the chimaeras compared to pTRAc.

In comparison to the optimization experiments (Figure 2, Table 4), the comparative time trial demonstrated similar trends in chimaera expression. The chloroplast-targeted L1/L2 chimaeras gave the highest yields (1040 - 1310 mg/kg; 2 - 3% TSP), improving chimaera expression by up to 28-fold in comparison to the

cytoplasm-targeting vector pTRAc (50 – 260 mg/kg; <1% TSP) and up to 7-fold in comparison to the self-replicative vector pRIC3 (190 - 660 mg/kg; <1% TSP).

Cytoplasm-targeted chimaera yields were improved up to 4-fold using the self-replicative vector pRIC3 in comparison to the non-replicative pTRAc vector. This suggests self-replication of the vector improves chimaera expression, although targeting to the chloroplast appears to be a superior strategy to increase chimaera expression in plants.

Although chloroplast-targeted HPV-16 L1 demonstrated higher average yields (1710 mg/kg, 4% TSP), the differences between the L1/L2 chimaeras and L1 were not statistically significant, indicating the L2 epitope substitutions do not appear to affect the expression and accumulation of recombinant protein in chloroplasts. However, western blotting (3a) and ELISA expression data (Figure 3b) consistently revealed higher levels of cytoplasm-localised L1/L2(108-120) and L1/L2(17-36) than L1/L2(56-81), suggesting L1/L2(108-120) and L1/L2(17-36) chimaeras with shorter L2 sequence replacements (13 and 20 aa respectively) may be better expressed and have a greater stability than L1/L2(56-81) with a 26 aa sequence replacement.

Assembly of the HPV proteins

Chimaera assembly into higher-order structures such as capsomeres or VLPs was assessed using H16.J4 (linear epitope-specific MAb) and H16.V5 (conformational epitope-specific MAb) capture ELISA. The amount of V5-detected conformational HPV protein was expressed as a percentage of the J4-detected total HPV protein for each of the vector constructs (Figure 4).

A low percentage of the expressed chimaeras assembled into H16.V5-detected higher-order structures. The NSs plant extract, used as a negative control, did not bind H16.J4 or H16.V5 MAb (data not shown). The low-expressing pTRAc chimaeras appear to have the highest proportion of assembled protein (11-18%), followed by the high-expressing pTRAc-rbcs1-cTP chimaeras (5-9%). The pRIC3 chimaeras did not contain a high percentage of assembled protein (< 2%). Although the pTRAc-rbcs1-cTP chimaeras did not contain the highest percentage of assembled protein, higher expression yields results in up to

40x and 4x more assembled protein than pTRAc and pRIC3 respectively. This provides further evidence that pTRAc-*rbcs1*-cTP is the best vector to use for the production of immunogenic L1/L2 chimaeras.

DISCUSSION

Optimisation of L1 chimaera transient expression in plants

All of the L1 chimaeras were successfully expressed in plants using an *Agrobacterium*-mediated transient system (Figure 2). Several methods were used to optimize chimaera expression in plants; including use of a NSs silencing suppressor, use of an agroinfiltration-delivered self-replicative viral vector and targeting of the expressed protein to the chloroplast.

Co-expression with the NSs silencing suppressor

Agrobacterium-mediated transient expression typically peaks 60-72 hours (~3 days) post-infiltration and then declines rapidly as a result of triggering post-transcriptional gene silencing (PTGS) in the host plant (Voinnet, 2001). PTGS is an adaptive anti-viral plant defense mechanism, where foreign RNA molecules are recognized and degraded in a sequence-specific manner (Meins, 2000; Sijen and Kooter, 2000). As a counter-defensive strategy, many plant viruses have evolved proteins that suppress various steps of the mechanism (Voinnet, 2001). Although PTGS responses reduce transgene mRNA accumulation in the plant cytoplasm and limit the efficiency of *Agrobacterium*-mediated transient expression (de Carvalho *et al.*, 1992; Van Blokland *et al.*, 1994), co-expression of proteins with viral silencing suppressors has been shown to repress PTGS responses and allow high level transient expression, resulting in higher yields (50-fold in some instances) and prolonged expression of the transgene (Voinnet *et al.*, 2003).

Co-infiltration with the tomato spotted wilt virus (TSWV) silencing suppressor NSs suppresses PTGS and increases transient expression (Takeda *et al.*, 2002). This effect was similarly observed in the transient expression of the L1/L2 chimaeras (Figure 2). Chimaeras typically displayed maximum expression levels 3-5 dpi without the presence of viral silencing suppressors. However, co-expression with NSs displayed a prolonged increase in the expression of the

chimaeras, whereby expression levels were increased by up to 16-fold and peaked 5-7 dpi.

The use of a self-replicative BeYDV vector

Cytoplasmic HPV-16 L1 yields have been improved by 50% using a self-replicative pRIC vector (Regnard *et al.*, 2010). As a result, a third-generation pRIC3 vector was examined as a potential strategy to improve chimaera yields. Three L1/L2 chimaeras were examined: L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36). All chimaeras demonstrated higher expression levels using pRIC3 (self-replicative vector), in comparison to pTRAc (non-replicative vector), suggesting transgene amplification improves L1/L2 yields in the plant cytoplasm (Figure 3a and b). However, chloroplast-targeting was more effective in the high-level accumulation of L1/L2 chimaeras (Figure 3b) and visible necrosis of the leaf tissue was observed in pRIC3-infiltrated leaves, suggesting the self-replication of the vector negatively affects plant health.

Chloroplast-targeting of L1 chimaeras

L1 chimaeras were targeted to the chloroplast using the pTRAc-*rbcs1*-cTP vector. The chloroplast transit peptide (cTP) is fused to the expressed chimaera and is cleaved by the chloroplast stromal processing peptidase (SPP) upon entry into the organelle (Robinson and Ellis, 1984). There are several factors responsible for the high level accumulation of protein in chloroplasts: (a) protection from cellular proteases, (b) different protein hydrolyzing machinery in the plastids and (c) protective plasmid-specific chaperones which assist in the correct folding of L1 and thus improve protein stability (Fernández-San Millán *et al.*, 2008). In this study, chloroplast-targeting was highly effective and increased L1/L2 chimaera yields by 40 to 80-fold in comparison to chimaeras targeted to the cytoplasm (Figure 3a).

The chloroplast-targeted chimaeras detected in the anti-L1 western blots produced bands of ~56 kDa (Figure 2), suggesting the signal peptide was effectively removed from the accumulated protein. Although L1/L2(108-120) runs higher than the other chimaeras on the western blot (Figure 2), this phenomenon is not caused by insufficient cleavage of the signal peptide, as the cytoplasm-

localised L1/L2(108-120) expressed with pTRAc, and insect cell-expressed L1/L2(108-120) analysed in parallel (data not shown), showed a similar banding pattern.

Higher molecular weight bands of ~65 kDa were detected for L1/L2(17-36) chimaeras (Figure 2), possibly as a result of glycosylation or insufficient denaturation of chimaeras. A glycosylated form of HPV-16 L1 produced in baby hamster kidney cells (BHK) was described by McLean *et al.* (1990), whereby CamVir1 detected 2 bands for L1: the 56 kDa L1 major band and a minor band of ~64 kDa. The additional band was subsequently removed from cell lysates when infected in the presence of the N-glycosylation inhibitor tunicamycin. Although plants do contain glycosylation pathways (Rybicki, 2009), subsequent western blots displayed a single ~56 kDa band, suggesting the L1/L2(17-36) chimaeras were partially denatured in initial experiments rather than glycosylated (Figure 3a).

Direct comparison of plant expression vectors

Two strategies have increased plant-expressed L1 yields to a maximum of 530 - 550 mg/kg (i) targeting the protein to the chloroplast (Maclean *et al.*, 2007) or (ii) the use of an agroinfiltration-delivered self-replicative BeYDV-derived expression vector (Regnard *et al.*, 2010). This was the first study which directly compared these strategies using the L1-based chimaeras. Chimaera expression levels using the plant expression vector pRIC3 (self-replicative, cytoplasm-targeting vector) was directly compared to pTRAc-rbcs1-cTP. Expression using the pTRAc (non-replicative, cytoplasm-targeting vector) was included for comparative purposes and HPV-16 L1 was used as a positive control (Figure 3a and b).

Chloroplast-targeting produced the highest yields for the majority of chimaeras (Figure 3a and b) and improved L1/L2 chimaera expression by up to 7-fold relative to pRIC3, and 28-fold relative to pTRAc, both which target the expressed protein to the cytoplasm (Figure 3b). Statistical analysis revealed that the chloroplast-targeted L1/L2 yields were significantly higher than the cytoplasm-targeted L1/L2 yields ($p < 0.01$). However, yield differences between chloroplast-targeted chimaeras and chimaeras expressed using the self-replicative pRIC3 vector were only significant for L1/L2(56-81). These results provide evidence that

pTRAc-rbcs1-cTP is the best vector to use for the high-level production of HPV chimaeras.

Expression of the L1/L2 chimaeras

Highly-expressed L1/L2 chimaeras containing the HPV-16 L2 epitopes

The L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36) chimaeras were highly-expressed, with yields up to 20-fold higher than the other chimaeras (Table 4). As a result, these three L1/L2 chimaeras were chosen as vaccine antigens for the mouse immunogenicity studies.

Chloroplast-targeted L1/L2 chimaeras consistently demonstrated the highest chimaera yields of ~ 1200 mg/kg plant tissue (2-3% TSP). Although HPV-16 L1 demonstrated higher yields than the L1/L2 chimaeras, the differences were not statistically significant (Figure 3b). This indicates that the L2 epitopes do not significantly affect the expression and accumulation of HPV protein in chloroplasts. Furthermore, the chimaera yields are ~2-fold higher than published HPV-16 L1 yields produced using an *Agrobacterium*-mediated tobacco expression system (Maclean *et al.*, 2007; Regnard *et al.*, 2010) and the production of these chimaeras is commercially viable (>1% TSP; Fischer *et al.*, 2004).

Assembly into higher-order structures is associated with a lower susceptibility to proteolysis (Chen *et al.*, 2000) and it was hypothesized that the high accumulation of L1/L2 may be as a result of assembly. The conformational-specific H16.V5 MAb binds assembled protein (Christensen *et al.*, 1996) and can be used to detect assembly into higher-order structures (Carter *et al.*, 2003; Wang *et al.* 2003; Ryding *et al.*, 2007). All plant-expressed L1/L2 chimaeras and the HPV-16 L1 control appeared to contain a low proportion of assembled protein (<20% TSP), suggesting majority of the protein exists as unassembled L1 monomers. However, both the L1/L2(56-81) and L1/L2(17-36) chimaeras contain L2 sequences overlapping the L1 C-terminal region aa 428-483 shown to be critical for the binding of H16.V5 (Varsani *et al.*, 2006b), suggesting this MAb may not be suitable for detection of chimaera assembly and cannot be used for comparable quantification.

Instability of the L1/L2 chimaera with the BPV L2 aa 1-88 epitope

The L1/L2 BPV(1-88) chimaera had low expression levels in comparison to the chimaeras containing the HPV-16 L2 epitopes (Figure 2). ELISA quantification estimated expression levels were similar to the NSs negative control, although L1/L2 BPV(1-88) was detected on western blots probed with H16.J4 MAb and showed low yields of 40 mg/kg plant tissue (Figure 2, ELISA data not shown). In addition, the chloroplast-targeted L1/L2 BPV(1-88) was partially degraded (Figure 2D), which has been previously described in several HPV L1 expression studies (Hagensee *et al.*, 1993; Sasagawa *et al.*, 1995; Li *et al.*, 1997; Kohl *et al.*, 2007).

These results provide evidence that L1/L2 BPV(1-88) is not well-expressed, neither is it stable in the present plant expression system. The L1/L2 BPV(1-88) chimaera contains the largest L2 sequence replacement and the 88 residue epitope replaced the entire C-terminal region of L1 (Table 1). The HPV L1 C-terminal region plays a role in VLP assembly (Zhou *et al.*, 1991b; Varsani *et al.*, 2006b; Bishop *et al.*, 2007), and deletion of the C-terminal region prevents assembly into higher-order structures which are less susceptible to degradation (Chen *et al.*, 2000). Sequence replacement of the L1 C-terminal region with foreign epitope sequences is not an effective strategy for HPV chimaera expression and plants are not a viable system for L1/L2 BPV(1-88) expression.

EXAMPLE 2: PURIFICATION AND ASSEMBLY OF HPV ANTIGENS

METHODS AND MATERIALS

Large-scale transient expression and extraction of antigens

N. benthamiana plants (2-4 weeks old) were vacuum-infiltrated with *A. tumefaciens* LBA4404 (pBIN-NSs) encoding the NSs silencing suppressor protein and the *Agrobacterium* GV3101 strain encoding HPV-16 L1 or the L1/L2 chimaeras, as described by Maclean *et al.* (2007). The plants were grown for 5 days in 16 hr light, 8 hr dark, at 22 °C.

The infiltrated leaves were harvested, weighed and ground in liquid nitrogen using a mortar and pestle. PBS extraction buffer, containing 0.5M NaCl and protease inhibitor (Roche Complete EDTA-free), was added at a ratio of 1:4

(w/v) and samples were homogenized in a Waring blender for 10 min on ice. The homogenate was sonicated on ice for 6x 20s intervals of sonication and rest (Macrotip sonication; Level 8; Heat Systems – Ultrasonics, Inc. Sonicator Cell Disruptor Model W-225 R), and the lysate was filtered through a double layer of Miracloth (CALBIOCHEM). The crude extract was clarified twice by centrifugation at 13,000 rpm for 10 min. Pellets were resuspended in 1ml PBS and stored at -70°C. The supernatant and pellet were examined by western blotting to check for localization of the HPV antigen to the supernatant.

Pilot purification of HPV antigens

Several methods were examined for the purification of plant-expressed L1 vaccine antigens. Size-based methods such as cross-flow microfiltration and ultracentrifugation using sucrose and caesium chloride density gradients were tested, as well as single-step cation-exchange and heparin chromatography for the rapid purification of L1 and L1-based chimaeras. L1 protein extracted in PBS containing 0.5M NaCl was diluted 10x in low-salt PBS (LS PBS: 10mM NaCl PBS, pH 7.4) prior to chromatography, to allow L1 binding to the columns. Ultrafiltration was utilized to concentrate antigens and desalt chromatography fractions for downstream application in mouse immunogenicity studies.

Overall, purification using heparin chromatography and diafiltration using Macrosep® ultrafiltration spin tubes were the best strategies to obtain partially-purified L1 and L1-based chimaeras, and these methods were used to prepare the vaccine antigens for subsequent mouse immunological experiments.

Purification of vaccine antigens

Sample preparation

HPV-16 L1 and L1-based chimaeras were expressed in *N.bethamiana* and extracted as described above in Example 1 using LS PBS as the extraction buffer. The double-clarified crude supernatant for L1/L2(108-120), L1/L2(56-81), L1/L2(17-36), HPV-16 L1 and the NSs-infiltrated plant extract (Vaccines 1-5 respectively) was filtered through a 0.22 µm Millipore filter prior to chromatography to remove any debris.

Heparin chromatography

Chromatography was performed using an ÄKTA Explorer System 10. The procedure was followed as recommended in the GE Healthcare column instruction manual and a flow rate of 0.5 ml/min was maintained. The column was equilibrated with 10 column volumes (cv) of low salt wash buffer (LS PBS: 10mM NaCl PBS) prior to loading the sample. The crude extract (10-20 ml) was loaded on a pre-packed 1ml HiTrap Heparin cation-exchange column (GE Healthcare, Amersham Biosciences AB, Sweden) and the column was washed with 10 cv of LS PBS wash buffer. The elution profile for each HPV antigen was optimized in a pilot experiment using a linear ionic strength gradient, whereby 10 cv of 0-100% of a high salt PBS (HS PBS) elution buffer containing 1.5M NaCl was applied to the column. Once it had been established that all antigens eluted when <50% HS PBS was applied to the column, a 50% step elution gradient (0.75M NaCl) was applied for purification of the vaccine antigens. The step elution gradient was 10 cv of 50% HS PBS, followed by 10 cv of 100% HS PBS. The column was finally washed with 5 cv of distilled water and 5 cv of 20% ethanol. Fractions (1ml) were collected and analyzed by western dot blots.

Western dot blot detection of purified HPV antigens

The dot blots were performed as described above in Example 1. CamVir1 (1:10000) was used to detect L1 and Cervarix was used as a positive control. Eluted fractions containing a high concentration of purified antigen were pooled and stored at -70°C. For the NSs-infiltrated plant extract (V5: negative control), the fractions which corresponded with the eluted protein peak were pooled and tested on the L1 positive control vaccine (V4) dot blot, to confirm it did not contain L1.

Desalting of purified antigen samples by ultrafiltration

The purified antigens in the 50% HS PBS elution buffer (0.75M NaCl), were desalted prior to mouse vaccinations. Ultrafiltration spin tubes with 10kDa MWCO filter (Macrosep® Centrifugal Devices, 10K Omega, PALL Life Sciences) were used to concentrate and desalt the purified L1 fractions rapidly by centrifuging the samples at 7000g for 15-30 min. The retentate containing the L1 antigens was diluted in LS PBS and re-concentrated by ultrafiltration several times as per the

manufacturer's instructions until the samples contained a NaCl concentration similar to that found in commercial PBS (~0.15M NaCl). Both the retentate and filtrate fractions were examined.

Analysis of antigen purity

Coomassie staining and western blot detection of HPV antigens to assess purification

The crude extract and purified sample for each of the vaccine antigens was compared by Coomassie staining and western blot analysis. Samples were prepared as described in Example 1 above and equal volumes were loaded into two 10% SDS-PAGE protein gels. One gel was stained with Coomassie solution overnight at room temperature and destained 2x 2hr at 37°C. The other gel was blotted onto nitrocellulose membrane and probed with CamVir1.

Total soluble protein quantification

The negative control vaccine (V5: NSs-infiltrated plant extract) cannot be quantified by anti-L1 western blotting. As a result, the amount of total soluble protein (TSP) was determined for each vaccine antigen using the Biorad Lowry protein assay (described in above in Example 1) to ascertain that the TSP was similar for all vaccines.

Detection of HPV antigens by capture ELISA to determine enrichment of antigen relative to the TSP

A capture ELISA was performed as described in Example 1, using the linear epitope-specific monoclonal antibody (MAb) H16.J4. The HPV antigen yields determined by ELISA were compared to corresponding TSP yields in both the crude and purified samples to determine antigen enrichment.

Western blot quantification of purified vaccine antigens

A dilution series of the vaccine Cervarix (containing 40 ug/ml of insect cell-produced HPV-16 L1) was used to quantify the plant-produced HPV antigens (V1-4). Several dilutions of the antigen were analyzed to ensure quantification occurred within the linear range of the standard curve. Equal volumes of purified

antigens and Cervarix dilutions were loaded into 10% SDS-PAGE gels, proteins were blotted onto nitrocellulose membrane and the HPV antigens were detected with CamVir1 (1:10000).

Densitometry (GeneTools, Syngene, Synoptics Ltd) was used to quantify the antigens (as done by Aires *et al.*, 2006; Bazan *et al.*, 2009) and the amount of HPV protein present in the samples was determined using the standard curve generated by the Cervarix dilution series. Quantified HPV antigens were stored at -70°C.

Cytoplasmic expression and extraction of antigens

In order to establish whether small virus like particles are also formed when chimaeric HPV L1/L2 proteins are targeted to the cytoplasm, as opposed to the chloroplasts, *N. benthamiana* plants were infiltrated with recombinant *Agrobacterium* harbouring pRIC L1/L2 (108-120); L1/L2 (56-81) and L1/L2 (17-36) together with the silencing suppressor NSs using the methods described above. After 3 to 5 days the infiltrated leaves were harvested, ground up and cell debris was removed by centrifugation.

Structural analysis by transmission electron microscopy

Aliquots of the purified vaccine antigens were pre-treated as if they were being prepared for mouse vaccinations. The antigens were defrosted overnight at 4°C, resuspended in PBS to the required concentration and incubated at 37°C for 30 min.

To determine the effect of purification, the pre-treated purified antigens and the crude plant extracts for each antigen were diluted 10x in PBS, immunotrapped using CamVir1 (1:1000), a linear epitope-specific HPV-16 L1 antibody which binds both L1 monomers and assembled L1 protein (McLean *et al.*, 1990), and captured on glow-discharged carbon-coated copper grids. The proteins were negatively stained with 2% uranyl acetate and viewed on a Zeiss 912 Omega Cryo EFTEM.

RESULTS

Purification of plant-expressed HPV antigens

Detection of HPV antigens in the clarified extract

The localisation of L1 and the L1/L2 chimaeras to the clarified supernatant was confirmed by western blot analysis. The Coomassie-stained protein gel indicated the abundant presence of Rubisco in the supernatant and the removal of several contaminating proteins present in the pellet (data not shown).

Pilot purification of HPV antigens

Purification using size-based techniques was largely unsuccessful and not reproducible between the vaccine antigens, as the L1/L2 chimaeras appear to assemble into a variety of structures in contrast to L1. In addition, protein degradation was detected and thus purification using chromatography was examined as an alternative method.

Although cation-exchange chromatography using the HiTrap SPFF or POROS 50HS column was unsuccessful in the purification of the L1-based chimaeras, heparin affinity chromatography purified all the vaccine antigens. The concentration and removal of salt in the chromatography fractions was examined using two ultrafiltration-based methods, either by cross-flow filtration or centrifugation spin columns. Although cross-flow ultrafiltration was effective, the method was costly with regard to time and significant protein degradation was detected, resulting in the preferential use of spin columns. Thus, heparin chromatography and centrifugation ultrafiltration were considered the best strategies to purify the vaccine antigens for subsequent mouse immunological experiments.

Purification using heparin chromatography

Vaccine antigens were purified from the clarified crude plant supernatant by heparin chromatography using a high NaCl gradient for elution of the HPV antigens. The step elution gradient was optimised for each HPV antigen using a linear 0-100% 1.5M NaCl gradient. All HPV antigens eluted between 0.45 - 0.75M

NaCl (data not shown). As a result, a 50% (0.75M NaCl) step gradient was used to purify the vaccine antigens for the mouse immunogenicity study. Detection of the purified HPV antigens in the eluate fractions was determined using CamVir1 dot blots.

An absorbance peak was detected when the HS PBS elution buffer was applied to the column and these fractions contained the purified HPV antigens. The chromatograms for the other vaccine antigens were similar, including the graph for the NSs-infiltrated plant extract (negative control). This indicates that the HPV antigens were co-purified with other contaminating plant proteins.

The fractions containing the partially-purified HPV antigens (or co-eluted plant proteins for the negative control) were pooled and then desalted using ultrafiltration spin columns. Western dot blots indicated that the HPV antigens were successfully retained and concentrated (data not shown).

Purity of the vaccine antigens

The purity of the vaccine antigens was examined by comparing the purified sample to the crude plant extract. This was done using Coomassie staining, to indicate total protein present in the samples (Figure 5A), and western blot analysis, to detect the HPV antigens and indicate the loss in antigen yield (Figure 5B). Note that the L1/L2(108-120) chimaera (V1) runs higher than the other L1/L2 chimaeras (V2-3) and the L1 control (V4).

Figure 5 shows the purified samples were enriched with L1 as a result of the purification procedure. The Coomassie-stained gel shows a large decrease in the total protein in the purified samples, while the western blot results indicate that there is only a small decrease in antigen yield after purification. The L1 antigen was not detected in the negative control (V5: NSs-infiltrated plant extract).

Samples were only partially-purified, as additional Coomassie-stained protein bands were detected in Figure 5A for purified antigens V1 and V3 (more concentrated than V2 and V4), thus demonstrating that the purified samples contain several contaminating plant proteins. Also, although the NSs negative control (V5) was not detected on the western blot, several similar Coomassie bands were seen in the purified NSs sample.

Enrichment of HPV antigens in purified samples

The TSP of the purified antigens was determined to: (a) ensure that the TSP was similar for the NSs negative control (containing plant proteins which were co-purified with the HPV antigens) in comparison to the other vaccine antigens, and (b) to determine HPV antigen enrichment after purification. The TSP for the purified HPV vaccine antigens (V1-4) was similar, however the TSP for the NSs plant extract negative control (V5) was almost 2-fold higher, possibly as a result of more eluate fractions being pooled or greater ultrafiltration concentration (data not shown). As a result, it was diluted accordingly in 1x PBS.

Capture ELISA, using the linear-specific H16.J4 MAb was used to estimate the amount of HPV antigen present in the crude and purified samples. To determine the effect of purification on the TSP and the enrichment of HPV antigens, the H16.J4-detected HPV yield was directly compared to the TSP yield for both the crude and purified samples (Figure 6).

Figure 6 demonstrates that purification of the plant extracts reduced both the TSP and total HPV protein, as expected. However, relative to the TSP, there is up to a 5-fold enrichment of HPV antigen in purified samples (V1-4), suggesting that heparin chromatography is effective in removing a large proportion of contaminating protein. The NSs-infiltrated plant extract (V5) showed a similar decrease in TSP with purification and the amount of TSP in the "purified" negative control lies within the levels obtained for the HPV vaccine antigens (V1-4).

Western blot quantification of purified HPV antigens

HPV antigens were quantified by western blotting using densitometry and the commercial vaccine Cervarix as the standard (data not shown). Some L1 protein degradation, visible as a ~45 kDa band, was detected in some of the purified antigen batches, particularly after several freeze-thaw cycles. However, only the full-length 56 kDa L1 band was quantified in the samples prepared for mouse immunogenicity studies.

Structural analysis of purified vaccine antigens

The structural assembly of L1 and the L1/L2 chimaeras produced in the chloroplasts of plants in both the crude and purified samples was analysed by immunocapture transmission electron microscopy (Figure 7). The structural assembly of the L1/L2 chimaeras produced in the cytoplasm were analysed by immunocapture transmission electron microscopy from crude samples (Figure 8). Antigen purification resulted in the removal of contaminating background protein, particularly for L1/L2(108-120) and the negative control (Figure 7A and E respectively). In comparison to the negative control (NSs-infiltrated plant extract), all the HPV antigens appeared to contain secondary HPV structures, either capsomeres (~10 nm), capsomere aggregates, small VLPs (~30 nm) or full-sized VLPs (55 nm).

Purified L1/L2(108-120) assembled into small chimaeric VLPs (cVLP) which were regular in shape but varied in size (~30 nm), while L1/L2(56-81) only appeared to contain capsomeres and some aggregates, although VLP-like structures were visible in the crude extract. Purified L1/L2(17-36) contained a mixed population of amorphous cVLPs and a high proportion of capsomere aggregates in contrast to the crude extract, suggesting purification promoted the formation of higher-order structures. Purified V4, the HPV-L1 positive control, assembled into distinct VLPs (~50 nm), as described in previous studies (Biemelt *et al.*, 2003; Maclean *et al.*, 2007).

DISCUSSION

Stringent purification is necessary for the commercial production of vaccines, although the stability of L1 is negatively affected by multiple purification steps. Heparin affinity chromatography can be utilized to selectively purify assembled L1, and a purification strategy using a one-step chromatography method would be ideal for the rapid and cost-effective production of HPV vaccines. This study reports the purification of plant-expressed HPV-16 L1 and three L1/L2 chimaeras using heparin chromatography for subsequent immunogenicity studies in mice.

Optimisation of L1/L2 chimaera purification

HPV-16 L1 and the L1-based chimaeras were localized to the crude extract supernatant and were purified using a variety of methods. Although size-dependent purification methods have been used to purify plant-expressed HPV L1 (Biemelt *et al.*, 2003; Maclean *et al.*, 2007; Fernández-San Millán *et al.*, 2008), these methods were inefficient for L1/L2 chimaera purification and were non-reproducible between antigens. The L1-based chimaeras were broadly detected in several fractions using both sucrose and CsCl density gradient ultracentrifugation, indicating that the L1/L2 chimaeras assembled into heterologous higher-order structures, such as capsomeres, aggregates and VLPs. Furthermore, the extent of assembly appeared to differ between the chimaeras and the L1 positive control. This was confirmed by transmission electron microscopy (Figure 7), which showed distinct differences between the different L1/L2 chimaeras and the L1 control.

Chromatography was the next strategy to selectively purify HPV L1; either on the basis of surface charge, or by affinity for the proteoglycan heparin. The use of cation-exchange chromatography for the purification of yeast-expressed HPV L1 has been demonstrated using P-11 phosphocellulose (Kim *et al.*, 2009, 2010) or a POROS 50HS column (Cook *et al.*, 1999). In contrast, the plant-expressed L1/L2 chimaeras were not purified efficiently using either the strong cation-exchange HiTrap SPFF column or the POROS 50HS column. The majority of L1/L2 protein did not bind to either column, although a small proportion of protein bound strongly and irreversibly to the POROS 50HS resin. This phenomenon has been described by Cook *et al.* (1999), whereby 10% of HPV-11 L1 did not bind the resin and 45-65% could not be recovered without stripping the POROS 50HS column using 0.5M NaOH.

As a result, cation-exchange chromatography was not pursued further, although the reason for the poor purification of L1/L2 chimaeras is not clear. There are two HPV-16 L1 basic C-terminal regions which contain positively charged residues: aa 473-488 and 492-505 (Zhou *et al.*, 1991b; Sun *et al.*, 1995, 2010). The L2 sequence insertions did not overlap the major basic regions in the C-terminal of L1 and replaced a maximum of 26 residues at aa 414-439. A possible explanation is that the overall surface charge of L1 was affected, either by the amino acid composition of the inserted L2 epitopes, or by differences in protein

assembly. In addition, the crude plant extract may have contained several contaminating proteins which bound more strongly to the columns and out-competed HPV L1 binding.

Purification of the vaccine antigens

Vaccine antigens were purified using heparin chromatography (described by Joyce *et al.*, 1999; Bazan *et al.*, 2009; Johnson *et al.*, 2009; Kim *et al.*, 2009, 2010) for subsequent immunogenicity studies in mice. Heparin reversibly bound both the L1 and L1/L2 chimaeras in a similar manner (data not shown), and all antigens eluted with 0.75M NaCl. This is comparable to other studies where heparin-bound HPV-16 L1 eluted between 0.5 - 1.2M NaCl (Bazan *et al.*, 2009; Kim *et al.*, 2010; Baek *et al.*, 2011).

Heparin selectively purifies assembled L1 protein by binding to a conformational motif which is not present on the C-terminal of L1 (Fleury *et al.*, 2009) and is unaffected by the L2 sequence replacements. This is particularly beneficial for vaccine production, as denatured L1 does not elicit the production of neutralising antibodies (Kirnbauer *et al.*, 1992; Suzich *et al.*, 1995; Breitburd *et al.*, 1995). Furthermore, Kim *et al.* (2010) demonstrated that purification of HPV-16 L1 using heparin chromatography gave high recovery yields (~60%) and produced immunogenic VLPs (25-65 nm in diameter).

The purity of the heparin-purified samples was examined by Coomassie staining and western blot detection of L1 using CamVir1 (Figure 5). The purified samples were enriched with L1 or L1/L2 chimaeras, as there was a significant decrease in total protein with a relatively small decrease in antigen yield when compared to the crude samples. This was confirmed by H16.J4 capture ELISA and TSP assays (Figure 6).

Samples were partially-purified and contained several contaminating plant proteins (V1 and V2, Figure 5A), also present in the purified negative control (V5, Figure 5A). Contaminants were also observed in the purification of yeast-expressed HPV-16 L1 using heparin chromatography (Kim *et al.*, 2010). As a result, a single step method using heparin chromatography is not sufficient to obtain highly-purified HPV L1 and L1/L2 chimaeras. Kim *et al.* (2010)

demonstrated that co-purified contaminating proteins from yeast were not completely removed by additional cation-exchange and hydrophobic interaction chromatography steps, suggesting many of the contaminants have similar isoelectric points and hydrophobicity profiles to L1. Furthermore, the additional chromatographic steps reduced L1 recovery to ~10%. However, pure HPV-16 L1 was obtained by ammonium sulphate precipitation of yeast-expressed HPV-16 L1 prior to heparin chromatography, a method which should be examined in further purification studies using plant-expressed HPV L1-based proteins.

Western blot quantification of antigens

The purified antigens were quantified by western blot analysis (discussed by Heidebrecht *et al.*, 2009) using densitometry to measure the intensity of the CamVir1-detected L1 bands and the commercial vaccine Cervarix as the HPV-16 L1 standard (data not shown).

L1 degradation was detected in some of the batches of purified antigen, particularly after several freeze-thaw cycles. This was seen at high concentrations of V1, V2 and V4. However, the majority of the antigen proteins were not degraded and only the full-sized 56 kDa L1 band was quantified to ensure mice were immunized with similar doses of full-length antigen. Other groups have reported similar HPV-16 L1 degradation patterns when expressed in insect cells (Kirnbauer *et al.*, 1992), yeast (Cook *et al.*, 1999) and bacteria (Zhang *et al.*, 1998). A consideration for future purification studies is the salt concentration of the extraction and diafiltration buffers, as VLP disassembly occurs in low-salt conditions (Murata *et al.*, 2009). Increasing the salt concentration to 0.5 or 1M NaCl may stabilize VLPs (Mach *et al.*, 2006) and reduce degradation observed in the purified samples.

Assembly of the vaccine antigens

The assembly of plant-derived HPV-16 L1 and the L1/L2 chimaeras produced in the chloroplasts of plant cells was analysed using immunocapture electron microscopy (Figure 7). Purification appeared to remove some background protein and all the plant-expressed L1/L2 chimaeras and the L1 positive control

assembled into higher-order structures such as capsomeres, aggregates and VLPs.

Plant-expressed HPV-16 L1 VLPs are typically ~55 nm in diameter when localised to the tobacco chloroplasts (Maclean *et al.*, 2007; Fernández-San Millán *et al.*, 2008; Lenzi *et al.*, 2008). In this study, HPV-16 L1 assembled into full-sized VLPs (~50 nm, Figure 7 Dii).

Assembly of chimaeras into VLPs appears to be affected by the length of the L2 epitope, with L1/L2(108-120), L1/L2(17-36) and L1/L2(56-81) containing 13, 20 and 26 residue epitope replacements respectively. Plant-expressed L1/L2(108-120), with the shortest L2 epitope, successfully assembled into distinct cVLPs of about ~30 nm in diameter, which is smaller than L1 VLPs (Chen *et al.*, 2000) (Figure 7A). In contrast, L1/L2(17-36) predominantly formed capsomere aggregates, although the presence of larger amorphous VLP-like structures suggest there may be partial-assembly of small cVLPs (Figure 7C). Finally, L1/L2(56-81) with the longest L2 epitope predominantly assembled into capsomeres (Figure 7B).

L1/L2(108-120) has also been expressed in insect cells and the CsCl-purified chimaera was shown to assemble into amorphous VLPs and capsomere aggregates (Varsani *et al.*, 2003a), rather than discrete cVLPs of ~30 nm diameter.

Chimaeras targeted to the cytoplasm as a result of infiltration of plants using the pRIC expression vector resulted in the formation of detectable L1/L2(56-81) VLPs (Figure 8). This indicates that the chimaeric VLPs described herein can also be formed in the cytoplasm of plants.

EXAMPLE 3: IMMUNOGENICITY OF L1/L2 CHIMAERAS

In this study, mice were immunized with plant-derived L1 and three L1/L2 chimaera candidate vaccines containing the cross-neutralising L2 epitopes aa 108-120, 56-81 and 17-36. The immunogenicity of the chimaeras was analysed with respect to chimaera assembly and their ability to elicit anti-L1, anti-L2 and

protective NAb against homologous HPV-16 and heterologous HPV-18, 45 and 52 PsVs was investigated.

METHODS AND MATERIALS

Immunisation of mice

Female C57/BL6 mice from the South African Vaccine Producers Animal Unit (Johannesburg, South Africa) were maintained under Biosafety Level 2 (BSL-2) conditions in the Animal Unit in the Health Science Faculty, University of Cape Town. Permission for this study was granted by the Research Ethics Committee, University of Cape Town (AEC 008/037).

Mice (7-8 weeks old) were immunised to test humoral antibody responses to plant-derived HPV-16 L1/L2 candidate vaccines. The controls included plant-expressed HPV-16 L1 (positive control) and NSs-infiltrated plant extract (negative control). The L1/L2(108-120) chimaera (published as SAF; Varsani *et al.*, 2003a) has been shown by our laboratory to illicit anti-L1 responses and thus served as an additional positive control. The vaccination details are shown in Table 5.

Table 5: Plant-derived vaccine antigens used in the immunogenicity study

Vaccine	Vaccine No. (*n=10)	Group No. (*n=5)	Antigen dose (μ g)	[†] TSP (mg/ml)
L1/L2(56-81)	V2	G3 & G4	10	0.14
L1/L2(17-36)	V3	G5 & G6	10	0.09
HPV-16 L1 (+)	V4	G7 & G8	10	0.33
Plant extract (-)	V5	G9 & G10	N/A	0.16

*n = number of mice

[†]TSP = total soluble protein

The purified vaccine antigens were adjusted to contain a 10 μ g dose in 100 μ l Dulbecco's PBS (DPBS; Sigma). The total soluble protein (TSP) in each vaccine was assessed using a Bradford protein assay as discussed in Example 1 above to ensure the negative vaccine control contained a similar TSP in comparison to the other HPV vaccines (Table 5). The vaccine was prepared by homogenization of the vaccine antigen in Freund's Incomplete Adjuvant (FIA) in a 1:1 volume ratio using the syringe-extrusion technique (Koh *et al.*, 2006).

Mice were divided up into 2 groups of 5 mice per vaccine and were subcutaneously injected into the right flank, left flank or the inguinal site. Pre-bleeds were taken 12 days prior to vaccination (Day 0) and mice were boosted on Day 13, 27, 41 and 48 (approximately every 2 weeks, except for Day 48 when it was decided to boost rather than obtain a test bleed) before obtaining the final bleeds at Day 62 (~9 weeks post-vaccination). Serum was isolated and stored at -70°C.

ELISA detection of anti-L1 antibodies in mouse sera

Preparation of the insect cell-produced HPV-16 L1

Insect cell-produced HPV-16 L1 was used as an ELISA antigen to detect anti-L1 antibodies in the mouse sera. Insect cell-expressed L1 was used instead of plant-expressed L1 to avoid the background detection of antibodies against contaminating plant proteins. *Spodoptera frugiperda* (Sf-9) cells were grown shaking in SF90011 serum-free medium (Gibco) at 27°C and infected at a multiplicity of infection (MOI) of 1.0 and a cell density of 1×10^6 cells/ml. Cells were harvested after 96 hrs by centrifugation (1000 x g for 5 min) and pellets were washed with DPBS and stored at -70°C.

HPV-16 L1 was extracted by resuspending cells to 4×10^6 cells/ml in high-salt PBS (0.8M NaCl 1x PBS) containing protease inhibitor (Roche Complete EDTA-free) and sonicating on ice for 5x 20s intervals of sonication and rest (Microtip sonication; Level 5; Heat Systems – Ultrasonics, Inc. Sonicator Cell Disruptor Model W-225 R). The cell lysate was clarified by centrifugation (5000g for 5 min) to remove cell debris and the centrifugation step was repeated using the supernatant. The commercial vaccine Cervarix (20 µg HPV-16 L1) was used as a HPV-16 L1 standard for western blot quantification of HPV-16 L1 (as described above) and L1 was detected with CamVir1 (1:10000; Abcam®).

ELISA detection of anti-L1 antibodies

The anti-L1 antibody titre was determined by direct ELISA. A 96-well Maxisorp microtitre plate (Nunc) was coated with 100µl/well (30 ng) of insect cell-produced HPV-16 L1 antigen diluted in 1x PBS and incubated overnight at

4°C. Plates were blocked with blocking buffer (1% skim milk in 1x PBS; 200ul / well) for 2 hrs at room temperature and then washed 4x with PBS.

Mouse sera were pooled into vaccines (10 mice / vaccine) for analysis. Final bleed mouse sera were diluted in blocking buffer in a 4-fold series in triplicate, ranging from a dilution of 1:50 to 1:51200. Pooled pre-bleed sera were tested at 1:50 dilution and served as a negative control. Diluted sera was added to the wells (100ul / well) and incubated for 2 hrs at room temperature. Positive controls wells contained 1:50 dilution of anti-L1 antibodies; both CamVir1 (Abcam®), which binds both linear and conformational epitopes (McLean *et al.*, 1990), and H16.V5 MAb, which binds specifically to conformational epitopes (Christensen *et al.*, 1996). Blank wells with no antibody were included as a background control.

After a 4x PBS washing step, goat anti-mouse horseradish peroxidase conjugate (1:2000; Sigma) diluted in blocking buffer was added to the wells (100 ul / well) and incubated for 1 hr at 37°C. Plates were washed 4x with PBS (200ul / well) and 100 ul of O-phenylenediamine dihydrochloride (OPD) (DAKO; Denmark) was added per well. Plates were developed in the dark for 30 min at room temperature, the reaction was stopped with 0.5M H₂SO₄ and the absorbance at 490nm was detected. The anti-L1 binding titres were expressed as a reciprocal of the maximum serum dilution which produces higher absorbance readings than that of the corresponding pre-bleed serum diluted at 1:50.

Statistical analysis

A two-tailed, non-paired t-test was used to calculate statistical significance of the final bleed anti-L1 response, as compared to the negative control vaccine ($p = 0.01$). One-way Analysis of Variance (ANOVA) was used to compare the vaccines and the Fisher LSD, Turkey HSD and Bonferroni tests were used to determine the significance ($p = 0.01$).

Western blot detection of anti-L2 antibodies

Preparation of *E. coli*-produced HPV-16 L2

His-tagged HPV-16 L2 protein produced using the pProEX htb vector in *E. coli* (provided by David Mutepefa) was used for the western blot detection of anti-L2 antibodies in mouse sera. *E. coli* cultures were grown shaking at 37°C to an OD₆₀₀ of 0.6 and then induced by the addition of 0.6 mM iso-propyl-β-thiogalactoside (IPTG). After 3 hrs, cells were harvested by centrifugation (3800g for 15 min at 4°C) and the pellet was retained and weighed.

The inclusion bodies were extracted by resuspension of the cells in 4 volumes of lysis buffer (50 mM Tris pH 8.5, 5 mM β-mercaptoethanol) and phenylmethanesulfonyl fluoride (PMSF) and lysozyme (Roche) was added to a final concentration of 0.4 mM and 0.08 µg/µl respectively. The cells were incubated on ice for 20 min, Triton-X was added to 1% and cells were further incubated for 20 min at 37°C until the solution was viscous. DNase and RNase were added to 4 µg/ml and 40 µg/ml respectively and cells were incubated for 30 min at room temperature until viscosity cleared.

The inclusion bodies were collected by centrifugation at 13,000 rpm in a microcentrifuge for 15 min at 4°C and the pellet resuspended in 1ml lysis buffer (2.5 mM Tris pH 8.0, 3.125 mM β-mercaptoethanol, 0.2 mM EDTA, 0.0025% Triton-X) and left to lyse for 10 min at room temperature. The sample was centrifuged at 13,000 rpm for 15 min at 4°C and pellets were washed 4x with PBS. The pellet was resuspended in 1 volume PBS of the weight of pellet, quantified by Coomassie staining using a bovine serum albumin (BSA) standard and stored at -20°C.

Western blot detection of anti-L2 antibodies

The *E. coli*-produced HPV-16 L2 antigen was incubated at 95°C for 5 min in 5x loading buffer and was loaded into a 10% SDS-PAGE gel. Instead of using a 10-well comb, a 2-well comb was used: a small well for the protein marker and a large well consisting of the 9 wells fused together, thus producing a single wide well which allowed the protein to spread equally across the width.

E. coli-expressed His-tagged HPV-16 L2 antigen (2.5 mg) was separated on a 10% SDS-PAGE gel (Sambrook *et al.*, 1989) and transferred onto a nitrocellulose membrane by semi-dry electroblotting as described in Example 1 above. The western blotting protocol was then modified, whereby the portion of the membrane between 55-130 kDa containing the L2 protein (~80 kDa) was divided into 12 similar-sized strips to probe with different sera. The membrane strips were transferred into individual wells in a 25-well tissue culture plate and incubated in blocking buffer for 4 hrs at room temperature.

Individual pre-bleed and final bleed mouse sera were pooled into vaccines (10 mice per vaccine) and the membrane strips were probed with positive control mouse anti-His antibody (1:2000, Serotech) or pooled mouse sera diluted 1:100 in blocking buffer. Sera were added to different wells and incubated with shaking overnight at room temperature. The strips were then washed 4x 10 min with blocking buffer and probed with secondary goat anti-mouse IgG antibody conjugated to alkaline phosphatase (1:5000; Sigma) for 2 hrs at room temperature. The individual strips were washed again for 4x 10 min with wash buffer and then developed with NBT/BCIP (Roche).

Densitometry (GeneTools, Syngene, Synoptics, Ltd) was used to measure the absorbance intensity of each L2 band. Values were normalized for non-specific background absorbance using the value associated with the negative control vaccine. Sera with L2 bands having absorbance values >2x the value observed in the HPV-16 L1 final bleeds elicited an anti-L2 response.

HPV pseudovirion neutralisation assays

Preparation for the neutralisation assays

The protocols used for the HPV pseudovirion (PsV) neutralisation assays are taken from Dr John Schiller's Lab of Cellular Oncology technical files and the HPV L1/L2 pSheLL plasmids and the pYSEAP reporter plasmid were kindly provided by Dr John Schiller.

The pYSEAP plasmid was checked using a *Sall* and *Bam*HI restriction enzyme digest (as described in Example 1 above). The HPV L1/L2 pSheLL plasmids were similar in size and have similar restriction enzyme sites, thus the

plasmids were sequenced to confirm their identity using two sets of pSheLL vector-specific primers which bind upstream and downstream of the HPV L1 and L2 genes (Table 6). Sequences were aligned with the HPV L1/L2 pSheLL plasmid sequence and HPV L1 or L2 gene sequences using DNAMAN sequence analysis software.

Table 6: pSheLL vector-specific sequencing primers

Sequencing target	Primer	Sequence	T _m (°C)	Size (nt)
HPV L1	L1 Fwd	TGACCTTATGGGACTTTCCTAC (SEQ ID NO 18)	56.3	22
	L1 Rvs	CACCATAAGCAGCCACAAT (SEQ ID NO 19)	55.5	19
HPV L2	L2 Fwd	TACCACCACGAACAAGCAC (SEQ ID NO 20)	57.5	19
	L2 Rvs	AAGCCATACGGGAAGCAA (SEQ ID NO 21)	55.4	18

Endotoxin-free plasmid DNA (NucleoBond® Xtra Midi EF, Macherey-Nagel) was prepared from *E. coli* cultures grown under the appropriate antibiotic selection for both the pYSEAP plasmid and HPV-16, 18, 45 and 52 pSheLL plasmids (Table 7) and DNA was stored at -70°C.

Table 7: HPV PsV neutralisation assay plasmid vectors used in this study

Plasmid	HPV type	Gene of interest	Size (bp)	Antibiotic resistance
p16 SheLL	HPV-16	L1 & L2	10827	Ampicillin (100 µg/ml)
p18 SheLL	HPV-18	L1 & L2	10723	Ampicillin (100 µg/ml)
p45 SheLL	HPV-45	L1 & L2	10814	Ampicillin (100 µg/ml)
p52 SheLL	HPV-52	L1 & L2	10725	Ampicillin (100 µg/ml)
pYSEAP	-	SEAP	5297	Blasticidin (75 µg/ml)

Transfection of HEK293TT cells

The HEK293TT cell line was kindly provided by Dr John Schiller. HPV PsVs were produced as described in the "Production of Papillomaviral Vectors (Pseudoviruses)" protocol revised in June 2010.

HEK293TT cells were cultured in complete high glucose Dulbecco's Modified Eagle Medium (cDMEM) containing 1% GlutaMAX™ (Gibco) and 10% fetal calf serum (Gibco). The cDMEM media was supplemented with 1%

non-essential amino acids (Gibco), 100 units/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 10 µg/ml FunginTM (InvivoGen) and 250 µg/ml Hygromycin B (Roche) to select for the TT antigen (cDMEM-Ab). The thawing and passaging of cells was done as described in the protocol.

Cells were pre-plated in cDMEM (without antibiotics or Hygromycin B) in a 175cm² flask to reach 50-70% confluence the following day. On the day of transfection, fresh cDMEM was added to the cells and aliquots of endotoxin-free plasmid DNA were thawed on ice. The transfection mix was prepared as follows: 175 µl FuGene6 (Roche) was added to 5.7 ml DMEM with GlutaMAX (serum-free media) in white-capped conical tubes (Sterilin) and incubated for 5 min at room temperature. A total of 40 µg DNA was added (20 µg of each plasmid), the mixture was incubated for a further 30 min at room temperature and then added dropwise to the cells. Flasks were incubated for 40-48 hrs at 37°C in a 5% CO₂ humidified incubator and the medium was changed 6 hrs post-transfection (cDMEM).

Extraction of pseudovirions

Pseudovirions were harvested 40-48 hrs post-transfection. Cells were collected by trypsinisation with 0.05% Trypsin-EDTA (Gibco) and inactivated by the addition of cDMEM. The cells were transferred to a conical-bottomed polystyrene Sterilin tube (as pseudovirions adsorb non-specifically to polypropylene tubes), counted and centrifuged at 1200 rpm x 8 min. The pellet was washed with 0.5ml DPBS (Invitrogen) and resuspended in 1.5 pellet volumes of DPBS-Mg (DPBS with an additional 9.5 mM MgCl₂) to achieve a high cell density of >100 x 10⁶ cells/ml.

10% Brij-58 (Sigma) was added to the resuspended pellet to a final concentration of 0.5% (w/v) and both Benzonase (Sigma) and Plasmid-SafeTM ATP-dependent DNase (Epicentre) were added to 0.5% (v/v) and 0.2% (v/v) respectively. Using Chris Buck's "Improved Maturation of HPV and Polyomavirus" protocol, sterile ammonium sulphate (1M, pH 9.0) was added to a final concentration of 25 mM to promote the formation of intermolecular L1 disulphide bonds. The mixture was incubated at 37°C for 15 min to allow lysis and then transferred to the preferred temperature for pseudovirion maturation overnight (25°C for HPV-16 and 18, 37°C for HPV-45 and 52).

The matured lysate was chilled on ice for 5 min and the final NaCl concentration of the lysate was adjusted to 850 mM and incubating on ice for a further 10 min. The lysate was clarified by centrifuging 3000 x g for 10 min at 4°C. The supernatant was collected and the pellet was re-extracted by resuspending in an equal pellet volume of high salt DPBS (0.8M NaCl) and re-centrifuging. The supernatants were pooled, re-centrifuged and transferred into white-capped polystyrene tubes and kept on ice.

Purification of pseudovirions

PsV are purified by Optiprep density gradient centrifugation. Optiprep (60% w/v iodixanol solution; Sigma) was diluted in DPBS to a 46% (w/v) Optiprep stock solution, and supplemented with 0.625M NaCl to a final concentration of 0.8M NaCl, CaCl₂ to 0.9mM, MgCl₂ to 0.5mM and KCl to 2.1mM. High salt DPBS (0.8M NaCl) was used to dilute the stock solution to 27%, 33% and 39% Optiprep, and the 3-step gradient was prepared by underlaying the Optiprep dilutions (27 – 39%) in 1.5ml steps in thin wall 5ml polyallomer ultracentrifuge tubes (Beckman). The gradient was left to diffuse at room temperature for 4 hrs. Double-clarified cell supernatant was layered onto the linearized Optiprep gradient and centrifuged in a Beckman SW55ti rotor at 50,000 rpm (234,000 x g) for 3.5 hrs at 16°C. The bottom of the tube was punctured with a syringe needle and fractions were collected in white-capped polystyrene tubes: the first fraction was ~0.75 ml, fraction 2-11 was ~0.25 ml each and fraction 12 contained the remainder of the gradient.

The protocol for screening fractions was modified to detect the presence of HPV L1, the major protein present in the capsid (Buck *et al.*, 2008), using HPV type-specific anti-L1 dot blots. Each fraction was spotted onto nitrocellulose membrane (0.5 µl) and Cervarix (HPV-16 L1), *E. coli*-produced His-tagged HPV-16 L2, or the clarified HPV-16, 18, 45 or 52 supernatant initially loaded onto the gradient was used as positive controls.

The membranes were blocked in blocking buffer for 30 min at room temperature and then probed overnight at room temperature with an appropriate primary anti-L1 antibody diluted in blocking buffer. CamVir1 (1:5000; Abcam) was used to detect HPV-16. In addition, rabbit anti HPV-16 L2 sera was available and used to detect L2 in the HPV-16 fractions (1:2000). The H16.I23, H45.N5, H52.C1

and H52.D11 MAb kindly provided by Dr Neil Christensen were used to detect HPV-18, 45 and 52 respectively (1:2000; Christensen *et al.*, 1996). Membranes were probed with 1:10,000 secondary antibody (goat anti-mouse IgG conjugated to alkaline phosphatase or goat anti-rabbit alkaline phosphatase conjugate; Sigma), washed and developed as described above. Peak fractions containing a high concentration of L1 were pooled in polystyrene tubes and stored at -70°C for titration.

Electron microscopy of pseudovirions

Purified HPV PsV were analyzed using electron microscopy. The PsV's (1:1000) were trapped on glow-discharged carbon-coated copper grids, stained with 2% uranyl acetate and viewed using a Zeiss EM 912 CRYO EFTEM.

Pseudovirion titration

The PsV titrations and neutralisation assays were based on the "Papillomavirus Neutralisation Assay" protocol, with the exception that no NAb were included in the titration. PsV stocks were titrated prior to the neutralisation assays in order to determine the minimum amount of PsV required for a robust signal in the SEAP assay.

HEK293TT cells were grown in cDMEM-Ab to 70-80% confluence, collected as described, washed with DPBS and diluted to 3.0×10^5 cells/ml in neutralisation media (High glucose cDMEM with HEPES and without phenol red or sodium pyruvate, supplemented with 10% fetal calf serum; Gibco). Cells were pre-plated into 96-well tissue culture treated plates (Corning Costar) with 100µl cell suspension in each internal well and 150µl DMEM with phenol red in the external wells to avoid evaporation from the inner wells. Cells were incubated for 3-4 hrs at 37°C before the addition of the PsVs.

Serial dilutions of PsVs were prepared in neutralisation media (doubling dilutions from 1:250 to 1:64000) in non-treated sterile 96-well polystyrene plates (Nunc) and tested in triplicate. The PsV dilutions were added to the pre-plated cells (100µl / well) as outlined in the Schiller protocol, and each plate contained 6 negative control wells with no pseudovirions (cell control). Plates were incubated for 72 hrs at 37°C in a humidified CO₂ incubator.

SEAP activity was detected using the Great EscAPe™ SEAP Chemiluminescence Kit 2.0 (Clontech Laboratories, Inc.) according to manual instructions, except volumes were adjusted to 0.6 volumes of those given in the manufacturer's protocol (as done in the revised Schiller protocol). Supernatant (125µl) was transferred into sterile untreated 96-well polystyrene plates (Nunc) and centrifuged at 1000 x g for 5 min. Clarified supernatant (15µl) was transferred into a white 96-well Optiplate (96F white maxisorb luminometer plates; Nunc), 45µl 1x dilution buffer was added to each well and the plate was incubated at 65°C for 30 min. Plates were chilled for 5 min on ice and then 60µl substrate was added per well and incubated at room temperature for 20 min. SEAP production was detected using a microplate luminometer (Digene DML 2000). The PsV dilution chosen for the neutralisation assay was one that used the minimum amount of PsVs occurring within the linear range of the titration curve. As the HPV-52 titre was very low, it was re-titred from 1:125 to 1:4000.

Pseudovirion neutralisation assay

An *in vitro* neutralisation assay was used to detect HPV-specific antibody responses in mouse sera and to determine endpoint neutralisation titres.

Controls included:

- (a) Cell control (negative infection control): Cells were incubated with neutralisation media only (no sera or pseudovirions) to give a background reading of the cell culture supernatant. The luminescent values associated with this control represented 0% PsV neutralisation.
- (b) PsV control (positive PsV infection control): PsVs were pre-incubated in neutralisation buffer prior to cell infection. The values associated with this control represented 100% PsV neutralisation.
- (c) MAb or antisera known to neutralise the HPV-type PsV used in the assay (positive neutralisation assay control): PsV's were pre-incubated with 6 dilutions which should span the pre-determined neutralisation titre (0-100% neutralisation).

- (d) Pre-bleeds: PsV's were pre-incubated with pooled mouse pre-bleeds (negative control).

The NAb positive controls (Table 8) were titrated prior to the test sera neutralisation assay in order to determine the neutralisation dilution range to be used in the PsV neutralisation assays. The HPV-16, 45 and 52 neutralisation controls were H16.V5, H45.N5, H52.C1 and H52.D11 MAb. The HPV-18 control was rabbit anti-Cervarix sera from our laboratory.

Table 8: HPV type-specific neutralising antibodies

Positive control antibody	HPV type neutralised	Fold dilution	Dilution range
Mouse H16.V5 ascites	HPV-16	10-fold	$2 \times 10^2 - 2 \times 10^7$
Rabbit anti-Cervarix sera	HPV-18	4-fold	50 – 51200
Mouse H45.N5 ascites	HPV-45	4-fold	800 – 819200
Mouse H52.C1 supernatant	HPV-52	10-fold	$2 \times 10^2 - 2 \times 10^7$
Mouse H52.D11 supernatant	HPV-52	10-fold	$2 \times 10^2 - 2 \times 10^7$

Sera from mice immunized with plant-produced HPV-16 chimaera candidate vaccines were pooled (10 mice/vaccine) and tested for neutralisation of HPV-16, as well as HPV-18, 45 and 52. Pooled vaccine sera was diluted 4-fold in triplicate in the range 1:50 to 1:12800. Pre-bleeds were also pooled and tested in triplicate as a negative control at the lowest dilution of 1:50. Serial dilutions of sera were prepared in sterile non-treated 96-well tissue culture plates (1:10 to 1:2560).

PsVs were diluted in neutralisation buffer to the concentration pre-determined in the titration assay. In another untreated 96-well plate, 100µl diluted PsVs were added to each well and 25µl of diluted sera (or neutralisation buffer for the PsV control wells) were added to the triplicate wells, resulting in a further 1:5 dilution of pre-diluted sera. The PsVs and sera were incubated at 4°C for 1hr to allow for the neutralisation of infectious PsVs, and then 100µl were added to each well in the pre-plated HEK-293TT plate (neutralisation buffer for the cell control wells). The plates were incubated for a further 72 hrs in a 37°C humidified CO₂ incubator.

The supernatant was harvested as described above and assayed for the presence of SEAP. The neutralisation titre was stated as the reciprocal of the

maximum serum dilution which reduces SEAP activity by at least 50% in comparison to the control sample not pre-incubated with serum.

RESULTS

Humoral immune response against HPV-16 L1

The detection of antibodies elicited against HPV-16 L1 was done by direct ELISA, using insect cell-expressed HPV-16 L1 as the coating antigen (Figure 9). The anti-L1 titres were expressed as the reciprocal of the maximum serum dilution containing higher absorbance readings than that of the corresponding pre-bleed serum at 1:50.

No anti-L1 response was detected for the L1/L2(56-81) chimaera and the negative control vaccine (V2 and V5; Figure 9A) as well as the vaccine pre-bleeds (Figure 9C). In comparison, the ELISA MAbs (H16.V5, CamVir1, Figure 9B) and the plant-derived L1 positive controls (V4, Figure 9A) showed a good response and both the plant-derived L1/L2(17-36) and L1/L2(108-120) chimaeras elicited anti-L1 titres of 200 and 12800 respectively (V3 and V1, Figure 9A). Although HPV-16 L1 elicited the highest anti-L1 titres (12800-51200), L1/L2(108-120) showed a similar response (V4 and V1 respectively, Figure 9A), suggesting the insertion of the L2 aa 108-120 epitope had less of an effect on L1 immunogenicity in comparison to the other chimaeras. Furthermore, the L1/L2(108-120) and HPV-16 L1 anti-L1 response was statistically significant from their corresponding pre-bleeds and the NSs-infiltrated plant extract ($p = 0.01$).

Humoral immune response against the HPV-16 L2 epitopes

The anti-L2 response against the *E. coli*-produced His-tagged HPV-16 L2 protein was determined using western blotting. Individual mouse sera were pooled for each of the vaccines and analysed for anti-L2 responses (Figure 10).

A non-specific band similar to the ~80 kDa L2 band was detected in both the antisera from the negative vaccine control (V5; plant extract) and the L1 vaccine control (V4; plant-expressed HPV-16 L1) which serves as an additional negative L2 control in this experiment (Figure 10). All chimaera vaccines (V1-3) appeared to give an anti-L2 response, as strong L2 bands were detected using the

chimaera antisera (Figure 10). However, only the L1/L2(108-120) and L1/L2(17-36) chimaeras (V1 and V3 respectively) gave a definitive anti-L2 response, with L2 bands >2X intensity of HPV-16 L1 (V4).

Neutralisation assays

Plasmid analysis

The identity of the pYSEAP and the HPV-16, 18, 45 and 52 L1/L2 pSheLL plasmids was confirmed using restriction enzyme digestion and sequencing (data not shown).

Optiprep purification and HPV PsV detection in purified fractions

HPV PsVs were purified from the clarified cell supernatant by density gradient ultracentrifugation on a 27-39% Optiprep linear gradient. A light grey band was faintly visible a third of the way up from the gradient and the fractions were collected from the bottom of the tube.

Fractions were screened for the presence of PsVs using HPV type-specific anti-L1 dot blots. CamVir1 and rabbit antisera against HPV-16 L2 was used to detect HPV-16 L1 and L2, using Cervarix and *E. coli*-produced His-tagged HPV-16 L2 as controls. The H16.I23, H45.N5, H52.C1 and H52.D11 MAb were used to detect HPV-18, 45 and 52 respectively, using the initial clarified cell supernatant as the HPV type-specific control (data not shown).

HPV-16 was detected in fraction 3-5 using H16.V5 and weakly detected with the HPV-16 L2 antisera, as the L2 protein is located internally to the L1 capsid surface in co-assembled L1/L2 VLPs (Buck *et al.*, 2008). HPV-18, 45 and 52 L1 was strongly detected in fractions 5-7, 4-6 and 6-10 respectively. PsV fractions were pooled, examined by electron microscopy and used in the neutralisation assays.

Electron microscopy analysis

The pooled PsV samples were examined by transmission electron microscopy to determine their assembly, morphology and purification (data not shown). All HPV types assembled into spherical PsVs (55 nm). HPV-45 PsVs

appeared to exist exclusively as fully-assembled PsV particles. HPV-16 and 18 PsVs were predominantly assembled, although some capsomeres and aggregates were visible. HPV-52 PsVs contained a large proportion of capsomere aggregates and partial PsVs, possibly as a result of low HPV-52 L1 and L2 expression in the HEK293TT cells.

HPV PsV titration

The purified PsVs were titrated to determine the PsV dilution to be used for the neutralisation assays. The dilution used was the minimum amount of PsVs giving a robust signal within the linear range of the titration curve.

For HPV-16 and 18 PsVs, the linear range of the titration curve occurred between dilutions 1:250 to 1:1000 (data not shown), and thus 1:500 was chosen for the neutralisation assays. HPV-45 PsVs had the highest titre, with the linear range occurred between dilutions of 1:500 and 1:2000, thus 1:1000 was chosen for further work (data not shown). HPV-52 PsVs had to be re-titred using lower dilutions. The linear range occurred between dilutions 1:125 to 1:250 (data not shown), and a 1:200 dilution was used in the HPV-52 neutralisation assay.

Titration of the positive control neutralising antibodies

The NAb positive controls were tested prior to the neutralisation assays with the mouse antisera, in order to check their neutralising ability and to determine a suitable dilution range. All positive control antibodies were neutralising and showed a linear relationship within the dilution range tested (Table 9).

Table 9: Titration of the positive control neutralising antibodies

HPV PsV type	Positive control	Dilution range	PsV neutralisation (%)
HPV-16	H16.V5	$2 \times 10^2 - 2 \times 10^7$	19 – 100
HPV-18	anti-Cervarix sera	50 – 51200	34 – 99
HPV-45	H45.N5	800 – 819200	29 – 100
HPV-52	H52.C1	$2 \times 10^2 - 2 \times 10^7$	0 – 98
	H52.D11	$2 \times 10^2 - 2 \times 10^7$	0 – 98

HPV PsV neutralisation assays

Sera from mice immunized with plant-produced HPV-16 L1 and L1/L2 chimaeras were tested for homologous neutralisation of HPV-16 PsVs and heterologous cross-protection against HPV-18, 45 and 52 PsVs (Figures 10-13). All positive control NAb successfully neutralised the HPV-16, 18, 45 and 52 PsVs (Figures 10-13F), demonstrating that the neutralisation assay results were valid. The neutralisation titre was defined as the highest dilution of serum which reduces SEAP activity by >50% in comparison to the control sample, which was not treated with serum.

HPV-16

The results from the HPV-16 PsV neutralisation assays are shown in Figure 11. Plant-derived HPV-16 L1 sera (V4; Figure 11D) mimicked the H16.V5 positive control (Figure 11F) and strongly neutralised HPV-16 PsV, followed by L1/L2(108-120) with a similar neutralisation curve (V1; Figure 11A). Both L1/L2(56-81) and L1/L2(17-36) did not appear to elicit HPV-16 NAb (V2-3; Figure 11B-C) showing similar neutralisation curves to the negative control (V5; Figure 11E).

HPV-18

The antisera from all the vaccines did not neutralise HPV-18 PsV (Figure 12). The L1/L2(56-81) and L1/L2(17-36) chimaeras (V2-3, Figure 12B-C) produced neutralisation curves similar to the type-specific HPV-16 L1 vaccine and the negative control (V4-5, Figure 12D-E). L1/L2(108-120) appeared to have some neutralising activity, with reciprocal sera dilutions of <800 reducing luminescent readings below that of the pre-bleed and the unneutralised HPV-18 PsV control (V1; Figure 12A). However, the chimaera did not reduce SEAP levels by >50%.

HPV-45

The results from the HPV-45 PsV neutralisation assay (Figure 13) suggest that none of the L1/L2 chimaera vaccines (V1, V2 and V3; Figure 13A-C) elicited significant titres of HPV-45 NAb, with neutralising curves similar to HPV-16 L1 and the negative vaccine control (V4-5; Figure 13D-E).

HPV-52

The HPV-52 PsV neutralisation assays (Figure 14) provide evidence that L1/L2(56-81) sera did not neutralise HPV-52 (L2; Figure 14C), as seen for HPV-16 L1 and the negative control sera (V4-5; Figure 14D-E). L1/L2(108-120) and L1/L2(17-36) chimaera vaccines appeared to have some neutralising activity at low reciprocal dilutions (50-200), reducing SEAP levels by >50% in comparison to the unneutralised HPV-52 PsV control (V1 and V3; Figure 14A and C).

Although the assay was successful, as shown by the H52.C1 NAb control (Figure 14F), there was a great deal more variation between triplicates samples and trend lines were difficult to establish. This may be attributed to the partial purification and low concentration of HPV-52 PsVs which may have exaggerated small differences between replicates. The values for the HPV-52 PsV infection control differ between vaccines as V1, V2 and V4 (Figure 14A-B and D) were analyzed on a different plate from V3, V5 and H52.C1 (Figure 14C and E-F). Time constraints prevented this assay from being repeated.

Table 10 summarizes the HPV-16, 18, 45 and 52 PsV neutralisation antibody titres elicited by the plant-derived vaccines. L1/L2(108-120) elicited homologous HPV-16 NAb and the antisera cross-neutralised heterologous HPV-52 PsV, suggesting this vaccine has the most potential for protection. L1/L2(17-36) chimaeras elicited low levels of cross-neutralising HPV-52 NAb, but homologous HPV-16 NAb were not detected, suggesting the immunogenicity against HPV-16 L1 may be compromised. L1/L2(56-81) did not elicit NAb. None of the HPV vaccines elicited cross-neutralising antibodies against phylogenically-related HPV types 18 and 45.

Table 10: Summary of the neutralisation titres for plant-derived L1 and the L1/L2 chimaera candidate vaccines

Vaccine	Chimaera	PsV neutralisation assay titres*			
		HPV-16	HPV-18	HPV-45	HPV-52
V1	L1/L2(108-120)	50-500	0-50	0-50	50-200
V2	L1/L2(36-58)	0-50	0-50	0-50	0-50
V3	L1/L2(17-36)	0-50	0-50	0-50	50-200
V4	HPV-16 L1	500-5000	0-50	0-50	0-50
V5	Plant extract	0-50	0-50	0-50	0-50
+ control	H16.V5	$2 \times 10^5 - 2 \times 10^6$			
	α -CamVir1		12800-51200		
	H45.N5			3200-12800	
	H52.C1				$2 \times 10^4 - 2 \times 10^5$
	H52.D11				$2 \times 10^5 - 2 \times 10^6$

Overview of vaccine immunogenicity

The structural assembly (see Example 2 above), the anti-L1 and L2 humoral responses and the HPV-type NAb detected in the L1/L2 chimaera antisera are summarized in Table 11. Assembly into VLPs appears to be associated with higher anti-L1 and HPV-16 PsV neutralisation titres, suggesting assembly is associated with L1 immunogenicity.

Table 11: Antibody responses for the L1 and L1/L2 chimaeric vaccines

Vaccine	Plant-expressed antigen	TEM structure*	Anti-L1 response**	Anti-L1 titres	Anti-L2 response***	HPV-16/18/45/52 neutralisation
V1	L1/L2(108-120)	VLPs	Y	12800	Y	HPV-16/52
V2	L1/L2(56-81)	C / CA	N	0-50	N	None
V3	L1/L2(17-36)	CA / VLPs	Y	200	Y	HPV-52
V4	HPV-16 L1 (+)	VLPs	Y	>12800	N	HPV-16
V5	Plant extract (-)	N/A	N	0-50	N	None

* TEM antigen assembly: C = capsomeres, CA = capsomere aggregates, VLPs = virus-like particles.

** ELISA detection of anti-L1 antibodies. Y = yes, N = no.

*** Western blot detection of anti-L2 antibodies.

DISCUSSION

Plant-derived HPV-16 L1 (Macleane *et al.*, 2007; Fernández-San Millán *et al.*, 2008) and L1-based chimaeras (Paz De la Rosa *et al.*, 2009) assemble into immunogenic VLPs and elicit the production of neutralising antibodies (NAb). In

this study, the immunogenicity of three plant-derived L1/L2 chimaeras containing cross-neutralising HPV-16 L2 aa 108-120, 56-81 or 17-36 epitopes in the h4 region of HPV-16 L1 were analysed. Mice were subcutaneously immunized with 10 µg of plant-derived antigen in Freund's incomplete adjuvant, and received 4 booster vaccinations within 7 weeks.

Humoral immune responses

The humoral anti-L1 and L2 responses elicited by the plant-derived L1/L2 chimaeras were analysed in this study, to determine if the L2 peptides are displayed and whether the L2 insertions compromise L1 immunogenicity.

The detection of L1 and L2 antibodies in mouse antisera was done by direct ELISA (Figure 9) and western blotting (Figure 10) respectively, using either insect cell-expressed HPV-16 L1 or *E. coli*-expressed His-tagged L2 antigen. Plant-derived HPV-16 L1 served as the anti-L1 positive control in the study and elicited the highest anti-L1 response, with titres of 12800 - 51200 (Figure 9A). These results are similar to other mouse immunogenicity studies using partially-purified plant-derived HPV-16 L1 VLPs (Titres = 20000 – 40960; Maclean *et al.*, 2007; Fernández-San Millán *et al.*, 2008).

The negative control vaccine (V5: NSs-infiltrated plant extract) and the vaccine pre-bleeds (V1-5 PB) did not give anti-L1 responses (Figure 9). However, antisera from the negative controls (V4-5, Figure 10) did detect the *E. coli*-expressed His-tagged HPV-16 L2 antigen, thus demonstrating the presence of non-specific antibodies in the sera which bound the His-tagged L2 protein. This is possibly due to the partial purification of antigens, which resulted in the vaccines containing contaminating plant proteins. Nevertheless, the negative control bands were less distinct than the bands for the L1/L2(108-120) and L1/L2(17-36) chimaeras, suggesting these L1/L2 chimaeras elicited an anti-L2 response.

L1/L2(108-120) assembled into distinctive ~30 nm cVLPs and was the most successful chimaera vaccine (Table 11), eliciting the highest anti-L1 response with titres of ~12800 (Figure 9A) and an anti-L2 response (Figure 10). Furthermore, only the L1/L2(108-120) and HPV-16 L1 antisera demonstrated significant anti-L1 responses ($p = 0.01$) in comparison to the pre-bleeds and the NSs-infiltrated plant

extract (negative control). The insect cell-expressed L1/L2(108-120) chimaera analysed by Varsani *et al.* (2003a) elicited higher anti-L1 titres (>204800) in comparison to the plant-derived chimaera, however a 10x higher dose was used (100 µg vs. 10 µg). Taken together, there is strong evidence that the L2 aa 108-120 peptide is effectively displayed on the surface of the L1 cVLPs.

The L1/L2(17-36) vaccine elicited a relatively weak anti-L1 response with titres of ~200 (Figure 9A) but elicited a strong anti-L2 response (Figure 10), suggesting that the L2 peptide is displayed on the surface of assembled L1. Similarly, fusion of a L2 aa 20-38 peptide to bacterial thioredoxin (Trx) elicited strong anti-L2 responses in comparison to other Trx-L2 peptides comprising of aa 56-120 (Rubio *et al.*, 2009) and the RG-1 MAb directed against the HPV-16 L2 aa 17-36 peptide has been shown to detect L2 in western blotting and ELISA (Gambhira *et al.*, 2007).

The L1/L2(56-81) capsomere vaccine did not elicit a detectable anti-L1 response at the lowest sera dilution 1:50 (Figure 9A) and the anti-L2 response was inconclusive (Figure 10), with both the anti-L1 and L2 responses similar to the vaccine pre-bleeds (V1-5 PB) and the negative controls (Figure 9-10). As a result, plant-derived L1/L2(56-81) do not appear to be immunogenic, unlike *E. coli*-expressed Trx-L2 fusion peptides (Rubio *et al.*, 2009) and insect cell-expressed L1/L2 chimaeras containing similar L2 epitopes in the DE loop of BPV-1 L1 VLPs (Slupetzky *et al.*, 2007; Schellenbacher *et al.*, 2009).

Pseudovirion neutralisation assays

The L1/L2 chimaeras, containing L2 epitopes aa 108-120, 56-81 and 17-36, were examined for their ability to elicit antibodies which neutralise HPV-16, 18, 45 and 52 PsVs. All of the L2 epitopes analysed in this study have been shown to elicit antibodies which neutralise homologous HPV-16 and cross-neutralise HPV-52 (Kawana *et al.*, 2003; Slupetzky *et al.*, 2007; Kondo *et al.*, 2007, 2008; Gambhira *et al.*, 2007; Schellenbacher *et al.*, 2009). Additionally, L2 aa 56-81 cross-neutralises HPV-18 and L2 aa 17-36 cross-neutralises both HPV-18 and 45 (Gambhira *et al.*, 2007; Kondo *et al.*, 2007, 2008; Alphs *et al.*, 2008; Schellenbacher *et al.*, 2009; Rubio *et al.*, 2009).

HPV-16 was chosen as HPV-16 L1 is the backbone of the chimaeric candidate vaccines and it causes the majority of cervical cancers, followed by phylogenically-related HPV-18 and HPV-45. HPV-16, 18 and 45 are associated with 48%, 23% and 10% of cervical cancers in Africa, and 61%, 10% and 6% of cervical cancers worldwide (de Sanjosé *et al.*, 2010). Although HPV-52 is only ranked 5th in Africa (3%) and 6th worldwide (6%), HPV-52 has been shown to be highly prevalent in low and high-grade cervical lesions in South African women and thus HPV-52 cross-neutralisation is of local significance (Allan *et al.*, 2008).

Homologous HPV-16 neutralisation

Plant-derived L1/L2(56-81) and L1/L2(17-36) did not elicit detectable HPV-16 NAb titres, giving results similar to the pre-bleeds and the NSs-infiltrated plant extract (Figure 11). Previous work has shown L1/L2 chimaeras containing HPV-16 L2 peptides aa 17-36, 18-38, 56-75 or 69-81 located in surface regions of BPV-1 or HPV-16 L1 elicited HPV-16 NAb (Slupetzkey *et al.*, 2007; Kondo *et al.*, 2008; Schellenbacher *et al.*, 2009); however, the insertion sites differed from those used in this study and the chimaeras assembled into cVLPs. Furthermore, MAb directed against HPV-16 L2 aa 73-84 were found to be non-neutralising and did not neutralise HPV-16 PsV (Gambhira *et al.*, 2007), similar to the results obtained for the L1/L2(56-81) chimaera in this study.

In this study, only L1/L2(108-120) and HPV-16 L1 neutralised HPV-16 PsV in a similar manner to H16.V5 (positive neutralisation control), giving titres of 50-500 and 500-5000 respectively (Table 10). These results are consistent with other mouse immunogenicity studies using plant-derived HPV L1 antigens. A similar or higher dose of plant-derived HPV-16 L1 VLPs elicited HPV-16 NAb titres of 400-1600 (Maclean *et al.*, 2007; Fernández-San Millán *et al.*, 2008) and plant-derived L1/E6/E7 cVLPs elicited HPV-16 NAb titres of ~400 using a hemagglutination assay (Paz De la Rosa *et al.*, 2009). Furthermore, immunisation of humans with the HPV-16 L2 aa 108-120 peptide has shown to elicit HPV-16 NAb titres of 100-1000 (Kawana *et al.*, 2003) and mouse antisera from L1/L2 chimaeras containing the L2 epitopes aa 108-120 (Slupetzkey *et al.*, 2007) or L2 aa 75-112 and 115-154 (Schellenbacher *et al.*, 2009) neutralised homologous HPV-16 PsVs with titres <1000. Therefore the titres obtained in the study are

within the range reported by L1/L2 chimaera vaccines produced in other expression systems.

Heterologous HPV-18, 45 and 52 neutralisation

Neutralising activity against phylogenically-related HPV-18 and 45 PsV was not detected for all the HPV vaccines (Figure 12-13). Similarly, the L1/L2(56-81) antisera did not neutralise HPV-52 PsV (Figure 14). Although L1/L2(108-120) and L1/L2(17-36) appeared to elicit low HPV-52 NAb titres (50-200), there was a great deal of variation in the assay, possibly due to the purification of partially-assembled PsVs, and the assay should be repeated to confirm results.

Previous work has demonstrated that L1/L2 chimaeras containing the L2 aa 56-81 peptide cross-neutralises both HPV-18 and 52 (Kondo *et al.*, 2008). However, the chimaeras were assembled into cVLPs unlike L1/L2(56-81), suggesting VLP assembly is important to induce the production of high NAb titres. Furthermore, L1/L2 chimaera containing L2 aa 17-36 or 18-36 (Kondo *et al.*, 2008; Schellenbacher *et al.*, 2009) elicits NAb against HPV-18, 45 and 52. However, the L2 peptides were inserted into the DE loop (Schellenbacher *et al.*, 2009) and the dosage was not stated for the study conducted by Kondo *et al.* (2008). In this study, the low HPV-52 NAb titres elicited by plant-derived L1/L2(17-36) in mice were comparable to titres elicited by a similar L1/L2 chimaera expressed in insect cells (Schellenbacher *et al.*, 2009), suggesting the expression system does not affect the ability of the antigen to cross-neutralise HPV-52.

Plant-derived L1/L2(108-120) chimaera appeared to elicit HPV-52 NAb and may have potential as a cross-protective HPV vaccine, supported by evidence that the L2 aa 108-120 peptide has been shown to elicit HPV-52 NAb titres of 50-1000 respectively in humans (Kawana *et al.*, 2003). There is no evidence that HPV-16 L2 aa 108-120 cross-neutralises HPV-45, however L1/L2 chimaeras containing similar L2 aa 96-115 or 75-112 epitopes cross-neutralised phylogenically-related HPV-18 (Kondo *et al.*, 2008; Schellenbacher *et al.*, 2009). However NAb titres reported in the studies were low (<100) and it is possible that elicited HPV-18 NAb were too low to detect in the L1/L2(108-120) antisera.

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CLAIMS

1. A chimaeric human papillomavirus (HPV) virus like particle (VLP) having a diameter of about 30nm, the chimaeric HPV VLP comprising a chimaeric HPV 16 L1/L2 polypeptide encoded by a human codon-optimised nucleotide sequence, the chimaeric HPV 16 L1/L2 polypeptide further comprising an HPV 16 L1 polypeptide that includes an HPV L2 peptide of between about 13 amino acids to about 26 amino acids inserted from residue 414 of the HPV 16 L1 polypeptide, and wherein the amino acids of the inserted HPV L2 peptide replace the corresponding amino acids of the HPV 16 L1 polypeptide.
2. The chimaeric HPV VLP of claim 1, wherein the inserted HPV L2 peptide is selected from the group consisting of:
 - (i) a 13 amino acid peptide of SEQ ID NO: 3 encoded by a human codon-optimised nucleotide sequence of SEQ ID NO: 7;
 - (ii) a 20 amino acid peptide of SEQ ID NO: 5 encoded by a human codon-optimised nucleotide sequence of SEQ ID NO: 9; and
 - (iii) a 26 amino acid peptide of SEQ ID NO: 4 encoded by a human codon-optimised nucleotide sequence of SEQ ID NO: 8.
3. The chimaeric HPV VLP of claim 1 or 2, wherein the human codon-optimised nucleotide sequence encoding the chimaeric HPV 16 L1/L2 polypeptide is modified to be nuclear localisation signal deficient.
4. The chimaeric HPV VLP of any one of claims 1 to 3, wherein the chimaeric HPV 16 L1/L2 polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 22, SEQ ID NO: 23 and SEQ ID NO: 24.
5. The chimaeric HPV VLP of claim 4 wherein the chimaeric HPV 16 L1/L2 polypeptide is encoded by a human codon-optimised nucleotide sequence selected from the group consisting of SEQ ID NO: 26, SEQ ID NO: 27 and SEQ ID NO: 28.

6. The chimaeric HPV VLP of any one of claims 1 to 5, wherein the chimaeric HPV 16 L1/L2 polypeptide is expressed in and recovered from a plant.
7. The chimaeric HPV VLP of claim 6, wherein the chimaeric HPV 16 L1/L2 polypeptide is targeted to a chloroplast of the plant.
8. A method of producing a chimaeric HPV VLP having a diameter of about 30nm, the method comprising the steps of:
 - (i) providing a chimaeric human codon-optimised nucleotide sequence encoding a chimaeric HPV 16 L1/L2 polypeptide, the chimaeric HPV 16 L1/L2 polypeptide comprising an HPV 16 L1 polypeptide having an HPV L2 peptide of between about 13 amino acids to about 26 amino acids inserted from residue 414 of the chimaeric HPV 16 L1/L2 polypeptide, wherein the amino acids of the inserted HPV L2 peptide replace the corresponding amino acids of the HPV 16 L1 polypeptide;
 - (ii) cloning the chimaeric human codon-optimised nucleotide sequence into an expression vector adapted to express a polypeptide in a plant;
 - (iii) transforming or infiltrating a plant cell with the expression vector of step (ii);
 - (iv) expressing the chimaeric HPV 16 L1/L2 polypeptide in the plant cell of step (iii) such that the expressed chimaeric HPV 16 L1/L2 polypeptide assembles into a chimaeric HPV VLP having a diameter of about 30nm; and
 - (v) recovering the chimaeric HPV VLP from the plant cell.
9. The method of claim 8, wherein the expression vector of step (ii) further includes targeting sequences encoding a polypeptide for directing the expressed chimaeric HPV 16 L1/L2 polypeptide from the cytoplasm to a chloroplast of the plant cell.

10. The method of claim 8 or 9, wherein the expression vector includes promoters and other regulators or the like, operably linked to the coding sequence of the expression vector.
11. The method of any one of claims 8 to 10, further including the step of co-infiltration or co-transformation of the plant cell with a suppressor protein adapted to inhibit post-transcriptional gene silencing in a plant.
12. The method of claim 11, wherein the suppressor protein is the NSs protein of the tomato spotted wilt virus or the p19 of tomato bushy stunt virus.
13. The method of any one of claims 8 to 12, wherein the inserted HPV L2 peptide is selected from the group consisting of:
 - (i) a 13 amino acid peptide of SEQ ID NO: 3 encoded by a human codon-optimised nucleotide sequence of SEQ ID NO: 7;
 - (ii) a 20 amino acid peptide of SEQ ID NO: 5 encoded by a human codon-optimised nucleotide sequence of SEQ ID NO: 9; and
 - (iii) a 26 amino acid peptide of SEQ ID NO: 4 encoded by a human codon-optimised nucleotide sequence of SEQ ID NO: 8.
14. A method of preventing or treating HPV infection or cervical cancer in a subject, the method comprising administering a therapeutically effective amount of the chimeric HPV VLP of any one of claims 1 to 7 to the subject.
15. The method of claim 14, further comprising a step of eliciting an immune response in the subject.
16. The method of claim 15, wherein the immune response is a neutralising antibody response or a cytotoxic T lymphocyte response.
17. The method of claim 15, wherein the immune response is a cross-protective immune response to multiple HPV types present in the subject.
18. The method of any one of claims 14 to 17, wherein the subject is human.

19. A chimaeric HPV VLP of any one of claims 1 to 7 for use in a method of preventing or treating HPV infection or cervical cancer in a subject, the method comprising administering a therapeutically effective amount of the chimaeric HPV VLP to the subject.
20. The chimaeric HPV VLP of claim 19, the method further comprising a step of eliciting an immune response in the subject.
21. The chimaeric HPV VLP of claim 20, wherein the immune response is a neutralising antibody response or a cytotoxic T lymphocyte response.
22. The chimaeric HPV VLP of claim 20, wherein the immune response is a cross-protective immune response to multiple HPV types present in the subject.
23. The chimaeric HPV VLP of any one of claims 19 to 22, wherein the subject is human.
24. The use of chimaeric HPV VLP of any one of claims 1 to 7 in the manufacture of a medicament for use in a method of preventing or treating HPV infection or cervical cancer in a subject, the method comprising administering a therapeutically effective amount of the medicament to the subject.
25. The use of claim 24, the method further comprising a step of eliciting an immune response in the subject.
26. The use of claim 25, wherein the immune response is a neutralising antibody response or a cytotoxic T lymphocyte response.
27. The use of claim 25, wherein the immune response is a cross-protective immune response to multiple HPV types present in the subject.
28. The use of any one of claims 24 to 27, wherein the subject is human.
29. A pharmaceutical composition comprising the chimaeric HPV VLP of any one of claims 1 to 7 and a pharmaceutically acceptable carrier or adjuvant.