Immunogenic assessment of plant-produced Human papillomavirus type 16 chimaeric L1:L2 virus-like particles and the production of an encapsidated therapeutic DNA vaccine candidate

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In memory of Priscilla Wanjiru Kairu

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Abstract

Immunogenic assessment of plant-produced Human papillomavirus type 16 chimaeric L1:L2 virus-like particles and the production of an encapsidated therapeutic DNA vaccine candidate

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Cervical cancer caused by infection with Human papillomavirus (HPV) is the 4th most common cancer in women globally, and results in an estimated 266 000 deaths every year. Current vaccines are based on the immunodominant L1 major capsid protein, which assembles into virus-like particles (VLPs) that are highly effective in type-specific prevention of cervical infection. However, these vaccines are produced in expensive cell culture systems, are type-specific and do not induce the regression of established infections. The cervical cancer burden (~80%) is mainly in developing countries due to limited healthcare resources, therefore there is a need for more broadly protective and affordable vaccines. Plants provide an alternative platform to produce cheaper vaccines, given their scalability, rapid production and low risk of contamination.

The L2 minor capsid protein has sequence regions that are highly conserved across several HPV types, and HPV-16 L2 peptides 108-120, 65-81, 56-81 and 17-36 have been shown to elicit cross-neutralising antibodies. To increase the immunogenicity of L2, second-generation L1:L2 chimaeric VLP (cVLP) vaccines have been investigated. In this study, the 4 L2 peptides above were used to generate plant-produced HPV-16-derived L1:L2 chimaeras. The L2 epitopes were substituted into the DE loop of HPV-16 L1 at position 131 (SAC) or between the helix 4 and β-J structural region at position 431 (SAE). All chimaeras were transiently expressed in *Nicotiana benthamiana* via *Agrobacterium*-mediated transfer. Optimisation of expression was conducted by comparing protein expression levels over several days using 4 plant expression vectors, with the highest yields obtained by targeting protein to the chloroplast or with the use of a self-replicating vector. The chloroplast targeted SAC chimaeras predominantly assembled into higher order structures (T=1 VLPs and T=7 VLPs), whereas SAE chimaeras assembled into capsomeres or formed aggregates, indicating that the length, sequence and substitution position of L2 epitopes affects VLP assembly.

All SAC chimaeras in addition to SAE 65-81 (smaller epitope not previously tested in chimaeras) were used in vaccination studies in mice, and their immunogenic potential analysed in pseudovirion-based

neutralisation assays (PBNAs). Of the 7 heterologous HPVs tested, cross-neutralisation was observed with HPV-11, -18 and -58. Only the anti-SAE 65-81 serum showed neutralisation of homologous HPV-16, suggesting that antibodies detected from all candidate vaccines were mostly non-neutralising, and that the position of the L2 epitope display is critical to maintaining L1-specific neutralising epitopes.

Lastly, to address the lack of therapeutic efficacy of current vaccines, I aimed to develop a novel E7 DNA vaccine delivered by plant-made pseudovirions (PsVs). A geminivirus-derived self-replicating plasmid encoding a shuffled E7 (E7SH) sequence that has no transformation ability but contains natural cytotoxic T-lymphocyte epitopes, was constructed using GoldenBraid technology and co-expressed in plants with HPV-16 or HPV-35 L1- and L2-encoding expression vectors. The pseudogenome was successfully encapsidated into plant-made PsVs. These PsVs were capable of infecting mammalian cells and encapsidated replicons expressed E7SH showing the promise of this candidate vaccine as a future combination prophylactic and therapeutic vaccine.

Abbreviations

aa	amino acid(s)
AAV	Adeno-associated virus
ACT	adoptive cell transfer
AHSV	African horse sickness virus
Ala	alanine
APC	antigen presenting cell(s)
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BeYDV	Bean yellow dwarf virus
BGH	bovine growth hormone
BM	basement membrane
bp	base pair(s)
BPV	Bovine papillomavirus
BSA	bovine serum albumin
BTV	Bluetongue virus
CaMV	Cauliflower mosaic virus
cDMEM	complete Dulbeco's Modified Eagle Medium
CHO	Chinese hamster ovary
CIN	cervical intraepithelial neoplasia
CMV	Cytomegalovirus
CO_2	carbon dioxide
CPV	Canine parvovirus
CRPV	Cottontail rabbit papillomavirus

CsCl	caesium chloride
CTB	cholera toxin B
CTL	cytotoxic T lymphocyte(s)
cVLP	chimaeric virus-like particle(s)
Cys	cysteine
DC	dendritic cell(s)
DMEM	Dulbeco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DPBS	Dulbeco's phosphate buffered saline
dpi	days post infiltration
ds	double stranded
DTT	dithiothreitol
ECM	extracellular matrix
eCPMV	empty Cowpea mosaic virus
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay(s)
ER	endoplasmic reticulum
FB	final bleed(s)
FBS	fetal bovine serum
FDA	Food and Drug Administration
FMDV	Foot and mouth disease virus
FW	fresh weight
g	gram(s)

GB	GoldenBraid
GC-SF	granulocyte macrophage colony-stimulating factor
GMP	Good Manufacturing Practice
h	hour(s)
HA	haemagglutinin
HBc	Hepatitis B core
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEK	human embryonic kidney
HIV	Human immunodeficiency virus
HLA	human leukocyte antigen
HPV	Human papillomavirus
HSIL	high-grade squamous intraepithelial lesion(s)
HSNaOAc	high salt sodium acetate
HSPBS	high salt phosphate buffered saline
HSPG	heparin sulphate proteoglycan(s)
HSV	Herpes simplex virus
HT	hypertranslational
ICC	invasive cervical cancer
IFA	incomplete Freund's adjuvant
IPTG	isopropylthio-β-D-galactoside
kb	kilobase(s)
kDa	kilodalton(s)

kg	kilogram(s)
KLH	keyhole limpet hemocyanin
KUN	kunjin
kV	kilovolt(s)
L	litre(s)
LA	Luria agar
LB	Luria broth
LC-MS	liquid chromatography - mass spectrometry
LCR	long control region
LIR	long intergenic region
LSIL	low-grade squamous intraepithelial lesion(s)
M	molar
mA	milliamperes
MAb	monoclonal antibody
MDSC	myeloid-derived suppressor cell
MES	2-morpholinoethanesulfonic acid
mg	milligram(s)
MHC	major histocompatibility complex
MHV	Mouse hepatitis virus
min	minute(s)
mL	millilitre(s)
mM	millimolar
MWCO	molecular weight cut-off
NAb	neutralising antibody

NaOAc	sodium acetate
NBT	nitro-blue tetrazolium
ng	nanogram(s)
nm	nanometre(s)
NV	Norwalk virus
OD	optical density
OVA	ovalalbumin
PAGE	polyacrylamide gel electrophoresis
PB	Pre-bleed(s)
PBNA	pseudovirion-based neutralisation assay(s)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
Phe	Phenylalanine
polyA	polyadenylation signal
pRb	retinoblastoma protein
PsV	pseudovirion(s)
PTGS	post-transcriptional gene silencing
PV	papillomavirus
PVX	Potato X virus
RCA	rolling circle amplification
RE	restriction enzyme(s)
REP	replication-associated protein
RNA	ribonucleic acid

ROPV	Rabbit oral papillomavirus
RSV	Respiratory syncytial virus
RuBisCo	ribulose-1,5-bisphosphate carboxylase/oxygenase
RV	Rabies virus
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulphate
SEAP	secreted alkaline phosphatase
sec	second(s)
Ser	serine
SIL	squamous intraepithelial lesion(s)
SIR	short intergenic region
siRNA	short interfering ribonucleic acid
shRNA	short hairpin ribonucleic acid
SIV	Simian immunodeficiency virus
SLP	synthetic long peptide
ss	single stranded
TAM	tumour-associated macrophage
TBSV	Tomato bushy stunt virus
TEM	transmission electron microscopy
T-DNA	transfer DNA
TLR	toll-like receptor
Treg	regulatory T cell(s)
Trx	thioredoxin
TSP	total soluble protein
TSWV	Tomato spotted wilt virus

ubi-1	ubiquitin-1 promoter
V	volt(s)
VIN	vulval intraepithelial neoplasia
vir	virulence
VLP	virus-like particle(s)
WNV	West Nile virus
w/v	weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Symbols

α	alpha
β	beta
γ	gamma
Ω	Ohm(s)
$	dollar(s)
µF	microfarad(s)
µg	microgram(s)
µL	microliter(s)
µm	micrometre(s)
µM	micromolar
°C	degrees Celsius
%	percentage

Chapter 1: Literature review

1.1. Introduction

Approximately 1 in 6 global deaths is due to cancer, with the economic cost estimated at US$1.2 trillion in 2010 (World Health Organisation, 2017). Cancer is the second leading cause of death (Abubakar et al., 2015) and it was estimated that Human papillomavirus (HPV) related cancers account for 5% of all human cancers (De Martel et al., 2012). HPV was first described by Harold zur Hausen as the cause of cervical cancer (zur Hausen, 1996), following extensive studies in the 1970s and 1980s (zur Hausen, 1977; zur Hausen, 1989; zur Hausen et al., 1976; zur Hausen et al., 1974). The isolation of HPV-16 and -18 types from cervical cancer biopsies (Cole and Danos, 1987; Seedorf et al., 1985), as well as genital warts, led to increased study of this field. Over the last 40 years, great strides have been made in understanding the virus life cycle, molecular mechanisms of the virus infection cycle, carcinogenesis, and risk factors for HPV infection. In addition, HPV screening has progressed from the traditional detection of abnormal cytology by the Papanicolaou (Pap) test, to DNA testing and colposcopy. Three prophylactic vaccines are currently on the market and have been shown to be effective for up to 9.4 years in preventing cervical disease (Naud et al., 2014; Roteli-Martins et al., 2012); however, the global burden of cervical cancer remains high, particularly in low-resource countries due to vaccine cost, induction of vaccine-type specific protection, and inadequate or lack of screening and treatment programmes. This review will discuss the involvement of HPV in several cancers, particularly cervical cancer, and vaccine strategies to date for the prevention of cervical cancer.

1.1.1. Structure of HPV

HPVs are small non-enveloped double-stranded (ds) DNA viruses with a genome size of approximately 8kb (de Villiers et al., 2004). Several genes are encoded for and are divided into early (E1, E2, E4, E5, E6 and E7) and late (L1 and L2) genes (Figure 1.1). The early genes encode proteins responsible for viral DNA replication, transcription and oncogenic transformation, and the late genes form the virus capsid (Brentjens et al., 2002; Münger and Howley, 2002). The genome is surrounded by an outer shell (the capsid) which is 50-60 nm in diameter. The capsid is arranged in a T=7 icosahedral formation and consists of major and minor capsid proteins, L1 and L2, respectively (Conway and Meyers, 2009). This is an interesting variation of a classic T=7 quasi icosahedral structure in that the structural units consist entirely of 360 pentameric capsomeres. A non-coding

region known as the long control region (LCR) contains control elements for transcription and replication. The major capsid protein consists of 360 copies of L1 that assembles into 72 pentamers and up to 72 copies of L2 can be integrated into each capsid (Buck et al., 2008; Buck et al., 2005a). L1 assembles into virus-like particles (VLPs) – structures that resemble the virus but lack the viral DNA – in the presence or absence of the L2 minor capsid protein and retain the immunogenic/antigenic properties of native papillomaviruses (PVs) (Casini et al., 2004; Hagensee et al., 1993; Kirnbauer et al., 1992).

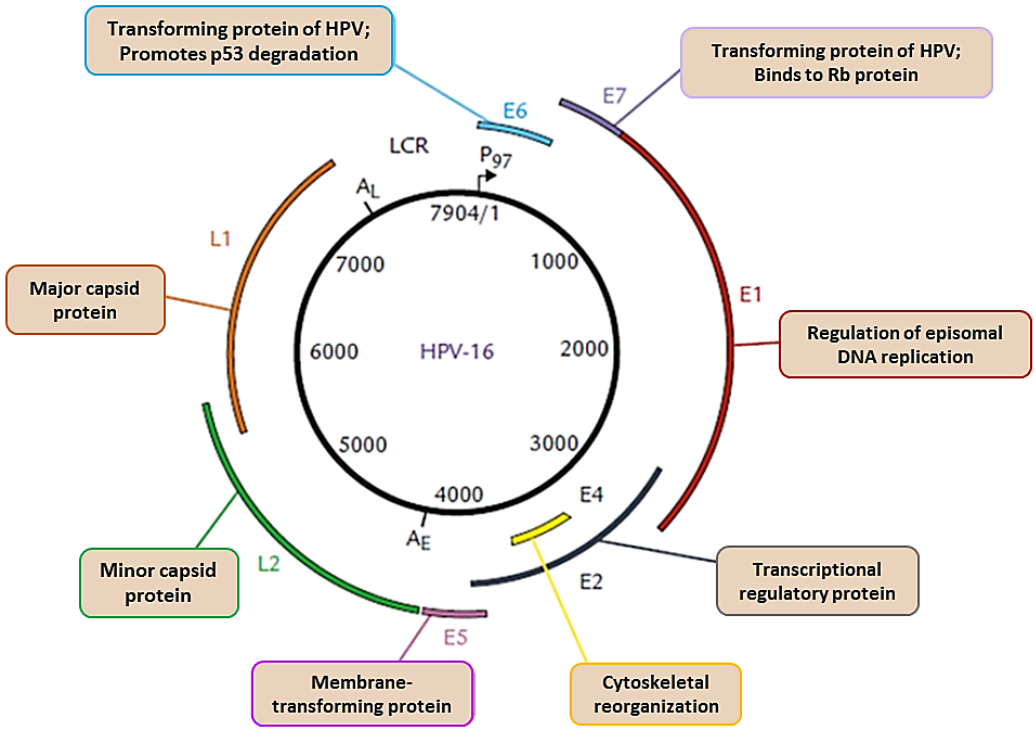


Figure 1.1: The HPV-16 genome and protein function. The genome contains 6 early genes, E1, E2, E4, E5, E6, E7, and two late genes, L1 and L2. The viral long control region (LCR) contains transcriptional and replication regulatory elements. P_{97} is the transcriptional promoter; A_E and A_L are the early and late polyadenylation sites, respectively. Image from Lin et al., 2010.

1.1.2. Classification of Papillomaviruses

PVs belong to the family *Papillomaviridae* which consists of 16 genera (Figure 1.2). PVs infect a range of hosts including birds and a variety of mammals such as rodents, rabbits, cattle and humans; however, HPV is the most intensively studied PV. HPV is classified into 5 genera: these are Alpha, Beta, Gamma, Mu and Nu PVs, based on sequence comparisons of the L1 major capsid protein open reading frame (de Villiers et al., 2004). The Alpha and Beta PVs are the two main genera of HPV and mainly cause lesions in squamous and cutaneous epithelial cells of the skin, the anogenital mucosa

and the upper respiratory tract. More than 40 types infect the anogenital tract (Muñoz et al., 2003; Parkin and Bray, 2006), while approximately 20% of upper respiratory tract cancers have DNA from the same HPV types (zur Hausen, 2000).

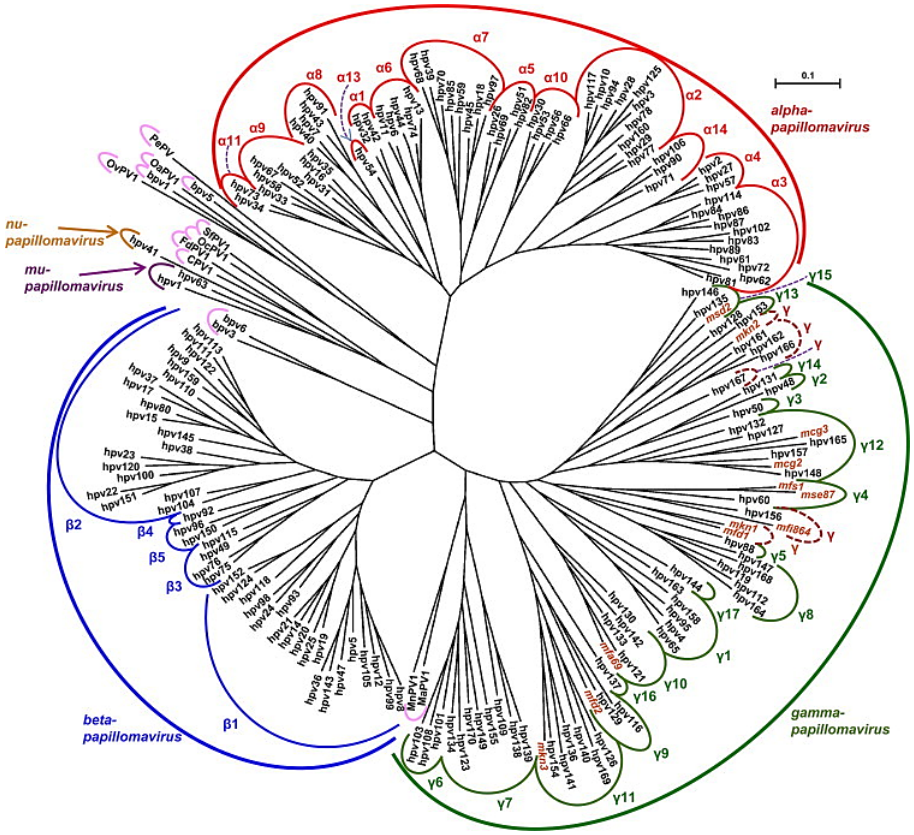


Figure 1.2: Classification of papillomaviruses. Phylogenetic tree of classifying papillomavirus genera and species based in L1 open reading frame. Image from de Villiers (2013).

There are over 170 HPV types which are divided into two main groups: these are low-risk HPV types (non-oncogenic) which cause benign condylomas such as genital warts, and include HPV-6/11/40/42/43/44/54/61/72 (Burd, 2003; zur Hausen, 2000); and high-risk types (oncogenic) which cause cancer and are detected in malignant lesions and include HPV-16/18/31/33/35/39/45/51/52/56/58/59/68 (de Sanjose et al., 2010; Li et al., 2011; Smith et al., 2007). These high-risk types are responsible for 99% of cervical cancers, with HPV-16 and -18 alone accounting for 70% of cervical cancers. HPVs are also responsible for many penile, vulvar and anal carcinomas and contribute to over 40% of oral cancers (Moody and Laimins, 2010; Walboomers et al., 1999). Persistent infection with high-risk HPVs results in the development of squamous intraepithelial lesions (SILs): in the cervix these are also called cervical intraepithelial neoplasia, CIN; and in the vulva, vulval intraepithelial neoplasia (VIN). SILs can progress to malignant cancers (zur Hausen, 2002).

1.1.3. HPV pathogenesis

HPV infects mucosal and cutaneous basal epithelial cells after tissue microtrauma (Kines et al., 2009) (Figure 1.3). Most infections are cleared by the immune system (Goodman et al., 2008; Rosa et al., 2008); however, some benign cervical lesions progress to invasive cervical cancer (ICC), caused predominantly by high-risk HPVs (zur Hausen, 2002). Similarly, other benign lesions can progress to penile, vulvar and anal carcinomas. Following entry of the virus into epithelial cells, the HPV genome is established as an episome and host cell machinery is used to mediate viral DNA synthesis (Moody and Laimins, 2010). The squamocolumnar junction is the most common site for cervical cancer to develop (Doorbar, 2006). Expression of the genome increases as the cells mature. Early genes are expressed in immature epithelial cells in the basal layer and late genes are expressed in terminally-differentiated cells, where encapsidated virions are released from the superficial zone (Figure 1.3) (Knoff et al., 2014). Continuous infection results in low-grade CIN 1 lesions. Progression to high-grade CIN 2/3 lesions caused by high-risk HPVs leads to ICC and other cancers, where the viral genome may integrate into the host genome.

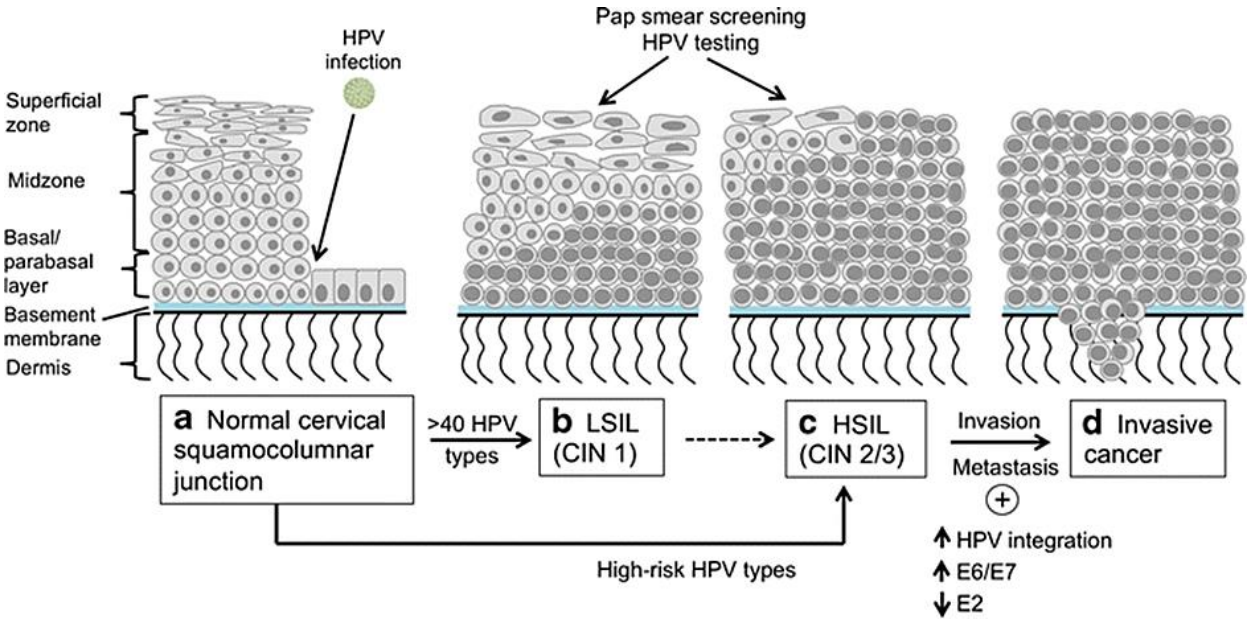


Figure 1.3: Cervical squamous intraepithelial lesions (SILs) and HPV-associated pathogenesis. **a)** The normal cervical squamocolumnar junction. The layer of basal cells that rests on the basement membrane is the normal barrier between the epithelium and the underlying stromal tissue. Normal squamous epithelium differentiates as shown, with the nuclear/cytoplasmic ratio decreasing closer to the surface. **b)** Productive infections produce low-grade squamous intraepithelial lesions (LSILs), in which the basaloid cells occupy the lower third of the epithelium. **c)** The cancerous precursor pathway is usually initiated by high-risk HPV infections and produces high-grade squamous intraepithelial lesions (HSILs). HSILs show less cellular differentiation, and the basaloid cells occupy at least the lower two-thirds and up to the full thickness of the epithelium. Pap smears and HPV tests can be used to detect SILs. **d)** If untreated, premalignant lesions can progress into microinvasive or invasive cancer, in which tumour cells breach the basement membrane. This process is associated with integration of the HPV genome into the host chromosomes, loss of E2, and upregulation of viral oncogene expression and genomic instability. Image from Knoff et al. (2014).

Integration into the host genome generally occurs with linearization of the genome by cleavage within the E2 gene, and results in the upregulation of the E6 and E7 oncogenes. E6 and E7 are essential for cell immortalization and transformation. E2 is involved in the initiation of viral DNA replication and genome segregation. In addition, E2 regulates the viral early promoter (P_{97} in HPV-16, Figure 1.1) and controls expression of E6 and E7 (Doorbar, 2006; Doorbar, 2016; Doorbar et al., 2012). E2 protein is the transcriptional repressor of E6 and E7, therefore when it is disrupted during genome linearization, E6 and E7 are constitutively expressed. The E6 protein degrades the p53 tumour suppressor, interfering with the regulation of the cell cycle, leading to proliferative cell growth (Scheffner et al., 1990; Werness et al., 1990). The E7 protein binds and inactivates the tumour suppressor, retinoblastoma protein (pRb) (Dyson et al., 1989) and leads to the transition of the cell life cycle to the S-phase stimulating replication and cell division (Chellappan et al., 1992), and contributing to tumorigenesis (zur Hausen, 2002).

1.2. Global importance

HPV is associated with over 99% of cervical cancers worldwide (Walboomers et al., 1999). Cervical cancer is the 4th most common cancer in women globally, and results in an estimated 528 000 cases and 266 000 deaths every year (Ferlay et al., 2015). About 80% of these cases occur in developing countries, largely due to limited healthcare resources (Parkin and Bray, 2006). HPV immunisation programmes have been implemented in 76 countries and territories worldwide; however, only 1% of women in low and low-middle income countries are covered by these programmes (Bruni et al., 2016). Cervical cancer is an important disease, more so than other cancers (breast, colorectal), as it affects women below the age of 45 resulting in more life years lost (Arbyn et al., 2011; Yang et al., 2004a). The precursor to cervical cancer is CIN, which about 1% of women suffer from (Da Silva et al., 2001), and if untreated leads to cervical cancer with the majority of cases resulting in squamous cell carcinomas (SCCs) (Agorastos et al., 2005). As previously mentioned, the majority of cervical cancer related deaths occur in the developing world in countries mainly in Central and South America, sub-Saharan Africa and South-East Asia. Figure 1.4 shows the GLOBOCAN 2012 estimated worldwide incidence and mortality rates for cervical cancer (World Health Organisation, 2015). There is an evident correlation between incidence rates and deaths. This coupled with an established link between HPV and cancer of the penis, anus, vulva, vagina, mouth and oropharynx (Parkin and Bray, 2006), show there is an urgent need for both prophylactic and therapeutic HPV vaccines.

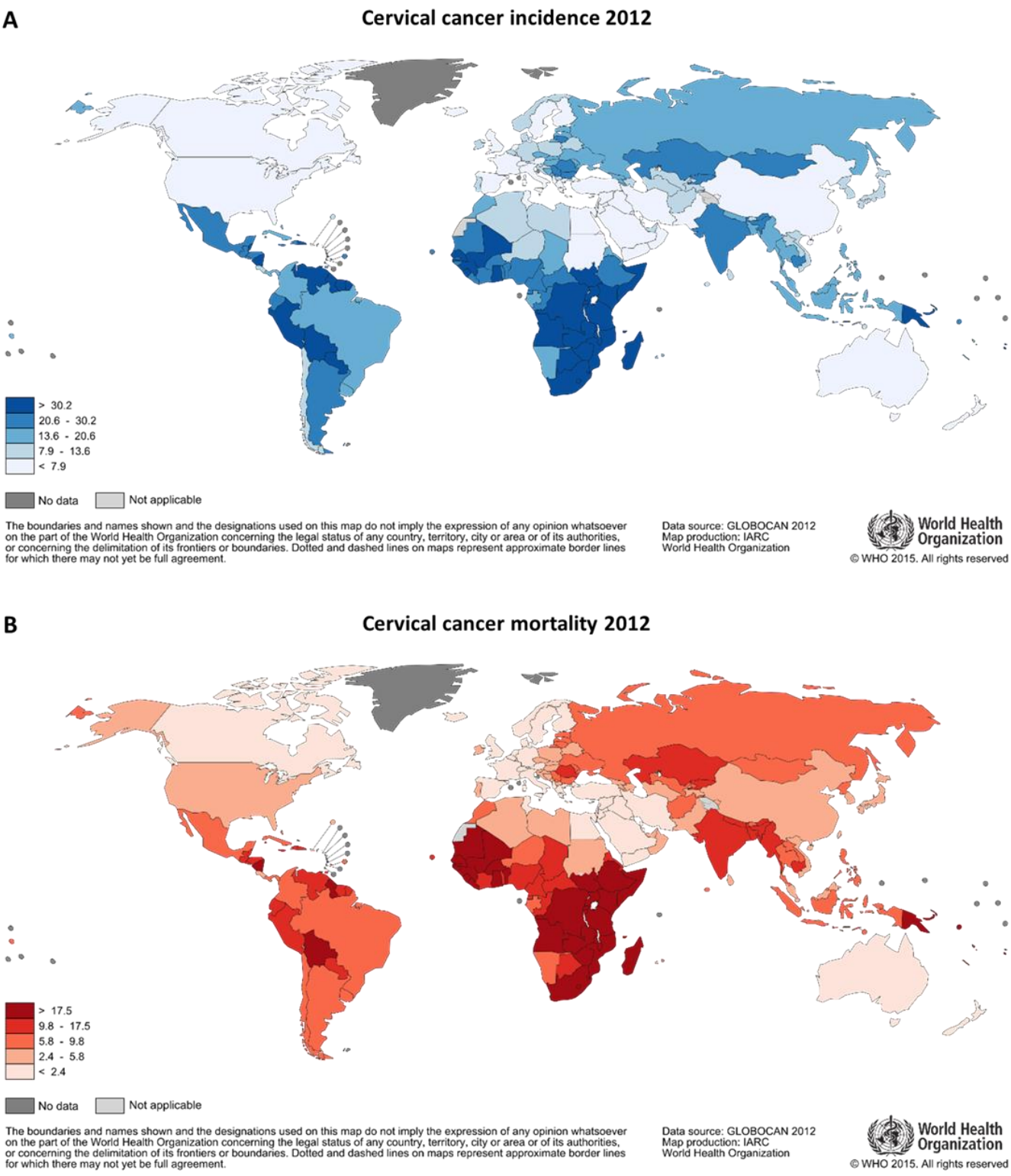


Figure 1.4: Estimated cervical cancer incidence (A) and mortality (B) worldwide in 2012. GLOBOCAN reported age standardised rates (world) per 100 000, (World Health Organisation, 2015).

The prevalence of HPV types in ICC differs geographically. Although HPV-16 and 18 infections are the most common cause of ICC cancer (Bosch et al., 2002; Bosch et al., 1995) – they are associated with 70% of cases (de Sanjose et al., 2010; Smith et al., 2007) – other high-risk types include HPV-

31/33/39/45/52/58/59. Several studies have described the type distribution of these HPV-types in meta- and pooled analyses (Clifford et al., 2003; de Sanjose et al., 2010; Li et al., 2011; Munoz et al., 2004; Smith et al., 2007) and showed that the prevalence of HPV-16/18 is more common in Europe, North America and Australia versus sub- Saharan Africa, South/ Central America and South Asia, and that the relative frequencies of the other high-risk HPV types differs in each geographical region.

For example, in a recent cross-sectional epidemiological study, Denny et al. (2014) assessed the prevalence and distribution of the most commonly detected HPV types in women from Ghana, Nigeria and South Africa. The authors found that in women with single and multiple HPV infections, the most commonly detected types were HPV-16 (51.2%), HPV-18 (17.2%), HPV-35 (8.7%), HPV-45 (7.4%), HPV-33 (4.0%) and HPV-52 (2.2%) and the incidence of these types differed to those observed globally (Figure 1.5). ICC in sub-Saharan Africa is the 2nd most common cancer, with the region accounting for some of the highest incidence and mortality rates worldwide (De Vuyst et al., 2013; Ferlay et al., 2015; World Health Organisation, 2015). Therefore, HPV vaccines should account for these differences in genotype distribution.

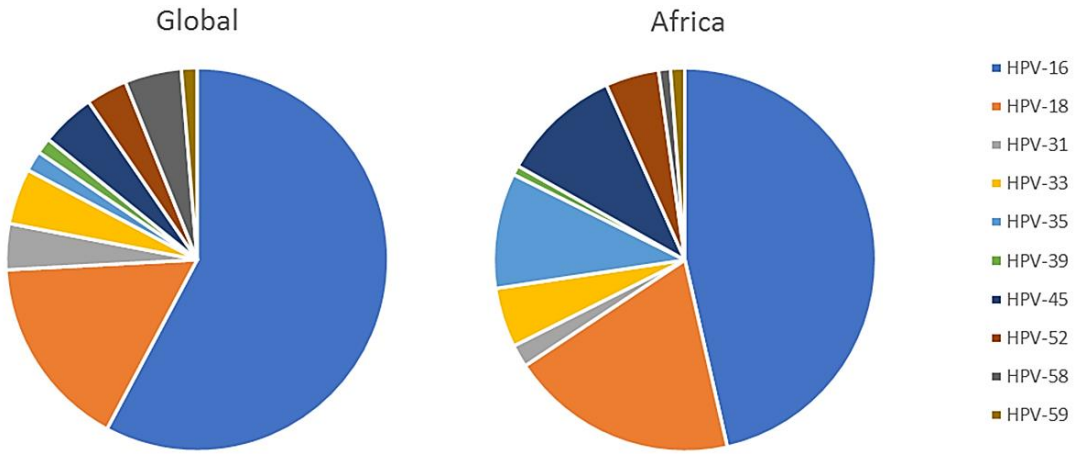


Figure 1.5: Comparison of HPV type prevalence globally and in Africa. Data adapted from Li et al. (2011), de Sanjose et al. (2010) and Denny et al. (2014).

Furthermore, the incidence of human immunodeficiency virus (HIV) has been shown to influence HPV acquisition, the prevalence of multiple HPV types, persistence of infection and alter the carcinogenicity of high-risk HPV types (Allan et al., 2008; Clifford et al., 2016; Massad et al., 2016; Massad et al., 2015; Rowhani-Rahbar et al., 2007). African HIV-positive women have the highest prevalence of HPV infection (Clifford et al., 2006; Clifford et al., 2017). A study in South African HIV-positive women showed increased disease severity, with HPV-16/35 (both 18.9%) and HPV-18 (7.6%)

predominant in women with HSILs (Allan et al., 2008). Other studies have also shown that in addition to HPV-16/18, HPV-35/45/51/52/58/68 are common in HIV-positive women (Denny et al., 2014; Denny et al., 2008; Marais et al., 2008; McDonald et al., 2014; Moodley et al., 2009). A meta-analysis by Clifford et al. (2017) looking at the carcinogenicity of HPV in HIV-positive women worldwide, recently showed HPV-16, -18 and particularly HPV-45 in African women accounted for a greater proportion of HPV infection in ICC compared to normal cytology, and other high-risk types accounted for important proportions of other low and high grade lesions.

Overall, this significant burden of HPV infection (and possible HIV-co-infection) in sub-Saharan Africa highlights the need for more rigorous screening programmes to decrease the burden of cervical cancer, as through early treatment and most importantly vaccination, mortality and morbidity rates can decrease.

1.3. Prophylactic vaccines

The L1 major capsid protein can spontaneously assemble into VLPs which are morphologically and immunogenically similar to native virions (Kirnbauer et al., 1992; Rose et al., 1994a; Zhou et al., 1991), and are the basis on which current vaccines are made. Immunization with VLPs elicits the production of neutralising antibodies (NAbs) which confer protection upon subsequent exposure to the virus. The ability of NAbs to protect animals from challenge (Breitburd et al., 1995; Christensen et al., 1996b; Jansen et al., 1995; Kirnbauer et al., 1996; Suzich et al., 1995), predominantly directed against type-specific conformational epitopes (Christensen and Kreider, 1990; Kirnbauer et al., 1992; Rose et al., 1994b) was first described over 20 years ago and laid the ground work for HPV vaccines.

The majority of HPV infections are cleared naturally by the immune system; however, a small number progress into persistent infections. The link between HPV infection and cervical cancer (Bosch et al., 2002; zur Hausen, 1996; zur Hausen, 2000) and the global incidence of infection (Parkin and Bray, 2006) led to the development of prophylactic vaccines. There are currently 3 licensed vaccines on the market. The first vaccine is Gardasil® (Merck & Co.), a quadrivalent HPV-6/11/16/18 VLP vaccine, which in addition to the most prevalent types includes HPV-6 and -11 that cause most genital warts. It is produced in a yeast expression system (*Saccharomyces cerevisiae*). The second vaccine is Cervarix® (GlaxoSmithKline), a bivalent HPV-16/18 VLP vaccine of the two virus types that cause most cervical cancers. It is produced in recombinant baculovirus in insect cells (*Trichoplusia ni*). The third and most recent vaccine Gardasil®9 (Merck & Co.), is a nonavalent HPV-

6/11/16/18/31/33/45/52/58 VLP vaccine against an additional 5 high-risk HPVs, also produced in *S. cerevisiae*. These vaccines are regarded as safe and effective (Future II Study Group, 2007a; Future II Study Group, 2007b; Garland et al., 2007; Joura et al., 2015; Schiller et al., 2008; Signorelli et al., 2017). These vaccines are all formulated to contain an aluminium salt adjuvant, with Cervarix® also containing monophosphoryl lipid A.

Multiple clinical trials showed that these vaccines consistently induced high titre and long-lasting NAb responses. The vaccines were found to be extremely effective at preventing infection and neoplastic disease (CIN/VIN) or genital warts by the targeted HPV types (Ault and Group, 2007; Future II Study Group, 2007b; Herrero et al., 2011; Joura et al., 2015; Kjaer et al., 2009; Lehtinen et al., 2012; Muñoz et al., 2010; Paavonen et al., 2009b; Signorelli et al., 2017), and a 9.4 year follow-up post-vaccination study showed that no new HPV-16/18 infections occurred and vaccine efficacy against HPV-16/18 was 95.6% (Naud et al., 2014). The highly repetitive display of L1 epitopes on the VLP surface, and their strong activation of B cells through B cell receptor cross-linking are thought to contribute to the strong antibody responses observed (Chackerian, 2007; Grgacic and Anderson, 2006; Schiller and Lowy, 2012). Pre-adolescent females are the primary target for HPV vaccines as there is a high incidence of cervical HPV infection within 3 years of first intercourse (Collins et al., 2002). In recent years there has been a push to vaccinate boys as they are also at risk for cancer (Centers for Disease Control Prevention, 2010).

1.3.1. Limitations of current vaccines

Despite the great success of these vaccines, the cervical cancer burden remains high, particularly in developing countries. Concerns with current vaccines include their type-restricted prophylactic efficacy, a lack of therapeutic efficacy and the high costs associated with these vaccines. Cervarix® and Gardasil® were released into the market in 2006 and 2007, respectively followed by Gardasil®9 8 years later in 2014. Low levels of cross-neutralising antibodies of HPV-16 to HPV-31/33/58 and HPV-18 to HPV-45 were elicited by the original L1 VLP vaccines (Bonanni et al., 2009; Brown et al., 2009; Harper, 2009; Paavonen et al., 2009a); however, titres were much lower than for the specific HPV-type and therefore cross-protection may be less durable (Schiller and Müller, 2015). The addition of a further 5 high-risk HPV types in Gardasil®9 should address the low cross-neutralisation observed with original vaccines. The introduction of Gardasil®9 may theoretically prevent 90% of cervical cancer; however, the 10% of cervical cancer caused by other high-risk types are still unpreventable. Also, the addition of more L1 VLP types has not decreased the cost of current vaccines. These vaccines are expensive to manufacture and deliver as they require cold storage. Currently,

Gardasil®9 is ~US$195/dose (Centers for Disease Control Prevention, 2017), with 2 or 3 doses required (dependent on patient age). This means the vaccine is still out of reach for majority of low-income countries. Furthermore, the current vaccines do not promote viral clearance or lesion regression and are therefore ineffective in individuals pre-exposed to HPV (Future II Study Group, 2007a; Hildesheim et al., 2016; Hildesheim et al., 2007). Hence, there is a need for next generation HPV vaccines that broadly target oncogenic HPV types, at reduced cost to women particularly in developing countries suffering most from cervical cancer.

1.4. Second generation prophylactic vaccines

Over 80% of cervical cancer cases occur in developing countries (Parkin and Bray, 2006) due to low vaccination rates and poor cytological screening programmes (Bruni et al., 2016). Additionally, the prevalence of oncogenic HPVs is not similar to those in the developed world and some of these types have only been included in the most recent Gardasil®9 vaccine. However, there still remain HPV types such as HPV-35, the 8th most prevalent type worldwide, but the 5th most prevalent in Africa and types HPV-39/59/68 which together are responsible for 3.2% of all cervical cancer cases (Li et al., 2011). Therefore, alternative HPV vaccination strategies are being developed in a number of centres, with emphasis both on expanding the HPV type coverage, and on reducing cost of the vaccines.

1.4.1. L2-based vaccines

The L2 minor capsid protein has been shown to be a promising antigen for prophylactic vaccination as it has highly conserved regions of sequence, particularly in the N-terminus (Lowe et al., 2008). Initial studies on L2 vaccine development were based on animal PV models as HPV did not produce disease in animals, was difficult to culture and no *in vitro* neutralisation assays were available (Galloway, 1994). Studies using rabbit and bovine PV models showed that L2 induced broad cross-protection *in vitro* and *in vivo*. Immunisation of cattle with full length L2 of bovine papillomavirus (BPV) type 4 showed protection against BPV viral challenge (Chandrachud et al., 1995; Gaukroger et al., 1996; McGarvie et al., 1994). In addition, vaccination with the BPV-4 L2 N-terminus-derived peptide (11-200) compared to a C-terminal peptide (327-524) was also shown to be protective against BPV-4 experimental challenge (Chandrachud et al., 1995). Rabbits challenged after vaccination with full length L2 or L2 peptides were protected from cutaneous challenge with cottontail rabbit papillomavirus (CRPV) (Christensen et al., 1991; Lin et al., 1992) or challenge with

rabbit oral papillomavirus (ROPV) (Embers et al., 2002; Gambhira et al., 2007a; Palmer et al., 2006). Following the development of pseudovirion (PsV) technology (Buck et al., 2004; Day et al., 2012; Pastrana et al., 2004; Roden et al., 1996) for use in *in vitro* neutralisation assays and challenge studies, testing of HPV L2 vaccine efficacy is now possible. PsVs differ from VLPs in that PsVs encapsidate plasmids that express a gene of interest, whereas VLPs are empty capsids that resemble the native virion.

Anti-L2 antibodies can neutralise a broad range of mucosal and cutaneous HPVs (Alphs et al., 2008; Pastrana et al., 2005), suggesting that an L2 vaccine could address the type-restrictive efficacy of L1 vaccines (Karanam et al., 2009). Roden et al. (2000) showed that HPV-6, -16 and -18 L2 polypeptides elicited antibodies that neutralised homologous HPV types *in vitro* as well as cross-neutralising heterologous types, showing the potential of L2 epitopes as broadly protective antigens.

1.4.1.1. L2 cross-neutralising epitopes

Several studies have investigated the use of HPV-16 L2 minor capsid protein as a potential prophylactic vaccine. Areas within the N-terminal region, especially amino acids (aa) 1-120, contain broadly cross-neutralising epitopes shared by cutaneous and mucosal HPV types and by types that infect divergent species (Alphs et al., 2008; Embers et al., 2004; Gambhira et al., 2007a; Gambhira et al., 2007b; Kawana et al., 2001; Kawana et al., 2003; Kawana et al., 1999; Kondo et al., 2007; Kondo et al., 2008; Palmer et al., 2006; Pastrana et al., 2005; Roden et al., 2000; Schellenbacher et al., 2009; Slupetzky et al., 2007). Several L2 epitopes in this region (Figure 1.6) are discussed below.

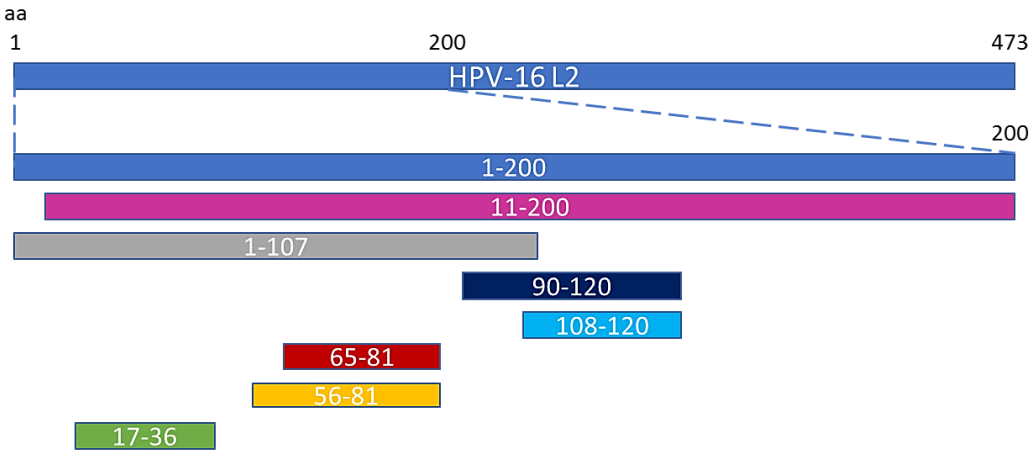


Figure 1.6: L2 neutralising epitopes. Overlapping L2 epitopes in HPV-16 L2 amino terminus with cross-neutralising potential.

Mapping studies have shown that several L2 peptides overlapping aa 90-120 are cross-neutralising. A monoclonal antibody (MAb) directed to the HPV-16 L2 aa 108-120 was first shown to be cross-neutralising to HPV-6 (Kawana et al., 1999) and HPV-52 PsVs (Kawana et al., 2003). In another study, this peptide was coupled to keyhole limpet Hemocyanin (KLH) and sera from immunized rabbits showed anti-L2 titres; however, did not cross-neutralise HPV-6/11/18 virions tested (Slupetzky et al., 2007). The authors reasoned this was due to low anti-L2 titres. HPV-16 L2 aa 96-115 have also been shown to cross-neutralise HPV-31/58 PsVs (Kondo et al., 2007). A longer L2 peptide aa 11-200 derived from CRPV was shown to protect rabbits against challenge with CRPV and ROPV and generated broadly cross-neutralising serum antibodies (Gambhira et al., 2007a), but aa 108-120 was not cross-protective (Embers et al., 2002), suggesting other cross-neutralising epitopes were present between aa 11-107. This was supported by Jagu et al. (2009) who found that antisera to HPV-16 L2 aa 1-107, 13-107, 11-200 and 13-200 neutralised HPV-16 and cross-neutralised HPV-18/31/45/58/5, and concatenated fusions of L2 peptides 11-200 from HPV types 6, 16, 18 induced strong cross-neutralisation to HPV-31/45/58. L2 aa 89-200 did not cross-neutralise any of the heterologous HPVs (Jagu et al., 2009). These results suggest an important epitope(s) may occur between aa 1-89.

Epitopes within L2 aa 56-81 have shown cross-neutralisation in several studies. Kawana et al. (1998) found that HPV-16 L2 peptide 69-81 was bound by 7 out of 11 anti-L2 MAbs produced from HPV-16 L1+L2 VLPs. This peptide also reacted with human sera positive for antibodies against HPV-16/18/58/6 L1 VLPs. Another linear peptide aa 63-75 was bound by 1 MAb, suggesting another epitope is present in this region (Kawana et al., 1998). Kondo et al. (2007) mapped L2 aa 56-75, 61-75 and 64-81 and found that antisera raised against these peptides cross-neutralised HPV-18/31/58 (L2 aa 56-75), and HPV-18/58 (L2 aa 61-75 and 64-81) suggesting aa 56-61 contains a cross-neutralisation epitope and that aa 75-81 may not be necessary. Jagu et al. (2013) also defined protective epitopes in this region and found that passive transfer of anti-L2 aa 65-81 serum significantly protected against HPV-16 entry in mice, but not residues 47-66, suggesting this region does not contain protective epitopes. However, these aa 47-66 were shown to be important for immunogenicity (Jagu et al., 2013).

HPV-16 L2 epitopes within aa 17-36 have shown the greatest cross-neutralisation potential. Generation of a MAb RG-1 that binds L2 aa 17-36 neutralised HPV-16 and cross-neutralised HPV-18, and anti-serum to the RG-1 peptide neutralised HPV-16 and cross-neutralised HPV-6/18/31/45/52/58 PsVs and HPV-11 virions (Gambhira et al., 2007b). In addition, cutaneous HPV-5 and BPV-1 PsVs were neutralised. Fusion of this peptide to a synthetic lipopeptide showed potent anti-L2 antibodies, neutralised HPV-16/18/45/5 and BPV-1, and protected mice from HPV-16

and -45 PsV challenge (Alphs et al., 2008). Jagu et al. (2013) also showed that passive transfer of aa 17-36 anti-serum significantly protected mice from vaginal HPV-16 challenge. Antiserum from aa 18-38 (Kondo et al., 2007) and aa 18-31 (Schellenbacher et al., 2009) have also been shown to be cross-neutralising. Another peptide aa 20-38 fused to bacterial thioredoxin (Trx) was shown to cross-neutralise HPV-18/31/45/58 (Rubio et al., 2009) Overall, L2 aa 17-36 has shown the greatest potential as a cross-neutralising epitope.

1.4.2. Capsid display vaccines

Although L2 peptide and protein vaccines have elicited broadly cross-neutralising antibodies in animal studies, they have shown low immunogenicity in clinical trials (de Jong et al., 2002; Karanam et al., 2009). L2 is also subdominant to L1, and the use of L1+L2 VLPs in vaccination does not confer more cross-protection in animals vaccinated with L1+L2 VLPs compared to L1 VLPs only (Roden et al., 2000). *In vivo,* L2 is not fully exposed on the surface of virions, until a conformational change occurs after virion binding to cell surface receptors and cleavage of the protein. Additionally, there are up to 72 L2 molecules in a typical virion, which is 5 times less than L1 molecules (Buck et al., 2008). It is likely that these factors contribute to the low immunogenicity observed, therefore scaffolded display of L2 peptides and the construction of chimaeric proteins with L1 has been used to overcome these limitations.

The structure and assembly of L1 has been well described (Bishop et al., 2007a; Chen et al., 2001; Chen et al., 2000c; Modis et al., 2002). L1 forms pentameric capsomeres which spontaneously assemble into VLPs in the presence or absence of L2 (Hagensee et al., 1993; Kirnbauer et al., 1992; Kirnbauer et al., 1993; Rose et al., 1993). L1 VLPs typically have a T=7 icosahedral formation, but can also form small T=1 VLPs containing 12 L1 pentamers as shown by *Escherichia coli* expressed L1 with an N-terminal deletion (Chen et al., 2000c). Assembly of pentamers into VLPs occurs by interaction of the C-terminal arms of L1 that project outwards to neighbouring pentamers, through intercapsomeric hydrophobic interactions (Bishop et al., 2007a; Chen et al., 2001; Chen et al., 2000c; Modis et al., 2002). L1 immunogenicity is dependent on its assembly into higher order structures and L1 surface loops (BC, DE, EF, FG and HI) which contain the conformational epitopes involved in the production of NAbs (Christensen et al., 1996a; Christensen et al., 1994; Roden et al., 1997; White et al., 1999). Figure 1.7 shows the L1 pentamer and monomeric structure and the position of the L1 surface loops.

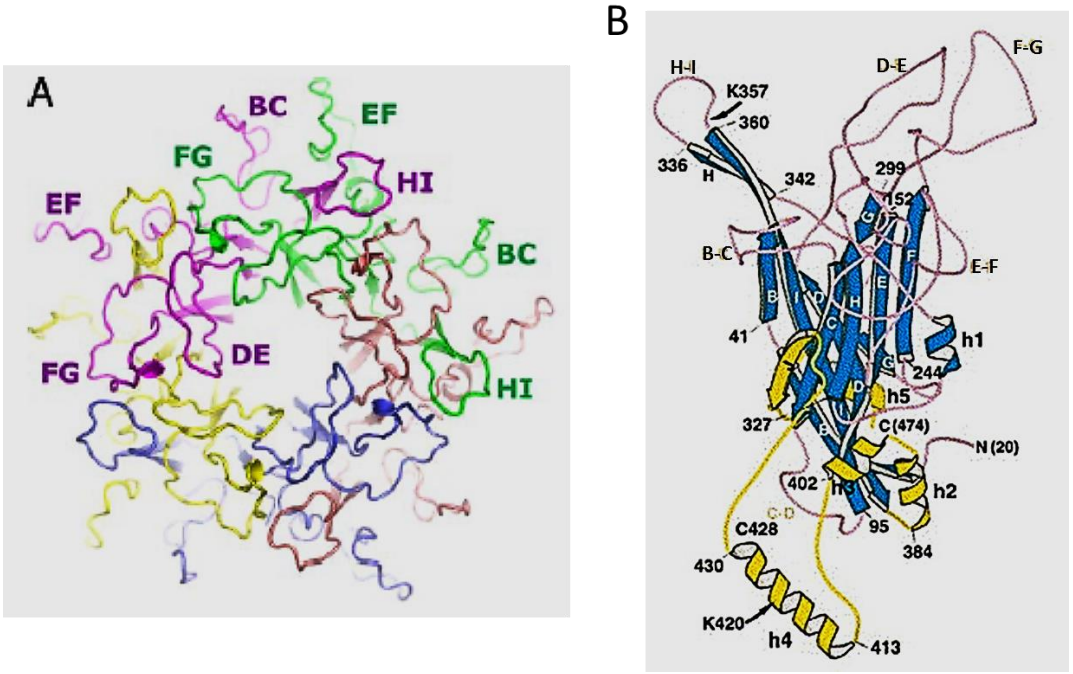


Figure 1.7: HPV-16 L1 structure. A) HPV-16 L1 pentamer comprising 5 L1 monomers with surface loops indicated on the diagram. Each L1 monomer is represented in different colours – yellow, purple, green, brown or blue (from Bishop et al. (2007b)). **B)** Detailed three-dimensional structure of the HPV-16 L1 monomer. β-strands are labelled B – J. The carboxy-terminal and α-helices (h) are shown in yellow, except short helix h1 in blue (from Chen et al. (2000c).

The type-specific nature of L1 NAbs is due to the variation in amino acid sequences found within the L1 surface loops of different HPV genotypes (Bishop et al., 2007b; Carter et al., 2003; Chen et al., 2000b). The exposed surface loops (e.g. BC and EF) show more sequence heterogeneity than the loops close to the lumen such as DE (Bissett et al., 2016). These surface loops are potential candidates for epitope display as they can present an antigen on the surface of L1 assemblies to the immune system. However, it is necessary that certain L1 functional domains are maintained to ensure VLP assembly. Chen et al. (2001) found that at least 10 aa of the N- terminus and 30 aa of the C- terminus of HPV-16 L1 could be deleted without affecting pentamer formation for HPV-16 L1 made in *E. coli* cells. The 10 aa N-terminal truncation can form small T=1 VLPs, while 9 aa N-terminal or 30 aa C-terminal truncations do not affect assembly into T=7 VLPs (Chen et al., 2000c). Varsani et al. (2003a) also found that aa 414-426 in the h4 helix are involved in HPV-16 VLP assembly in insect cells. Helix-helix interactions at the C-terminal mediate interpentameric bonds, with h2 and h3 being necessary for pentamer assembly and h4 critical for assembly of T=7 VLPs (Bishop et al., 2007a). Deletion of the h4 helix was shown to abolish the assembly of VLPs (Chen et al., 2001). Furthermore, the formation of disulphide bonds between the highly conserved cysteine residues 175 and 428 is important for VLP assembly and stability (Bishop et al., 2007a). Mutations in these regions have been shown to result in the preferential formation of capsomeres and not VLPs (Fligge et al., 2001; Li et

al., 1998; McCarthy et al., 1998; Sapp et al., 1998; Varsani et al., 2006a). Therefore, the position of epitope display is important for the maintenance of L1 epitopes required for the induction of NAbs, as well as for the formation of VLPs.

1.4.2.1. L1 surface display sites

The insertion or substitution of several peptides into several L1 surface loops has been shown not to affect chimaeric VLP (cVLP) assembly (Chackerian et al., 2008; Sadeyen et al., 2003; Schellenbacher et al., 2009; Slupetzky et al., 2007; Slupetzky et al., 2001; Varsani et al., 2003a). Sadeyen et al. (2003) inserted aa 78-83 of the hepatitis B core (HBc) antigen into the BC, DE, EF, FG, and HI loops of HPV-16 L1 and found that all chimaeras were capable of assembly into VLPs, and could elicit the generation of anti-L1 NAbs, albeit at reduced levels to native L1 VLPs. Anti-HBc antibodies were also detected, indicating exposure of the HBc epitope on the VLP surface. Slupetzky et al. (2001) also showed that insertion of an 8 aa B cell epitope in the FG loop of HPV-16 L1 or the DE, FG and HI loops of BPV-L1 did not affect assembly into pentamers or VLPs, and pentamers displayed some of the neutralisation epitopes found on fully assembled VLPs. It is however important to note that cVLP assembly may be peptide dependent and should be determined on a case by case basis.

1.4.2.2. L1:L2 chimaeras

The DE loop has widely been used for the insertion of L2 epitopes and has been shown to elicit anti-L1 responses as well as immune responses to the epitope inserted. HPV-16 L2 peptides 69-81 or 108-120 were inserted into the DE surface loop of BPV-1 L1 and these chimaeras assembled into pentamers or cVLPs which induced L2-specific serum antibodies and could partially neutralise HPV-16 PsVs, with L2 aa 69-81 partially cross-neutralising HPV-11 PsVs (Slupetzky et al., 2007). Schellenbacher et al. (2009) expounded on this study and inserted 9 overlapping L2 peptides (2-22, 13-107, 18-31, 17-36, 35-75, 75-112, 115-154, 149-175 and 172-200) into the DE surface loop of BPV-L1. The authors observed that except for cVLPs of aa 35-75 and 13-107, all chimaeras assembled into VLPs. Vaccination of rabbits with cVLPs plus adjuvant induced higher L2-specific antibody titres than with denatured protein, and immune sera to cVLPs of 13-155 neutralised HPV-16 PsVs and cVLP 17-36 cross-neutralised HPV-5/11/18/31/45/52/58 PsVs (Schellenbacher et al., 2009). The L2 peptide aa 17-36 (RG-1) has shown the most promise as a candidate cVLP vaccine as it has been shown to protect mice against challenge with high-risk mucosal PsV types HPV-16/18/45/31/33/52/58/35/39/51/59/68/56/73/26/53/66/34 and low-risk types HPV-6/43/44, with protection observed one year after vaccination (Schellenbacher et al., 2013). This candidate

vaccine is expected to enter phase I clinical trial in 2017 (Buchman et al., 2016). Boxus et al. (2016) also inserted HPV-33RG-1 into the HPV-18 L1 DE loop and found that this cVLP protected against HPV-6/11/16/31/35/39/45/58/59 as PsVs or quasivirions in both mouse and rabbit challenge models and that protection in rabbits was further enhanced when the cVLP was formulated with L1 VLPs from HPV-16/18.

The RG-1 peptide of other HPV-types has also been used to address the HPV types involved in cervical adenocarcinomas (Huber et al., 2015) and cutaneous infections such as non-melanoma skin cancer (Huber et al., 2017), which are not covered by current vaccines. In the first study, a HPV-18 L1-45RG-1 chimaera showed high titre neutralising antibodies to HPV-18 and cross-neutralised HPV-39/45/68. Passive immunisation protected mice from vaginal challenge with HPV-18/39/45/68 (Huber et al., 2015). In the second study, RG-1 homologs of beta HPV-17/5/4 were inserted into the DE loops of HPV-1/5/16/18, and tested against 12 novel beta HPV PsVs. Cross-neutralising humoral responses were observed for cVLPs with the HPV-17RG-1 epitope, but not for HPV-5. Passive immunisation showed that HPV-16RG-1 sera cross-protected beta HPV5/20/24/38/96/16, while HPV5L1-17RG-1 antisera cross-protected HPV20/24/96 only, and sera to HPV1L1-4RG-1 cross-protected HPV4 challenge (Huber et al., 2017).

In our laboratory, Varsani et al. (2003a) have explored the substitution (as opposed to insertion) of L2 aa 108-120 in the CD, DE and EF L1 surface loops, as well as in the C-terminal in the h4 helix and between h4 and β-J structural regions. High anti-L1 immune responses were observed for all chimaeras and only the CD-loop chimaera did not elicit an L2 immune response. Substitution of L2 in the DE loop and in the C-terminal between the h4 and β-J structural region resulted in the formation of VLPs, with substitution in the h4 helix showing the formation of capsomeres and relatively disordered aggregate structures. The h4 helix chimaera however showed the highest anti-L1 titres (Varsani et al., 2003a). The h4 helix chimaera was also shown to neutralise homologous HPV-16 PsVs (McGrath et al., 2013) and to elicit antibodies that cross-neutralised HPV-18/31/52 PsVs i*n vitro* (Megan Hendrikse et al., personal communication). The neutralisation potential of the other chimaeras was not tested, however.

1.4.2.3. Other L2 chimaeras

HPV-16 L2 aa 17-31 displayed on the surface of RNA bacteriophage PP7 VLPs generated a robust anti-L2 immune response and protected mice from genital challenge with HPV-16 and -45 PsVs (do Carmo Caldeira et al., 2010). In another study, 8 PP7 cVLPs (L2 aa 17-31 from

HPV-1/5/6/16/18/45/58) also elicited high anti-L2 antibodies and mice were protected from genital challenge with 8 diverse HPV PsVs types and cutaneous challenge with HPV-5 PsVs (Tumban et al., 2011). Insertion of L2 peptides into the N-terminus of a MS2 bacteriophage coat protein could also elicit broadly cross-protective antibodies to several heterologous HPV types (Tumban et al., 2012). Adeno-associated virus (AAV) can assemble into VLPs with co-expression of VP3 (Sonntag et al., 2010). Nieto et al. (2012) presented HPV-16 and -31 L2 aa 17-36 at positions 587 and 453, respectively, in VP3 and showed that sera induced high titres of HPV-16 L2 antibodies and cross neutralised HPV-18/45/52/58 PsVs. Additionally, passive transfer of sera protected mice from challenge with HPV-16 PsVs. Furthermore, robust protection was observed in rabbits 6 and 12 months post-immunisation after concurrent challenge with HPV16/31/35/39/45/58/59 quasivirions or CRPV (Jagu et al., 2015).

Trx is a small, highly soluble protein that has been used as a vaccine scaffold to display L2 multi-peptides. Rubio et al. (2009) investigated the immunogenicity and neutralisation potential of 6 HPV-16 L2 peptides: 20-38, 28-42, 56-75, 64-81, 96-115 and 108-120. Trx-L2 peptides were tested as fusions with one or multiple copies each peptide and fusion to Trx was shown to confer strong immunogenicity, with multipeptide forms showing increased immunogenicity compared to monopeptides. All Trx-L2 peptides also induced HPV-16 NAbs with Trx-L2(2-38) showing the most effective neutralisation of HPV-16 PsVs and heterologous HPV-18/45/58 PsVs. In a follow-on study, Trx-L2 was designed to include L2 sequences from divergent HPV-31 and -51 to extend cross-neutralisation. A comparison between multivalent antigens from all 3 HPV types on a single Trx scaffold and a mixture of the 3 monovalent Trx-L2 antigens, showed that the mixture conferred higher magnitude and more robust cross-reactivity to HPV-31 and -51, with similar anti-HPV-16 neutralisation titres (Seitz et al., 2014). Furthermore, Seitz et al. (2015) showed that low doses of trivalent Trx-L2, formulated in human compatible adjuvant induced robust cross-neutralisation of 12 out of the 13 oncogenic HPV types and that heat purified Trx-L2 immunogens were highly thermostable.

1.5. Therapeutic vaccines

Current HPV vaccines elicit strong humoral immune responses; however, they are prophylactic and do not clear established infections (Markowitz, 2007; Schiller et al., 2008). Treatment of HPV-induced lesions involves methods such as laser treatment, radiation, chemotherapy, or surgical intervention (American Cancer Society, 2017), which is invasive and not always successful. There is

therefore an urgent need for therapeutic vaccines to treat current infections, through the induction of the cell-mediated immune response, which can have an immediate effect on the prevalence of HPV-associated lesions. The E6 and E7 early genes are ideal targets for vaccine therapy due to their role in disruption of the cell cycle and their constitutive expression in premalignant and malignant tissues (zur Hausen, 2002). Several strategies have been investigated for the development of therapeutic vaccines using peptides, protein-based subunits, nucleic acid based vaccines, live vector vaccines, and cell-based vaccines (Figure 1.8).

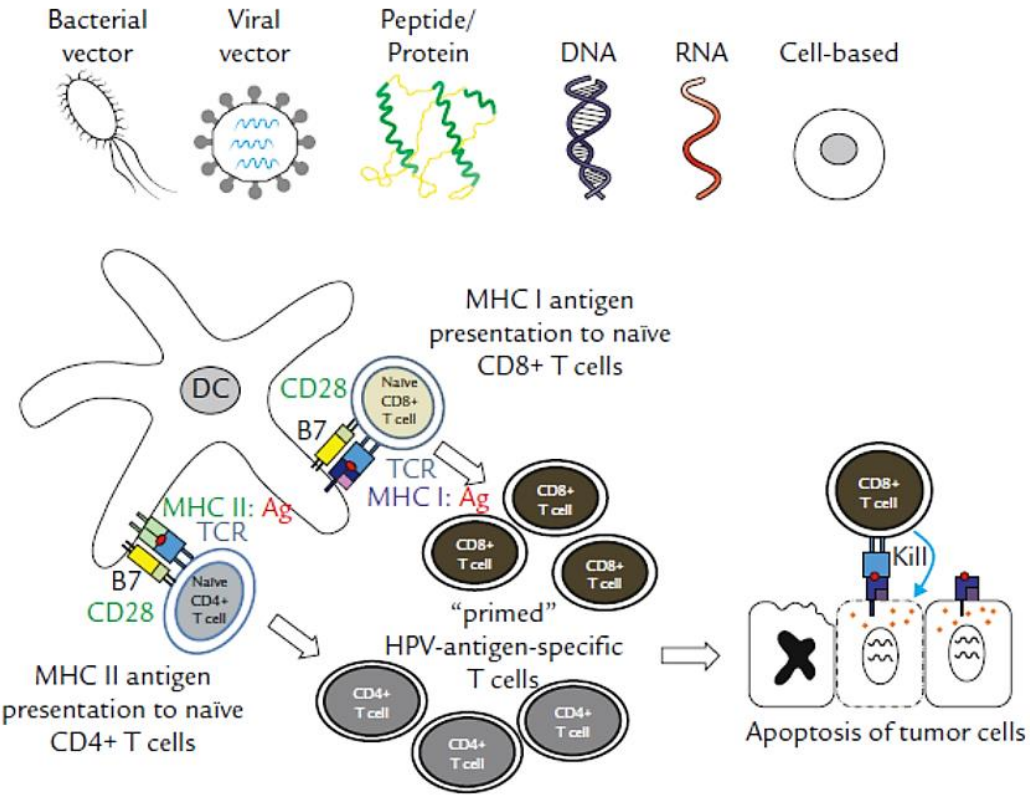


Figure 1.8: Methodologies for production and delivery of HPV therapeutic vaccines and their immunological activity. HPV E6 and/or E7 antigens have been developed and delivered using live vector-based vaccines, peptide/protein-based vaccines, nucleic acid-based vaccines and cell-based vaccines. Dendritic cells (DCs) prime naïve T cells through MHC: Antigen (Ag) complex with the help of costimulatory molecules (B7 on the DC and CD28 on the T cell). Antigens are processed and presented to CD4+ T cells via MHC class II pathway and presented to CD8+ T cells via MHC class I pathway. The primed effector T cells are subsequently HPV-antigen-specific T cells. Activated CD8+ T cells kill tumour cells by inducing apoptosis in the target cells. Induction of CD4+ T cell help can augment the CD8+ T cell immune response, supplementing tumour killing. Image from (Lin et al., 2010).

There is no licenced HPV therapeutic vaccine on the market to date. The progression of candidate vaccines to clinical trial is discussed below.

1.5.1. Live vector vaccines

Live vector vaccines are bacterial or viral vectors that replicate in the host cells and result in the spread of the antigen. Antigens are delivered to antigen presenting cells (APCs) and presented via major histocompatibility complex (MHC) class I or II complexes, inducing CD8+ or CD4+ effector T cells (Figure 1.8).

1.5.1.1. Bacterial vectors

Bacterial vector species include *Listeria monocytogenes* (Sewell et al., 2008; Souders et al., 2007), *Lactobacillus casei* (Kawana et al., 2014), *Lactobacillus lactis* (Bermúdez-Humarán et al., 2004) and *Salmonella* (Krul et al., 1996). *Listeria*-based vaccines were first used in clinical study by Maciag et al. (2009). *Listeria* is a promising vector due to properties such as its ability to infect macrophages and evasion of phagosomal lysis through secretion of listeriolysin O (LLO) (Schnupf and Portnoy, 2007). A phase I clinical trial of an E7-based vaccine called Lm-LLO-E7 in 15 patients with metastatic or advanced cervical cancer, showed an increase in E7-specific IFNγ+ T cells in 3 patients and reduction in tumour size in 4 patients (Maciag et al., 2009). Based on this study, design and planning of additional phase I and/or II clinical trials in patients with metastatic anal cancer, SCC of the rectum, metastatic cervical cancer, head and neck cancer or squamous or non-squamous cell carcinoma or the cervix, are in development (Yang et al., 2016). Oral administration of a *L. casei* bacterial vector vaccine, GLBL101c (modified HPV-16 E7), was recently tested in a phase I/IIa clinical trial in 17 patients with HPV16+ CIN3 and showed a significant increase in E7 cell mediated immunity, with 9 patients showing regression to CIN2, and 5 progressing to LSIL. No adverse side effects were experienced by any patients and this study was the first report of a therapeutic HPV vaccine to induce anti-neoplasm mucosal immunity (Kawana et al., 2014).

1.5.1.2. Viral vectors

Similarly, the efficacy of viral vector vaccines has been investigated in clinical trials. Recombinant modified vaccinia virus viral vectors based on HPV-16 or -18 E6 or E7 (TA-HPV) showed HPV-specific cytotoxic T lymphocyte (CTL) responses in 28% of patients with advanced cervical cancer in a phase I/II study (Borysiewicz et al., 1996; Kaufmann et al., 2002), and at least a 40% reduction in lesions in 83% of patients (aged 42-54) with high-grade vulval or vaginal intraepithelial neoplasia in a phase II study (Baldwin et al., 2003). Most recently, a phase III study using a vaccine based on HPV-16 E2 (MVA E2) was shown to have 90% efficacy in the treatment of HPV-induced anogenital

intraepithelial lesions in 1356 male and female patients (Rosales et al., 2014). Additionally, all males showed complete eradication of lesions, and HPV-specific CTL T cell responses were observed. E2 is a protein inhibitor for the expression of E6 and E7 (Doorbar, 2016; Doorbar et al., 2012), and has been shown to arrest cell growth and induced apoptosis of cancer cells (Desaintes et al., 1997), therefore vaccination with E2 may suppress E6 and E7 activity in the infected host, thereby reducing the transformation ability of infected cells and survival of HPV tumour cells.

However, there remain challenges in the use of live vector vaccines due to potential dominance of the immune response to the viral vector instead of the HPV antigen, pre-existing immunity and the generation of NAbs which restrict repeated therapy (Lin et al., 2010).

1.5.2. Peptide and protein-based vaccines

Peptides and proteins from HPV antigens (mainly E6 and E7) are taken up by dendritic cells (DCs), processed and expressed via MHC I and II complexes to appropriate effector cells (Figure 1.8).

1.5.2.1. Peptide vaccines

Peptide vaccines are stable safe and easy to produce; however, they are often MHC specific and need to match the patient's human leukocyte antigen (HLA) type for effective presentation, therefore immunogenic epitopes need to be identified for each individual making large scale production and treatment of disease more difficult (Kast et al., 1994; Su et al., 2010). In addition, they have poor immunogenicity and require administration with molecules or adjuvants such as cytokines and Toll-like receptor (TLR) ligands, to improve vaccine potency for strong CD8+ T cell responses (Lin et al., 2010; Yang et al., 2016). The use of overlapping long peptide vaccines that contain E6/E7 peptides to overcome HLA specificity has been shown to improve T cell responses in preclinical models (Vambutas et al., 2005; Zwaveling et al., 2002), and several vaccines have advanced to clinical trials showing strong HPV-specific T cell responses. A phase II clinical trial using the HPV-16 synthetic long-peptide vaccine (HPV-16 SLP – E6 and E7 overlapping peptides plus adjuvant) showed that women positive for HPV-16 VIN 3 induced the activation of CD4+ T cells and CTL responses, with 5 patients showing complete regression of lesions (Kenter et al., 2009). Another phase II study showed lasting HPV-16 specific T cell responses (up to 1 year) and that low-dose vaccinations were significant in generating this response (van Steenwijk et al., 2014). A phase I clinical study using PepCan, a vaccine that consist of 4 HPV-16 E6 synthetic peptides and a novel adjuvant Candin, in patients with HSIL showed that 45% of patients had regression of disease, and

significant decreases in viral load (Coleman et al., 2016). Additional trials are in progress to assess the efficacy of these and other peptide-based vaccines (Yang et al., 2016). The future of peptide-based vaccines is dependent on their immunogenicity and antigen presentation.

1.5.2.2. Protein-based vaccines

Subunit protein-based vaccines contain all antigenic HLA epitopes therefore are not MHC restricted like peptide vaccines; however, they show low immunogenicity and promote antibody responses over T cell responses due to presentation to the MHC II complex (Su et al., 2010). Efforts to increase immunogenicity and presentation to the MHC I pathway and activation of CD8+ T cells include creation of fusion proteins to target antigen to DCs and the use of adjuvants. TA-CIN is a subunit vaccine (fusion protein composed of HPV-16 L2, E6, and E7) and has been proven safe in a number of clinical trials (Davidson et al., 2004; de Jong et al., 2002; van der Burg et al., 2001). In a phase II trial treating VIN2/3, it was shown that after vaccination with TA-CIN there was an increase in CD4+ and CD8+ T cells and complete regression of VIN in 63% of patients 1 year after vaccination (Daayana et al., 2010). Fusion proteins targeting proteins to the endoplasmic reticulum (ER) have also shown improved CTL responses. A HPV-16 E7 fusion peptide (The Vax Genetic Vaccine Company), with GP100 adjuvant, demonstrated a strong HPV-specific E7 CTL response and protected mice against tumour challenge (Skeate et al., 2016). A phase IIa clinical trial is currently underway in patients with HSIL (NCT02576561). A HPV-16 E6/E7 fusion protein mixed with ISCOMATRIX adjuvant was immunogenic in a phase I study, showing a significant increase in E6 and E7 specific CD8+ T cell responses in patients with CIN compared to placebo recipients (Frazer et al., 2004). A fusion protein, HspE7 (HPV-16 E7 and Hsp65 from *Mycobacterium bovis*) showed complete regression of CIN 3 in 22% of patients in a phase II trial (Chu et al., 2000). Several other clinical trials are ongoing testing the potential of therapeutic protein vaccines (Yang et al., 2016). Overall, the enhancement of immunogenicity and CD8+ T cell responses is key to the future of protein-based vaccines.

1.5.3. Nucleic acid vaccines

1.5.3.1. DNA vaccines

DNA vaccines are safe, easy to manufacture and purify, promote MHC I antigen presentation and unlike live vector and protein vaccines, do not produce NAbs to the vector, allowing for repeated vaccination (Lee et al., 2016). DNA vaccines have extensively been studied and proven to be safe in several clinical studies. The risk of DNA plasmids integrating into the host genome for HPV vaccines

in particular has been addressed with the use of modified E6 and E7 genes that do not encode proteins with oncogenic transformation properties. However, the vaccines have been found to be poorly immunogenic. To increase their potency, strategies such as increasing the number of antigen expressing/antigen-loaded DCs, improving antigen presentation and processing, and enhancing DC and T cell interaction have been developed (Hung and Wu, 2003; Tsen et al., 2007). Examples of DNA vaccines include pNGVL4a-CRT/E7(detox) (HPV-16 E7 fused to calreticulin) in which a phase I trial showed that 30% of patients with HPV-16+ CIN 2/3 regressed to CIN 1 or less and more CD8+ T cells were induced when vaccination was via intralesional injection (Alvarez et al., 2016). Another phase I trial has recently been completed to evaluate the safety and immunogenicity of pNGVL4a-CRT/E7(detox) (NCT00988559).

Fusion of signal sequences targeting protein to the secretory pathway can enhance vaccine potency and promote antigen presentation. GX-188E (Genexine, Inc.), is a DNA vaccine of HPV-16/18 E6/E7 fused Flt3L (Fms-like tyrosine kinase-3 ligand extracellular domain) and signal sequence of plasminogen activator (tpa). A phase I study in 9 patients with HPV-16/18+ CIN 3 showed HPV-specific polyfunctional CD8+ T cell responses, with 7 patients showing complete lesion regression at the end of the study (Kim et al., 2014). A phase II trial in women with HPV-16/18+ CIN 3 lesions (NCT02139267) has been completed and another phase II trial in women with HPV-16/18+ CIN 2, CIN2/3 or CIN 3 (NCT02596243) is ongoing with expected completion in 2018. A phase Ib/II trial to investigate the safety and efficacy of GX-188E administered intramuscularly with local administration of immunomodulators GX-I7 or Imiquimod in women with CIN 3, is currently recruiting participants (NCT03206138).

Enhanced delivery methods such as microencapsulation or electroporation can increase the number of antigen-expressing/antigen-loaded DCs (Huang et al., 2010). VGX-3100 (Inovio Pharmaceuticals, Inc.), is a DNA vaccine based on HPV-16/18 E6/E7, is delivered via intramuscular injection followed by electroporation for the delivery of a small electrical charge. A phase I clinical trial in 18 patients who had been previously treated for CIN 2/3, showed that 14 patients (78%) induced HPV-specific CD8+ T cell responses, 17 patients (94%) had increased HPV-16 E7 antibody titres and all patients had increased HPV-18 E7 antibody titres. Furthermore, 12 patients (67%) and 7 patients (39%) had increased HPV-16 E6 or HPV-18 E6 antibody titres, respectively (Bagarazzi et al., 2012). A follow-up phase IIb trial was conducted based on the robust antigen-specific immune responses observed and the potential to contribute to the eradication of HPV-infected cells and lesion regression. In a randomized, double blind, placebo controlled study, patients with CIN 2/3 lesions showed greater HPV-specific humoral and T cell immune responses and lesion regression accompanied by viral

clearance (Trimble et al., 2015). VGX-3100 is the most successful DNA vaccine to date and is being tested in a phase I/IIa trial in patients with HPV- associated head and neck cancer (NCT02163057) (Aggarwal et al., 2015), as well as a phase I/IIa trial in women with new, recurrent or persistent cervical cancer (NCT02172911). A phase III randomized, double blind, placebo controlled study in women with confirmed CIN 2 or 3 is expected to start in 2017 (NCT03185013) (Kim, 2017).

1.5.3.2. RNA vaccines

RNA vaccines have similar properties to DNA vaccines in that they are safe, do not generate NAbs and therefore can be administered multiple times. Additionally, they can replicate in various cell types (Varnavski et al., 2000) and pose no risk of chromosomal integration or cellular transformation. However, they are difficult to make, are unstable, and also cannot spread intracellularly. Naked RNA replicon vaccines can be derived from RNA viruses e.g. Sindbis virus (Cheng et al., 2002) and Venezuelan equine encephalitis virus (Cassetti et al., 2004). RNA replicons are capable of self-replication, resulting in sustained antigen expression and thus an increase in immunogenicity (an advantage over DNA vaccines) (Lin et al., 2010). However, the unstable nature of RNA replicons does not make them ideal vaccine candidates. Suicidal DNA vaccines, a combination of the RNA replicon and DNA vaccines, have been designed to overcome this problem. Suicidal DNA is translated into RNA in transfected cells and triggers apoptosis, ensuring genomic integration cannot occur. However, apoptosis results in poor immunogenicity of DCs in cells transfected with the replicons (Yang et al., 2016). The inclusion of an anti-apoptotic gene in suicidal DNA to enhance survival of APCs has been described by Kim et al. (2004) as well as the use of flavivirus Kunjin (KUN) vector (Herd et al., 2004; Varnavski et al., 2000), which allows and prolongs direct presentation by transfected DCs. The latter approach elicited E7-specific T cell responses and was protective in mice challenged with an E7-expressing tumour (Herd et al., 2004).

RNA vaccines in other cancers have progressed to clinical trials (Sebastian et al., 2014); however, there remains a lot of work to be done in the development of HPV RNA replicon vaccines. To date no RNA HPV vaccine candidate has progressed to clinical trial.

1.5.4. Cell-based vaccines

Cell based vaccines involve the isolation of target cells such as DCs or T cells from the patient, manipulation *ex vivo*, and transfer back to the patient for treatment.

1.5.4.1. Dendritic cell-based vaccines

DCs are the major APCs and can efficiently present antigens that activate CD4+ and CD8+ T cells. DCs are advantageous as they can act as natural adjuvants and increase the potency of the specific antigen (Santin et al., 2005). DCs can either be loaded with HPV-specific peptide/protein antigens or transduced to express antigen vaccines, which are then delivered back to the patient (Palucka and Banchereau, 2014). A phase I study with full length HPV-16 and -18 E7 and KLH, showed an increase in E7-specific CD4+ T cells in patients with stage Ib or IIa cervical cancer and 8 out of 10 patients showed E7-specific CD8+ T cells (Santin et al., 2008). Additionally, the vaccine was well tolerated in all patients. In a similar study of DCs loaded with HPV-16 or -18 E7 co-administered with IL-2, specific CD4+ T cell responses were detected in 2 out of 4 patients and E7-specific CD8+ responses observed in all patients (Santin et al., 2006).

However, DC-based vaccines have several limitations as they are restricted in their capacity for large-scale production due to the requirement of sufficient DCs from each patient, do not have a defined route for vaccination (critical for priming of T cells), and they have a limited lifespan due to T-cell mediated apoptosis (Skeate et al., 2016; Yang et al., 2016). To address their short life span, short interfering RNA (siRNAs) targeting pro-apoptotic molecules have been explored, which have shown enhanced E7-specifc CD8+ activation and anti-tumour effects in mice (Ahn et al., 2015; Kim et al., 2009; Peng et al., 2005).

1.5.4.2. Adoptive cell transfer

Adoptive cell transfer (ACT) involves the generation of antigen-specific CTLs *ex vivo*, which are then used *in vivo* to enhance immunogenicity. This technique is advantageous as it generates antigen-specific CTLs that can be produced in large quantities *in vitro*; CTLs can be engineered or activated *ex vivo*; and it allows for manipulation of the host before cell transfer to eliminate suppressor cells (e.g. regulatory T cells, Tregs) (Skeate et al., 2016). A pilot study in 9 patients with metastatic cervical cancer showed complete regression in 2 patients after treatment with HPV -16 E6 and E7 reactive CTLs (Stevanović et al., 2015). In another study, T cell receptors against E6 were introduced into CTLs, which killed HPV+ cells from cervical and head and neck cancer cell lines (Draper et al., 2015) and a phase I/II clinical trial has recently been completed (NCT02280811). However, it is thought that CTLs alone may not be enough to eliminate cancer cells in patients with advanced disease, as HPV cancers evade the immune system by mechanisms that remain unknown (Skeate et al., 2016).

1.5.5. Combinational approach

1.5.5.1. Prime-boost regimens

Prime-boost regimens can be used to enhance vaccine potency: for example, one may prime the immune system with a DNA-based vaccine, followed by a boost with a virus-based vaccine. Greater CD8+ T cell responses have been observed in mice primed with a HPV-16 E6/E7 DNA vaccine and boosted with recombinant HPV-16 E6/E7 vaccinia (Chen et al., 2000a) or adenovirus (Wlazlo et al., 2004) produced vaccines compared to a DNA vaccine only.

Evaluation in clinical trials of prime-boost regimens has also been explored. In a phase II clinical trial, TA-CIN fusion protein vaccine was boosted with recombinant vaccinia virus TA-HPV in 29 patients with anogenital intraepithelial neoplasia, with 5 patients showing increased HPV-16 antigen-specific T cell mediated immune responses (Fiander et al., 2006; Smyth et al., 2004). However, this result was not significant over TA-HPV vaccination alone. In another study, 10 patients with HPV-16+ high grade VIN were this time primed with TA-HPV and boosted with TA-CIN. Nine patients showed HPV-16 specific T-cell responses and 3 patients had significant reduction in lesions. However, these results did not show a direct correlation between clinical and immunological responses (Davidson et al., 2004). More recently in a phase I trial, the use of pNGVL4a-sig/E7(detox)/HSP70 DNA prime followed by TA-HPV boost was shown to elicit systemic HPV-specific CD8 T-cell responses in women with CIN 2/3 that could traffic to the lesion and regression was observed in some patients (NCT00788164).

1.5.5.2. Tumour microenvironment

Vaccines with immunomodulatory agents that influence the tumour microenvironment can increase the success of therapeutic vaccines. There are several targets for immune modulation including Tregs, tumour-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) (Sica and Bronte, 2007). For example, Tregs release immunosuppressive cytokines (e.g. IL-10) and transforming growth factors which can affect T cell function. Depletion of CD4+ and CD25+ Tregs have been shown to enhance E7-HSP70 vaccine potency (Chuang et al., 2009a). Therefore, controlling the effects of these factors could deprive tumour cells of important growth factors and enhance the antitumour response of therapeutic vaccine candidates (Kim et al., 2006; Pittet, 2009; Sica and Bronte, 2007).

1.5.5.3. Therapeutic vaccines with other therapies

Therapeutic treatments such as chemotherapy and radiotherapy have been used in conjunction with candidate therapeutic vaccines. Apigenin, a chemotherapeutic agent that induces tumour cell death was used in conjunction with a HPV-16 E7 DNA vaccine fused to HSP70, in mice with E7-expressing tumours. Results showed enhanced E7-specific CD8+ T cell responses and potent antitumour effects in the combination group versus E7-HSP70 vaccine only (Chuang et al., 2009b). Similarly, low dose radiotherapy combined with CRT/E7(detox) showed the highest E7-specific CD8+ T cell responses in mice and was significantly enhanced when both radiation, chemotherapy and CRT/E7(detox) were used (Tseng et al., 2009). Clinically, in a phase II trial, women with VIN 2 and 3 were treated with the topical immunomodulator imiquimod, followed by 3 doses of TA-CIN. One year after the last immunization, 63% of patients showed lesion clearance, and had increased CD4+ and CD8+ T cells compared to non-responders. In addition, 36% of patients showed HPV-16 clearance and 79% were symptom-free (Daayana et al., 2010).

1.6. Combination prophylactic and therapeutic vaccines

Combination vaccines would be beneficial in providing immediate impact and long-term protection through mass immunization of pre-adolescents and older women who have previously been exposed to HPV infection (Schiller and Davies, 2004). It would be particularly impactful in low-income countries where vaccines are expensive and screening programmes are limited or not available. cVLPs and PsVs delivering a DNA vaccine are strategies to produce a combination vaccine.

1.6.1. Chimaeric vaccines

Several studies have developed L1/E7 chimaeras where full length or N-terminal regions of E7 have been fused to the L1 C-terminal (Bian et al., 2008; Freyschmidt et al., 2004; Jochmus et al., 1999; Kaufmann et al., 2001; Kaufmann et al., 2007; Kuck et al., 2006; Müller et al., 1997; Schäfer et al., 1999) or fused to the C-terminal of L2 to create L1/L2/E7 chimaeras (Da Silva et al., 2003; Greenstone et al., 1998; Qian et al., 2006; Rudolf et al., 2001; Wakabayashi et al., 2002).

A clinical study in 39 women with CIN 2/3 by Kaufmann et al. (2007) showed that vaccination with L1:E7 cVLPs induced high anti-L1 antibody titres and low anti-E7 antibody titres, in addition to cellular immune responses to both L1 and E7. Improvement to CIN1 or normal tissue was seen in 39% of patients compared to 25% in the placebo group, with 56% of patients being HPV-16 DNA

negative at the end of the trial. However, clinical efficacy was not significant. Bian et al. (2008) showed that capsomeres can have prophylactic and therapeutic efficacy in mice. The L1:E7 capsomeres were able to induce NAbs against VLPs, elicited L1- and E7-specific cell-mediated responses and protected mice from tumour challenge. Chimaeric HPV-16 VLPs displaying full length HPV-16 E7 and E2 (VLP-E7E2) have also been tested in HLA-A2 transgenic mice with different immunomodulators. E7 CTL responses were significantly enhanced when mice were vaccinated with VLP-E7E2 and coinfected with GC-CSF (Granulocyte macrophage colony-stimulating factor) and anti-CD40 antibodies; however, low CD8+ T cell responses to L2 were detected (Qian et al., 2006). A prime-boost with VLP-E7E2 and E2 peptides was performed to enhance the E2 T cell response and showed an improved E2 CTL response compared to immunisation with E2 peptides alone. Additionally, anti-L1 antibodies were detected and titres were similar in mice immunized with VLP-E7E2 with/ without immunomodulators (Qian et al., 2006).

The subunit vaccine TA-CIN as discussed earlier, has also been shown to induce HPV-16 specific T-cell responses (de Jong et al., 2002); however, no reliable antibody titres could be determined.

1.6.2. PsVs in gene delivery

PsVs have emerged as a means for DNA and RNA gene delivery into several cell types and tissues. To this end, PsVs have the potential to be used in several applications such as virion characterisation, *in vitro* and *in vivo* measurement of protective NAb titres and use in medical applications e.g. gene therapy (Ma et al., 2011). PsVs have several advantages as they have been shown to act as adjuvants and can facilitate the activation and maturation of APCs such as DCs (Lenz et al., 2001; Peng et al., 2010; Rudolf et al., 2001; Yang et al., 2004b), and do not have safety concerns, unlike live vector vaccines (Peng et al., 2010). Packaging of plasmid DNA allows for stimulation of both humoral and cellular immunity (Gurunathan et al., 2000). *In vivo*, PsVs have been used in gene therapy experiments for ovarian cancer. HPV-16 PsVs were used to deliver a herpes simplex thymidine kinase (HSV-tk) gene to ovarian tumour cells in mice and were shown to have antitumour effects (Hung et al., 2012). Delivery of the model ovalalbumin (OVA) antigen in HPV-16 PsVs was also shown to generate OVA-specific CD8+ T cells immune responses in mice, the highest number of OVA-specific CD8+ T cells compared to DNA delivery by other methods (Peng et al., 2011; Peng et al., 2010), and potent neutralising responses when DNA was co-administered with capsid proteins (Yang et al., 2015). In another study, the delivery of genes to the vaginal epithelium where majority of HIV infections occur was demonstrated by Gordon et al. (2012). Simian immunodeficiency virus (SIV) Gag DNA in HPV-16, -45 and -58 PsVs was delivered to the vaginal tract of macaques and induced Gag-

specific antibodies in blood, the vaginal mucosa and draining lymph nodes. Mucosal delivery of M and M2 genes of the respiratory syncytial virus (RSV) encapsidated in HPV-16 PsVs has also been shown to elicit local and systemic M/M2-specific CD8+ T-cell responses in mice, that were comparable to a 10 000-fold higher dose of naked DNA (Graham et al., 2010).

Alternative approaches using short hairpin RNA (shRNA) or microneedles have also been reported. HPV-31 PsVs encoding shRNA against E6 or E7 were used to silence E6 or E7 expression in cervical carcinoma cells. shRNA was delivered to CaSki and TC1 HPV-positive cells and resulted in the degradation of E6 and E7 mRNAs, with more significant cell death observed when E7 expression was suppressed, and *in vivo* testing in mice resulting in the dramatic inhibition of tumour growth (Bousarghin et al., 2009). The use of microneedles to deliver HPV-16 PsV-encapsidating M/M2 and F protein of RSV was recently described by Kines et al. (2015). Post-immunization, dose-dependent HPV and F-specific responses were detected, and M/M2-specific T-cell responses were detected post RSV challenge. The authors also suggested that microneedle delivery of lyophilized PsVs could provide a thermostable combined vaccine approach for use in clinical evaluation, as microneedles are safe, strong, dissolvable and highly reproducible (Kim et al., 2012b).

1.7. Reducing vaccine production cost

Developing countries account for the majority of HPV-related disease and death. Despite the success of current prophylactic vaccines, there is still an urgent need for vaccines that are more affordable. Plant-made vaccines have been said to have the potential to increase global health in the context of the 2015 Millennium Development Goals and beyond (Penney et al., 2011).

1.7.1. Advantages of plant-based systems

Plants offer several advantages for the expression of heterologous proteins due to their scalability, rapid production and low risk of contamination (Fischer et al., 2004; Merlin et al., 2014; Rybicki, 2010), compared to traditional microbial fermentation or mammalian/ insect cell expression systems. Additionally, they contain the necessary eukaryotic machinery for protein modification such as protein folding and glycosylation (Fischer et al., 2004). The scalability and rapid production for the emergency response to influenza outbreaks has been demonstrated by Medicago Inc. in the production of a plant-produced H7N9 influenza VLP vaccine. The vaccine was available in 3 weeks from the date of release of the viral sequence, in comparison to the 4-6 months required for

traditional egg-based vaccine production (World Health Organisation, 2009). Further, Medicago Inc. have also shown the ability to produce 10 million doses of an H1N1 VLP vaccine in approximately 1 month (Rybicki, 2014). It has been estimated that there could be a 31% reduction in vaccine cost, particularly associated with upstream processes, by using plants for production of vaccines (Rybicki, 2009).

1.7.2. Plant expression systems

Over the last 30 years, several biological products, vaccines and therapeutics have been produced in plant-based systems (Ma et al., 2003; Rybicki et al., 2012; Scotti and Rybicki, 2013). Production of a pharmaceutically relevant protein in plants was first described by Barta et al. (1986) in the production of chimaeric human growth hormone in transgenic tobacco and sunflower. This was followed by production of fully-assembled MAbs (Hiatt et al., 1989) and hepatitis B surface antigen (Mason et al., 1992) in transgenic tobacco. Since then, several classes of recombinant proteins including: MAbs, vaccine antigens, enzymes and biopolymers (Tiwari et al., 2009; Xu et al., 2012), have been produced.

Several approaches have been developed for the expression of heterologous proteins in plants. Plant expression systems can be divided into transgenic (stable) or transient expression (Figure 1.9) (Obembe et al., 2011; Scotti and Rybicki, 2013). Transgenic expression requires the integration of a transgene into the nuclear or plastid genome of plants. This is via transformation with the soil pathogen *Agrobacterium tumefaciens*, or biolistics (e.g. microparticle bombardment) (Hansen and Wright, 1999; Lessard et al., 2002).

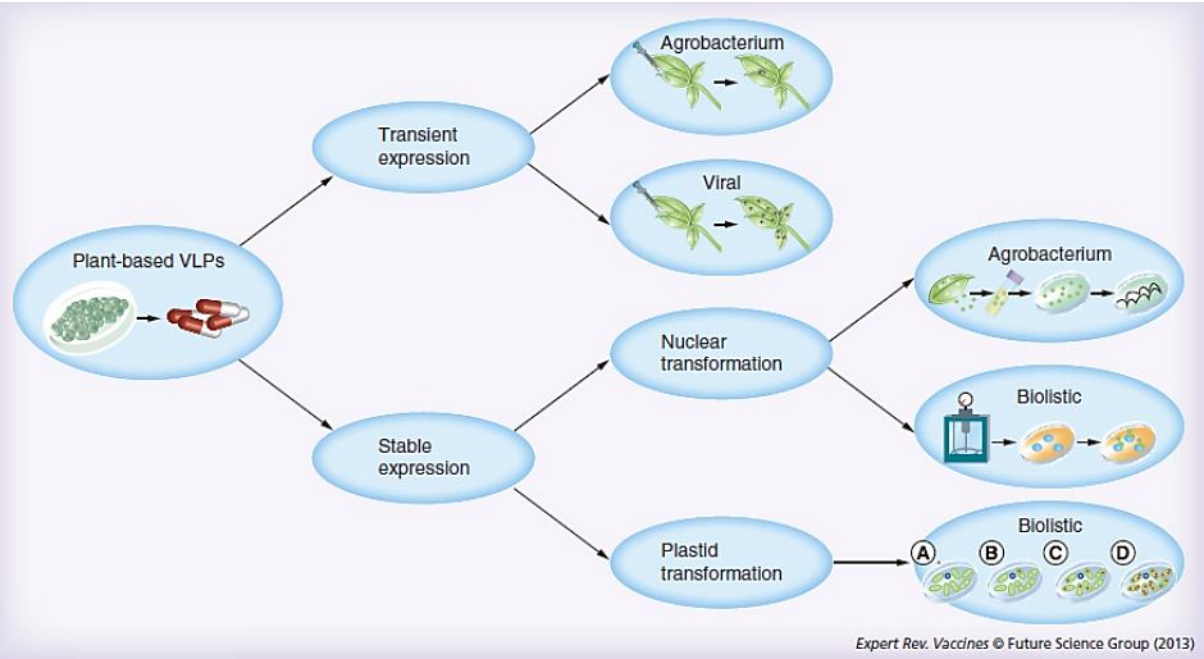


Figure 1.9: Summary of methods for the expression of heterologous proteins in plants. Image from Scotti and Rybicki (2013).

Transient expression of recombinant protein is mainly through *Agrobacterium*-mediated gene transfer, where a transferred DNA (T-DNA) from the tumour inducing (Ti) plasmid is transferred into the plant host genome with the help of *vir*ulence (*vir*) genes (Tzfira et al., 2004). This system is advantageous as it allows for rapid protein production several days after cloning (compared to months needed for transgenic expression), plants are not affected by transgene insertions, and higher protein expression levels have been obtained compared to transgenics (Fischer et al., 2004; Kapila et al., 1997). Virus-based transient expression is another method for transient expression and allows for systemic spread of the virus. It involves the use of infectious modified plant viruses (e.g. geminiviruses, tobomaviruses and bromoviruses (Komarova et al., 2010)) to deliver a transgene to the cell, without the need for stable integration (Porta and Lomonossoff, 2002). However, this method shows low infectivity. This method was improved upon by using *A. tumefaciens* as a vector to deliver viral replicons. The MagnICON® system described by Gleba et al. (2005), enables amplification of the viral replicons and has been shown to produce very high protein yields (up to 80% total soluble protein) (Marillonnet et al., 2004). Our research group has also developed a self-replicating viral vector derived from the bean yellow dwarf mastrevirus (BeYDV; family *Geminiviridae*), which has been shown to significantly increase protein expression up to 50% (Regnard et al., 2010).

Additionally, *in vitro* cell culture systems where plant biomass is cultured under sterile conditions have been used to produce recombinant proteins. Expression of proteins can be transgenic or transient as mentioned above, with the latter performed by co-cultivation with *A. tumefaciens* or particle bombardment (Hellwig et al., 2004; Xu et al., 2012). This system is advantageous in that production can occur under Good Manufacturing Practice (GMP) conditions; cultivation media are also very simple and cheap; however, in terms of infrastructure cost there is no advantage over other cell culture systems (Rybicki, 2009). Regardless, it is a highly successful method and to date one cultured plant cell-made product has been approved by the Food and Drug Administration (FDA) for use in humans – Protalix's carrot cell-produced Gaucher disease therapeutic enzyme glucocerebroside (ELELYSO™) (Shaaltiel et al., 2007). GMP procedures have recently been established on transgenic and transient expression systems for recombinant protein production; this should lead to an increase in the number of plant-made vaccines and products that enter clinical trials (Fischer et al., 2012).

Low yields of recombinant protein have been reported using plant expression systems, especially when transgenic systems are used, but also with transient systems. However, several methods to address this issue have been developed. These include the use of strong plant promoters to enhance transcription, such as the cauliflower mosaic virus 35S (CaMV 35S) or maize ubiquitin-1 (*ubi*-1) promoters (Obembe et al., 2011; Twyman et al., 2003); codon-optimisation of the gene of interest (Biemelt et al., 2003; Maclean et al., 2007; Pereira, 2008), as codon bias may be rate-limiting in translation (Gustafsson et al., 2004); co-expression of silencing suppressor proteins to enhance transcript stability and transgene expression (Takeda et al., 2002; Voinnet et al., 2003); subcellular targeting of recombinant proteins (Maclean et al., 2007; Twyman et al., 2003), which can improve the yield and stability of proteins; and fusion of signal sequences for targeting and/or retention in secretory pathways (Franconi et al., 2006; Torrent et al., 2009). Expression of the Zera® domain (Zip® Solutions, Spain), derived from the γ-zein storage protein in maize kernel, has been shown to result in the high-level accumulation of recombinant proteins in the ER in large, stable protein bodies that are protected from degradation and are easy to purify (Geli et al., 1994; Torrent et al., 2009; Whitehead et al., 2014).

1.7.3. Plant-derived products

The choice of host for production of recombinant proteins is important for success as economic factors such as total biomass, set up costs, value of protein of interest, downstream processing cost, and many more need to be considered (Fischer et al., 2004; Schillberg et al., 2005). Plant-derived

products have been produced in several hosts including: tobacco, maize, spinach, lettuce, alfalfa, tomato, potato, *Arabidopsis*, rice and duckweed (Chen and Lai, 2013; Obembe et al., 2011); however, generic tobacco (*Nicotiana* spp.) remains the preferred plant species for molecular farming due to its high biomass yield, well established expression protocols and non-food status, so has a minimal risk for contamination of food supplies (Biemelt and Sonnewald, 2005; Stoger et al., 2002).

Nicotiana benthamiana is routinely used in transient expression of recombinant proteins for both developmental research and large-scale production (D'Aoust et al., 2010; Goodin et al., 2008; Lomonossoff and D'Aoust, 2016). Several GMP-compliant pharmaceutical products using *N. benthamiana* are in clinical trials (Fischer et al., 2012), and include influenza virus VLP vaccine (Medicago Inc.) (D'Aoust et al., 2010), influenza virus haemagglutinin (HA) subunit vaccine (Fraunhofer CMB) (Shoji et al., 2011; Shoji et al., 2015) and MAPP66 – Herpes simplex virus (HSV)/ HIV microbicide (Bayer/ ICON). The most recent development, however, was the use of ZMapp, the experimental cocktail of three chimaeric MAbs used for the treatment of Ebola. It was approved for use by the FDA during the 2014 Ebola outbreak in West Africa, after having shown positive results in a study using non-human primates (Qiu et al., 2014). Transient expression in *N. benthamiana* allowed for the rapid production of ZMapp. The cocktail is being developed further by Leaf Biopharmaceutical (subsidiary of Mapp Biopharmaceutical) (Hiatt et al., 2015).

1.7.4. VLPs in plants

VLPs are a powerful tool as vaccine candidates or as drug delivery vehicles as they maintain the morphology of the native virion (lacking infectious genetic material) and retain and display the immunogenic antigens required to elicit a strong immune response. VLPs can stimulate both the humoral and cell-mediated responses as antigens are efficiently taken up by APCs and presented by both MHC I and II molecules, inducing potent CD4+ and CD8+ responses (Chackerian, 2007; Grgacic and Anderson, 2006; Kushnir et al., 2012; Schirmbeck et al., 1996). VLPs of Hepatitis B virus (HBV) (Mechtcheriakova et al., 2006), BPV (Love et al., 2012), Bluetongue virus (BTV) (Thuenemann et al., 2013; van Zyl et al., 2016), Norwalk virus (NV) (Mason et al., 1996; Mathew et al., 2014), Influenza virus (D'Aoust et al., 2008; Landry et al., 2010; Shoji et al., 2011) and most recently African horse sickness virus (AHSV) (Dennis et al., 2017), have successfully been produced in plants and have been shown to elicit positive immune responses in animals and in some instances protection from virus challenge (D'Aoust et al., 2008; Landry et al., 2010; Shoji et al., 2013; Thuenemann et al., 2013). cVLPs of West Nile virus (WNV), HIV and Hepatitis B small antigen (HBsAg) have also been expressed in plants, with an additional 15+ studies showing the use of plant viruses as carriers for antigens of

Foot and mouth disease virus (FMDV), Hepatitis C virus (HCV), HIV, Rabies virus (RV), Canine parvovirus (PV), CRPV, mouse hepatitis virus (MHV) or RSV as vaccine candidates. These studies have shown virus-specific antibodies in animals and strong T cell mediated immune responses (Chen and Lai, 2013; Rybicki, 2014; Scotti and Rybicki, 2013).

Additionally, empty RNA-free Cowpea mosaic virus (eCPMV) VLPs have been produced in *N. benthamiana* (Saunders et al., 2009), with the aim of using them in bio-nanotechnology for applications such as epitope display and cell-specific targeting of drugs (Sainsbury et al., 2011; Wen et al., 2012). eCPMV VLPs have recently been used as targets for tumour immunotherapy and were shown to induce potent anti-tumour immune responses in melanoma, ovarian, colon and breast cancer models (Lizotte et al., 2016).

1.7.5. HPV expression in plants

1.7.5.1. VLPs and PsVs

HPV VLPs have been successfully produced in plants via transgenic (Biemelt et al., 2003; Fernandez-San et al., 2008; Kohl et al., 2007; Lenzi et al., 2008; Liu et al., 2005; Varsani et al., 2003b; Waheed et al., 2011; Warzecha et al., 2003) or transient expression (Maclean et al., 2007; Matic et al., 2011; Pineo et al., 2013; Regnard et al., 2010; Varsani et al., 2006b), and have been shown to be immunogenic and protective in animal models (Kohl et al., 2006). Furthermore, L1:L2 cVLPs produced by Pineo et al. (2013) were shown to assemble into higher order structures, and elicit anti-L1 and -L2 antibody responses which neutralised HPV-16 and -52 PsVs.

HPV PsVs have also been produced in plants for the first time (Lamprecht et al., 2016), and were shown to be capable of infecting mammalian cells, could deliver of a functional reporter plasmid for use in pseudovirion-based neutralisation assays (PBNAs) – the standard in vitro assay used for the detection of HPV-specific antibody responses (Buck et al., 2005a; Pastrana et al., 2004) – and could be neutralised by anti-L1 MAbs. Plant made PsVs could offer a cheaper alternative to current PsV production methods that require cultured mammalian cells and expensive media and transfection reagents. Moreover, plant production is probably far more scalable than mammalian cell production, which involves simultaneous transfection with three plasmids.

1.7.5.2. Therapeutic vaccines

Candidate HPV therapeutic vaccines based on the E7 gene have also been produced in plants and have been shown to elicit humoral and cell-mediated immune responses, and protection against tumour challenge in mice. Franconi et al. (2002) expressed HPV-16 E7 using potato virus X (PVX) vector in *N. benthamiana* and showed E7-specific CTL responses. Mice showed both Th-1 and Th-2 responses, and after challenge with C3 cells, tumour growth was inhibited in 40% of mice. Tumour growth was further inhibited in 80% of mice in another study by the same group, where E7 expression was enhanced 5-fold by targeting to the plant secretory pathway (Franconi et al., 2006). The authors also suggested that plant extracts may have adjuvanting properties as mice showed a stronger immune response when vaccinated with the leaf extracts (without adjuvant) and at a 20-fold lower dose than is known to prevent tumour growth, compared to the positive control group vaccinated with *E. coli* produced E7 and Quil A adjuvant (Franconi et al., 2006). In another study, expression of an HPV-16 E7 fusion mutant, E7GGG (E7 mutant without a Rb binding site fused to *Clostridium thermocellum* β-1,3- 1,4-glucanase) also expressed with PVX in tobacco, showed strong humoral and cell mediated responses and inhibited tumour growth in all vaccinated mice (Massa et al., 2007; Venuti et al., 2009). Furthermore, expression of E7GGG in chloroplasts of the unicellular alga *Chlamydomonas reinhardtii*, protected 60% of mice from tumour growth and slowed tumour growth in mice that did develop tumours compared to the control group (Demurtas et al., 2013). Additionally, the E7 protein in this study was soluble compared to most other E7 proteins that are insoluble, giving it the advantage of more cost-effective downstream processing.

Recently, HPV-16 E7 protein bodies made in *N. benthamiana* were shown to cause tumour regression in mice (Whitehead et al., 2014). A shuffled E7 sequence that has no transformation ability but contains natural CTL epitopes (Ohlschlager et al., 2006)) was fused to Zera®, and stimulated humoral and potent cellular immune responses in a murine model. Results were comparable to a DNA vaccine and the use of Incomplete Freund's adjuvant (IFA) in the presence of Zera® did not enhance immune responses significantly, suggesting Zera® has adjuvating properties (Whitehead et al., 2014).

1.7.5.3. Combination prophylactic and therapeutic VLPs

L1:E6:E7 cVLPs with the potential to be both prophylactic and therapeutic have been produced in transgenic tomato by Paz De la et al. (2009). The cVLPs elicited NAbs (tested in haemagglutination inhibition assays) and elicited cytotoxic T cell responses to L1 and E6/E7 in mice. In a follow up study

looking at the persistence of specific IgG antibodies and the therapeutic potential of L1:E6:E7 cVLPs, IgG antibodies were shown to be persistent for over 1 year and there was a 57% reduction in tumour growth in immunized mice (Monroy-Garcia et al., 2014).

1.8. Project rationale

There is an urgent need to address the high burden of cervical cancer in developing countries. Existing HPV L1 VLP vaccines are expensive, type-restrictive and lack therapeutic efficacy, putting them out of reach where they are most needed. Additionally, the prevalence of HPV types differs geographically, meaning the HPV types included in current vaccines are not all-inclusive or are potentially ill-suited to regions like Africa, where HPV-35 is more important (~5% of ICC) than several of the constituents of Gardasil®9, for example. A low-cost vaccine that is cross-protective against multiple oncogenic HPVs would ideally address this need. The L2 minor capsid protein contains several peptides that are cross-neutralising against several HPV types; however, L2 is subdominant to L1 resulting in low neutralising titres. The display of L2 epitopes on the L1 capsid could facilitate its repetitive display, increasing exposure of L2 to the immune system and generation of potent NAbs. However, prophylactic vaccines to do not induce the regression of pre-existing infections. The development of a combination prophylactic and therapeutic vaccine could provide immediate and long-term protection. The delivery of a DNA vaccine in PsVs could induce potent CD8+ T cell responses as DNA vaccines promote MHC I antigen presentation, while the capsid can elicit the production of NAbs. E6 and E7 are ideal targets for HPV therapeutics due to their constitutive expression in premalignant and malignant tissues. Plants have shown their capability as a vaccine production platform with several GMP-compliant products in clinical trials. Plants offer several advantages over other production systems with the potential to provide low-cost products, that are desperately needed in resource poor settings.

1.9. Study aims

The main aims of this study were the production and evaluation of candidate HPV-16 L1:L2 chimaeric VLP prophylactic vaccines made in *N. benthamiana*, and the design of a self-replicating DNA vaccine for encapsidation in plant-made PsVs as a potential combination prophylactic and therapeutic vaccine. These aims were further broken down into 4 objectives:

1) The first objective of this study was to optimise the transient expression of HPV-16 L1:L2 chimaeras. The L2 peptides were substituted into L1 in the DE loop at position 131 (here called SAC) or between the h4 and β-J structural region at position 431 (called SAE). The L2 peptides are: 108-120 (Kawana et al., 1999), 65-81 (Jagu et al., 2013), 56-81 (Kawana et al., 1998) and 17-36 (Gambhira et al., 2007b). The substitution positions were selected as they have been shown to induce high anti-L1 and L2 responses and have the best potential to form VLPs (Varsani et al., 2003a). All genes were human codon optimised as described by Maclean et al. (2007) for maximum protein expression. Four plant expression vectors were used to compare expression levels using: i) non-replicating vector, ii) non-replicating chloroplast-targeting vector, iii) geminivirus-derived self-replicating vector, and iv) non-replicating vector with a hypertranslational expression system and silencing suppressor on the same plasmid. Assembly of chimaeras into higher order structures was analysed by transmission electron microscopy (TEM).

2) The second objective was the purification of the plant made L1:L2 chimaeras. Several strategies were investigated to purify cVLPs that assembled into higher order structures. The effect of L2 substitution on L1 epitope display was evaluated by indirect enzyme-linked immunosorbent assays (ELISA).

3) Thirdly, to assess the immunogenic potential of candidate L1:L2 cVLPs, mice were vaccinated and anti-L1 and -L2 responses analysed. The cross-protective ability of the cVLPs was determined using the standard L1 and L2-specific PBNAs. Eight HPV types: 6/11/16/18/31/45/52 and 58 were selected for production based on HPVs the L2 epitopes that are known to cross-neutralise.

4) Lastly, I aimed to construct a novel DNA vaccine encapsidated in PsVs as a combination prophylactic and therapeutic vaccine. The E7 gene is an ideal candidate for vaccine therapy due to its role in the inactivation of the pRb tumour suppressor. A shuffled E7 (E7SH) sequence that has no transformation ability but contains natural CTL epitopes (Ohlschlager et al., 2006) was fused to Zera® (Whitehead et al., 2014), a peptide that causes fusion proteins to accumulate at high levels in the ER. A geminivirus-derived (Regnard et al., 2010) self-replicating Zera®E7SH plasmid was constructed using the unique Goldenbraid cloning strategy based on type IIS restriction sites (Sarrion-Perdigones et al., 2011). Plant-made HPV-

16 and -35 PsVs were used to encapsidate and deliver the Zera®E7SH pseudogenome into mammalian cells and expression of the replicons evaluated.

Chapter 2: Transient expression optimisation of HPV-16 L1:L2 chimaeras in *Nicotiana benthamiana*

2.1. Introduction

Cervical cancer is the 4th most common cancer in women globally and results in an estimated 528 000 cases and 266 000 deaths every year (Ferlay et al., 2015). Current vaccines are based on the immunodominant L1 major capsid protein, which has been found to self-assemble into VLPs and produces high titres of NAbs (Christensen et al., 1994; Roden et al., 2000). These vaccines are however type-specific, non-therapeutic and expensive. They are therefore available to a limited population, mainly in developed countries. As of December 2014, 76 countries and territories worldwide have implemented HPV immunisation programs, however only 1% of women in low and low-middle income countries were covered by these programs (Bruni et al., 2016). Although >70% of cervical cancers are caused by HPV-16 and -18, at least 12 other non-cross-protective high-risk HPVs cause cancer (Parkin and Bray, 2006; zur Hausen, 2002) and their prevalence can differ geographically. For example, in Africa, HPV-35 is more common than in Europe and North America (Li et al., 2011; Smith et al., 2007). The most recent prophylactic vaccine Gardasil®9 contains L1 VLPs of 9 HPV types (HPV-6/11/16/18/31/33/45/52/58) (Cuzick, 2015), but this does not include HPV-35. Thus, particularly in an African context, there is a need for more broadly protecting and cheaper vaccines.

The use of the L2 minor capsid protein can potentially address this issue as it has sequence regions conserved across several HPV types, which can elicit antibodies with the ability to cross-neutralise other HPV types (Gambhira et al., 2007b; Kawana et al., 1999; Kondo et al., 2008; Slupetzky et al., 2007). Next-generation vaccines using L2 peptides to generate more cross-protective responses have been investigated by many researchers (Schellenbacher et al., 2017). The N-terminus of HPV-16 L2 has a highly conserved region from aa 1-120 (Lowe et al., 2008), of which 4 L2 cross-neutralising epitopes are of interest in this study: these are peptides spanning aa 108-120 (Kawana et al., 2003; Kawana et al., 1999), aa 56-81 (Kawana et al., 1998; Kondo et al., 2007; Kondo et al., 2008; Slupetzky et al., 2007) and aa 17-36 (Alphs et al., 2008; Boxus et al., 2016; Gambhira et al., 2007b; Kondo et al., 2007; Kondo et al., 2008; Schellenbacher et al., 2009). More recently, a shorter peptide, aa 65-81, has been shown to elicit NAbs that provided significant protection against passive challenge (Jagu et al., 2013). L1:L2 chimaeras that can assemble into VLPs displaying L2 on their surface and are

capable of generating NAbs could be ideal vaccine candidates because they may be cross-protective against several HPV types. This concept has been explored in our laboratory, with Varsani et al. (2003a) using the 108-120 L2 peptide substituted into several L1 locations, and McGrath et al. (2013) and Pineo et al. (2013) substituting additional L2 peptides 56-81 and 17-36 in the h4 helix. The substitution positions of L2 peptides in L1 are important as certain substitutions may affect particle assembly. Varsani et al. (2003a) demonstrated that substitution of L2 108-120 in the DE L1 surface loop and between the β-J structural and h4 region formed recognizable VLPs and capsomeres, substitution into the h4 region formed VLPs in a state of disassembly, and substitution in the CD and EF L1 surface loops formed pentameric aggregates. Therefore, the L1 DE loop and the area between the h4 and β-J structural region, are potential areas for epitope display and were explored in this study.

Plants provide a convenient protein production platform to potentially reduce the cost of vaccine production. Their production is easily scalable, they are eukaryotes that contain the necessary machinery for mammal-like post-translational modification, and they have no risk of contamination by human pathogens (Biemelt et al., 2003; Fischer et al., 2004; Rybicki, 2010). Plant expression systems include transgenic (stable) expression and transient expression systems (Rybicki, 2009). Transient expression of recombinant protein via *Agrobacterium*-mediated transfer is a quick and efficient method, both for small-scale testing of expression, and for full production scale. It involves the transfer of T-DNA from the Ti plasmid into the host cells' nuclei with the help of *vir* genes (Tzfira et al., 2004), where it can be maintained as an episome or – rarely – integrated covalently. This results in rapid protein production several days after induction, with high protein expression levels reported compared to stable transformation (Fischer et al., 2004; Kapila et al., 1997). Virus-based transient expression has also been shown to produce very high protein yields (up to 80% total soluble protein) (Marillonnet et al., 2004; Regnard et al., 2010).

HPV VLPs have previously been transgenically expressed in tobacco, *Arabidopsis*, tomatoes and potatoes (Biemelt et al., 2003; Kohl et al., 2007; Varsani et al., 2003b; Warzecha et al., 2003); however, protein expression yields were low and elicited weak immune responses. Codon-optimisation of HPV-16 L1 (Maclean et al., 2007) and HPV-11 L1 (Mossadegh et al., 2004) has been shown to significantly improve protein expression. Maclean et al. (2007) showed that human codon optimised genes resulted in higher protein accumulation when compared to wild-type and plant codon optimised genes. Additionally, the authors found that signal peptides targeting proteins to different cellular locations can significantly increase protein levels. Chloroplast-targeted HPV-16 L1 was expressed at higher levels compared to cytoplasmic localisation or retention in the ER. L1 of

COPV has also been shown to have higher expression levels when targeted to the chloroplast (Azhakanandam et al., 2007). In an effort to increase protein expression levels, a self-replicating viral vector based on the geminivirus BeYDV has been explored. Regnard et al. (2010) found that an increase in vector replication was associated with an increase in protein expression. When expressing HPV-16 L1 using this vector, there was a 50% increase in L1 expression compared to using a non-replicating vector.

Protein expression levels can be affected by post-transcriptional gene silencing (PTGS), an RNA-degradation defence mechanism employed by plants in response to virus infection that results in degradation of specific sequences. Silencing suppressors have been used to counter PTGS. There are several plant viruses that encode silencing suppressors such as Tomato bushy stunt virus (TBSV) and Tomato spotted wilt virus (TSWV). The p19 protein of TBSV and NSs protein of TSWV have been shown to suppress transgene PTGS, with p19 showing up to 50-fold enhanced expression for several proteins (Takeda et al., 2002; Voinnet et al., 2003). Co-infiltration of plants with recombinant *A. tumefaciens* that encode the gene of interest and the silencing suppressor in *trans* have significantly increased expression of several proteins (Maclean et al., 2007; Meyers et al., 2008; Regnard et al., 2010). Expression of a silencing suppressor and the gene of interest on a single plasmid has been shown by Sainsbury et al. (2009) to greatly enhance protein expression.

The ability of L1 to form VLPs, the cross-protective activity of L2 and the use of plant systems for production offer the opportunity to develop a broadly protecting affordable vaccine against HPV. In this study, 8 HPV-16 L1:L2 chimaeras were transiently expressed in *N. benthamiana* via *Agrobacterium*-mediated transfer. Optimisation of expression was conducted by comparing protein expression levels using 4 plant expression vectors: these were pTRAc, a cytoplasmic expression vector; pTRAkc-rbcs1-cTP, a chloroplast targeting expression vector; pRIC3, a self-replicating geminivirus-derived vector; and pEAQ-*HT*, a hypertranslational expression system that can increase protein expression without the need for viral replication, and allows the expression of the gene of interest and silencing suppressor from the same plasmid (Sainsbury et al., 2009). Protein expression levels were monitored over several days, and the effect on expression of protein targeting to different cellular compartments examined. The use of a self-replicating vector and gene silencing suppressors on the same or different plasmids, to increase protein expression was evaluated. Lastly, the ability of the various L1:L2 proteins to assemble into cVLPs was also investigated.

2.2. Materials and methods

2.2.1. Plasmid isolation, restriction enzyme digestion and gel extraction

DNA from all plasmids in this study was isolated using the QIAprep® Spin Miniprep kit (Qiagen). All restriction enzyme (RE) digests were performed using REs from Fermentas (ThermoFisher Scientific) or Roche as per the manufacturer's instructions. DNA gel extractions were performed using the QIAquick® Gel Extraction kit (Qiagen) as per the manufacturer's instructions.

2.2.2. Ligation and transformation of *E. coli*

Ligation of DNA fragments was performed using T4 DNA Ligase (ThermoFisher Scientific) as per the manufacturer's instructions. DH5-α chemically competent *E. coli* cells (E. cloni™, Lucigen) were used to transform ligation reactions. Transformed cells were plated on Luria agar (LA) plates with the appropriate antibiotics (Table 2.1 and 2.2) and incubated at 37°C overnight.

2.2.3. Synthesis of L2 peptides and generation of chimaeric genes

Conserved HPV-16 L2 peptides from the N-terminal region – aa sequences 108-120 (Kawana et al., 1999), 56-81 (Kawana et al., 1998) and 17-36 (Gambhira et al., 2007b) – were used to replace the corresponding numbers of aa in the HPV-16 L1 ORF from aa position 131 in the DE loop (SAC) or 431 between the h4 and β-J structural region (SAE), and were ordered from GENEART (Germany). The chimaera L1:L2 65-81 (Jagu et al., 2013) was ordered from GenScript USA Inc (USA) (Table 2.1). All L1:L2 coding sequences were human codon optimised (HPV-16 L1 human codon optimised genes have been shown to result in higher protein accumulation compared to wild-type and plant codon optimised genes (Maclean et al., 2007)). REs were added to the 5' and 3' end of L1:L2 peptides to facilitate subcloning into HPV-16 L1 (Table 2.1). Figure 2.1 shows a schematic of where these regions are in the L1 monomer. In total, 4 SAC and 4 SAE L1:L2 chimaeras were generated.

Table 2.1: Summary of L2 amino acid regions synthesised for generation of chimaeras

Synthesised L1:L2 amino acid region	Size of gene (bp)	Origin of insert	Original plasmid	Antibiotic resistance	5' restriction sites	3' restriction sites	Designed by
SAC 108-120	513	GENEART	pMA	Ampicillin (100 µg/mL)	*Hind*III, *Eco*RI, *Mlu*I, *Bsp*HI	*Pvu*II	Inga Hitzeroth
SAC 56-81	513	GENEART	pMA	Ampicillin (100 µg/mL)	*Hind*III, *Eco*RI, *Mlu*I, *Bsp*HI	*Pvu*II	Inga Hitzeroth
SAC 65-81	513	GenScript	pUC57	Ampicillin (100 µg/mL)	*Hind*III, *Eco*RI, *Age*I, *Mlu*I, *Bsp*HI	*Pvu*II	Aleyo Chabeda
SAC 17-36	513	GENEART	pMA-T	Ampicillin (100 µg/mL)	*Hind*III, *Eco*RI, *Mlu*I, *Bsp*HI	*Pvu*II	Inga Hitzeroth
SAE 108-120	313	GENEART	pMA-T	Ampicillin (100 µg/mL)	*Pst*I	*Xho*I	Inga Hitzeroth
SAE 56-81	313	GENEART	pMA-T	Ampicillin (100 µg/mL)	*Pst*I	*Xho*I	Inga Hitzeroth
SAE 65-81	313	GenScript	pUC57	Ampicillin (100 µg/mL)	*Pst*I	*Xho*I	Aleyo Chabeda
SAE 17-36	313	GENEART	pMA-T	Ampicillin (100 µg/mL)	*Pst*I	*Xho*I	Inga Hitzeroth

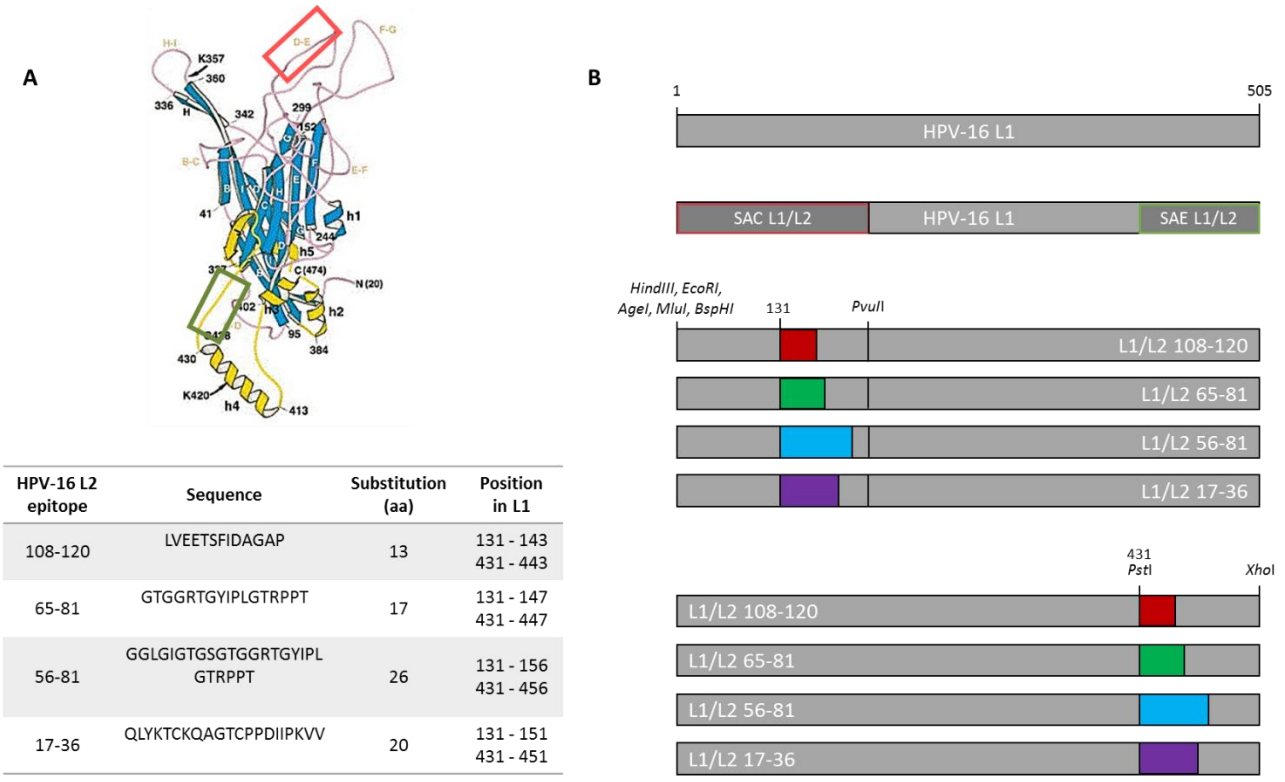


Figure 2.1: Schematic of HPV-16 L1:L2 chimaera construction. A) HPV-16 L2 aa regions 108-120, 65-81, 56-81 and 17-36 were substituted into HPV-16 L1 **(B)** at aa positions 131 in the DE loop (SAC) or 431 between the h4 and β-J structural region (SAE), to generate L1:L2 chimaeras. Not drawn to scale. Drawing showing L1 monomer and surface loops from (Chen et al., 2000c).

2.2.3.1. Construction of SAE L1:L2 chimaeras

The SAE chimaeras were constructed by Alexandra Field (Biopharming Research Unit). Briefly, each L1:L2 encoding sequence was subcloned into intermediary vector pGA4-SAF-MOD (Table 2.2), with the 3' end of the SAF gene being replaced with the L1:L2 peptide. The SAF gene is the South African HPV-16 L1 isolate gene sequence (GenBank accession no. AY177679). This gene contained the L2 aa 108-120 in the h4 helix of HPV-16 L1 (Varsani et al., 2003a) and has a *Pst*I site just 5' to the insertion site of the L2. The SAE L1:L2 genes were excised from their respective plasmids using 5' *Pst*I and 3' *Xho*I REs and subcloned into pGA4-SAF-MOD, replacing the 108-120 sequence with other L2 peptides.

Table 2.2: Subcloning of HPV-16 L1:L2 chimaeras into the four plant expression vectors pTRAc, pTRAkc-rbcs1-cP, pRIC3 and pEAQ-*HT*

	Intermediary vector	**5' restriction enzyme**	**3' restriction enzyme**	***E. coli* strain**	**Antibiotic resistance**	***Agrobacterium* strain**	**Antibiotic resistance**
	pGA4-SAE L1:L2	*BspH*I, *Mlu*I, *Age*I	*Xho*I	E. cloni™	Ampicillin (100 µg/mL)	n/a	n/a
	pNEW-L1:L2	*BspH*I, *Mlu*I, *Age*I	*Xho*I	E. cloni™	Ampicillin (100 µg/mL)	n/a	n/a
	Plant expression vector	**5' restriction enzyme**	**3' restriction enzyme**	***E. coli* strain**	**Antibiotic resistance**	***Agrobacterium* strain**	**Antibiotic resistance**
Destination vectors	pTRAc	*Afl*III	*Xho*I	E. cloni™	Ampicillin (100 µg/mL)	GV3101::pMP90RK	Rifampicin (50 µg/mL) Kanamycin (30 µg/mL) Carbenicillin (50 µg/mL)
	pTRAkc-rbcs1-cTP	*Mlu*I	*Xho*I	E. cloni™	Ampicillin (100 µg/mL)	GV3101::pMP90RK	Rifampicin (50 µg/mL) Kanamycin (30 µg/mL) Carbenicillin (50 µg/mL)
	pRIC3	*Afl*III	*Xho*I	E. cloni™	Ampicillin (100 µg/mL)	GV3101::pMP90RK	Rifampicin (50 µg/mL) Kanamycin (30 µg/mL) Carbenicillin (50 µg/mL)
	pEAQ-*HT*	*Age*I	*Xho*I	E. cloni™	Kanamycin (50 µg/mL)	LBA4404	Rifampicin (50 µg/mL) Kanamycin (30 µg/mL)

2.2.3.2. Construction of SAC L1:L2 chimaeras

The generation of SAC chimaeras required subcloning into 2 intermediary vectors. The SAC region is in the 5' region of the L1 gene and substitution using pGA4-SAF-MOD would result in the formation of a double chimaera. To overcome this, pMA-SAF-MOD was used. The SAF portion of the plasmid was replaced with hL1 (human codon optimised L1) obtained from pFastbac hL1, a plasmid that contains the full hL1 sequence (provided by Mark Whitehead, Biopharming Research Unit) (Figure 2.2). The plasmids were digested with *Pst*I and *Xho*I to form pMA-hL1. An additional *Pvu*II RE site is

present in the pMA backbone. *Pvu*II is required for the substitution of L1 with the SAC L1:L2 genes, therefore the entire hL1 gene was excised with *EcoR*I and *Xho*I and ligated into pTRAc (called pNEW so as not to confuse it with other pTRAc recombinants) (Figure 2.2A). pNEW and the SAC L1:L2 plasmids were digested with *EcoR*I and *Pvu*II and subsequently ligated to form pNEW SAC L1:L2 where L2 is either SAC L2 108-120, 65-81, 56-81 or 17-36 (Figure 2.2B).

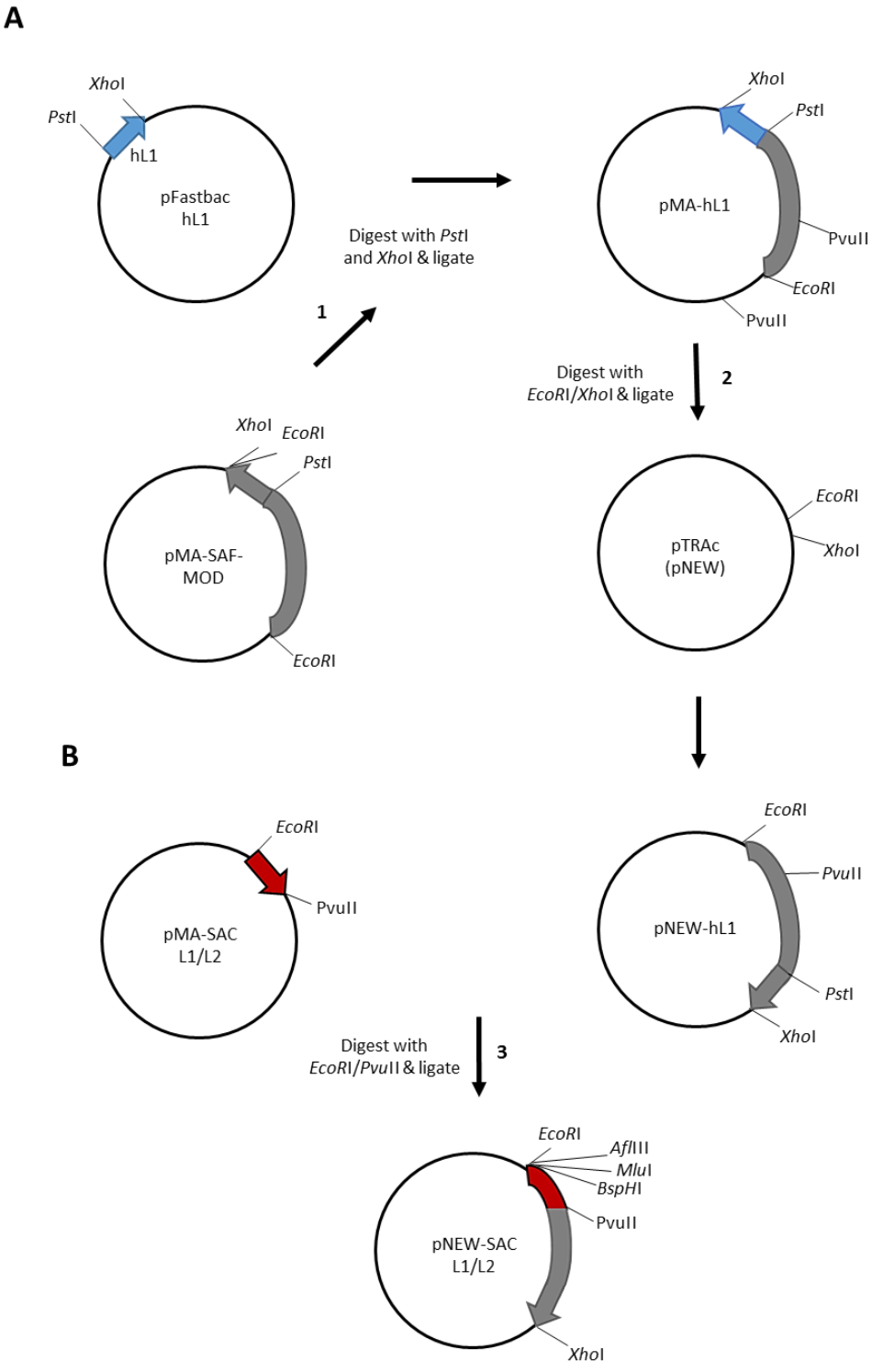


Figure 2.2 (on previous page): Schematic of generation of SAC L1:L2 chimaeras. A) To prevent the formation of a double chimaera pMA-SAF-MOD was used, where SAF was replaced with hL1 by RE digestion with *Pst*I and *Xho*I (1). The pMA backbone contains a *Pvu*II site and to allow cloning of L1:L2 peptide into 5' end of L1 using *Pvu*II, hL1-MOD was excised with *EcoR*I and *Xho*I and ligated into pTRAc (then subsequently named pNEW (2). **B)** pNEW-hL1 and the SAC L1:L2 plasmids were digested with *EcoR*I and *Pvu*II (3) to form pNEW SAC L1:L2 (L2 108-120, 65-81, 56-81 or 17-36). Not drawn to scale.

2.2.4 Subcloning of L1:L2 chimaeras into plant expression vectors

Each L1:L2 SAC and SAE chimaera was cloned into 4 plant expression vectors: these were pTRAc, a cytoplasmic expression vector; pTRAkc-rbcs1-cTP, a chloroplast targeting expression vector (both provided by Prof. Rainer Fischer, Fraunhofer Institute for Molecular Biology and Applied Ecology, Germany); pRIC3, a geminivirus-based self-replicating viral vector (Regnard et al., 2010); and pEAQ-*HT* (provided by Prof. George Lomonossoff, John Innes Centre, UK), a hypertranslational expression system that has been shown to increase protein expression without the need for viral replication (Sainsbury et al., 2009) (Figure 2.3). pEAQ-*HT* also has a p19 silencing suppressor sequence incorporated into the T-DNA, allowing for the expression of the gene and silencing suppressor from the same plasmid. The L1:L2 chimaeric genes were excised from the pGA4 and pNEW vectors (5' *BspH*I, *Mlu*I or *Age*I and 3' *Xho*I sites) and directionally subcloned into the plant expression vectors using *Afl*III/*Xho*I for cloning into pTRAc and pRIC3; *Mlu*I/*Xho*I for pTRAkc-rbcs1-cTP and *Age*I/*Xho*I for pEAQ-*HT*. E. cloni™ cells were transformed with the plasmid constructs and recombinants selected using antibiotic selection (Table 2.2). Recombinant clones were screened by colony PCR, using vector-specific primers or chimaera-specific primers that bind to different L2 peptides (Table 2.3). Recombinants were also verified by RE digest and sequencing.

The cloning of SAE 108-120, SAE 56-81 and SAE 17-36 into pGA4-SAFMOD and subsequent cloning into pTRAc and pTRAkc-rbcs1-cTP was completed by Alexandra Field (Field, 2011). All other recombinant clones were constructed in this study.

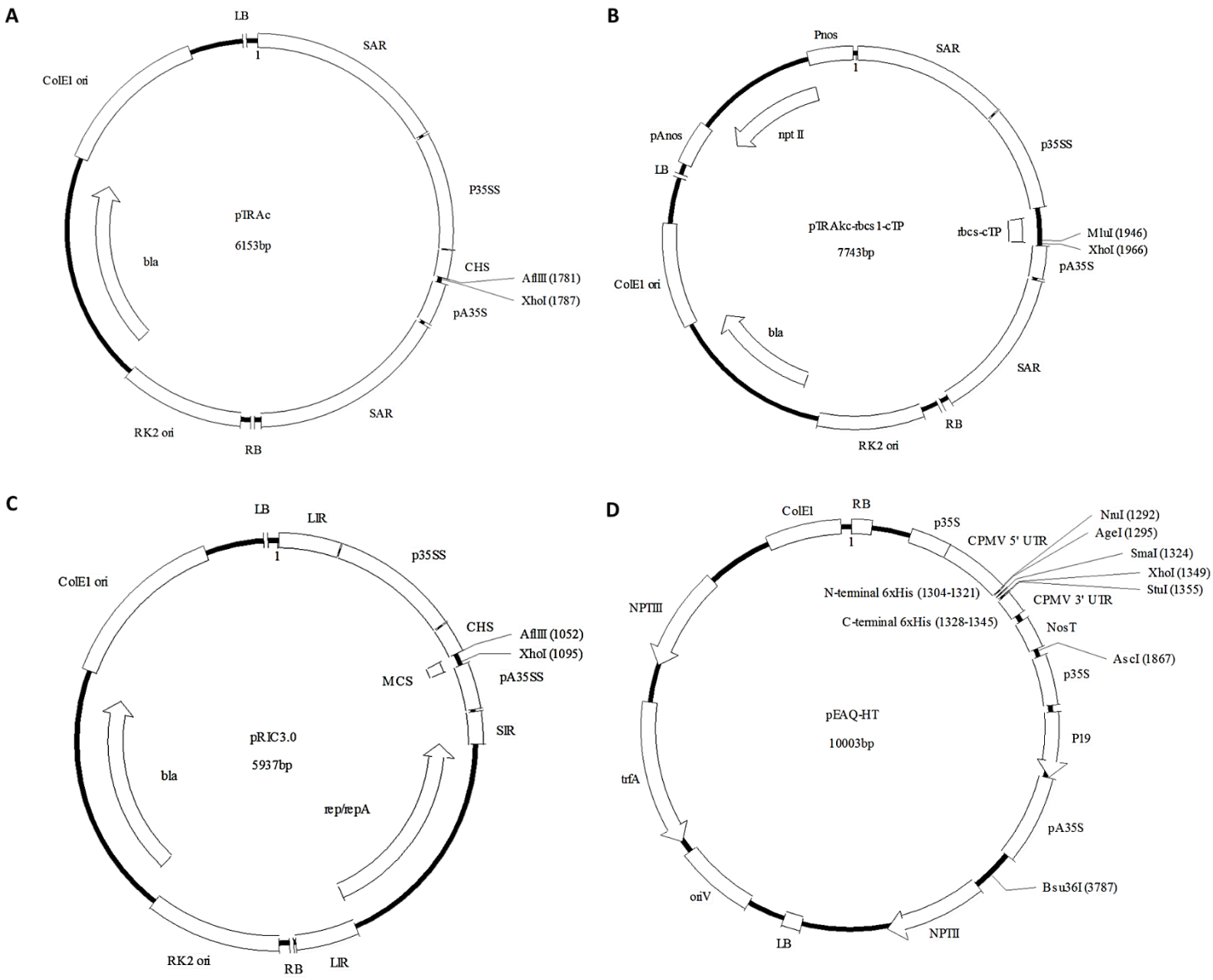


Figure 2.3: Plant expression vectors used for cloning and expression of L1:L2 chimaeras. The vector elements of each plant expression vector are shown for **A)** pTRAc, **B)** pTRAkc-rbcs1-cTP, **C)** pRIC3 and **D)** pEAQ-*HT*. The pTRA and pRIC3 vectors contain: P35SS, CaMV 35S promoter containing 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 pTRAkc-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 (Maclean et al., 2007). The pRIC3 vector contains: LIR, BeYDV long intergenic region; SIR, BeYDV short intergenic region; and Rep/RepA, BeYDV rep gene (Regnard et al., 2010). The pEAQ-*HT* vector contains: LB/RB, left and right borders for T-DNA integration; 5'UTR, modified 5' UTR from CPMV RNA-2, 3'UTR from CPMV RNA-2; NosT, nopaline synthase terminator; P19, suppressor of gene silencing from TBSV; 35S terminator from CaMV; nptII, kanamycin resistance gene; OriV, pRK2 origin of replication; TrfA, replication essential locus; and ColE1, pBR322 *E. coli* origin of replication (Sainsbury et al., 2009).

2.2.5. Confirmation of L1:L2 chimaeras by PCR and RE digests

Colony 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 $MgCl_2$ concentration of 1.5 mM. O'GeneRuler™ 1 kb DNA Ladder (#SM1163, ThermoFisher Scientific) was used as molecular weight marker for all agarose gels in this study.

2.2.6. Sequencing of recombinant clones

Recombinant clones were all sequenced (Macrogen Inc, Netherlands) using primers that flanked the L1:L2 sequence (Table 2.3). Sequences were analysed and aligned using CLC Main Workbench 6 (Qiagen).

Table 2.3: Primers used to confirm L1:L2 recombinant clones by colony PCR and sequencing

	Primer name	5'-3' sequence	Tm (°C)	Used for
pTRAc, pTRAkc-rbcs1-cTP & pRIC3 primers	pTRA For	CATTTCATTTGGAGAGGACACG	59	Sequencing and colony PCR
	pTRA Rev	GAACTACTCACACATTATTCTGG	59	
pEAQ-*HT* primers	pEAQ For A	GCAATATCTCTACTTCTGCTTGACG	67	Sequencing and colony PCR
	pEAQ Rev	GACCGCTCACCAAACATAGAAATG		
	*Age*I For	TCG**ACCGGT**TCATGAGCCTGTGGCTGCC	57	Addition of ***Age***I site to 5' of genes
	XhoI Rev	GCTGGAGCTCCTCGAGG	53	Reverse primer for *Age*I PCR
L2 specific primers	SAC L2 108-120 Rev	CGTACTCATCGGTGCTCACCAC	65	PCR - gene specific reverse primer
	SAC L2 65-81 Rev	CGACTCCCACGCATGCCCAG	63	
	SAC L2 56-81 Rev	CGGCGTGGTAGTAGATGTTGGTC	67	
	SAC L2 17-36 Rev	CACGTACTCATCGGTGCTCAC	61	
	VEET Rev (SAE 108)	GATGAAGCTGGTCTCCTCC	55	
	SAE L2 65-81 Rev	GCTGTGGTAGATGTGCTGCTGG	65	
	SAE L2 56-81 Rev	CTGTTCTGCCTCCTGTGCCAG	63	
	SAE L2 17-36 Rev	ACCTTGGGGATGATGTCGGG	59	
SAF-MOD primers	FwdModNew	CGACGACCTGTACATCAAGG	57	Colony PCR
	VEET Rev	GATGAAGCTGGTCTCCTCC	55	

2.2.7. *Agrobacterium* transformation

A. tumefaciens GV3101::pMP90RK or LBA4404 were made electrocompetent using the method described (Shen and Forde, 1989). Electrocompetent *A. tumefaciens* GV3101::pMP90RK or LBA4404 were transformed with the chimaeric constructs using 200-400 ng plasmid DNA. One hundred microlitres of cells was pipetted into a pre-cooled 1 mm gap electroporation cuvette, DNA added and chilled on ice for 5 min. Electroporation was carried out under the following conditions: 1.8 kV,

25 µF and 200 Ω after which 900 µl LB (Luria Bertani) media was added to the cells. Cells were transferred to a microfuge tube and incubated at 27°C for 2 h. After incubation, the cells were plated onto LA plates with the appropriate antibiotics (Table 2.2), incubated at 27°C for 2-3 days, after which colonies were screened by PCR using primers in Table 2.3. Recombinant clones were verified by RE digest and sequencing, after back transformation into *E. coli*.

2.2.8. *Agrobacterium*-mediated transient expression

To determine the optimal cell concentration required for the best protein expression, leaves (six weeks old) of *N. benthamiana* were syringe infiltrated with the recombinant *A. tumefaciens* cultures at 3 cell densities (OD_{600}): 0.25, 0.5 and 1.0, and with the silencing suppressor NSs at 0.25. Only pTRAc, pTRAkc-rbsc1-cTP and pRIC3 clones were co-infiltrated with NSs. Ten millilitre overnight cultures in induction medium (Maclean et al., 2007) were diluted to required densities (with or without NSs) in infiltration medium (10 mM 2-morpholinoethanesulfonic acid [MES], 10 mM $MgCl_2$, 3% sucrose and 200 µM acetosyringone, pH 5.6) to the required OD_{600}. The diluted cultures were incubated for 2 h at room temperature to allow induction of the *vir* genes by acetosyringone. The cell suspension was introduced into the abaxial spaces of the leaves by making a small hole with a needle and placing a blunt-end syringe over it to make the introduction of cell suspension easier. The plants were grown at 22°C under 16 h/ 8 h light/ dark cycles until harvested.

2.2.9. Small scale protein extraction and western blot analysis

Three leaf discs were harvested for each recombinant clone at 3, 5, and 7 days post-infiltration (dpi) and ground in liquid nitrogen. One hundred microlitres of extraction buffer, 1x high salt phosphate buffered saline, HSPBS (0.5 M sodium chloride (NaCl), 10 mM disodium hydrogen phosphate (Na_2HPO_4), 2.7 mM potassium chloride (KCl), 2 mM potassium dihydrogen phosphate (KH_2PO_4), pH 7.4) and 1x Complete Mini EDTA-free protease inhibitor cocktail (Roche) was added to each sample and mixed by vortexing. The samples were clarified by centrifugation at 11 000 x *g* for 5 min in a benchtop centrifuge. The supernatant was collected and re-clarified as described by Maclean et al. (2007).

Samples were prepared for western blotting by denaturation at 90°C for 10 min in 5x sample application buffer (Sambrook et al., 1989). The PageRuler Prestained Ladder (#SM0671, ThermoFisher Scientific) was used as the molecular weight marker for all western blots in this study. Proteins were separated on 10% SDS polyacrylamide gels and transferred onto nitrocellulose

membranes at 15 V for 1.5 h in a Trans-blot® semi-dry transfer cell (Bio-Rad). The membranes were blocked for 30 min in blocking buffer (5% non-fat dairy milk powder (NFDM), 10% 10x PBS (pH 7.4) and 0.1% Tween-20). After blocking the membranes were incubated at 4°C overnight, with shaking, in 1: 10000 Camvir-1 (ab69, Abcam) (recognises linear L1 epitope aa 204-210) diluted in blocking buffer. The next day, the membranes were washed 4x with blocking buffer for 15 min each. Following the washes, membranes were incubated in anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (Sigma Aldrich), diluted 1:10000 in blocking buffer, for 1 h at 37°C. The membranes were washed 4x in 1x PBS-T (blocking buffer without milk powder) for 15 min each. Detection was performed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro-blue tetrazolium (NBT) phosphatase substrate (BCIP/NBT 1-component, KPL).

2.2.10. TEM of cVLPs in crude plant extract

TEM was used to visualise the formation of cVLPs after expression using the different plant expression vectors. Carbon coated copper grids were glow discharged, and made hydrophilic, at 25 mA for 30 s using a Model 900 SmartSet Cold Stage Controller (Electron Microscopy Sciences). The grids were placed on a 20 µl drop of sample for 3 min and washed 5x in double distilled water. The samples were negatively stained for 1 min with 2% w/v uranyl acetate and viewed using a FEI Tecnai 20 equipped with a LaB6 emitter and operated at 200 kV.

2.2.11. Immuno-gold labelling of whole leaf sections for *in situ* TEM

Agroinfiltrated leaves harvested on day 5 were cut into leaf slices (0.5 cm x 0.5 cm). Leaf slices were placed in 2.5% glutaraldehyde (25% glutaraldehyde diluted 1:10 in 0.1 M phosphate buffer (20 mM sodium dihydrogen (ortho) phosphate (NaH_2PO_4) and 80 mM disodium hydrogen (ortho) phosphate (Na_2HPO_4, pH 7.4)) and incubated overnight at 4°C. Slices were transferred to a 1.5 mL tube and washed 3x for 5 min in 0.1 M phosphate buffer. Samples were fixed for 1 h in one part osmium tetraoxide and one part 0.2 M phosphate buffer (40 mM NaH_2PO_4 and 160 mM Na_2HPO_4, pH 7.4), after which they were washed 3x for 5 min in 0.1M phosphate buffer and incubated overnight. Samples were sequentially dehydrated for 5 min in 30%, 50%, 70%, 90%, and 95% ethanol, with a final 100% ethanol step for 2x 5 min and incubated overnight. The next day, the samples were placed in one part ethanol and one part London Resin (LR) white resin (50% resin solution) and incubated for 4 h. The ethanol was gradually replaced with LR resin by removing half the solution and replacing it with pure resin (75% resin solution), and repeated once more (87.5% resin solution), before placing the samples in pure LR resin. The samples were incubated in each resin solution for

4 h. Leaf samples were embedded in BEEM capsules by placing them at the bottom of the capsule and filling the capsule to the top with resin. All capsules were incubated at 60°C for 24 h. The embedded leaf specimens were cut into ultrathin sections with a diamond knife and collected onto copper grids.

To gold label embedded leaf sections, grids were placed on 150 mM glycine (1.1%) in PBS for 10 min, followed by 10 min in 1x PBS supplemented with 1% Bovine serum albumin (BSA) (Sigma Aldrich) and 1% glycine. The grids were washed 3x in 1x PBS, followed by incubation for 3 h at room temperature in primary antibody Camvir-1 (1:1000) diluted in 1x PBS (with 0.1% BSA). The grids were washed 7x for 1 min in 0.05% PBS-T and placed on anti-mouse IgG gold antibody (10 nm gold beads) (Sigma Aldrich) for 30 min. Grids were washed 7x for 1 min in 0.05% PBS-T, followed by 3x 1 min in 1x PBS. The grids were then placed on 2% glutaraldehyde diluted in 1x PBS for 4 min, and finally washed for 4 min in double distilled water. Grids were stained with uranyl acetate for 5 min after which they were washed 5x for 15 sec with double distilled water. The grids were blotted dry and transferred to lead citrate for 10 min after which they were washed with double distilled water and blotted dry. Gold-labelled sections were viewed using a Philips Tecnai F20 equipped with a field emission gun and operated at 200 kV.

2.2.12. Total soluble protein comparison of crude extracts

Total soluble protein (TSP) of each construct was determined using the Bio-Rad DC Protein Assay kit, as per the manufacturer's instructions. BSA (Sigma Aldrich) was used to generate the protein standard curve. Absorbance was read at 750 nm using a Bio-Tek Powerwave XS spectrophotometer. Crude extracts were quantified by indirect ELISA (as described in Chapter 3, section 3.2.6) and the amount of L1 in each sample (mg) expressed as chimaera per kilogram (kg) of plant tissue and as a percentage of TSP.

2.3. Results

2.3.1. Creation and confirmation of recombinant L1:L2 chimaeras

Generation of SAC chimaeras which is a replacement in the 5' region of the L1 gene required the L2 peptide in the 3' end of L1 (SAF-MOD) to be removed to create hL1 (Figure 2.2A). The replacement of SAF-MOD with hL1 and was confirmed by colony PCR and RE digest (Figure 2.4). Colonies of pMA-

hL1 were screened by colony PCR with primers FwdModNew and a peptide specific primer VEET Rev (Table 2.3) and the absence of a band indicated the replacement of L2 peptide in SAF-MOD with L1 sequence, creating hL1 (Figure 2.4A). This was further confirmed by digestion of pMA-hL1 with *EcoR*I (Figure 2.4B). pMA SAF-MOD has two *EcoR*I sites, one in SAF-MOD and one in the vector backbone. Two bands of ~1.5 kb and ~2.4 kb indicate that SAF-MOD was still present. Positive pMA-hL1 clones produced a linear product of ~3.9 kb.

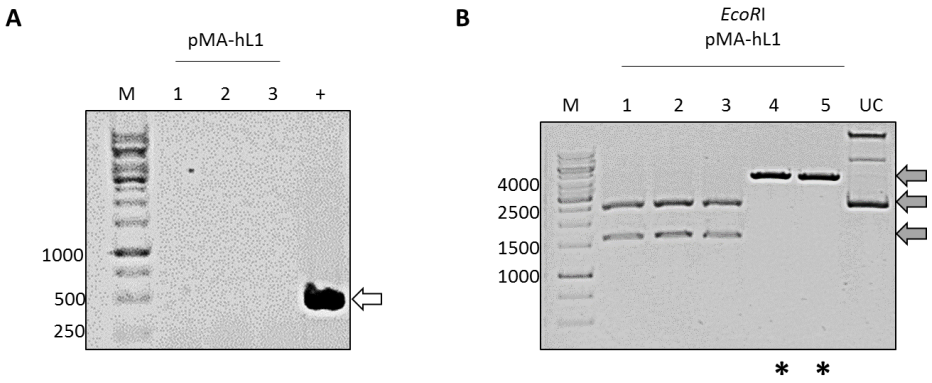


Figure 2.4: Colony PCR and RE digest of pMA hL1 recombinants. A) PCR of pMA-hL1 colonies with primers specific to SAF-MOD. No product indicates hL1 has replaced SAF-MOD. +ve control, pMA-SAF-MOD; white arrow, SAF-MOD PCR product (~300 bp). **B)** RE digest of pMA hL1 with *EcoR*I. The presence of 2 bands (~1.5 kb and ~2.4 kb) in lanes 1 – 3 indicates that SAF-MOD is still present. Lanes 4 and 5 contain hL1 as only one band (~3.9 kb). Labels: * positive pMA-hL1 recombinants; M, molecular weight marker; grey arrows, fragments from RE digest.

The hL1 gene was excised with *EcoR*I and *Xho*I and ligated into pTRAc and called pNEW (Figure 2.2B). The SAC 108-120, 65-81, 56-81 17-36 peptides, were successfully substituted into 5' end of pNEW-hL1 by RE digest with *EcoR*I and *Pvu*II. Positive clones were confirmed by RE digest, colony PCR and sequencing. pNEW L1:L2 SAC 108-120 was digested with RE *Bsa*I (~0.6, 3.4 and 3.5 kb) and *BspH*I (~1.0, 2.8 and 3.8 kb). pNEW L1:L2 SAC 56-81 and 17-36 were digested with *BspH*I (~1.0, 2.8, 3.8 kb), and *EcoR*I (~7.6 kb) (Figure 2.5). Positive clones were additionally confirmed by colony PCR with pTRA primers: pTRA For and Rev (Table 2.3) The expected gene insert of ~1.5 kb was observed for SAC 108-120, 56-81 and 17-36 (data not shown).

pNEW SAC and pGA4 SAE L1:L2 65-81 were digested with RE's *Mlu*I and *Xho*I and gene inserts of ~1.5 kb were ligated into plant expression vectors. The clones were also confirmed by colony PCR using chimaera specific reverse primers and the pTRA For primer (Table 2.3) giving products of ~0.3 and ~1.5 kb for SAC and SAE L1:L2 chimaeras, respectively (data not shown).

All clones were sequenced and analysed using CLC Main Workbench 6 (Qiagen; data not shown), before proceeding to subcloning in the plant expression vectors.

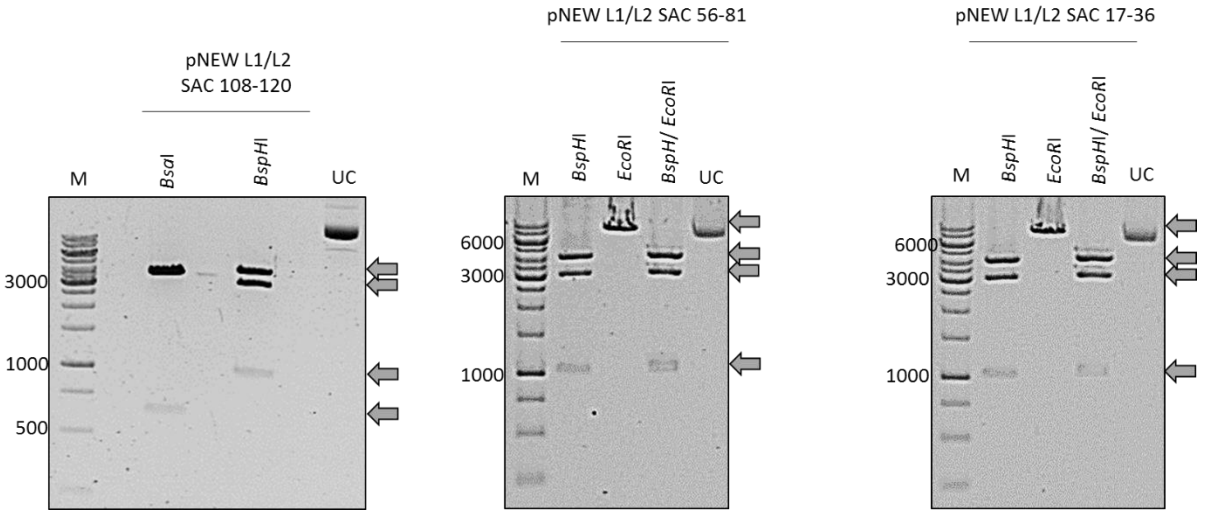


Figure 2.5: Confirmation of pNEW L1:L2 chimaeras. RE digests of pNEW L1:L2 SAC 108-120, *Bsa*I (~0.6, 3.4, 3.5 kb), *BspH*I (~1.0, 2.8, 3.8 kb); 56-81, *BspH*I (~1.0, 2.8, 3.8 kb), *EcoR*I (~7.6 kb); and 17-36, *BspH*I (~1.0, 2.8, 3.8 kb), *EcoR*I (~7.6 kb).

2.3.1.1. pTRAc and pTRAkc-rbcs1-cTP

The L1:L2 chimaeras were subcloned into pTRAc and pTRAkc-rbcs1-cTP with *Af*lIII/*Xho*I and *Mlu*I/*Xho*I, respectively. pTRAc and pTRAkc-rbcs1-cTP L1:L2 SAC 108-120, 56-81 and 17-36 clones were confirmed by RE digest with *Pvu*II and *Xho*I yielding two bands of ~1.2 and ~7 kb for pTRAc clones, and ~1.5 and ~7.5 kb for pTRAkc-rbcs1-cTP clones (data not shown).

2.3.1.2. pRIC3 and pEAQ-HT

L1:L2 chimaeras were subcloned into pRIC3 and pEAQ-*HT* with *Af*lIII/*Xho*I and *Age*I/*Xho*I respectively. pRIC3 recombinant clones were confirmed by RE digest with *EcoR*V and *Xho*I for L1:L2 SAC 108-120, 56-81 and 17-36 chimaeras yielding bands of ~1.7 and 5.7 kb. pRIC3 L1:L2 SAC 65-81 and SAE 65-81 clones were digested with *EcoR*V and *Xho*I (~2.1 and 5.3 kb) and *Pst*I and *Xho*I (~0.3 and 7.1 kb) respectively (data not shown). pEAQ-*HT* clones were confirmed by RE digest with *Age*I and *Xho*I, except for SAE 56-81. Digests with *Age*I and *Xho*I gave expected bands of ~1.6, and 10.4 kb**.** L1:L2 SAE 56-81 was digested with *BspH*I and *Xho*I, giving expected product sizes of ~1.6, 2.9 and 7.5 kb. L1:L2 SAC and SAE 65-81 clones were confirmed by colony PCR with pEAQ For A and L2 specific

primers SAC L2 65-81 Rev and SAE L2 65-81 Rev (Table 2.3), giving products of ~0.4 and 1.5 kb respectively (data not shown).

2.3.2. Confirmation of *Agrobacterium* transformation

Recombinant pTRAc, pTRAkc-rbcs1-cTP and pRIC3 constructs were electroporated into *A. tumefaciens* GV3101::pMP90RK and pEAQ-*HT into A. tumefaciens* LBA4404. Colonies were screened and confirmed by colony PCR and RE digest after plasmid DNA from a positive clone was back-transformed into *E. coli* (data not shown).

2.3.3. Expression optimisation of L1:L2 chimaeras in plants

Expression studies were performed by syringe-infiltrating leaves with recombinant *Agrobacterium* cultures of different concentrations, defined by OD_{600} 0.25, 0.5 or 1.0, into *N. benthamiana* since it had been shown by Wroblewski et al. (2005) that transgene expression can be increased by an increase in *Agrobacterium* cell concentration. Expression of recombinant clones was evaluated over 7 days by western blotting. Ten percent SDS-PAGE gels were loaded with equal protein or equal volume samples and the L1 protein (56 kDa) was detected with anti-L1 antibody, Camvir-1 (Figure 2.9). pTRAc-hL1 was loaded as a positive control. All chimaeras expressed at OD_{600} 1.0 and harvested 7 dpi as well as negative control samples (empty vector), were loaded on separate gels as all samples could not fit on one gel. Protein detection at 7 dpi was lower in comparison to other time points (data not shown). Expression of chimaeras was observed without NSs (data not shown); however, expression was improved with co-infiltration. Expression time trials were performed in triplicate for each clone. Data presented represents one experiment.

pTRAc recombinant clones for all the SAC constructs tested (Figure 2.9A) showed very poor protein expression with little to no protein being detected after 3 dpi (black arrow), even with increasing concentration of *Agrobacterium* cultures. In contrast, pTRAc SAE 17-36 protein was only detected 7 dpi, with the highest levels obtained using an infiltration OD_{600} of 0.5. Expression of pTRAc SAE 108-120 resulted in the highest protein accumulation levels at 3 dpi when using infiltration OD_{600} of 0.5 and 1.0, and after 3 dpi expression decreased. At an OD_{600} of 0.25 of SAE 108-120, highest protein levels were detected at 5 dpi with no protein visible at 7 dpi. Overall, a culture concentration of OD_{600} 1.0 resulted in the highest protein expression levels. No L1 bands were observed for SAE 56-81 expression at any of the *Agrobacterium* culture concentrations tested. Degradation products were present in all chimaeras (white arrows), but to a lesser extent in pTRAc SAE 108-120 and SAE 17-36.

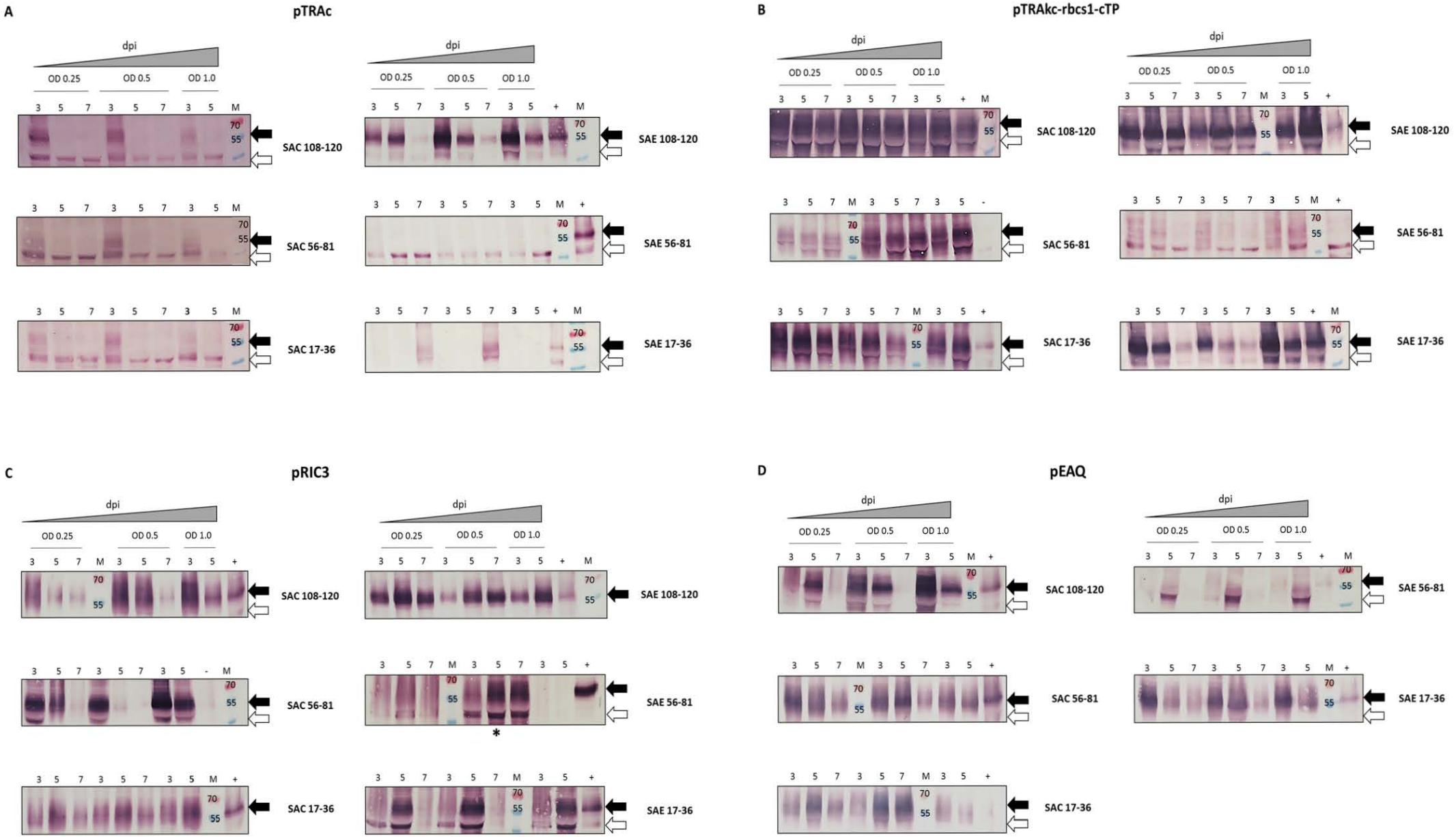
The pTRAkc-rbcs1-cTP expression vector has previously been shown to yield high protein levels by targeting of HPV16-L1 proteins to the chloroplast (Maclean et al., 2007). Protein expression was observed in all chimaeras, except SAE 56-81 (similar to pTRAc) where only degradation products were present in western blots (Figure 2.9B). pTRAkc-rbcs1-cTP SAC 108-120 and SAE 108-120 showed the highest protein accumulation seen by strong band intensities at 3, 5 and 7 dpi at all *Agrobacterium* culture concentrations tested. Leaves harvested 7 dpi at infiltration OD_{600} 1.0 also showed protein expression (data not shown); however, accumulation was highest at 3 or 5 dpi. SAC 56-81 expression showed low protein detection at OD_{600} 0.25, with an increase in detection at infiltration OD_{600} 0.5 and 1.0, with OD_{600} 1.0 at 5 dpi having the highest detection. SAC 17-36 protein expression was highest at 5 dpi at OD_{600} 0.25, with overall expression decreasing at infiltration OD_{600} 0.5 and 1.0. SAE 17-36 protein expression was detected at all infiltration ODs with highest levels at 3 dpi. Protein expression appeared to decrease at OD_{600} 0.5, and increase again at OD_{600} 1.0.

Regnard et al. (2010) found that an increase in expression vector copy number caused by replication correlated with an increase in protein expression. pRIC3 recombinant clones (Figure 2.9C) showed protein expression for all constructs. pRIC3 SAC 108-120 and SAC 56-81 showed highest protein accumulation at 3 dpi at infiltration OD_{600} 1.0, while SAC 17-36 showed highest expression at the same infiltration OD_{600} but at 5 dpi. The L1 bands observed for SAC 17-36 appeared as a smear, suggesting that protein was not denatured completely or may have been overloaded. However, even at lower protein concentrations loaded, smears were still observed (data not shown). pRIC3 SAE 108-120 expression peaked at 5 dpi for all infiltration ODs with OD_{600} 1.0 showing the highest expression. No L1 degradation products were present. pRIC3 SAE 56-81 protein expression was low at infiltration OD_{600} 0.25, increasing and peaking at OD_{600} 0.5, with no expression observed at OD_{600} 1.0. Expression at infiltration OD_{600} 0.25 produced smears. Expression of SAE 17-36 showed the best protein expression at 5 dpi for all infiltration ODs, with OD_{600} 0.5 showing the highest accumulation, but smears for 3 and 7 dpi similar to SAC 17-36 and SAE 56-81 were observed.

pEAQ-*HT* recombinant chimaeras showed protein expression with all chimaeras with the best expression profile for SAC 108-120 where highest expression was observed at 3 dpi at infiltration OD_{600} 1.0 (Figure 2.9D). pEAQ-*HT* S*AC* 56-81 and SAC 17-36 showed expression at all concentrations tested with highest protein accumulation at 5 dpi at OD_{600} 0.5 in both constructs. Poor expression of SAE 56-81 was observed only at 5 dpi at OD_{600} 0.25, 0.5 and 1.0; however, majority of the protein was degradation products (white arrow). SAE 17-36 expression was observed at all infiltration ODs tested, with expression peaking at 3 dpi, and the highest protein accumulation at OD_{600} 1.0. pEAQ-*HT* SAE 108-120 expression was not evaluated due to difficulties in electroporation of *Agrobacterium*

LBA4404. Electroporation of this clone was performed at the same time as other pEAQ-*HT* clones and after unsuccessful attempts using several strategies (range of DNA concentrations, fresh isolation of plasmid DNA, fresh antibiotics, new electrocompetent *Agrobacterium*, and use of fewer cells), it was decided not to continue with this clone.

Figure 2.6 (continued on next page): Western blots of L1:L2 chimaera expression time trials in *N. benthamiana*. A) pTRAc, **B)** pTRAkc-rbcs1-cTP, **C)** pRIC3 and **D)** pEAQ-*HT*. L1:L2 chimaera protein (56 kDa) was probed with Camvir-1 (1:10000) and anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (1:10000). Labels: M, molecular weight marker (kDa); 3, 5, 7 – days post infiltration; black arrows, L1/ L2 chimaera (56 kDa); white arrow, degraded protein; +, pTRAc hL1 positive control.


A
pTRAc
dpi
OD 0.25
OD 0.5
OD 1.0
SAC 108-120
SAE 108-120
SAC 56-81
SAE 56-81
SAC 17-36
SAE 17-36
B
pTRAkc-rbcs1-cTP
C
pRIC3
D
pEAQ
70
55

The L1:L2 SAC and SAE 65-81 chimaeras were constructed after all other chimaera expression time trials had been completed. It was decided that these two chimaeras would be expressed using the pTRAkc-rbcs1-cTP vector as this was the vector of choice for further study of the other chimaeras (Figure 2.7). SAC 65-81 showed protein expression at OD_{600} 0.25 and 1.0, with poor expression at OD_{600} 0.5. The highest protein accumulation was at 5 dpi at OD 1.0. SAE 65-81 expression was evident at 3 dpi at OD_{600} 0.25, after which it decreased. Expression at OD_{600} 0.5 and 1.0 peaked at 5 dpi with overall highest protein accumulation at 5 dpi at OD_{600} 0.5. L1 degradation products were only observed in SAE 65-81 (white arrow).

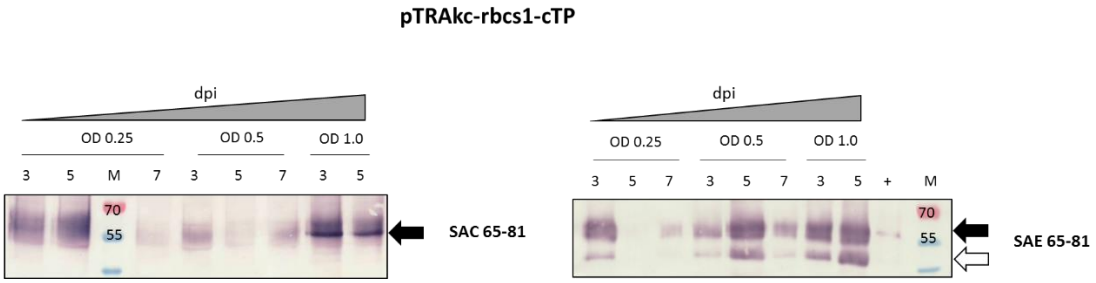


Figure 2.7: Western blots of pTRAksc-rbcs1-cTP SAC and SAE L1:L2 chimaera expression time trials in *N. benthamiana*. L1:L2 chimaera protein (56 kDa) was probed with Camvir-1 (1:10000) and anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (1:10000). Labels: M, molecular weight marker (kDa); 3, 5, 7 – days post infiltration; black arrows, L1/ L2 chimaera (56 kDa); white arrow, degraded protein; +, pTRAc hL1 positive control.

Based on time trial expression results, pTRAkc-rbcs1-cTP and pRIC3 constructs were chosen to move forward with, and expressed at OD_{600} 0.5 or 1. pTRAkc-rbcs1-cTP and pRIC3 constructs were harvested 5 and 3 dpi, respectively. Table 2.4 summaries the protein expression levels of pTRAkc-rbcs1-cTP and pRIC3 constructs. ELISA quantitation of crude extracts was performed using HPV-16 L1 VLPs as the standard (described in Chapter 3, section 3.2.6), and using the Camvir-1 antibody to detect L1:L2 chimaeras. There was no significant improvement in expression when protein was targeted to the chloroplast or the cytoplasm; however, expression of SAE 56-81 was improved 3-fold when localised to the cytoplasm, indicating self-replication of the vector in this instance improved expression. SAC and SAE 108-120 chimaeras showed the highest yields (420-480 mg/kg) and accounted for the highest percentage protein of TSP (2.2-3.6%) and represent the shortest L2 peptide substitution used to generate the L1:L2 chimaeras, suggesting that expression of chimaeras is favoured with shorter L2 peptide substitutions. Overall, pRIC3 chimaeras showed higher yields of L1 than the pTRAkc-rbcs1-cTP chimaeras.

Table 2.4: Summary of pTRAkc-rbcs1-cTP and pRIC3 chimaera expression

Chimaera	pTRAkc-rbcs1-cTP		pRIC3		Fold increase pRIC3 vs pTRAkc-rbcs1-cTP
	Yield (mg/kg)	Yield (% TSP)	Yield (mg/kg)	Yield (% TSP)	
L1:L2 SAC 108-120	470	2.2	420	2.5	0.9
L1:L2 SAC 65-81	310	1.1	-	-	n/a
L1:L2 SAC 56-81	360	1.0	423	2.6	1.2
L1:L2 SAC 17-36	325	0.8	290	1.9	0.9
L1:L2 SAE 108-120	480	3.6	450	3.1	0.9
L1:L2 SAE 65-81	280	0.9	-	-	n/a
L1:L2 SAE 56-81	90	0.3	280	1.6	3.1
L1:L2 SAE 17-36	340	2.6	410	1.8	1.2

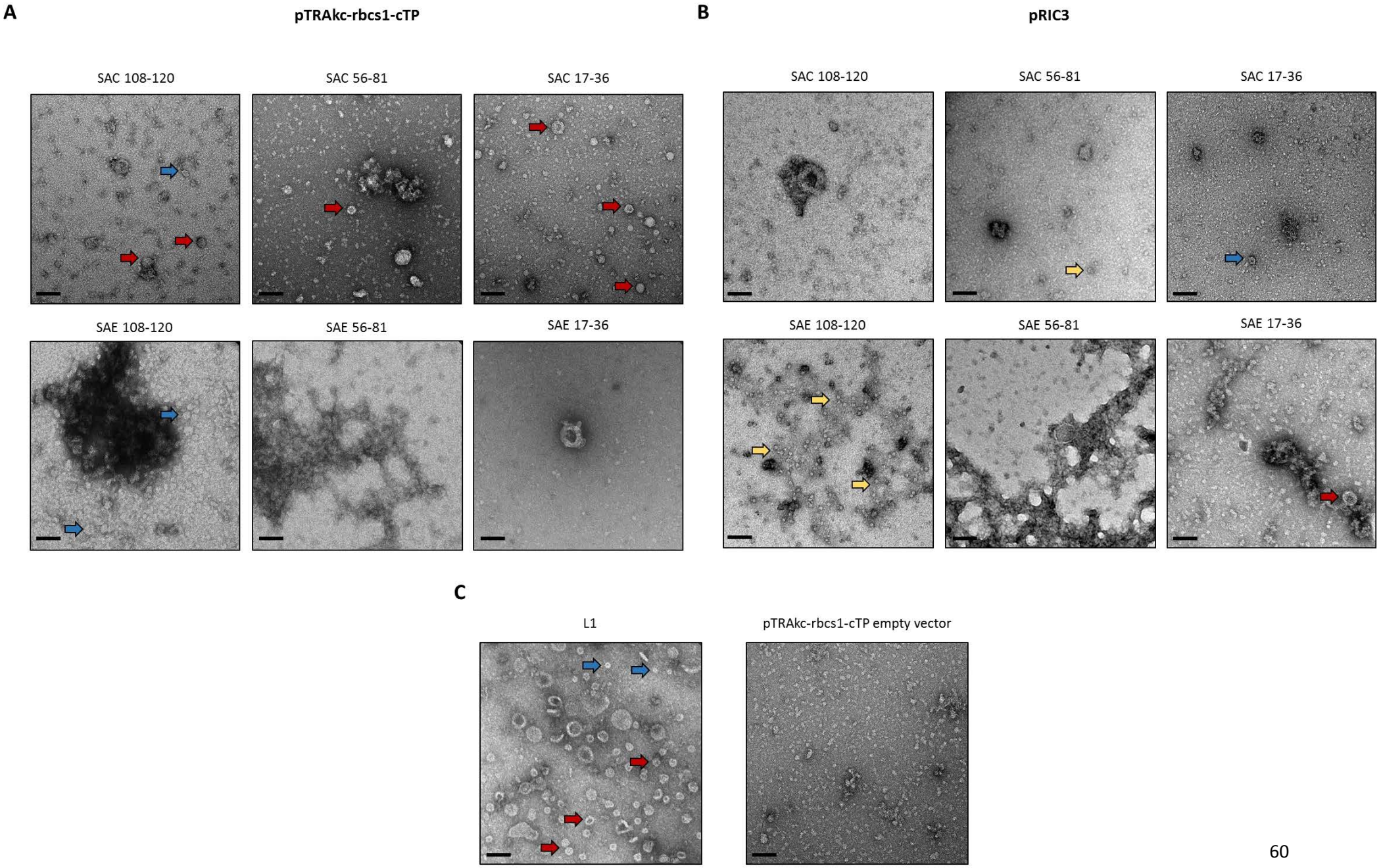
2.3.4. Analysis of cVLP formation in crude plant extracts

Previous studies using pTRAkc-rbcs1-cTP (Maclean et al., 2007) and pRIC3 (Regnard et al., 2010) have shown that high yields of recombinant protein could be achieved when using these expression vectors. To determine whether high protein yield of the chimeric constructs resulted in cVLP formation, crude samples of leaf material were harvested 3 or 5 dpi, immuno-trapped onto copper grids using Camvir-1 as trapping antibody and viewed under TEM. TEM images of the pTRAc and pEAQ-*HT*-expressed crude extract cVLPs showed protein aggregates, but no cVLPs were observed (data not shown) and these results correlate with the low protein expression levels observed in western blots in figures 2.6A and 2.6D.

TEM analysis of pTRAkc-rbcs1-cTP (Figure 2.8A) and pRIC3 (Figure 2.8B) expressed cVLPs showed the presence of aggregates; however, structures resembling L1 VLPs (Figure 2.8C) were observed. Discrete particles were observed particularly for pTRAkc-rbcs1-cTP SAC chimaeras and ranged in size from 25-40 nm (small cVLPs) (blue arrows) to 50-60 nm cVLPs (red arrows). pTRAkc-rbcs1-cTP SAE cVLPs showed small VLPs (SAE 108-120), protein aggregates (SAE 56-81) and small and large structures (SAE 17-36) which are thought to be plant material. pRIC3 chimaeras showed capsomeres (yellow arrows) in SAC 56-81 and SAE 108-120, with very few small cVLPs and cVLPs observed (SAC and SAE 17-36). Aggregates were mostly present in pRIC3 SAC 108-120 and SAE 56-81 crude extract preparations. HPV-16 L1 VLPs and an empty vector control were also analysed (Figure 2.8C) as positive and negative controls, respectively. The L1 VLPs showed a heterogeneous population of VLPs ranging in size from 25-60 nm, with larger shapes which may be plant debris as grids were prepared from crude plant extracts. The pTRAkc-rbcs1-cTP empty vector control showed aggregates which are also thought to be plant material aggregates, but no structures resembling VLPs were

observed. Gradient purification of the cVLP preparations and the detection of conformational surface epitopes were conducted and are described in Chapter 3, sections 3.3.1 and 3.3.3.

Figure 2.8 (continued on next page): Transmission electron micrographs of crude plant extracts. Crude samples of leaf discs extracted in HSPBS. Samples were negatively stained with 2% uranyl acetate. **A)** pTRAkc-rbcs1-cTP chimaeras, **B)** pRIC3 chimaeras, **C)** L1 positive control and empty vector negative control. Labels: Red arrows, cVLPs 50 – 60 nm; blue arrows, small cVLPs 25 – 40 nm; yellow arrows, capsomeres, ~10 nm. Scale bar (100 nm) indicated bottom of each image.

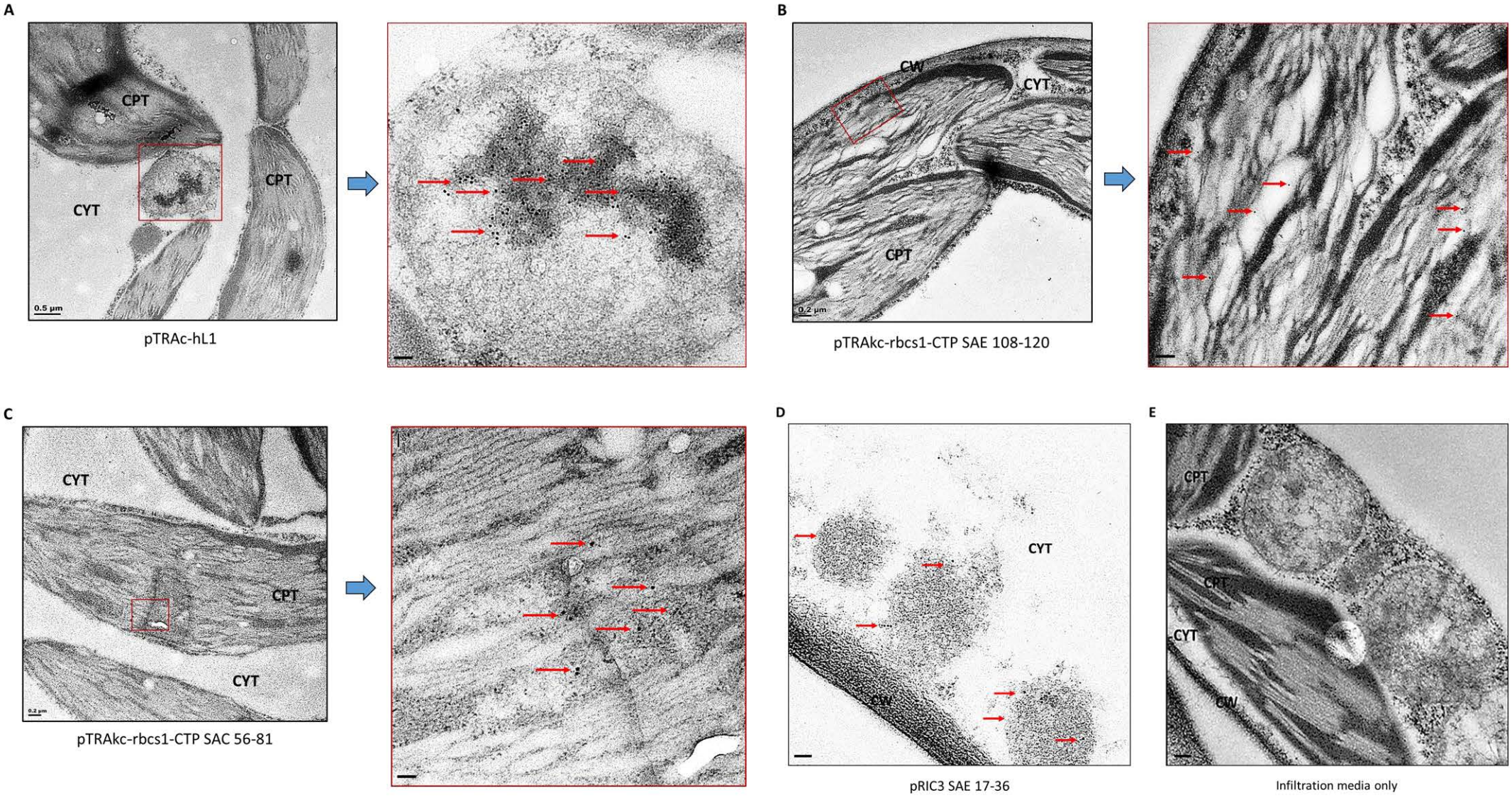

A
pTRAkc-rbcs1-cTP
SAC 108-120
SAC 56-81
SAC 17-36
SAE 108-120
SAE 56-81
SAE 17-36
B
pRIC3
SAC 108-120
SAC 56-81
SAC 17-36
SAE 108-120
SAE 56-81
SAE 17-36
C
L1
pTRAkc-rbcs1-cTP empty vector

2.3.5. *In situ* whole-leaf sections of immunogold labelled cVLPs

Attempts to view cVLPs *in situ* to confirm subcellular localisation proved difficult, especially for chloroplast targeted cVLPs due to the densely-packed chloroplasts (data not shown). The challenge of distinguishing between cVLPs and plant structures was presented due to the small size of the cVLPs. Therefore, immunogold labelling of leaf sections was investigated as an alternative option to visualise these particles *in planta*.

Based on expression time-trial results (Figure 2.6) it was determined that protein expression was best at 5 dpi at using an infiltration OD_{600} of 0.5 or 1. Leaves were harvested 5 dpi, embedded in LR white resin, labelled with 10 nm gold beads and viewed using TEM. Figure 2.9 shows representative results of pTRAkc-rbcs1-cTP and pRIC3 cVLPs. pTRAkc-rbcs1-cTP SAE 108-120 (Figure 2.9B) and SAC 56-81 (Figure 2.9C) showed gold beads (red arrows) spread through the chloroplast. Particles were distinguished from other plant structures due to the perfectly round nature of the gold beads. Labelling was not observed in the cytoplasm. pRIC3 SAE 17-36 cVLPs expressed in the cytoplasm (Figure 2.9D), showed clustering of gold beads, and this was also observed in cytoplasm expressed L1 VLPs (Figure 2.9A). Gold labelling was not observed in the chloroplasts as expected. The negative control was infiltrated with infiltration media only (Figure 2.9E) and no gold beads were observed as with HPV16-L1 and the chimaeric constructs. An immunogold-labelled secondary antibody only control was also tested and showed 1-2 random gold beads per section viewed (data not shown), indicating that this observed binding was non-specific.

Figure 2.9 (continued on next page): Transmission electron micrographs of immunogold labelled plant sections. Leaf sections were labelled with gold beads to determine the cellular localisation of L1:L2 chimaera protein *in planta*. Sections were stained with 2% uranyl acetate and lead citrate. **A)** pTRAc hL1 - positive control, **B)** pTRAkc-rbcs1-cTP SAE 108-120, **C)** pTRAkc-rbcs1-cTP SAC 56-81, **D)** pRIC3 SAE 17-36, **E)** Infiltration media only – negative control. Labels: CYT, cytoplasm; CPT, chloroplast; CW, cell wall; Red arrows, gold beads 10 nm in diameter. Scale bar indicated bottom of each image: A) 0.5 and 0.1 µm, B) 0.2 and 0.1 µm, C) 0.5 and 0.1 µm, D and E) 0.1 µm.


A
CPT
CPT
CYT
0.5 µm
pTRAc-hL1
B
CW
CYT
CPT
0.2 µm
pTRAkc-rbcs1-CTP SAE 108-120
C
CYT
CPT
CYT
0.2 µm
pTRAkc-rbcs1-CTP SAC 56-81
D
CYT
CW
pRIC3 SAE 17-36
E
CPT
CPT
CYT
CW
Infiltration media only

2.4. Discussion

Transient expression via *Agrobacterium*-mediated transfer has been shown to be a quick and efficient method to express heterologous proteins, in comparison to stable expression (Fischer et al., 1999; Rybicki, 2009). In this chapter, transient expression of HPV-16 L1:L2 chimaeras was optimised by several methods: these were co-expression in the presence of a silencing suppressor (from the same or a different plasmid), comparison of expression of cytoplasmic and chloroplast targeting proteins, as well as investigating the use of a self-replicating viral vector. All chimaeras were successfully cloned and transiently expressed in plants (Figures 2.6 and 2.7) with the exception of pEAQ-*HT* L1:L2 SAE 108-120, where most likely the introduction of the plasmid into the *Agrobacterium* was not stable and the plasmid was ejected from the cells.

Previous studies in our lab have investigated the use of silencing suppressors to reduce gene silencing for several proteins. The NSs protein has been shown to suppress PTGS and increase transient protein expression (Takeda et al., 2002). Co-expression of NSs with HPV-16 L1 (Regnard et al., 2010), haemagglutinin (H5) (Mortimer et al., 2012) and HPV-16 L1:L2 chimaeras (Pineo et al., 2013) in *N. benthamiana* showed an increase in protein levels from 5 -7 dpi. In this study, increased protein expression of L1:L2 chimaeras was at 3 or 5 dpi (Figures 2.6 and 2.7) and *N. benthamiana* leaves were healthy with minimal chlorosis. Infiltrated plant tissue exhibited signs of necrosis from 7 dpi (data not shown), especially for pRIC3 chimaeras suggesting that plant tissue health may be affected due to the self-replicating nature of the vector.

Protein expression of L1:L2 chimaeras was investigated by comparing the use of four different plant expression vectors and by varying the cell density used for infiltration, Figure 2.6 shows the expression levels for each L1:L2 chimaera in pTRAc, pTRAkc-rbcs1-cTP, pRIC3 and pEAQ-*HT*. Expression in pTRAc (Figure 2.6A), where expression was localised in the cytoplasm, was low and this was observed even after an increase in *A. tumefaciens* cell culture concentrations. The presence of a band below the expected 56 kDa of L1 was more prominent, suggesting the majority of the L1 was degraded (white arrows), even when a broad-based protease inhibitor cocktail containing cysteine and serine protease inhibitors was used during the extraction process. This suggests that degradation occurred *in planta* where there are many plant proteases (Doran, 2006) whose functions have not been identified, but may have been responsible for this observed degradation.

Targeting of proteins to different cellular compartments for isolation, purification and post-translational modification can increase the yield of recombinant protein (Maclean et al., 2007;

Twyman et al., 2003). The targeted compartment can affect processes such as folding, assembly and stability of the protein (reviewed by Schillberg et al. (2002)). Some antibodies and single chain variable fragments have been shown to accumulate to significantly higher levels in the ER or apoplast (Schillberg et al., 1999; Zimmermann et al., 1998) than in the cytosol. Chloroplast targeting of proteins has also been shown to have increased yields of recombinant protein. Fernandez-San et al. (2008) showed that L1 expressed in transgenic tobacco chloroplasts yielded 24% TSP and Maclean et al. (2007) showed that transient expression of HPV16-L1 localised to the chloroplast yielded 17% TSP.

The rbcs1 signal peptide of pTRAkc-rbcs1-cTP is the chloroplast-transit peptide (cTP) sequence of the potato Rubisco small subunit gene (rbcS1) (Wolter et al., 1988). Maclean et al. (2007) postulated that an increase in protein expression levels was due to less proteolytic activity in the chloroplast and to sequestration compared to the cytoplasm, or more stable mRNA or protein due to the rbcs1 signal. L1:L2 expression using pTRAkc-rbcs1-cTP resulted in high protein accumulation (Figure 2.6B) compared to expression in the cytoplasm in the pTRAc and pEAQ-*HT* non-replicating vectors (Figure 2.6A and D). Black arrows show a 56 kDa band (Figure 2.6B) suggesting that the cTP signal of rbcs1 was cleaved during membrane translocation as it has been shown that cTP is cleaved by the chloroplast stromal peptidase upon entry into the chloroplast (Robinson and Ellis, 1984). L1 degradation products were also present in pTRAkc-rbcs1-cTP chimaeras (white arrows, Figure 2.6B and 2.7) suggesting that protein is not fully protected from proteolytic activity. Yields of 0.3-2.6% TSP were obtained for L1:L2 chimaeras (Table 2.4), similar to yields of 1.7-3.7% TSP obtained by Pineo et al. (2013) in the expression of 3 HPV-16 L1:L2 chimaeras. The L1:L2 108-120 chimaeras had the highest expression levels (Table 2.4), also shown by Pineo et al. (2013), where the L1:L2 108-120 chimaera, in which L2 was substituted into the h4 helix of L1 at aa position 414, showed the highest level of expression when targeted to the chloroplast. Chloroplast localisation was confirmed by immunogold labelling of ultrathin plant sections (Figure 2.9 B and C).

It has been shown that HPV-16 L1 yields can be increased by 50% when using a self-replicating viral vector pRIC3, compared to the non-replicating vector pTRAc (Regnard et al., 2010). Expression of L1:L2 chimaeras was increased when using pRIC3 (Figure 2.6C) in comparison to pTRAc (Figure 2.6A), suggesting that increased gene amplification can result in increased protein yields in the cytoplasm. Although L1:L2 chimaera yields were overall higher than yields using pTRAkc-rbcs1-cTP (Table 2.4), yields were not significantly improved, implying that an increase in transcript levels does not necessarily lead to an increase in translation to protein. This may be due oversaturation of plant transcription and translation machinery. Furthermore, plants infiltrated with pTRAkc-rbcs1-cTP

showed less tissue necrosis when compared to expression in pRIC3 (data not shown), suggesting that the chloroplast provides an environment that reduces that toxicity of foreign protein expression. Daniell et al. (2001) showed that expression of cholera toxin B (CTB) subunit gene in transgenic tobacco chloroplasts assembled into functional oligomers that were identical to native CTB, compared to nuclear transgenic plants, supporting the idea that sequestration of protein to the chloroplast is advantageous. pRIC3 chimaera expression was localised to the cytoplasm where the high protein expression levels may have had undesirable effects on plant health. This was also seen by Pineo (2011) in the expression of L1:L2 chimaeras. Immunogold labelled sections of pRIC3 L1:L2 SAE 17-36 (Figure 2.9D) confirmed the accumulation of the chimaera in the cytoplasm.

Similarly, as with pTRAc and pRIC3, protein expressed using pEAQ-*HT* is localised in the cytoplasm. The pEAQ-*HT* vector contains the p19 silencing suppressor on the same T-DNA as the gene of interest and has been shown to increase the expression of heterologous genes (Sainsbury et al., 2009). In contrast, the pTRAc and pRIC3 vectors were co-infiltrated with the NSs silencing suppressor. van Zyl et al. (2016) found that the expression of VP2, VP3, VP5 and VP7 of Bluetongue virus serotype 8 using pEAQ-*HT* versus the pTRA expression vectors, resulted in the expression of all 4 viral proteins required to form VLPs. It was suggested that because the gene of interest and the silencing suppressor are on the same T-DNA and therefore transferred to the same plant host cells, that PTGS and gene expression occur at the same time. The positioning of the gene of interest between the 5' and 3' UTR of RNA-2 is also thought to produce a stable mRNA for translation, resulting in hypertranslation (Sainsbury and Lomonossoff, 2008). pEAQ-*HT* L1:L2 chimaeras showed highest expression levels at 3 or 5 dpi using *A. tumefaciens* cell culture concentrations of OD_{600} 0.5 or 0.1 (Figure 2.6D); however, higher expression levels were still obtained with chloroplast targeting further supporting the advantage of protein sequestration to organelles.

L1 can spontaneously self-assemble into VLPs, in both the presence and absence of the minor capsid protein L2 (Hagensee et al., 1993; Kirnbauer et al., 1992; Kirnbauer et al., 1993). It has also been shown that pentamers can self-assemble into capsids in a protein concentration dependent manner (Casini et al., 2004). This suggests that an increase in L1 protein expression may lead to improved capsid formation. Both capsomeres (Rose et al., 1998; Thones et al., 2008) and VLPs (Christensen et al., 1996b; Kirnbauer et al., 1992) have been shown to induce the production of NAbs. The L1 surface loops BC, DE, EF, FG and HI (Figure 2.1) contain the conformational epitopes that are essential for binding by NAbs (Christensen et al., 1996a; Roden et al., 1997) therefore, these regions are potential candidates for epitope display. The insertion or substitution of several peptides into some of these surface loops has been shown not to affect VLP assembly (Sadeyen et al., 2003; Slupetzky et al.,

2001; Varsani et al., 2003a). Although expression using the pRIC3 vector showed the highest yields, TEM analysis showed that mostly capsomeres were present (Figure 2.8D). Crude extracts of pTRAkc-rbcs1-cTP SAC chimaeras (Figure 2.8A) showed cVLPs (50-60 nm) and small cVLPs (25-40 nm) compared to capsomeres and aggregates observed for the SAE chimaeras. It has been suggested that the assembly of L1 and L1:L2 into higher order structures has been linked to reduced proteolysis (Chen et al., 2000c), which has been stated as an advantage of protein targeting to and expression in chloroplasts (Daniell et al., 2001; Maclean et al., 2007). Therefore, pTRAkc-rbcs1-cTP SAC chimaeras may have preferentially assembled into cVLPs for this reason (the detection of surface conformational epitopes on these cVLPs using MAbs was conducted and is described in Chapter 3, section 3.3.3).

The L2 peptides in the SAC chimaeras are substituted at aa position 131 (DE loop) and in the SAE chimaeras at aa position 431 (C-terminal). Although the SAE substitution region is not in a L1 surface loop as it falls outside of the h4 helix region where aa 414-426 are thought to be involved in VLP assembly (Varsani et al., 2003a), this C-terminal region has demonstrated anti-L1 and anti-L2 responses when aa 108-120 was substituted with L1 (Varsani et al., 2003a), suggesting it was a candidate for epitope display. SAE chimaeras may not have assembled into VLPs due to the size of the L2 peptides substituted at position 431. L2 aa 108-120 replaces 13 codons, while aa 56-81 and 17-36 replace 26 and 20 codons respectively. Varsani et al. (2003a) showed VLP formation when L2 108-120 was substituted at position 431. It may be possible then, that substitution of larger peptides may affect capsid assembly. Position 431 is next to a Cys^{428} residue, which has been shown to be important in disulphide cross-linking with Cys^{175} and stability of the virion (Bishop et al., 2007a). Perhaps these larger substitutions may affect this disulphide cross-linkage, thereby only allowing pentamer formation, and VLP formation might be hindered.

In conclusion, expression studies showed that chloroplast targeting or the use of a self-replicating viral vector in the expression of L1:L2 chimaeras were the best options. The pTRAkc-rbcs1-cTP L1:L2 SAC chimaeras showed the ability to form higher order structures with particles resembling L1 VLPs. These SAC chimaeras are ideal for scale up and vaccine production, since it was shown that VLPs induce higher antibody titres than capsomeres (Thones et al., 2008). Therefore, pTRAkc-rbcs1-cTP SAC chimaeras that formed VLPs, in addition to SAE 65-81 (smaller peptide not previously tested in chimaeras) were used in subsequent purification and vaccination studies.

Chapter 3: Large-scale expression, purification and assembly of HPV-16 L1:L2 chimaeric virus-like particles

3.1. Introduction

VLPs are self-assembled, non-infectious particles that resemble the native virion but do not contain viral genetic material. VLPs have been produced for a variety of viruses in a variety of systems, such as mammalian, insect, yeast, bacterial and plant expression systems (Kushnir et al., 2012; Roldão et al., 2010; Santi et al., 2006). The immunogenicity of L1 depends on its ability to assemble into higher order structures such as capsomeres, small VLPs (T=1, 30-40 nm) or VLPs (T=7, 50-60 nm), which have been shown to elicit humoral and cellular immune responses (Christensen et al., 1996b; Kirnbauer et al., 1992; Rose et al., 1998; Schadlich et al., 2009; Thones et al., 2008). The C-terminal arm of L1 is critical for assembly into VLPs (Li et al., 1997) and also contains conformational epitopes which are bound by NAbs on the surface of the virion (Chen et al., 2000c; Christensen et al., 1996a; Kirnbauer et al., 1992; Modis et al., 2002). Disulphide cross-linking of residues Cys^{175} and Cys^{428} contributes to the stability of the virion (Bishop et al., 2007a) and mutations in these regions have been shown to result in the preferential formation of capsomeres and not of VLPs (Fligge et al., 2001; Li et al., 1998; McCarthy et al., 1998; Sapp et al., 1998; Varsani et al., 2006a). The position at which the L2 epitope is substituted in L1 is therefore important, as was described in Chapter 2, as this may affect correct folding and thus the display of epitopes that may elicit NAbs.

HPV L1 VLPs have been produced in mammalian (Mossadegh et al., 2004), yeast (Cook et al., 1999; Neeper et al., 1996), insect cell (Kirnbauer et al., 1992), bacterial (Zhou et al., 1991) and plant (Maclean et al., 2007) expression systems. Current HPV L1 VLP-based vaccines are produced in eukaryotic cell culture (yeast and insect cells) and have been shown to be both highly immunogenic and to elicit the production of NAbs with high neutralisation titres (Schiller et al., 2008). Although the aim of these vaccines was to address the high burden of cervical cancer worldwide, their cost of production and purification helps to make them expensive. Plants offer an alternative solution for vaccine production, due to potentially low costs, and to very flexible scalability and rapid production (Fischer et al., 2009; Marsian and Lomonossoff, 2016; Rybicki, 2014; Scotti and Rybicki, 2013; Tiwari et al., 2009). In a recent review by Merlin et al. (2014), a case study of 4 molecules (Human glutamic acid decarboxylase, NV VLPs, anti-HIV MAb 2G12 and human IL-6) showed the advantages of using plant-based production systems. These advantages include increased yields, the potential for use of products for oral delivery, rapid production and facilitation of complex posttranslational

modification. Overall, the reduction of production cost has particularly been associated with upstream production, such as the generation of biomass, extraction of the proteins of interest and sample pre-treatment (Tiwari et al., 2009). Downstream processes have yet to catch up with cost reductions that may be possible as they rely on purification methods developed for other production systems. Efforts are being made to develop processes that are particularly suitable for plant-produced products (Wilken and Nikolov, 2012).

Purification of VLPs is dependent on factors such as whether they are enveloped versus non-enveloped, and their size, molecular weight and stability. HPV L1 VLPs have traditionally been laboratory purified by ultracentrifugation in caesium chloride (CsCl) or sucrose gradients (Biemelt et al., 2003; Fernandez-San et al., 2008; Kirnbauer et al., 1992; Maclean et al., 2007; Varsani et al., 2006b; Waheed et al., 2011), but chromatography is usually required for the removal of host cell contaminants (Kim et al., 2010). Purification using several chromatography steps (size exclusion, cation exchange, heparin) and repeated dialysis are required, and have been found to be time-consuming and can result in protein loss and degradation (Kim et al., 2010; Park et al., 2008). Heparin chromatography has been explored as a method to purify VLPs, as cell-surface heparin sulphate proteoglycans (HSPGs) are the main receptors for attachment of HPV to cells (Joyce et al., 1999). Heparin is a highly sulphated form of heparan sulphate and binds a conformational motif of L1, aiding in the purification of higher order structures as this motif is only present in VLPs and capsomeres and not L1 monomers (Rommel et al., 2005). High yields have been achieved in the purification of HPV-16 VLPs (up to 60%) (Kim et al., 2010) and cVLPs (up to 600 mg/kg FW) (Pineo et al., 2013) and the purified antigens were shown to be appropriately immunogenic. Cation-exchange chromatography to purify L1 VLPs has shown improved purity (Kim et al., 2007) and improved yields of up to 10% (Cook et al., 1999). The use of a one-step chromatography method (Kim et al., 2010) has improved the efficiency of purification of L1 VLPs. Comparison studies using these two methods showed that heparin chromatography was better than cation-exchange chromatography in selecting functional HPV-16 VLPs that elicited neutralising antibodies (Kim et al., 2012a), implying the choice of purification technique is important.

Although these methods have been shown to be efficient, chromatographic purification of plant-produced proteins has proven difficult due to the lack of optimised extraction and purification protocols. A protocol to scale the production of L1 and VLPs from tobacco plants has recently been developed, using several salt and pH mediated buffers (Zahin et al., 2016). A density-based purification method for VLPs produced in plants has been suggested by Peyret (2015) for VLPs that may require careful handling or that do not have native surface properties. Additionally, van Zyl and

Hitzeroth (2016) have developed a simple method for VLP purification from plants based on the density or the size and rate of sedimentation of particles.

Disassembly and reassembly of VLPs (the method by which Gardasil® vaccines are made) has also been extensively studied, with factors such as pH, ionic strength, and oxidizing and reducing agents affecting particle assembly (Hanslip et al., 2006; Mach et al., 2006; Mukherjee et al., 2008). Low pH and high salt concentrations can favour the assembly of L1 into VLPs (Mach et al., 2006; McCarthy et al., 1998) and Chen et al. (2001) showed that capsomeres in a pH 5.4/0.5 M NaCl buffer supported the assembly of HPV-11 VLPs. HPV VLPs are relatively easily disrupted and their correct assembly is important to prevent aggregation, as well as for correct antigenicity (Shi et al., 2005).

In this Chapter, strategies to determine the best option for simple but efficient purification of plant-produced cVLPs that resemble VLPs are described. Assembly of purified cVLPs was analysed by TEM, and preparations were used in immunogenicity studies in mice (Chapter 4).

3.2. Materials and methods

3.2.1. Large-scale expression of L1:L2 chimaeras in *N. benthamiana*

Ten millilitre starter cultures of recombinant *A. tumefaciens* of each pTRAkc-rbsc1-cTP expressed vaccine candidate (Table 3.1) were grown in enriched induction media (Maclean et al., 2007) supplemented with appropriate antibiotics (Chapter 2, Table 2.2) and incubated overnight at 28°C with agitation. The starter cultures were transferred to a bigger flask (without rifampicin) and incubated overnight. The cultures were prepared for infiltration (as described in Chapter 2, section 2.2.8) in infiltration media without sucrose. Twenty to twenty-five *N. benthamiana* plants were vacuum infiltrated with pTRAkc-rbcs-1-cTP recombinant constructs (OD_{600} 0.5-1) and grown for 5 days at 22 °C under 16hrs/8hrs light/dark cycle.

Table 3.1. Final pTRAkc-rbsc1-cTP constructs purified for use in animal studies

Vaccine Group	Vaccine candidate
G1	SAC 108-120
G2	SAC 65-81
G3	SAC 56-81
G4	SAC 17-36
G5	SAE 65-81
G6	hL1
G7	Empty vector

3.2.2. Purification optimisation of cVLPs

Figure 3.1 shows a flow chart of the strategies investigated for purification of cVLPs. Whole leaves were harvested and thoroughly homogenised with Waring-type blender in cold extraction buffer at a w/v ratio of 1:1 (see section 3.2.2.1.), supplemented with 1x Complete Mini EDTA-free protease inhibitor cocktail (Roche). Homogenates were incubated at 4°C with shaking for 1.5 h, filtered through 4 layers of Miracloth (Calbiochem) and clarified two times at 10 000 *x g* for 10 min at 4°C. The clarified extracts were subsequently loaded onto gradients where density or size-based centrifugation purification methods were investigated (see section 3.2.2.2.).

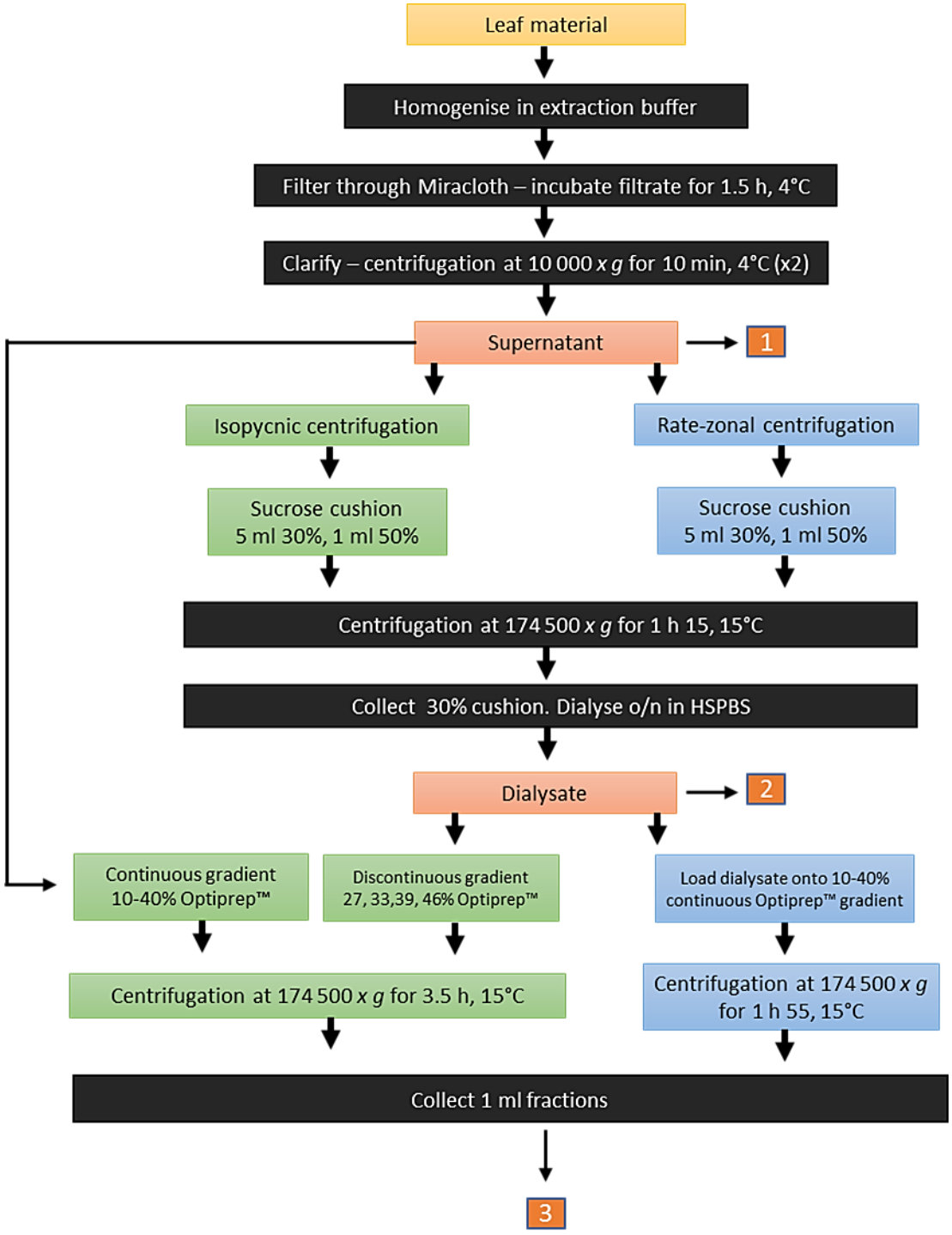


Figure 3.1: Flow chart of purification strategies for transiently expressed cVLPs and hL1 VLPs. Numbers 1-3 indicate sampling points for dot blot and TEM analysis.

3.2.2.1. Buffer optimisation

Two buffers were investigated for extraction and purification of cVLPs from plants: these were high salt 1x PBS (HSPBS, 0.5 M NaCl, pH 7.4) (Maclean et al., 2007) or high salt 0.1 M sodium acetate (HSNaOAc, 0.5 M NaCl, pH 5.2) (A. R. van Zyl, personal communication).

3.2.2.2. Isopycnic vs. rate-zonal centrifugation

While L1 VLPs and L1:L2 cVLPs have previously been purified by sucrose and CsCl ultracentrifugation (Maclean et al., 2007; Schellenbacher et al., 2009; Varsani et al., 2003a), in this study, Optiprep™ – a sterile endotoxin-free iodixanol density gradient medium (Axis-Shield PoC AS, 2016) – was used for the purification of our cVLPs. Optiprep™ is routinely used for the purification of PsVs and VLPs. Density gradient (isopycnic) and rate-zonal centrifugation methods were investigated for purification of cVLPs.

For isopycnic centrifugation (Figure 3.1), plant tissue was homogenised in either in HSPBS or HSNaOAc. The clarified extracts were centrifuged in pre-formed discontinuous (5 ml 27%, 5ml 33%, 2 ml 39 and 1.2 ml 46%) or continuous (10-40%) Optiprep™ gradients (made in HSPBS or HSNaOAc) for 3.5 h at 174 500 *x g* in a SW 32 Ti rotor (Beckman). Alternatively, clarified extracts were first centrifuged on a double sucrose cushion (5 ml 30% and 1 ml 50%) for 1 h 15 min at 174 500 *x g* in a SW 32 Ti rotor, followed by overnight dialysis of the 30% fraction containing putative cVLPs in HSPBS and then centrifuged on discontinuous or continuous Optiprep™ gradients as described above. Purified fractions were collected from the bottom of the tubes and analysed by dot blots and TEM (as described in Chapter 2, section 2.2.9/10). For rate-zonal centrifugation, clarified plant extracts were partially purified on double sucrose cushions, followed by dialysis of the 30% fraction in HSPBS as described above. The dialysates were loaded on to 10-40% continuous Optiprep™ gradients and centrifuged for 1 h 55 min at 174 500 *x g* in a SW 32 Ti rotor (Figure 3.1). Fractions were collected from the bottom of the tube and analysed by dot blots and TEM.

Additionally, maturation of cVLPs was explored to improve particle assembly. After centrifugation on a double sucrose cushion and overnight dialysis in HSPBS, 0.5% Triton X-100 and 25 mM ammonium sulphate (pH 9) were added to the lysate and the lysate incubated overnight at 37°C as described (Cardone et al., 2014). The lysate was then purified on a discontinuous Optiprep™ gradient as shown in Figure 3.1. SDS-PAGE gradient gels of 3-10% Tris acetate (Cardone et al., 2014) were poured and

run to visualise different L1 species (monomers, dimers and trimers), with samples prepared in non-reducing and/or non-denaturing conditions.

3.2.2.3. Concentration of purified cVLPs

For precipitation of cVLPs, a polyethylene glycol (PEG, molecular weight 8000) working solution at 30% w/v was prepared in HSPBS and added to the purified sample to final concentration of 10% in a volume of 10 ml. The solution was incubated overnight at 4°C with agitation. The solution was centrifuged for 30 min at 11 200 *x g* after which the supernatant was collected and the pellet (not visible) was resuspended in HSPBS. Both the supernatant and pellet were analysed by western blotting and TEM. For ultrafiltration, Amicon® Ultracentrifugal filter units with a MWCO of 100 kDa (Merck) were used as per the manufacturer's instructions. The eluted sample was analysed by western blotting and TEM.

3.2.3. Purification of vaccine antigens

The final chimaeras selected for purification to be used in animal studies are shown in Table 3.1, in addition to a positive and negative controls (G1-G7). Biomass from large scale infiltration of *N. benthamiana* was extracted in HSNaOAc buffer at a ratio of 1:1 (w/v). The homogenate was incubated for 1.5 h at 4°C with shaking, filtered through 4 layers of Miracloth and clarified two times at 10 000 *x g* for 10 min at 4°C. The clarified extract was loaded onto discontinuous Optiprep™ gradients and centrifuged for 3.5 h at 174 500 *x g*, 15°C, in a SW 32 Ti rotor, after which 1 mL fractions were collected from the bottom of the tubes. Fractions 1-4 were pooled, added to a 5 mL ultracentrifuge tube (Ultra-Clear Thinwall TUBE, Beckman) and centrifuged for 1 h at 183 548 *x g* at 15°C, in a SW 55 Ti rotor (Beckman). The opaque band visible after centrifugation was collected using a needle and syringe and quantified by indirect ELISA.

3.2.4. Mass spectrometry

To determine if the purified protein was indeed HPV-16 L1:L2, samples were run on SDS-PAGE gels, Coomassie stained, and the relevant bands cut out for liquid chromatography - mass spectrometry (LC-MS) analysis. This was performed by the Centre for Proteomic and Genomic Research (CPGR, Cape Town, South Africa). Gel pieces were washed and fragmented by in-gel trypsin digestion as per the protocol described by Shevchenko et al. (2007). The peptide solution was analysed using a Dionex Ultimate 3000 nano-HPLC system (ThermoFisher Scientific, USA) coupled to a Q Exactive™

Hybrid Quadrupole-Orbitrap Mass Spectrometer (ThermoFisher Scientific, USA). Byonic Software (Protein Metrics USA) was used for comparison of the spectra with sequences retrieved from the UniProt Swissprot protein database. Samples were analysed against profiles from *Nicotiana* spp, *Agrobacterium* spp and HPV proteomes.

3.2.5. TEM of purified cVLPs

Purified samples were negatively stained with 2% uranyl acetate. Grids were viewed as described in Chapter 2, section 2.2.10.

3.2.6. Quantitation of purified cVLPs by indirect ELISA

The 5 chimaeras and hL1 positive control were quantified by indirect ELISA. Ninety-six well plates (Nunc Maxisorp, ThermoFisher Scientific) were coated with a) 80 ng purified HPV-16 L1 VLPs (100 µL/well) serially diluted 2-fold in coating buffer (10 mM Tris, pH 8.5) to generate a standard curve, or b) 100 µL vaccine antigen serially diluted 2-fold from 1:50 – 1:400 in coating buffer, and incubated overnight at 4°C with gentle shaking. Plates were blocked with 300 µL blocking buffer (1x Tris-Cl (TBS), pH 7.5, 5% NFDM) for 2 h at room temperature after which they were washed 4x with 1x TST (1x TBS, 0.05% Tween 20) wash buffer using an EL_x50 Autostrip washer (Bio-Tek Instruments Inc). A volume of 100 µL of Camvir-1 (1:15000) primary antibody was added to each well and the plates incubated at 37°C for 1 h. The plates were washed 4x with 1x TST, followed by the addition of 100 µL alkaline phosphatase-conjugated anti-mouse IgG secondary antibody (1:10000) to each well and incubated at 37°C for 1 h. For the final washes, plates were washed with 1x TBS (pH 9) after which 200 µL *SIGMAFAST*™ *p*-nitrophenyl phosphate (Sigma Aldrich) substrate was added to each well and incubated in the dark for 30 min. The absorbance was detected at 405 nm using a Bio-Tek Powerwave XS spectrophotometer. Total L1 yield of each vaccine antigen was calculated using the average absorbance values obtained and the equation of the chart generated from the standard curve. The negative control was quantified by TSP using the Bio-Rad DC Protein Assay (described in Chapter 2, section 2.2.12.) as there was no L1 antigen to detect in the vector only control.

3.2.7. Characterisation of cVLP epitope display by indirect ELISA

One hundred nanograms (SAC 108-120, SAC 65-81, SAC 17-36 and hL1) or 50 ng (SAC and SAE 65-81) of native cVLPs or hL1 VLPs prepared in 100 µL coating buffer, were coated onto 96-well plates (Nunc Maxisorp, ThermoFisher Scientific) and incubated overnight at 4°C with gentle shaking. For

denaturing conditions cVLPs or hL1 VLPs were dried onto the 96-well plates without a lid in 0.2 M $NaHCO_3$ (pH 10.6) + 0.01 M freshly added dithiothreitol (DTT) buffer overnight at 37°C. The next day, plates were blocked with 300 µL blocking buffer for 2 h at room temperature, followed by 4x washes with 1x TST wash buffer. Five-fold serial dilutions of antibodies (1:200 – 1:125000) in blocking buffer were added to the wells in triplicate (100 µL/well) and incubated at 37°C for 1 h. Antibodies used were neutralising MAbs H16:V5, H16.E70, H16.U4, H16.9A, H16.J4 and L2 4B4 (kindly provided by Dr. Neil Christensen), linear non-neutralising commercial MAb Camvir-1, or rabbit serum raised against HPV-16 L2. Plates were then washed again and 100 µL alkaline phosphatase-conjugated goat anti-mouse (1:10000) or goat anti-rabbit (1:5000) secondary antibody added, and incubated at 37°C for 1 h. Final washes and detection were performed as described in section 3.2.6.

3.3. Results

3.3.1. Optimisation of cVLP purification

Several strategies were investigated to determine a suitable and efficient method to purify cVLPs. Based on the successful purification of insect cell produced cVLPs (Varsani et al., 2003a), and plant-made PsVs (Lamprecht et al., 2016) by density ultracentrifugation, candidate vaccines were purified using Optiprep™ density medium. For each method tested, hL1 VLPs were also purified as a positive control and for comparison of VLP assembly. Two buffers were used to compare their effect on extraction and purification: these were HSPBS (Maclean et al., 2007; Varsani et al., 2003a) and HSNaOAc (A.R van Zyl, personal communication).

3.3.1.1 Initial purification

Leaf material containing hL1 and the L1:L2 chimaeras was initially extracted in HSPBS, centrifuged on a double sucrose cushion, followed by centrifugation on a continuous or discontinuous gradient (Figure 3.1). Dot bot analysis of collected fractions (crude extract, 30% cushion, fractions from gradients) showed the detection of L1; however, for cVLPs, L1 was only detected in the crude extract and 30% cushion. No L1 signal was observed in purified fractions obtained after density gradient centrifugation (data not shown), suggesting a loss of protein during dialysis. Based on these results, after plant tissue extraction, cVLPs were directly purified on continuous or discontinuous density gradients.

Samples were extracted and purified in HSPBS or HSNaOAc on continuous 10-40% or discontinuous 27-46% Optiprep™ gradients. Upon visual inspection of the gradients, the HSNaOAc clarified extract appeared to contain less green pigment (blue arrows, Figure 3.2) than the HSPBS clarified extract, possibly indicating that the low pH buffer facilitated the removal of major plant proteins such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo). Two distinct opaque bands were present in the HSNaOAc discontinuous gradient (orange arrows, Figure 3.2B) which may represent two populations of VLPs. No opaque bands were observed in the HSPBS discontinuous gradient. Dot blot analysis of collected fractions showed that L1 was distributed across more fractions in the continuous versus the discontinuous gradient (data not shown), suggesting cVLPs of varying densities were present. TEM analysis of hL1 VLPs or cVLPs (Figure 3.2 C and D, respectively) extracted in either HSPBS or HSNaOAc, and subsequently purified on continuous or discontinuous Optiprep™ gradients, showed that L1 VLPs (50-60 nm) were successfully purified whether extracted in HSPBS or HSNaOAc and purified on a continuous or discontinuous gradient (red arrows Figure 3.2C). cVLPs extracted in either buffer and purified on a continuous gradient showed very few cVLPs measuring ~25 nm in size (purple arrows, Figure 3.2D), that were irregular in shape as well as protein aggregates (green arrows, Figure 3.2D), while cVLPs purified on a discontinuous gradient in HSPBS showed particles resembling L1 VLPs (red arrows, Figure 3.2D). Extraction in HSNaOAc showed that smaller particles of 25-40 nm (blue arrows, Figure 3.2D) were purified. HPV particles have been shown to form particles of varying sizes from 25-60 nm (Kim et al., 2010; Kim et al., 2012a; Maclean et al., 2007; Matic et al., 2011), therefore these results are not entirely unexpected. Table 3.2 summarises the results for the purification of the chimaeric vaccine antigens.

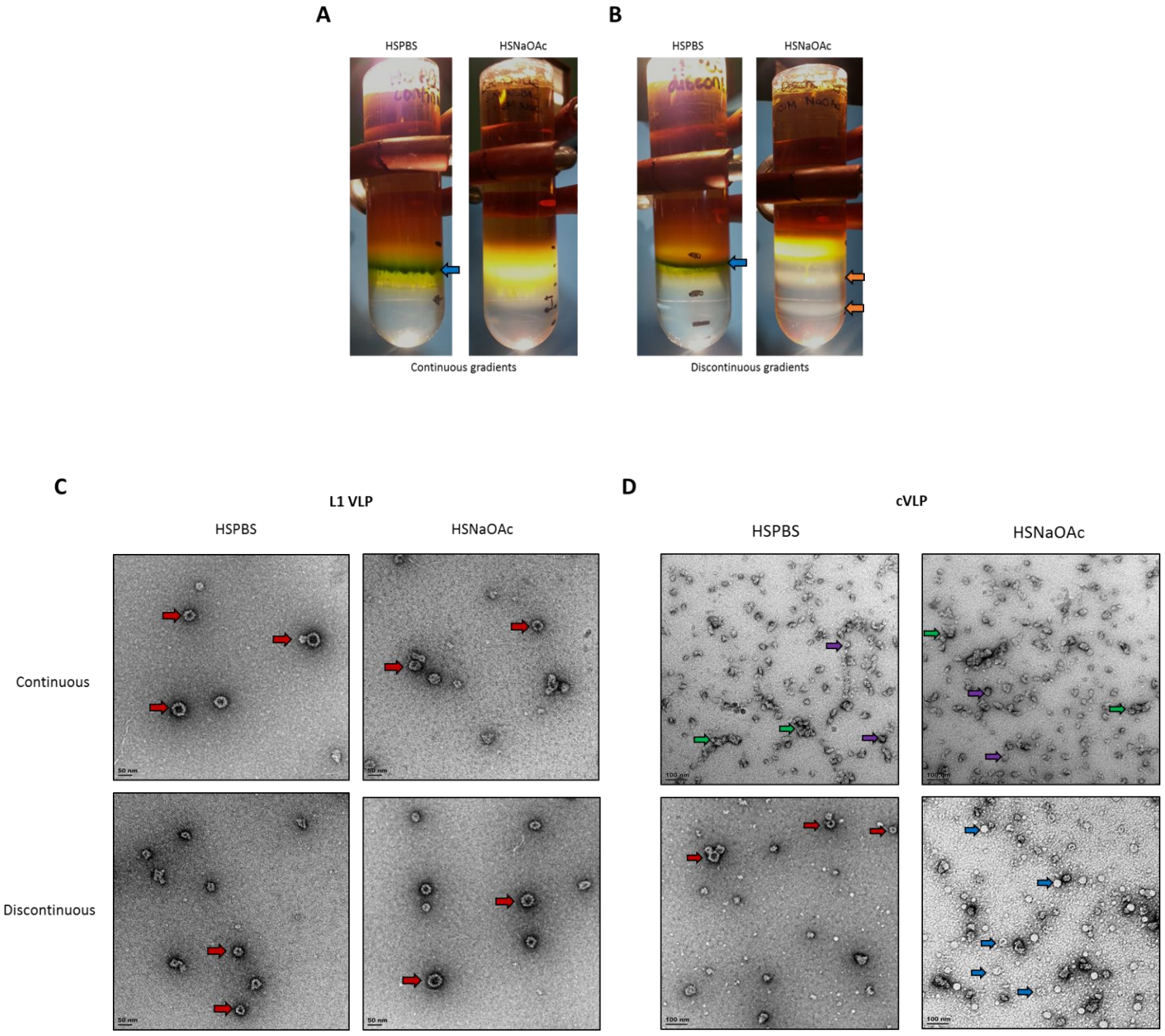


Figure 3.2: Comparison of buffers for extraction of cVLPs on continuous and discontinuous Optiprep™ gradients. A) Continuous 10-40% Optiprep™ gradient. **B)** Discontinuous 27-46% Optiprep™ gradient. Labels: blue arrows, plant material; orange arrows, opaque bands of two possible cVLP populations. **C)** TEM of L1 VLPs from continuous or discontinuous HSPBS or HSNaOAc gradients. **D)** TEM of SAC 56-81 cVLPs from discontinuous HSPBS or HSNaOAc gradients. Scale bar indicated bottom left in each image. Labels: red arrows VLPs; blue/purple arrows, small cVLPs; green arrows, aggregates.

Table 3.2: Summary of methods investigated for the purification of chimaeric vaccine candidates

Method	Gradient	Sample extraction buffer	Details	Results
Buffer optimisation	Continuous or discontinuous gradient	HSNaOAc or HSPBS	Extraction in HSNaOAc or HSPBS	Extraction in HSNaOAc is better than HSPBS in removal of RuBisCo.
Isopycnic centrifugation	Continuous gradient	HSNaOAc or HSPBS	10% to 40% continuous Optiprep™ gradient made in HSNaOAc or HSPBS	L1 in cVLPs was detected in a wide range of fractions from dot blots analysis. Few cVLPs observed with aggregates present. No noticeable difference in extraction in HSPBS or HSNaOAc.
	Discontinuous gradient	HSNaOAc or HSPBS	27%, 33%, 39% and 46% discontinuous Optiprep™ gradient made in HSNaOAc or HSPBS	Dot blots show L1 of cVLPs concentrated in fewer fractions. Extraction in HSPBS showed cVLPs more similar to L1 VLPs than HSNaOAc; however, yield was lower in HSPBS.
Rate-zonal centrifugation	Continuous gradient	HSNaOAc or HSPBS	30% and 50% sucrose cushions made in HSNaOAc followed by overnight dialysis in HSPBS and then continuous 10% to 40% Optiprep™ gradient made in HSPBS	Sample required dilution in order to load on gradient. Dot blots showed detection of L1 for almost all fractions. cVLP yields were low. Not reproducible for different chimaeras.
Concentrating cVLPs	Amicon® Ultra centrifugal filter units Ultra-15, MWCO 100 kDa	HSPBS	Sample diluted, added to Amicon® receiving chamber and centrifuged.	Degradation of L1. Significant loss in L1 yields.
	PEG precipitation	HSPBS	Sample incubated in PEG 8000 10% w/v and centrifuged to pellet cVLPs	cVLPs did precipitate, however western blot and TEM analysis showed that L1 was degraded and cVLPs lost structural integrity.

Although rate-zonal centrifugation of hL1 VLPs has previously been shown to separate L1 VLPs efficiently, low yields were obtained, making it an ineffective method to characterize the population of VLPs (van Zyl and Hitzeroth, 2016). Rate-zonal centrifugation was not successful for the purification of chimaeras in this study (Table 3.2) and this could be due to the presence of aggregates or the formation of misassembled particles. Additionally, this method is not scalable, but remains useful as an analytical tool.

To improve the assembly of cVLPs, maturation of particles was performed as described by Cardone et al. (2014). Matured samples were purified, treated in non-reducing and/or non-denaturing conditions and resolved on non-reducing SDS-PAGE gels. Different L1 species (monomers, dimers or

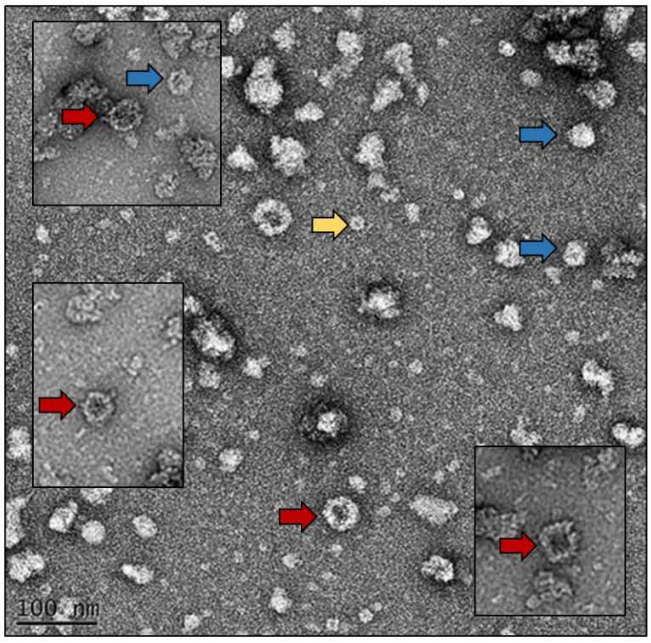
trimers) were not observed, but instead smears were observed in the lanes of the gel (data not shown).

Quantitation of chimaeras by gel densitometry was unsuccessful as samples run on the gels produced smears, even after dilution of the purified samples (data not shown). Analysis of these smears by LC-MS showed significant hits to the HPV-16 L1 major capsid protein based on the best Log probability score, total intensity and number of unique peptides; however, several *Nicotiana* spp. associated proteins were also detected, showing that some plant proteins were co-purified with L1.

3.3.1.2. Final purification of vaccine antigens

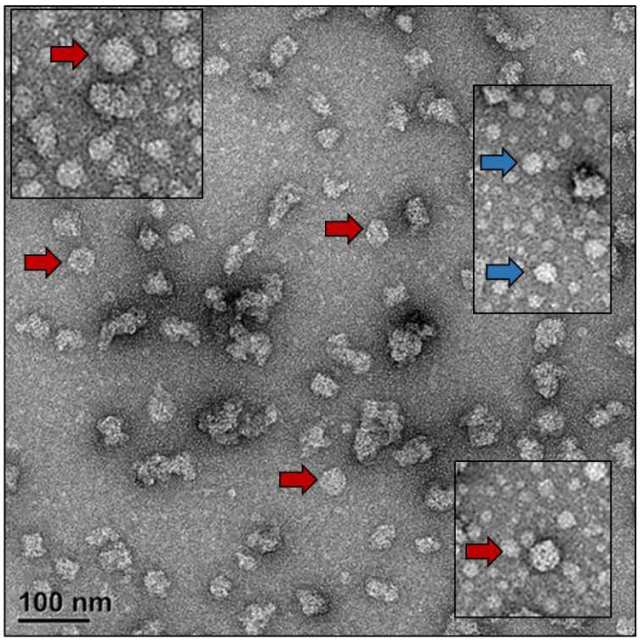
cVLPs and hL1 VLPs were extracted and purified in HSNaOAc on discontinuous Optiprep™ density gradients. Concentration of cVLPs by ultrafiltration and precipitation were unsuccessful (Table 3.2) therefore, fractions 1-4 were pooled and centrifuged again to concentrate the particles. The cVLPs and VLPs were observed as an opaque band which was then collected with a needle. Purified cVLPs were visualised by TEM to determine their structural integrity prior to vaccination (Figure 3.3). Chimaeras of SAC 108-120, SAC 65-81, SAC 56-81 and SAC 17-36 (Figure 3.3 A-D, respectively) showed cVLPs that ranged in size from 50-60 nm (red arrows), small cVLPs (25-40 nm, blue arrows) and capsomeres (10 nm, yellow arrows), with SAC 108-120 (Figure 3.4A) cVLPs being the most similar to purified HPV16-hL1 VLPs (Figure 3.3F). SAE 65-81 (Figure 3.3E) showed few cVLPs with mostly aggregates present. HPV-16 hL1 (Figure 3.4F) assembled into particles measuring 50-60 nm in size, with a few small VLPs present. The empty vector control (Figure 3.4G) showed no VLPs or structures resembling capsomeres. In comparison to cVLPs previously purified in our group by heparin chromatography (Pineo et al., 2013) and cation exchange chromatography (McGrath et al., 2013), the cVLPs were more rounded and similar to HPV-16 hL1 VLPs.

A



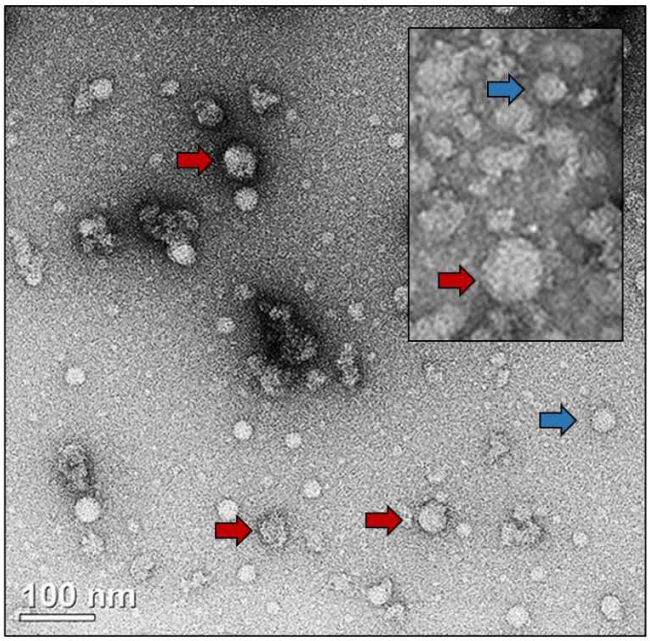
SAC 108-120

B



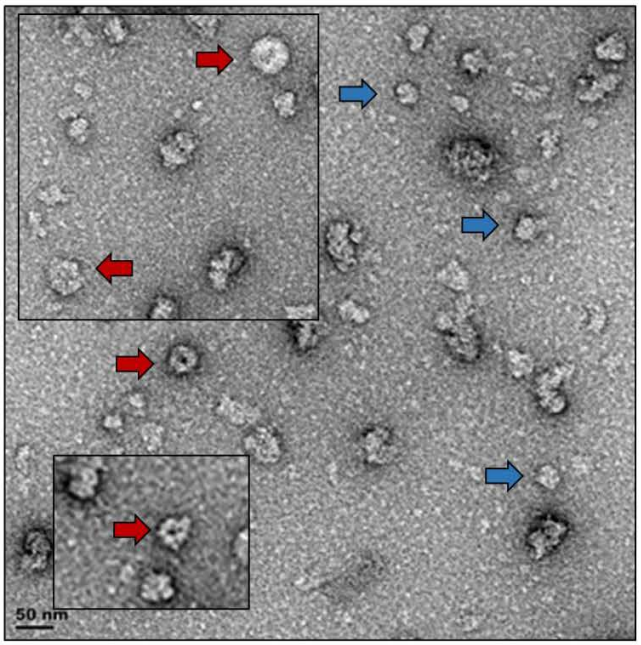
SAC 65-81

C



SAC 56-81

D



SAC 17-36

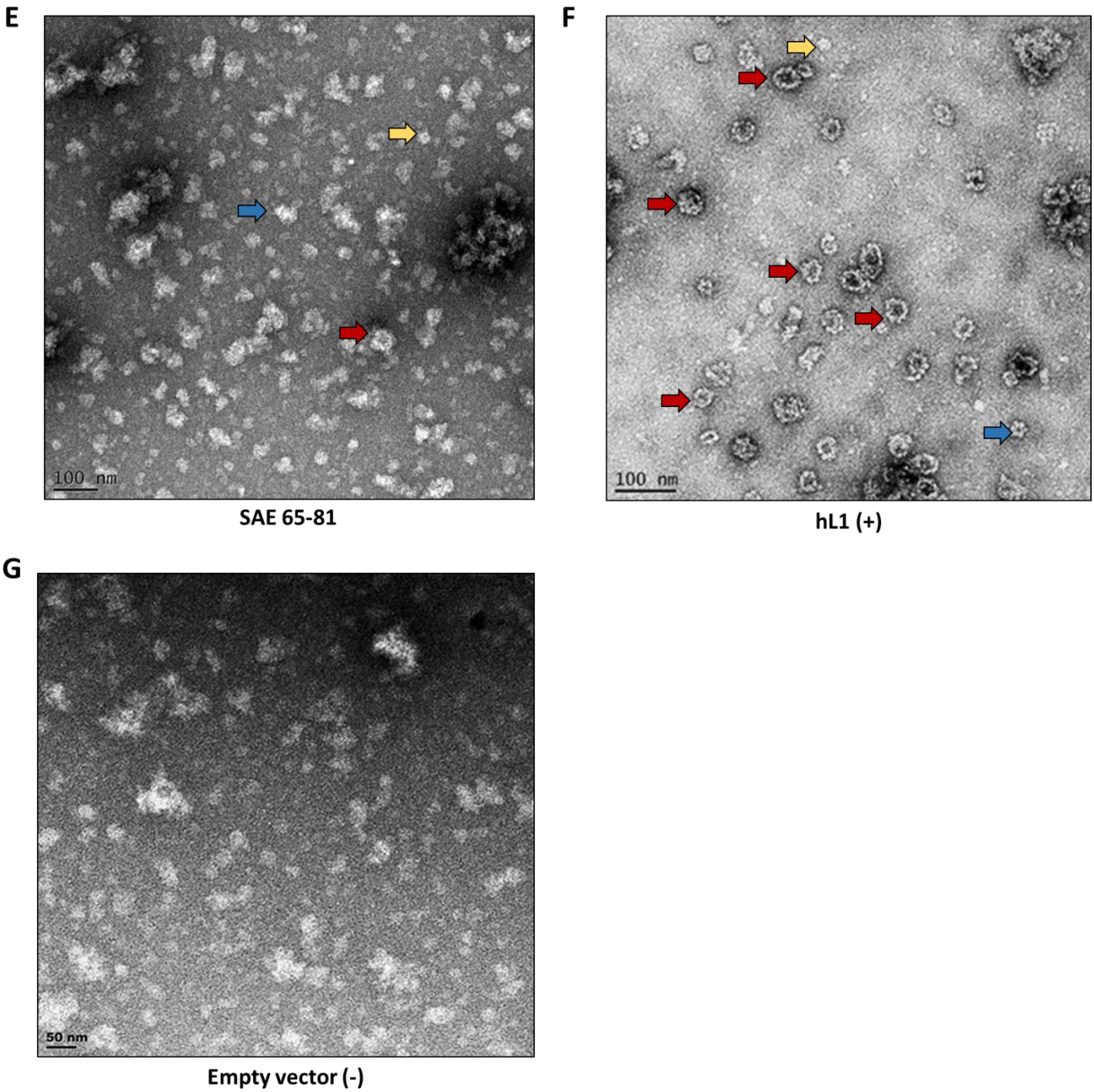


Figure 3.3: Transmission electron micrographs of purified cVLPs used in animal trials. Purification of cVLPs in HSNaOAc buffer, followed centrifugation on a discontinuous Optiprep™ gradient. **A)** SAC 108-120, **B)** SAC 65-81, **C)** SAC 56-81, **D)** SAC 17-36, **E)** SAE 65-81, **F)** HPV-16 hL1 and **G)** empty vector control. Labels: Red arrows, cVLPs 50-60 nm; blue arrows, small cVLPs 25-40 nm; yellow arrows, capsomeres ~10 nm. Scale bar indicated bottom left in each image.

3.3.2. Indirect ELISA quantitation of purified vaccine antigens

Vaccine antigens were purified on discontinuous Optiprep™ gradients in HSNaOAc, followed by concentration. Purified cVLPs were subsequently quantified by indirect ELISA. Figure 3.4 shows the yields obtained for each chimaera after purification and concentration. Total L1 protein was detected using Camvir-1 MAb (McLean et al., 1990) that recognises the linear L1 epitope 204-210, as none of the L2 substitutions affected this site. SAC 108-120 had the highest yield of 145 mg/kg, which was comparable to hL1 at 142 mg/kg. SAC 56-81 had the second highest yields of 43 mg/kg, followed by SAC 17-36 (29 mg/kg), SAC 65-81 (7.8 mg/kg) and SAE 65-81 (1.2 mg/kg). A minimum yield of 50 mg/kg (black dashed line) was required to have at least 5µg/100 µL dose/mouse in animal trials. Despite several attempts at concentrating the cVLPs, Figure 3.3 represents the highest yields obtained after the purification of the vaccine antigens.

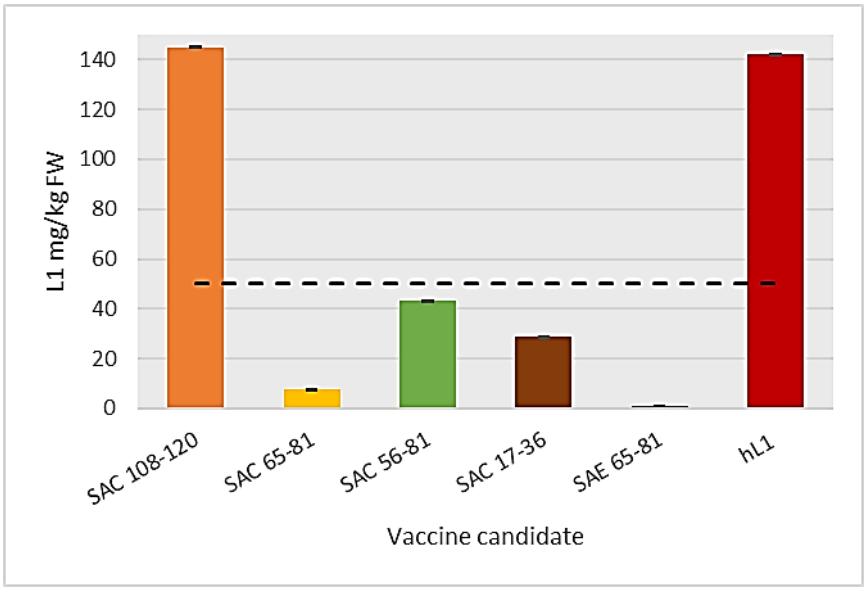


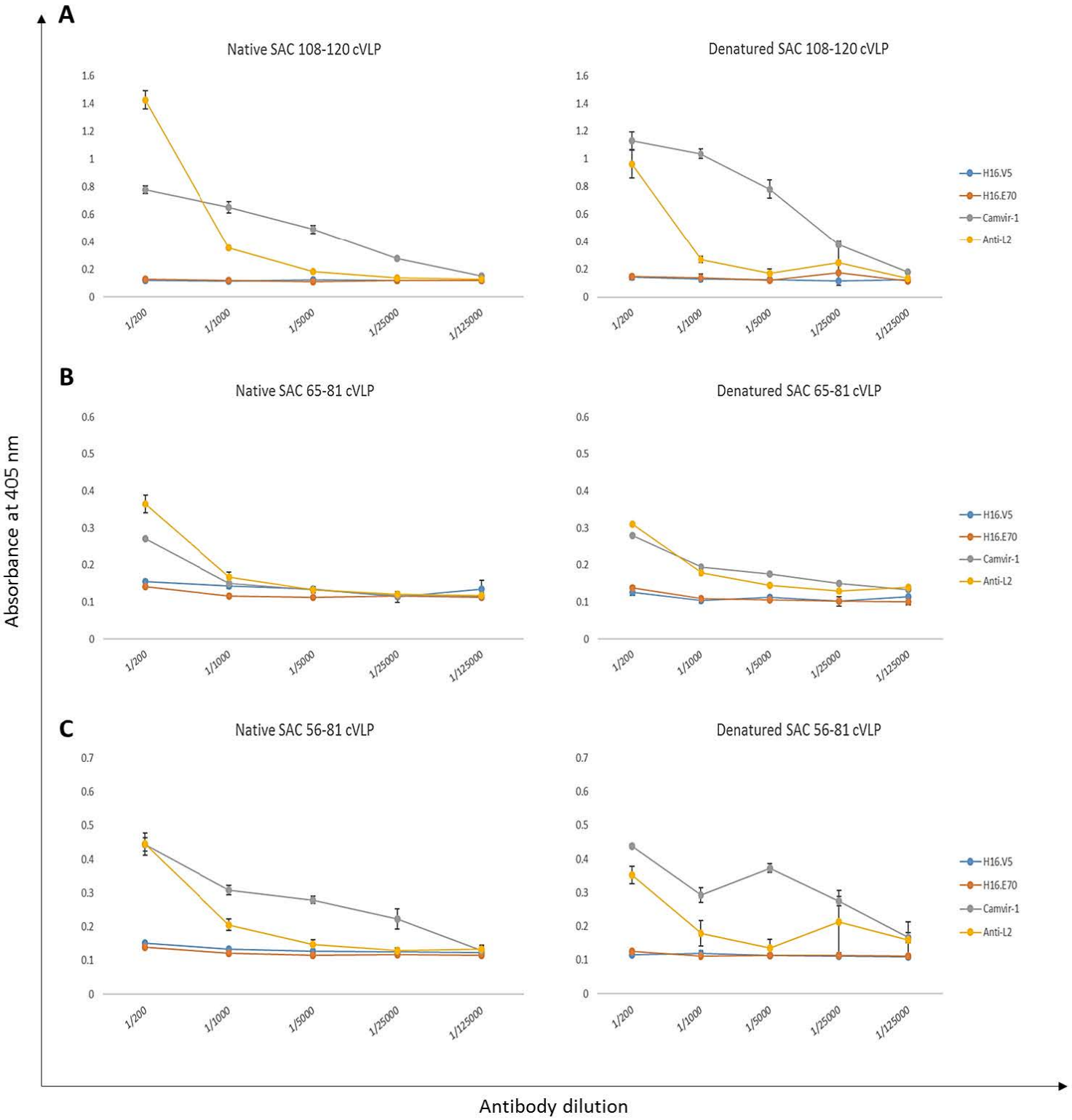
Figure 3.4: Purification yields of vaccine candidates based on L1 indirect ELISA. Final purification yields of cVLPs to be used in animal studies. cVLPs were detected using Camvir-1 MAb. Error bars indicate standard deviation between replicates. Horizontal dashed line, cut off needed to have 5 µg doses for animal studies.

3.3.3. L1 and L2 epitope display on the cVLP scaffold

The antigenicity of cVLPs and their ability to present the substituted L2 peptides was analysed by indirect ELISA using a panel of MAbs and anti-L2 polyclonal serum (Figures 3.5 and 3.6). Under native conditions, conformationally dependent neutralising MAbs H16.V5 and H16.E70 did not bind the L1:L2 cVLPs (Figures 3.5 A-E, left panels), indicating the disruption or steric hindrance of the V5 and E70 neutralising epitopes by substitution of the L2 peptides. The anti-L2 polyclonal serum reacted with all cVLPs in native form indicating the L2 peptides were displayed on the virion surface, with

strongest binding observed for SAC 108-120 (Figure 3.5A, left panel). Additionally, binding of the MAb L2-4B4, which recognises the L2 peptide 108-120 (Figure 3.6A, left panel), showed strong binding only to SAC 108-120 cVLPs. Strong binding was also seen by the non-neutralising MAb Camvir-1, that recognises a linear epitope L1 aa 204-210, preferentially under denaturing conditions (Figures 3.5 A-F, right panels). As expected, native hL1 VLPs were not bound by anti-L2 polyclonal serum. hL1 VLPs were bound by H16.V5 and H16.E70, but binding was diminished under denaturing conditions (Figure 3.5F, compare left and right panels).

Similarly, neutralising MAbs H16.9A and H16.J4 bound to all native SAC cVLPs (Figures 3.6 A-D, left panels). H16.U4 however showed decreased binding for SAC 108-120, SAC 56-81 and SAC 17-36 cVLPs (Figures 3.6 A, C and D, respectively). No binding of these MAbs to native SAE 65-81 cVLPs (Figure 3.6E, left panel) was observed, and this may be due to the poor assembly of cVLPs as observed by TEM (Figure 3.3E) and/or disruption or steric hindrance as mentioned above. Under denaturing conditions, all cVLPs were not bound by H16.U4, H16.9A conformational MAbs, but showed binding by H16.J4, which recognises the linear epitope aa 261-280 (Figures 3.6 A-D, right panels). Likewise, as observed for H16.V5 and H16.E70 MAbs, H16.U4, H16.9A and H16.J4 MAbs strongly bound HPV-16 hL1 VLPs, but showed decreased binding when denatured (Figure 3.6F, compare left and right), with H16.J4 showing slightly better binding.


A
Native SAC 108-120 cVLP
Denatured SAC 108-120 cVLP
B
Native SAC 65-81 cVLP
Denatured SAC 65-81 cVLP
C
Native SAC 56-81 cVLP
Denatured SAC 56-81 cVLP
H16.V5
H16.E70
Camvir-1
Anti-L2
1/200
1/1000
1/5000
1/25000
1/125000
Absorbance at 405 nm
Antibody dilution

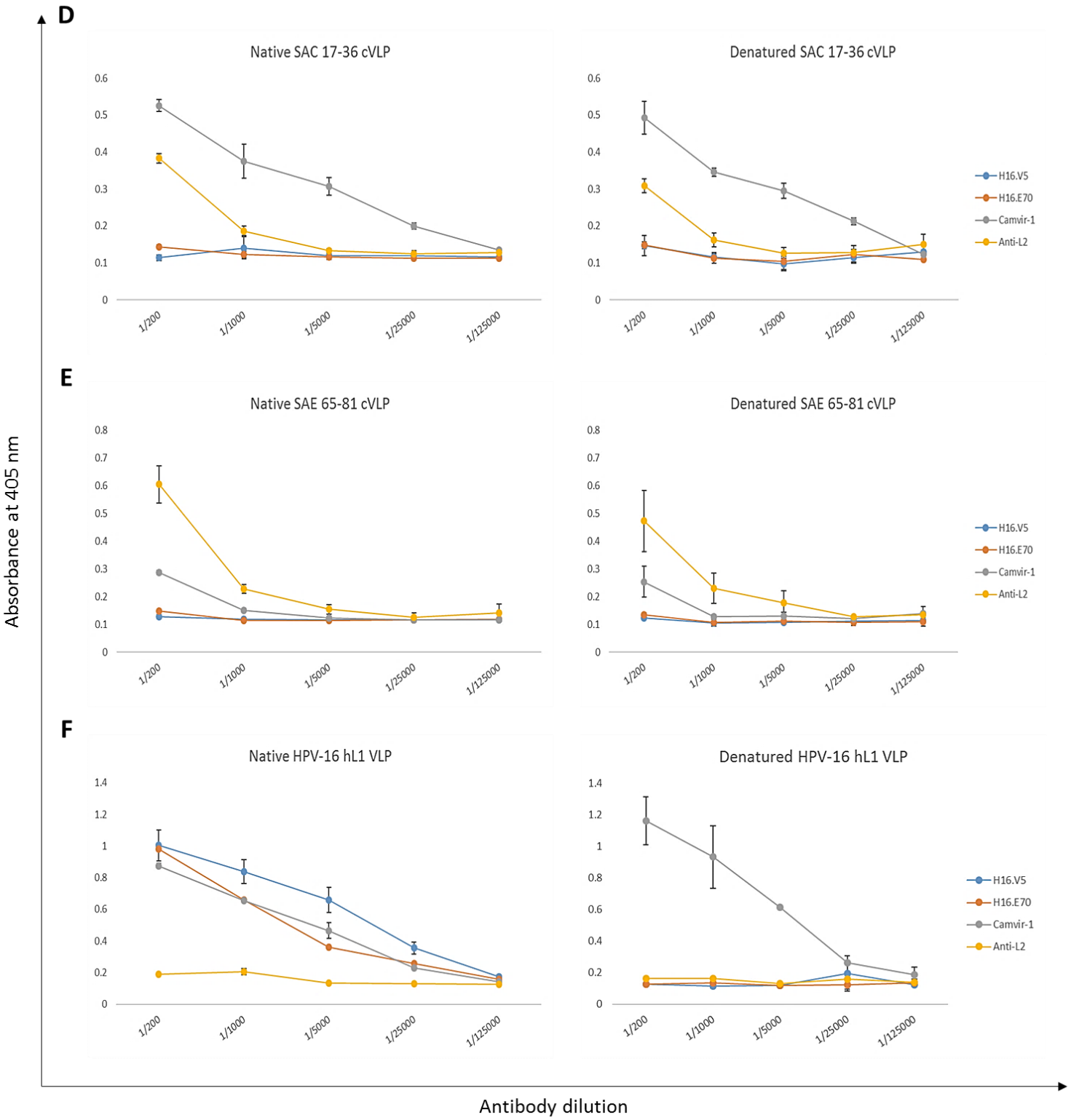
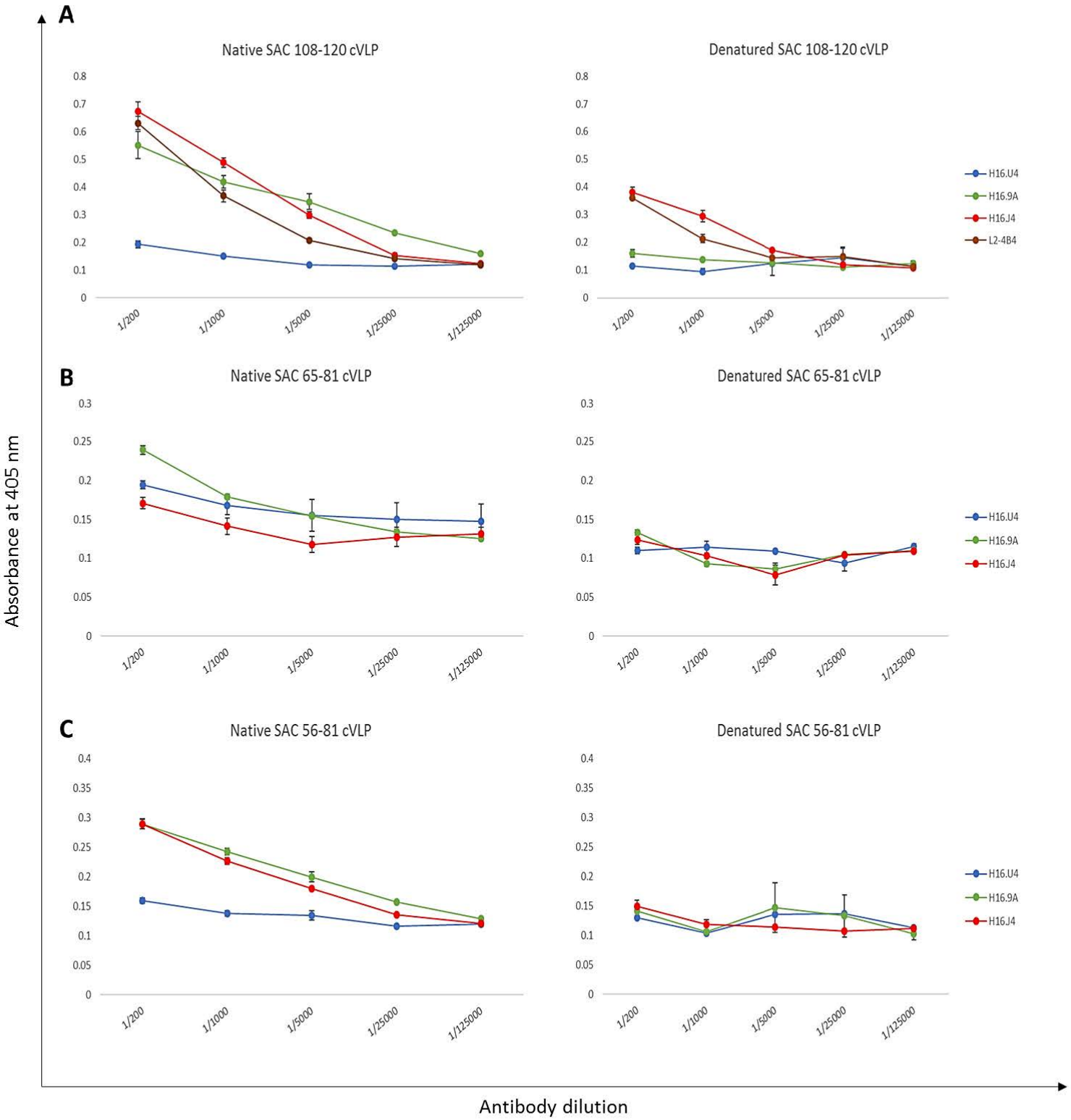


Figure 3.5: Characterisation of cVLP epitope display by indirect ELISA. Binding of monoclonal and polyclonal antibodies to HPV-16 L1:L2 cVLPs: **A)** SAC 108-120, **B)** SAC 65-81, **C)** SAC 56-81, **D**) SAC 17-36 and **E)** SAE 65-81 were compared to HPV-16 hL1 VLPs **(F)** under native and denaturing conditions using neutralising MAbs H16.V5, H16.E70, non-neutralising MAb Camvir-1 and polyclonal anti-L2 serum.


A
Native SAC 108-120 cVLP
Denatured SAC 108-120 cVLP
B
Native SAC 65-81 cVLP
Denatured SAC 65-81 cVLP
C
Native SAC 56-81 cVLP
Denatured SAC 56-81 cVLP
H16.U4
H16.9A
H16.J4
L2-4B4
1/200
1/1000
1/5000
1/25000
1/125000
Absorbance at 405 nm
Antibody dilution

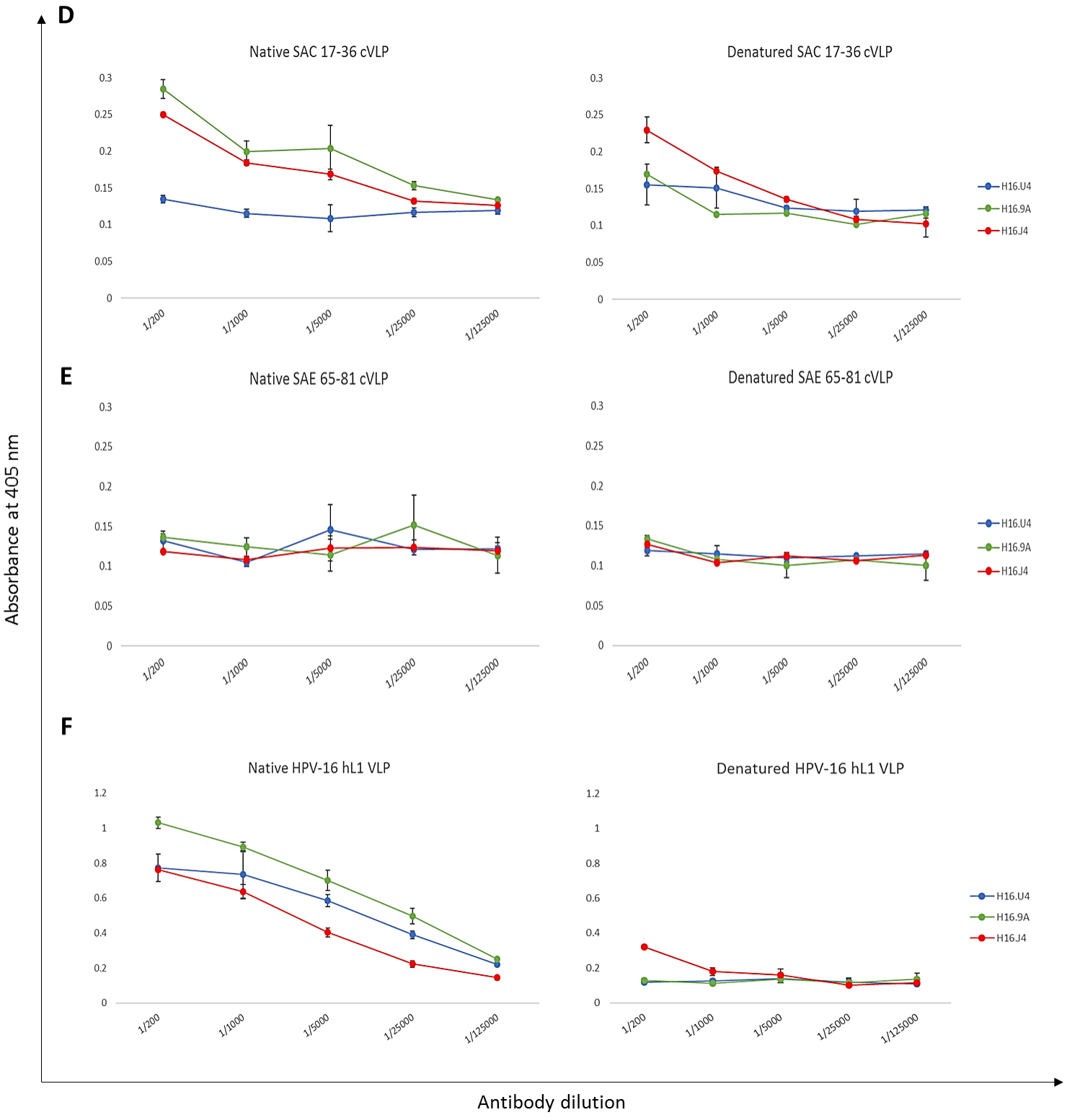


Figure 3.6: Additional characterisation of cVLP epitope display using MAbs by indirect ELISA. Binding of MAbs to HPV-16 L1:L2 cVLPs: **A)** SAC 108-120, **B)** SAC 65-81, **C)** SAC 56-81, **D**) SAC 17-36 and **E)** SAE 65-81 were compared to HPV-16 hL1 VLPs **(F)** under native and denaturing conditions using neutralising MAbs H16.U4, H16.9A and linear neutralising MAb H16.J4. MAb L2-4B4 (L2 aa 108-120) was also used to analyse binding to SAC 108-120 cVLPs.

3.4. Discussion

Purification of VLPs that maintain the display of epitopes that elicit binding and NAbs is important for induction of an immune response in vaccination studies. L1 VLPs have been extensively purified by various methods from a variety of host expression systems. Commercial HPV vaccines are produced in yeast or insect cells, and are expensive due to extensive production and purification protocols. Low-cost production and purification systems are therefore key in the production of more affordable vaccines.

Heparin chromatography has been successful in the purification of yeast-derived L1 VLPs (Kim et al., 2010; Kim et al., 2012a); however, co-purification of contaminants was observed. Similarly, purification by this method of plant-made L1 VLPs (A. R. van Zyl, personal communication) or L1:L2 chimaeras (Pineo et al., 2013) resulted in the presence of plant contaminants, suggesting that heparin can bind miscellaneous proteins. Cation-exchange chromatography has also been used to purify yeast-expressed VLPs (Cook et al., 1999; Kim et al., 2010; Kim et al., 2012a) or insect-cell produced L1:L2 chimaeras (McGrath et al., 2013), but purification of plant-made L1:L2 chimaeras was unsuccessful as the majority of the protein remained unbound by the column (Pineo, 2011). Therefore, density or size-based purification methods were investigated.

Density-based ultracentrifugation is routinely used for the purification of VLPs from several expression systems. L1 VLPs have successfully been expressed and purified from plants (Biemelt et al., 2003; Fernandez-San et al., 2008; Maclean et al., 2007). L1:L2 chimaeras ranging in size from 50-60 nm have successfully been produced in insect cells and purified by ultracentrifugation on sucrose-PBS and CsCl-PBS density gradients (Huber et al., 2015; Huber et al., 2017; Schellenbacher et al., 2013; Schellenbacher et al., 2009; Varsani et al., 2003a). In this study, positive control L1 VLPs and L1:L2 cVLPs were purified by ultracentrifugation based on density or size in Optiprep™ density medium. Optiprep™ is a sterile and endotoxin-free iso-osmotic and isotonic solution, which reduces destabilisation of proteins and is suitable for use in animals (Axis-Shield PoC AS, 2016). Extraction of chimaeras in HSNaOAc was beneficial in the partial removal of plant proteins (Figure 3.2 A and B), mainly RuBisCo, as no signal was detected in purified fractions probed with anti-RuBisCo in dot blots (data not shown). Additionally, the low pH and high salt concentration of the buffer appeared to help maintain the integrity of the cVLPs during the extraction and purification process (Figure 3.2 D).

Purified cVLPs of 25-55 nm in size were observed by TEM (Figure 3.3), and were similar to cVLPs purified in CsCl by (Varsani et al., 2003a). Although aggregates were observed in these preparations,

Huber et al. (2017) have also observed tubular baculovirus structures, aggregates and pentamers in purification of their cVLPs. cVLPs of SAC 108-120 (Figure 3.3A) were most similar in appearance to hL1 VLPs (Figure 3.3F), and also resembled insect cell produced cVLPs where L2 108-120 was substituted into the DE loop or between the h4 and β-J region (Varsani et al., 2003a). However, the substitution of the 3 other L2 epitopes in these positions showed a mixed population of cVLPs, capsomeres or aggregates, suggesting that epitope substitution affects particle assembly (Figure 3.3 B-F). Notably, SAE 65-81 showed the poorest assembly into VLP-like structures (Figure 3.3E), which may be due to structural alterations in the C-terminal arm of L1, where the h4 and β-J region is located, and which is critical for assembly into VLPs (Li et al., 1997). Differences in assembly of cVLPs could further be attributed to the length and amino acid sequence of the L2 peptide used. Cys residues 175 and 428 have been shown to be critical for the formation of disulphide bonds between capsomeres for the formation of VLPs and mutations of these residues prevents the formation of VLPs (Fligge et al., 2001; Li et al., 1998; McCarthy et al., 1998; Sapp et al., 1998; Varsani et al., 2006a). Figure 3.7 shows a multiple sequence amino acid alignment of the substituted L2 epitopes against the HPV-16 L1 sequence. Although the Cys^{175} and Cys^{428} (blue boxes) are not lost due to L2 peptide substitution, the rate of formation of disulphide bonds between neighbouring L1 capsomeres may have been decreased due to a slow kinetic thio-disulphide interchange rate (Nagy, 2013).

A

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HPV16       PLLNKLDDTENASAYAANAGVDNRECISMDYKQTQLCLIGCKPPIGEHWGKGSPCTNVAV  180
108-120     PLLNKLDDTELVEETSFIDAGAPRECISMDYKQTQLCLIGCKPPIGEHWGKGSPCTNVAV  180
65-81       PLLNKLDDTEGTGGRTGYIPLGTRPPTSMDYKQTQLCLIGCKPPIGEHWGKGSPCTNVAV  180
56-81       PLLNKLDDTEGGLGIGTGSGTGGRTGYIPLGTRPPTCLIGCKPPIGEHWGKGSPCTNVAV  180
17-36       PLLNKLDDTEQLYKTCKQAGTCPPDIIPKVVKQTQLCLIGCKPPIGEHWGKGSPCTNVAV  180
            **********                  .:   ********************:*****
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B

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HPV16       VTSQAIACQKHTPPAPKEDPLKKYTFWEVNLKEKFSADLDQFPLGRKFLLQAGLKAKPKF  480
108-120     VTSQAIACQKLVEETSFIDAGAPYTFWEVNLKEKFSADLDQFPLGRKFLLQAGLKAKPKF  480
65-81       VTSQAIACQKGTGGRTGYIPLGTRPPTEVNLKEKFSADLDQFPLGRKFLLQAGLKAKPKF  480
            **********  .               **********************************
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Figure 3.7: ClustalOmega alignment of HPV-16 L1 with substitution of L2 epitopes into DE loop (SAC) **(A)**, or between the h4 and β-J structural region (SAE) **(B)** of HPV-16 L1. The homologous regions are in black. Red regions are the 4 L2 peptides. Blue boxes indicate Cys residues 175 and 428. Alignment performed using multiple sequence alignment tool found at: http://www.ebi.ac.uk/Tools/msa/clustalo/.

L2 108-120 is the shortest epitope of the 4 substitutions, suggesting that longer epitopes may be detrimental to complete particle assembly and thus epitope display, wherever they are situated. This

was also observed by Pineo et al. (2013) and McGrath et al. (2013) who investigated the production of cVLPs in plant and insect cell systems, respectively. In these studies, L2 epitopes were substituted in the h4 helix from position 414 in the C-terminal region and although L2 108-120 substitution did not eliminate the Cys^{428} residue, substitution of L2 56-81 and 17-36 did, resulting in the formation of capsomeres and aggregates. In contrast, Matic et al. (2011) produced plant-made HPV-16 L1 chimaeras containing influenza virus type A M2e epitopes in the h4 helix and between the h4 and β-J region, and found that a longer epitope (23 residues) was better than a shorter epitope (8 residues) in the display of the M2e epitope. Additionally, the amino acid sequence composition can affect interaction with other residues (due to charge and size) and therefore folding. Specifically, the addition of two Cys residues in the L2 epitope 17-36 may form disulphide bonds with Cys^{175} or Cys^{428} in the L1 backbone, accounting for the particles observed in Figure 3.3D. These data suggest that protein modelling of the interactions of substituted epitopes of varying length and sequence with L1 residues requires further investigation. Although protein modelling of chimaeras was not part of the scope of this study, the L2 substitution positions were selected based on protein models of L2 108-120 in 5 HPV-16 L1 surface loops described by Varsani (2003).

A characterisation of L2 epitope display in L1 by Varsani et al. (2003a) showed that the L2 108-120 epitope substituted in the DE loop or between the h4 and β-J structural region was bound by a polyclonal antiserum raised against the L2 epitope. Epitope display characterised by binding of MAbs to cVLPs was also investigated in my study and showed diminished binding by H16.V5 and H16.E70 (Figure 3.5). This lack of binding by H16.V5 and H16.E70 was also observed by Varsani et al. (2003a) and Huber et al. (2017) where substitution of L2 aa 108-120 or insertion of L2 aa 17-36 respectively, in the DE loop of HPV-16 L1, resulted in weak or no binding of these MAbs, and indicates disruption of these conformational epitopes. However, binding by MAbs H16.U4, H16.9A and H16.J4 was observed (Figure 3.6) and suggests that several epitopes required for the generation of NAbs were maintained. Unexpectedly, the SAE 65-81 cVLPs showed no binding by the conformational MAbs (Figure 3.5E and 3.6E), even though substitution of L2 between the h4 and β-J structural region should not affect display of these epitopes. This suggests that folding of the SAE 65 cVLPs may have been incorrect for binding by conformational MAbs. However, binding of anti-L2 polyclonal serum to all cVLPs (Figures 3.5 A-E) and L2 4B4 MAb to SAC 108-120 cVLPs (Figure 3.6A) confirmed the display of L2 peptides on the L1 surface. Although anti-L2 serum was able to detect the L2 peptides on native cVLPs, L2 peptide ELISAs as described by Huber et al. (2017), using L2 peptides as coating antigen and cVLP antisera as the primary antibody, could be considered for future characterisation studies, for a more specific evaluation of L2 epitope display.

Purified chimaera yields of 1.2 mg/kg to 145 mg/kg (Figure 3.4) were similar to yields of 45 to 120 mg/kg of L1/M2e chimaeras produced by Matic et al. (2011); however, these were much lower than yields of up to 600 mg/kg reported by Pineo et al. (2013) for other plant produced HPV L1:L2 chimaeras. cVLPs produced by Pineo et al. (2013) were purified by heparin chromatography, suggesting that this method was more successful at antigen enrichment, with L2 108-120 cVLPs showing the highest L1 yield. Interestingly, L2 108-120 cVLPs in this study also had the highest yields, suggesting that there may be discrimination in the expression and subsequent purification of cVLPs depending on the length and position of the epitope substitution. Pineo's work also indicated that even small changes in sequence of L1, due to substitutions as small as 5 aa, could cause reductions in yield of cVLPs of up to several orders of magnitude, which suggests that yield in these circumstances is empirically determined, and not subject to prediction.

Although purification of cVLPs by heparin chromatography resulted in higher L1 yields (Pineo et al., 2013), co-purification of plant proteins was still evident, and repeated attempts in purification of L1 VLPs were unsuccessful with no L1 detected by SDS-PAGE or TEM analysis (A.R. van Zyl, personal communication). This indicates that there remains a lot of work in optimising the purification of plant-made VLPs. Additionally, it should be determined if the method of purification affects VLP stability. L1 VLPs purified by ultracentrifugation in this study were visually more similar to L1 VLPs produced in other systems, than plant-made heparin purified VLPs (Pineo et al., 2013). Therefore, we need to find a balance between yield and VLP integrity. The correct display of epitopes to the immune system is critical for the induction of the humoral immune response for the generation of potent NAbs. Optimisation of disassembly and reassembly of plant-produced VLPs may be an avenue to explore to improve the balance between yield and VLP integrity as it has been shown to prevent aggregation of VLPs and result in homogenous sample preparations (Mach et al., 2006).

At least 5 µg/mouse of vaccine antigen was required for animal studies based on doses used by McGrath et al. (2013). However, due to low yields obtained from cVLP purification, with the exception of SAC 108-120 and hL1, all other chimaeras were used at the maximum dose possible (0.8-4.5 µg/dose – see Table 4.1, Chapter 4, section 4.2.1). However, Kim et al. (2012a) have shown high anti-HPV-16 L1 IgG titres and NAbs using L1 VLP vaccine doses of 8 ng and 1 µg; therefore we proceeded to animal studies.

In conclusion, a simple method for the purification of cVLPs was developed, with the use of a high salt/ low pH buffer advantageous in the removal of host cell proteins. cVLPs displayed L1 and L2

epitopes showing their potential to induce the generation of NAbs. Small VLPs (T=1) have been shown to elicit humoral immune responses that were similar to VLPs (T=7) (Schadlich et al., 2009). Therefore, the cVLPs purified in this study were used in immunogenicity studies and the neutralising potential of sera were investigated in Chapter 4.

Chapter 4: Immunogenic assessment and cross-neutralising potential of HPV-16 L1:L2 chimaeric virus-like particles

4.1. Introduction

Knowledge of viral and immunological mechanisms that govern immunity to a particular pathogen is key to the development of effective vaccines (Stanley, 2008). The L1 major capsid protein of PVs can spontaneously assemble into VLPs (Hagensee et al., 1993; Kirnbauer et al., 1992; Rose et al., 1993), and has been shown to elicit strong HPV type-specific NAbs (Christensen et al., 1994; Kirnbauer et al., 1992). L1 VLPs are the basis of the commercialised HPV vaccines Cervarix® (bivalent), Gardasil® (quadrivalent) and Gardasil®9 (nonavalent). These vaccines elicit robust humoral immune responses, with an efficacy of >90% (Cuzick, 2015; Joura et al., 2015; Kjaer et al., 2009; Paavonen et al., 2009a; Schiller et al., 2008); however, despite increasing valency, Gardasil®9 and other next-generation vaccines are unlikely to cost less. Vaccine use in developing countries which have the highest cervical cancer burden will therefore be limited (Parkin and Bray, 2006). Second generation vaccines based on the L2 minor capsid protein present an alternative to L1 VLP vaccines. L2-based vaccines are promising due to low antigenic variation across several HPV types, meaning they can potentially be broadly protective (Roden et al., 2000). The N-terminus of HPV-16 L2 has highly conserved regions (Lowe et al., 2008) which in particular have been explored as targets for vaccine development.

L2-based vaccines have been shown to induce broad cross protection both *in vitro* and *in vivo* in animal papillomavirus models (Karanam et al., 2009). However, L2 vaccines in clinical trials have shown weak serum antibody responses and low titres of L2-specific cross-neutralising antibodies (de Jong et al., 2002; Kawana et al., 2003). A strategy to increase the immunogenicity of L2 neutralising epitopes is the generation of cVLPs. Several studies have explored the presentation of L2 epitopes on larger molecules as it may increase their immunogenicity. The most promise has been seen with chimaeric RG1-VLPs (Schellenbacher et al., 2013; Schellenbacher et al., 2009), where HPV-16 L2 aa 17-36 was presented in HPV-16 L1 DE-surface loop. Mice were protected against challenge with high-risk mucosal PsV types HPV-16/18/45/31/33/52/58/35/39/51/59/68/56/73/26/53/66/34 and low-risk types HPV-6/43/44, with protection observed one year after vaccination. GMP production of RG1-VLPs is underway (Buchman et al., 2016) and a phase I clinical study is expected to begin in 2017.

There is no challenge model to test HPV vaccine efficacy; however, neutralisation of HPV PsVs *in vitro* has been used as the standard method to detect HPV-specific antibody responses. Buck et al. (2004) developed a system for the intracellular assembly of PV PsVs, and with this technology also developed a high throughput technique to analyse sera in PBNAs (Buck et al., 2005a; Pastrana et al., 2004). PsVs are produced in mammalian cells by transient transfection of codon optimised L1 and L2 structural genes and a reporter plasmid into HEK 293TT cells, where the SV40 large T antigen is overexpressed and causes replication of the reporter plasmid, which increases PsV yield. A secreted alkaline phosphatase (SEAP)-encoding reporter plasmid is used to monitor 293TT infection by enzyme activity assay in the cell culture supernatant. PsVs incubated with neutralising virus-specific sera and MAbs are not capable of cell infection and thus SEAP expression, resulting in a reduction in SEAP detection (Buck et al., 2005a). However, detection of antibodies produced by L2-based vaccines has proven difficult in standard *in vitro* PBNAs, even though protection has been shown *in vivo* in several animal challenge models and mouse challenge with HPV (Karanam et al., 2009). Although these results are promising for potential L2 vaccines, they are not suited for routine use in clinical trials as procedures are too cumbersome (Day et al., 2012). To address this issue, an *in vitro* assay based on the mechanism of HPV infection *in vivo* was developed by Day et al. (2012) to increase assay sensitivity for L2 detection.

In vivo, the main L2 cross-neutralising epitopes are not fully exposed on the outer surface of the capsid, but instead buried within the PsV, and inaccessible to NAbs (Buck et al., 2008; Gambhira et al., 2007b). L1 binds HSPGs on the epithelial basement membrane (BM) which is exposed following epithelial wounding, which induces a conformational change in the PsV capsid, exposing the N-terminus of L2 to cleavage by furin and a proprotein convertase (Kines et al., 2009). A second conformational change occurs further exposing cross neutralising epitopes of L2 (Kines et al., 2009). Binding of L2 NAbs at this step prevents the capsid from associating with keratinocyte cell surface receptors and prevents infection (Day et al., 2010; Day et al., 2008). The extended exposure of neutralising epitopes on the BM (for several hours before transcription initiation) may account for the effectiveness of L2-based vaccines (Day et al., 2010; Schiller et al., 2010). *In vitro*, this process is replicated by association of PsVs with the extracellular matrix (ECM) of specific cell types (e.g. HaCaT), and PsVs bind HSPGs on the cell surface essential for infection (Day et al., 2007; Giroglou et al., 2001; Kines et al., 2009). The ECM is similar to the BM as PsVs can bind it *in vitro* however, this interaction is not critical for infection as PsVs directly bound to the cells is enough to facilitate infection. L2 epitope exposure by furin cleavage can also take place on the cell surface, therefore conformational changes can occur *in vitro*. This assay was shown to be highly sensitive for the detection of protective anti-L2 antibodies (Day et al., 2012).

In this study, mice were immunised with 5 candidate cVLPs vaccines composed of chimaeric L1-based VLPs with substitutions of various L2-derived peptides in different surface features of L1, as well as a positive HPV-16 L1 VLP control and two negative controls. The immunogenic potential of the plant-produced chimaeras was analysed by their anti-L1 and anti-L2 humoral responses and the ability of NAbs to neutralise homologous HPV-16 and heterologous HPVs in L1 and L2-specific PBNAs.

4.2. Materials and methods

4.2.1. Immunisation of mice

Mice were immunised with 5 plant-derived candidate cVLPs vaccines, as well as a positive HPV-16 L1 VLP control and two negative controls (PBS and plant extract from *Agrobacterium* infected plants containing empty vector). Table 4.1 shows the vaccine groups used in this study. Animal ethics was obtained and approved by the Faculty of Health Sciences Animal Ethics Committee, University of Cape Town (AEC 014/024).

Table 4.1: Vaccine group information for immunisation study

Vaccine Group	Vaccine construct	Protein content	Mice per group	Antigen dose (µg)
G1	SAC 108-120	HPV-16 L1:L2 SAC 108-120	5	5
G2	SAC 65-81	HPV-16 L1:L2 SAC 65-81	5	0.8
G3	SAC 56-81	HPV-16 L1:L2 SAC 56-81	5	4.5
G4	SAC 17-36	HPV-16 L1:L2 SAC 17-36	5	2.85
G5	SAE 65-81	HPV-16 L1:L2 SAE 65-81	5	0.26
G6	hL1	HPV-16 L1	5	5
G7	Empty vector	-	5	n/a
G8	PBS	-	5	n/a

Forty female Balb/c mice, 8-10 weeks old, from the Research Animal Facility (Faculty of Health Sciences, University of Cape Town) were housed in a biosafety level 2 (BSL-2) facility. The antigen doses were adjusted to 100 µL with Dulbeco's PBS (DBPS, Sigma Aldrich) and administered according to Table 4.1. Each mouse was injected subcutaneously in the left or right flank. Pre-bleed sera (PBs) were collected 3 days prior (D -3) to vaccination on Day 0. Mice were boosted with the same doses on Day 0, Day 14 and Day 28, and a test bleed collected on Day 42 to ascertain if an additional boost was required. An additional boost was administered on Day 45 and final bleed sera (FBs) were collected by cardiac puncture on Day 59. Serum was isolated from the blood by centrifugation in

microtainer tubes (BD Microtainer® tube with BD Microgard™ Closure SST™ Gold) at 10 000 *x g* for 10 min, after which it was stored at -80°C.

4.2.2. Western blot detection of anti-L1 and -L2 antibodies in mouse sera

One microgram of purified HPV-16 L1 protein was loaded into wells of 10% SDS-PAGE gels and transferred onto nitrocellulose membranes as described in Chapter 2, section 2.2.9. Membranes were cut into individual strips and probed with pooled sera from each vaccine group at 1:100. Camvir-1 was used as positive control antibody at 1:20000 dilution.

HPV-16 L2 was expressed in *N. benthamiana* using a cell density of OD_{600} 0.6 for infiltration. Biomass was harvested 3 dpi. L2 protein was homogenised in 8 M urea with 1x Complete Mini EDTA-free protease inhibitor cocktail (Roche) at a ratio of 1:1.5 (w/v). The homogenate was filtered through Miracloth™ and clarified by centrifugation at 10 000 *x g* for 10 min at 4°C. TSP in the supernatant was quantified using the Bio-Rad DC Protein Assay (described in Chapter 2, section 2.2.12). One hundred micrograms total protein was loaded into the wells of 10% SDS-PAGE gels, and transferred to nitrocellulose membrane, and strips probed with pooled sera from each vaccine group at 1:100. Rabbit-raised anti-L2 serum was used as positive control antibody at 1:1000. Strips were probed with anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (1:10000), except for the L2 positive control which was probed with goat anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody (1:5000).

4.2.3. ELISA detection of anti-L1 antibodies in mouse sera

Indirect ELISAs were performed as described in Chapter 3, section 3.2.6. Ninety-six-well plates (Nunc Maxisorp, ThermoFisher Scientific) were coated with 100 ng purified HPV-16 L1 protein per well and incubated overnight at 4°C. Pre- and final bleed serum of individual mice was used as primary antibody (1:100), and alkaline phosphatase-conjugated anti-mouse IgG (1:10000) as secondary antibody.

4.2.4. Statistical analysis

Statistical significance was calculated between the vaccine groups (G1-G6) and the negative controls (G7 and G8) using the non-parametric Kruskal-Wallis test and Dunn's multiple comparison test to determine significance ($p<0.05$). Analyses were performed using GraphPad Prism 7.03 software.

4.2.5. Pseudovirion production

PsVs of 8 different HPV types (HPV-6, 11, 16, 18, 31, 45, 52 and 58) were produced as described in Production of Papillomaviral Vectors (Pseudoviruses), revised June 2015 (Laboratory of Cellular Oncology, https://home.ccr.cancer.gov/lco/pseudovirusproduction.htm). The HPV plasmid names and antibiotic resistance are shown in Table 4.2. Endotoxin-free plasmid DNA (NucleoBond® Xtra Midi EF, Macherey-Nagel) of all HPV types and the pYSEAP reporter plasmid (Pastrana et al., 2004) were prepared according to the manufacturer's instructions.

Table 4.2: Plasmids used to make PsVs

Plasmid	HPV type	Gene	Antibiotic resistance
p6 SHELL	HPV-6	L1 & L2	Ampicillin (100 µg/mL)
p11L1w	HPV-11	L1	Kanamycin (30 µg/mL)
p11L2w	HPV-11	L2	Kanamycin (30 µg/mL)
p16 SHELL	HPV-16	L1 & L2	Ampicillin (100 µg/mL)
p18 SHELL	HPV-18	L1 & L2	Ampicillin (100 µg/mL)
p31 SHELL	HPV-31	L1 & L2	Ampicillin (100 µg/mL)
p45 SHELL	HPV-45	L1 & L2	Ampicillin (100 µg/mL)
p52 SHELL	HPV-52	L1 & L2	Ampicillin (100 µg/mL)
p58 SHELL	HPV-58	L1 & L2	Ampicillin (100 µg/mL)
pYSEAP	n/a	SEAP	Blasticidin (75 µg/mL)

293TT cells were cultured in complete Dulbeco's Modified Eagle Medium (cDMEM) with 1% GlutaMAX (Life Technologies). cDMEM was supplemented with 10% fetal bovine serum (Hyclone™ FBS, Separations), 1% non-essential amino acids (Gibco), Penicillin-Streptomycin (100 units/mL penicillin, 100 µg/mL streptomycin) (Sigma Aldrich) and 250 µg/mL Hygromycin B (Roche). Cells were incubated at 37°C in a 5% CO_2 incubator.

4.2.5.1. Transfection of 293TT cells

293TT cells that reached 50-70% confluency were transfected follows: a total of 75 µg plasmid DNA (37.5 µg of each HPV plasmid and 37.5 µg of pYSEAP) was mixed in 3 mL of DMEM (serum free). In a separate tube, 160 µL of Lipofectamine®2000 was added to 3 mL DMEM. The DNA and Lipofectamine®2000 mixtures were incubated separately at room temperature for 20 min, combined and incubated for an additional 20 min. The resulting lipid/DNA complexes were added to the pre-plated cells and the media replaced with cDMEM 6 h post-transfection. Transfected cells were incubated at 37°C, 5% CO_2 for 40-48 h.

4.2.5.2. Harvest and maturation of PsVs

Culture medium was collected and cells in suspension pelleted by centrifugation for 5 min at 2000 x *g*. The cells adhered to the flask were collected by trypsinisation (0.05% Trypsin-EDTA, Gibco) and added to the previous tube and pelleted again by centrifugation. The supernatant was discarded and the pellet partially resuspended in residual fluid. The suspension was transferred to a 1.5 mL siliconized microcentrifuge tube and the original tube rinsed with 0.5 mL DBPS. This was added to the resuspended cells, after which the cells were centrifuged again and the supernatant discarded.

For PsV maturation, the pellet was partially resuspended by flicking the microcentrifuge tube, after which 1.5 mL DPBS supplemented with Penicillin-Streptomycin (100 units/mL penicillin, 100 µg/mL streptomycin) (Sigma Aldrich) and 9.5 mM $MgCl_2$ was added. Cells were lysed in $1/20^{th}$ volume 10% Triton X-100, $1/1000^{th}$ RNase mix (Ambion) and $1/40^{th}$ 1 M ammonium sulphate (pH 9) and the lysate incubated at 37°C for 20-24 h and mixed by gentle inversion twice for the first few hours. After maturation, the lysate was chilled on ice and clarified by centrifugation at 4°C for 5 min at 5000 *x g*. The supernatant was transferred to a siliconized tube and the remaining pellet washed in two pellet volumes of DPBS and re-centrifuged. This supernatant was combined with the first supernatant. The procedure was repeated twice more, with the first wash using one-pellet volume DPBS, and the addition of 0.8 M NaCl for the second wash. All the pooled supernatants were further re-clarified and used for the purification of PsVs.

4.2.5.3. PsV purification and fraction collection

PsVs were purified by ultracentrifugation on discontinuous Optiprep™ gradients of 46%, 39%, 33% and 27%. Gradients were left to diffuse for 1-4 h before addition of the clarified supernatant. The gradients were centrifuged at 16°C for 3.5 h at 234 000 *x g* in an SW 55 Ti rotor. Fractions were collected in siliconized microcentrifuge tubes by puncturing the bottom of the tube with a needle. The first fraction collected was 750 µL, after which 250 µL was collected for fractions 2-10 and the final 2 mL in fraction 11 and 12. Purified fractions were analysed on dot blots. One microlitre drops were spotted onto nitrocellulose membranes and probed with 1:2000 dilutions of the MAbs (kindly provided by Dr. Neil Christensen) described in Table 4.3. Anti-mouse IgG alkaline phosphatase-conjugated antibody (1:10000) was used as secondary antibody and detection was performed as described in Chapter 2, section 2.2.9.

4.2.6. Pseudovirion-based neutralisation assays

293TT cells were maintained as described in section 4.2.5. The L1 PBNA was performed as described in: Papillomavirus Neutralisation Assay (Laboratory of Cellular Oncology, https://home.ccr.cancer.gov/lco/neutralizationassay.htm). Titrations of PsVs were carried out to determine the amount to be used in the L1 assay. Additionally, the MAbs (positive neutralisation control) required to neutralise each HPV PsV type were titrated to determine optimal antibody dilutions.

4.2.6.1. PsV and monoclonal antibody titration

293TT cells were trypsinised and resuspended in neutralisation media (DMEM without phenol red, 10% FBS, 1% non-essential amino acids, Penicillin-Streptomycin). Cells were diluted to 3 x 10^5 cells/mL and 96-well tissue culture treated plates (TPP®, Sigma Aldrich) seeded with 100 µL/well of the cell suspension. To avoid evaporation, the outer wells of the plate were not used and instead filled with 150 µL/well of DMEM containing phenol red. The cells were incubated for at least 4 h before addition of the PsVs. All PsV and MAb dilutions were tested in triplicate. Doubling dilutions of PsVs preparations, ranging from 1:250-1:16000, were made in neutralisation media in untreated 96-well plates (Nunc, ThermoFisher Scientific). One hundred microlitres of each PsV dilution was added to the cells. To wells with cells only, 100 µL of neutralisation media was added and the plates incubated for 72 h at 37°C, 5% CO_2.

For the positive neutralisation control, the MAb was diluted so that it was 5-fold more concentrated than its known 95% neutralising dilution. Twenty-five microlitres of the diluted antibody in neutralisation media was added to the PsVs (total volume 125 µL) and incubated for 1 h on ice. The 125 µL mixture was then added to cells and incubated for 72 h at 37°C, 5% CO_2.

4.2.6.2. Detection of secreted alkaline phosphatase

After the 72 h incubation, SEAP was detected using the Great Escape kit (Great EscAPe™ SEAP Chemiluminescence Kit 2.0 (Clontech Laboratories, Inc.)) as per the manufacturer's instructions with a few modifications. The volumes of the dilution and substrate buffer were adjusted to 0.6 volumes of the stated instructions (described in Papillomavirus Neutralisation Assay protocol). Briefly, 15 µL of the supernatant was transferred to a white porvair 96-well plate (Porvair Sciences) and 45 µL 1x dilution buffer added to each well. The plate was covered with plastic coverfilm and foil and

incubated for 30 min at 65 °C, followed by incubation on ice for 2-5 min, after which 60 µL room temperature 1 x assay substrate was added to each well. The plate was incubated for 20 min at room temperature and SEAP signal read using a Modulus Microplate Reader (Promega).

4.2.6.3. L1-based neutralisation assay

Antibody responses and endpoint neutralisation titres in mouse sera were determined in this *in vitro* neutralisation assay. Controls for the assay included: Cell control, which gives the background cell culture supernatant reading; PsV only control (0% neutralisation); and MAb positive controls (100% PsV neutralisation). PsV were used at a concentration determined from the titration in section 4.2.6.1. MAbs were diluted in the ranges shown in Table 4.3, based on titrations performed in section 4.2.6.1. Due to the number of serum samples that required testing, pre-bleeds (PB) were tested at a dilution of 1:50 and final bleeds (FB) at 1:50 and 1:200 dilutions.

Table 4.3: HPV type-specific neutralising antibodies

HPV type	Monoclonal antibody	Dilution range	Fold dilution
HPV-6	H6.C6	250 – 16000	4-fold
HPV-11	H11.B2	250 – 16000	4-fold
HPV-16	H16.V5	$2x10^2$ – $2x10^6$	10-fold
HPV-18	H18.J4	$2x10^2$ – $2x10^6$	10-fold
HPV-31	H31.A6	$2x10^2$ – $2x10^6$	10-fold
HPV-45	H45.N5	$1x10^2$ – $1x10^6$	10-fold
HPV-52	H52.D11	$1x10^2$ – $1x10^6$	10-fold
HPV-58	H58.J6.3	$2x10^4$ – $2x10^8$	10-fold

293TT cells were prepared as described in section 4.2.6.1. One hundred microlitres of PsVs was added to sterile, untreated 96-well plates, after which 25 µL of MAb dilutions, PB and FB sera were added to the wells. Plates were incubated for 1 h at 4°C. The PsV/sera/MAb mixtures were added to the cells and the plates incubated for 72 h at 37°C, 5% CO_2. To PsV only and cell only control wells, 25 µL and 125 µL neutralisation media, respectively, was added to the wells. After incubation, the SEAP assay was performed as described in section 4.2.5.2. Neutralisation titres were stated as the reciprocal of the maximum serum dilution which reduced SEAP activity by >50% in comparison to the PsV control sample, which was not treated with serum.

4.2.7. L2-based PBNA

4.2.7.1. Cell maintenance

Human mammary epithelial cells (MCF10A) were cultured in DMEM:F-12A (Life Technologies) supplemented with 5% horse serum (The Scientific Group), 1% glutamine, 1% Penicillin-Streptomycin, 10 µg/mL insulin (Biochrom), 500 ng/mL hydrocortisone (Sigma Aldrich) and 20 ng/mL epidermal growth factor (Sigma Aldrich). Chinese hamster ovary cells, CHOΔfurin and pgsa-745 cells (defective in xylosyltransferase activity and do not produce any glycosaminoglycans) were cultured in DMEM supplemented with 10% FBS, 200 µM L-proline (Sigma Aldrich) and 1% Penicillin-Streptomycin. L2 assay medium: DMEM, 10% FBS, 200 µM proline and 1% Antimycotic Antimycotic (Life Technologies) was used for dilution of HPV PsVs, mouse sera as well as the L2 antibodies, K4L2 and K18L2 positive neutralization controls (kindly provided by Martin Müller's Lab, DKFZ, Heidelberg, Germany).

4.2.7.2. L2 neutralisation assay

The L2 assay was carried out according to the method described by Day et al. (2012). On day 1, 1 x 10^6 CHOΔfurin cells were seeded in a T75 cm^2 flask with 17 mL media and grown to confluency for 4 days. On day 4, a 96-well cell culture plate was seeded with 2 x 10^4 MCF10A cells/well in 100 µL and incubated overnight. The ECM was prepared from the MCF10A cells on day 5. The medium was removed from the cells and washed 2x with 100 µL 1x PBS. Fifty microlitres pre-warmed lysis buffer was added to each well and incubated for 5 min at room temperature. One hundred microlitres 1x PBS was gently added to each well on top of the lysis buffer, followed by the removal of 100 µL liquid. Fresh 100 µL 1x PBS was added to the wells, the total volume of 150 µL in the wells was removed leaving behind the ECM. The ECM was washed 2x with 100 µL 1 x PBS and the second wash left in the wells (for up to 60 min) until the CHOΔfurin cells were prepared. The conditioned media (cell culture supernatant) from the CHOΔfurin cells was centrifuged at 2000 x *g* for 5 min, and the clarified furin supernatant poured into a new centrifuge tube. Heparin (H-4784, Sigma Aldrich) was added to the furin supernatant to a final concentration of 8 µg/mL. The 100 µL 1x PBS that remained on the ECM was removed after which 70 µL furin-heparin supernatant and 50 µL of each HPV PsV dilution (diluted in L2 assay medium) was added to each well. Plates were incubated overnight at 37°C, 5% CO_2. The next day (day 6), the furin-supernatant virus mix was removed from each well, and the wells rinsed 2x with 100 µL 1x PBS, with the second wash left in the wells to prevent drying out while the sera dilutions were prepared. One hundred microlitres of each serum sample and L2 neutralisation control was added to the appropriate wells and the plates incubated at 37°C, 5% CO_2

for 2 h (minimum 1 h incubation). After incubation, pgsa-745 cells were trypsinised using 0.05% trypsin and centrifuged for 5 min at 2000 x *g*. The cell pellet was resuspended in L2 assay medium and the cell concentration adjusted to 1.6 x 10^5/mL. Fifty microlitres of cells were added to each well and the plates incubated for 48 h at 37°C, 5% CO_2. On day 8, the SEAP assay was performed as described in section 4.2.5.2. Neutralisation titres were expressed as the reciprocal of the maximum serum dilution which reduced SEAP activity by at least 50% in comparison to the PsV control sample, which was not treated with serum.

4.3. Results

4.3.1. Anti-L1 and -L2 humoral responses

4.3.1.1. Western blot detection of HPV-16 L1 and L2

The anti-L1 and L2 humoral immune responses of the cVLPs were analysed on western blots using purified L1 or partially purified L2 as antigen. PB and FB sera for all individual mice in the different vaccine groups (G1-G8) were pooled and analysed for anti-L1 and -L2 immune responses. In Figure 4.1, anti-L1 Camvir-1 MAb and rabbit-raised anti-L2 sera, which were used as positive control antibodies, successfully detected both L1 (~56 kDa, Figure 4.1A) and L2 (~80 kDa, Figure 4.1B). These proteins were not detected when probed with PB sera of G1-G8. A band representing L1 (~56 kDa, black arrow) was successfully detected by FB sera of SAC 108-120, SAC 56-81 and the L1 positive control vaccine (G1, G3, and G6, respectively) on anti-L1 western blots (Figure 4.1A). FB sera of SAC 65-81 and SAC 17-36 (G2 and G4, respectively) detected bands of lower molecular weight than the expected ~56 kDa for L1. These bands were also observed when probed with CamVir-1 MAb (+). The presence of these bands suggests that L1 protein may have been degraded during the extraction/purification processes, and linear epitopes on these degraded products were still detected by sera. Additionally, these bands were also present when probed with sera from G1, G3 and G6. It is important to note that vaccination doses for G1, G3 and G6, were higher (5, 4.5 and 5 µg, respectively), compared to G2 and G4 (0.8 and 2.85 µg, respectively), suggesting that more antibodies were generated in mice vaccinated with higher antigen doses and were better at the detection of full length L1 protein. Antiserum to SAE 65-81 (G5) came from mice which had the lowest concentration of antigen (0.26 µg) for vaccination, and did not detect any L1 protein. As expected, no L1 protein or smaller products were detected with FB sera of the empty vector and PBS negative control vaccines (G7 and G8, respectively).

In anti-L2 western blots (Figure 4.2B), an expected band of ~80 kDa (black arrow) was only detected using the positive control anti-L2 serum (+). No L2 was detected when probed with FB sera from G1-G8. As L2 is the subdominant antigen, levels of anti-L2 antibodies may be too low for detection of antigen on western blots. FB sera from all groups, except G8, showed background signal in all strips, suggesting that there may have been cross-reactivity with plant proteins. The L2 protein used for detection on western blots was partially purified from plants as the use of *E. coli* expressed L2 protein resulted in dark smears over the entire membrane when probed with anti-L2 sera (data not shown). Pineo et al. (2013) reported the presence of a band at ~80 kDa when detecting *E. coli*-expressed L2 protein using negative control sera (plant extract) from vaccinated mice, supporting the idea that there may be reactivity with co-purified plant proteins.

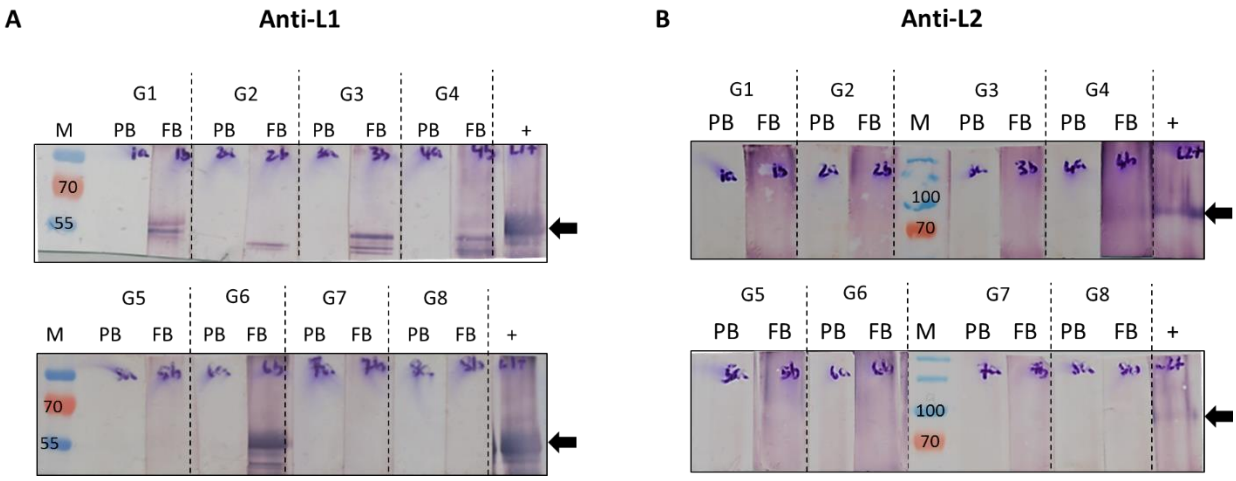

Figure 4.1: Anti-L1 and -L2 western blots using mouse sera. A) HPV-16 L1 protein or **B)** HPV-16 L2 protein was probed with pre-bleed (PB) or final bleed (FB) sera at 1:100 from the 8 vaccine groups. A band at 56kDa is expected for L1 protein and 80 kDa for L2 protein (black arrows). Labels: G1, SAC 108-120; G2, SAC 65-81; G3, SAC 56-81; G4, SAC 17-36; G5, SAE 65-81; G6, hL1; G7, pTRAkc-rbcs1-cTP only; G8, PBS; M, molecular weight marker (kDa); + L1 or L2 positive control, detected with Camvir-1 (1:20000) and anti-L2 sera (1:1000), respectively.

4.3.1.2. Indirect ELISA detection of anti-L1 antibodies

Detection of anti-L1 antibodies from individual mice in each vaccine group was tested by indirect ELISA using purified HPV-16 L1 as the coating antigen (Figure 4.2). A box-whisker plot analysis of anti-L1 responses elicited by SAC 108 (G1) and SAC 65-81 (G3) shows that the immune responses were statistically significant in comparison to PBS (G8) negative control (p=0.043 and p=0.047, respectively). hL1 (G6) anti-L1 responses were statistically significant in comparison to both empty vector (G7, p=0.029) and PBS (G8, p=0.0002) negative vaccine controls. Anti-L1 responses were not significant for SAC 56-81 (G2), SAC 17-36 (G4) and SAE 65-81 (G5) in comparison to G7 and G8, or in

comparison to each other, suggesting there is a vaccination dose-dependent relationship for the detection of anti-L1 antibodies. G1 and G6 were both used in animal experiments at 5 µg, in comparison to G2, G3, G4 and G5 experimental groups at <5 µg. One mouse vaccinated with SAC 17-36 (G4) was an outlier and had an anti-L1 response (OD_{405} ~1.01), similar to that of hL1 (G6) responses.

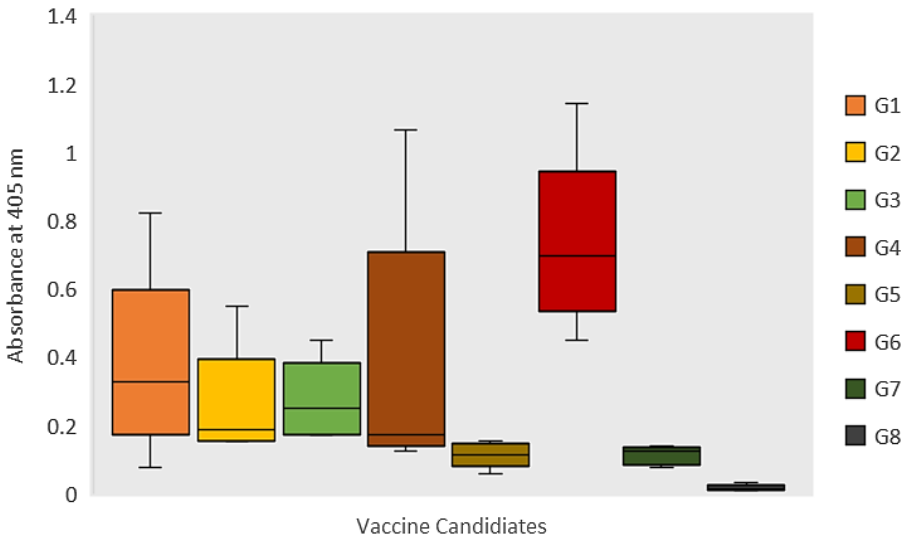

Figure 4.2: Box whisker plot comparing anti-L1 responses in vaccine groups to control groups. Indirect ELISA of individual mouse serum. Plates were coated with purified L1 antigen. Horizontal line indicates the exclusive median value with minimum and maximum values indicated by whiskers. Statistical significance was calculated using Kruskal-Wallis test. *p* values were calculated based on the Dunn's multiple comparisons test with 95% CI. Labels: G1, SAC 108-120; G2, SAC 65-81; G3, SAC 56-81; G4, SAC 17-36; G5, SAE 65-81; G6, hL1; G7, Empty vector; G8, PBS.

4.3.2. Determination of anti-L1 titres

Sera from individual mice were pooled and anti-L1 antibody titres determined by indirect ELISAs (Figure 4.3). All anti-L1 titres are stated as the reciprocal of the maximum dilution with higher absorbance readings than the corresponding PB serum at 1:50. Figure 4.3A shows that no anti-L1 response was detected for PB sera of all groups (G1-G8) or for the FB of negative control sera, PBS (G8) (Figure 4.3B). A titre of 50 was observed for empty vector (G7) FB serum (Figure 4.3B), which may be due to co-purification of plant proteins as mentioned in Section 4.3.1.2 above. The highest anti-L1 titres of 6400 were obtained for positive control hL1 sera (G6). SAC 108-120 and SAC 17-36 (G1 and G4, respectively) had anti-L1 titres of 1350, with sera of SAC 65-81 and SAC 56-81 (G2 and G3, respectively) with titres of 450. The lowest titre of 150 was observed for SAE 65-81 (G5). These data correspond to the western blots in Figure 4.1 where L1 protein (~56 kDa) was detected in G1,

G3 and G6, smaller proteins by G2 and G4 sera, and no protein by G5. Together, these data suggest L1 immunogenicity was affected in a dose-dependent manner.

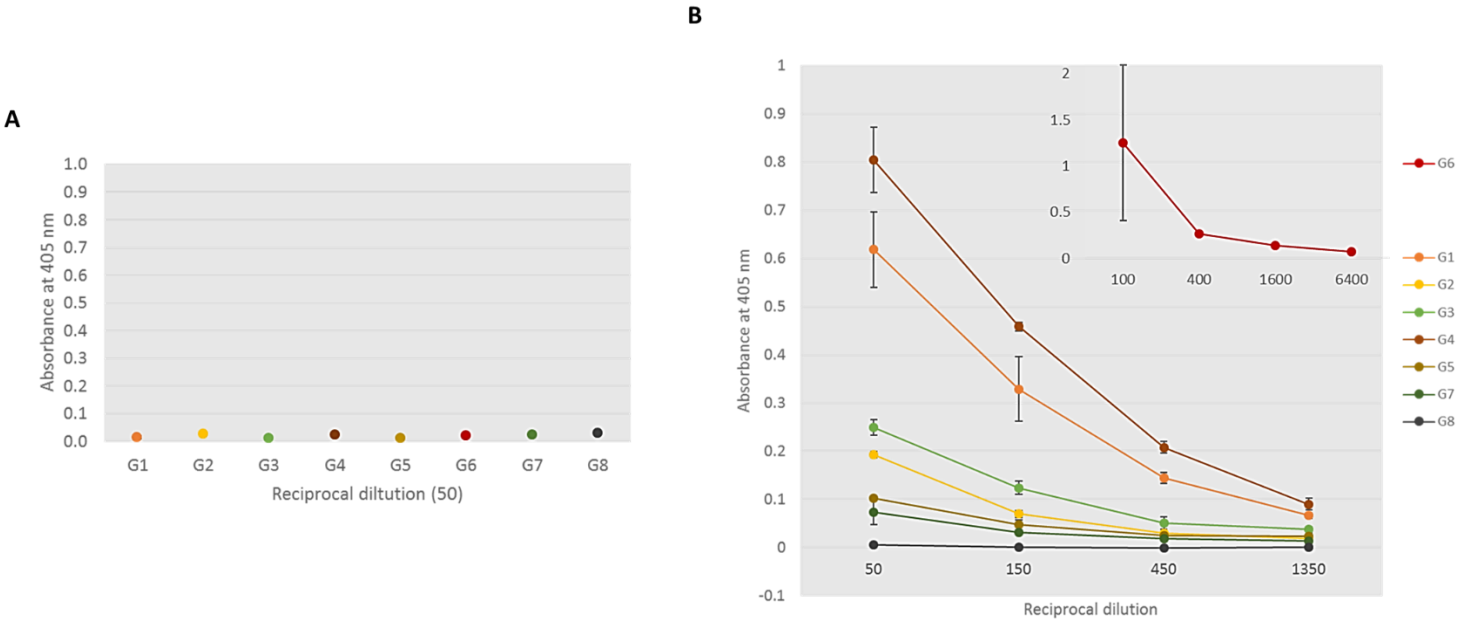

Figure 4.3: Anti-L1 indirect ELISA. A) Pre-bleed absorbance values at 1:50 dilution. **B)** Titration of pooled mouse anti-sera. Plates were coated with purified L1 antigen. Labels: G1, SAC 108-120; G2, SAC 65-81; G3, SAC 56-81; G4, SAC 17-36; G5, SAE 65-81; G6, hL1; G7, pTRAkc-rbcs1-cTP empty vector; G8, PBS. Error bars indicate standard deviation obtained from triplicate readings.

4.3.3 Detection and visualisation of purified HPV PsVs

4.3.3.1. Dot blot detection of purified PsVs

293TT produced PsVs were purified on discontinuous Optiprep™ gradients, after which fractions were screened for the presence of L1 on dot blots (Figure 4.4). L1 was detected with HPV type-specific anti-L1 conformational MAbs (Table 4.3). L1 was strongly detected in F3-F6 for HPV-6, 11, 16 and 31 (Figure 4.4 A, B, C and E, respectively). For HPV-18, 45 and 58 L1 was strongly detected in F3-F4, F9-F11 and F5-F9 (Figure 4.4 D, F and H, respectively). Low signal L1 signal was seen in HPV-52 PsVs (Figure 4.4G), with the strongest signal observed in F3. This may be due to low yields of assembled PsVs (TEM analysis, Figure 4.5). The PsV fractions with the strongest signals were pooled, examined by TEM and stored at -80°C. PsVs were not probed for the presence of L2, as L2 is not exposed on the outer surface of assembled capsids (Buck et al., 2008).

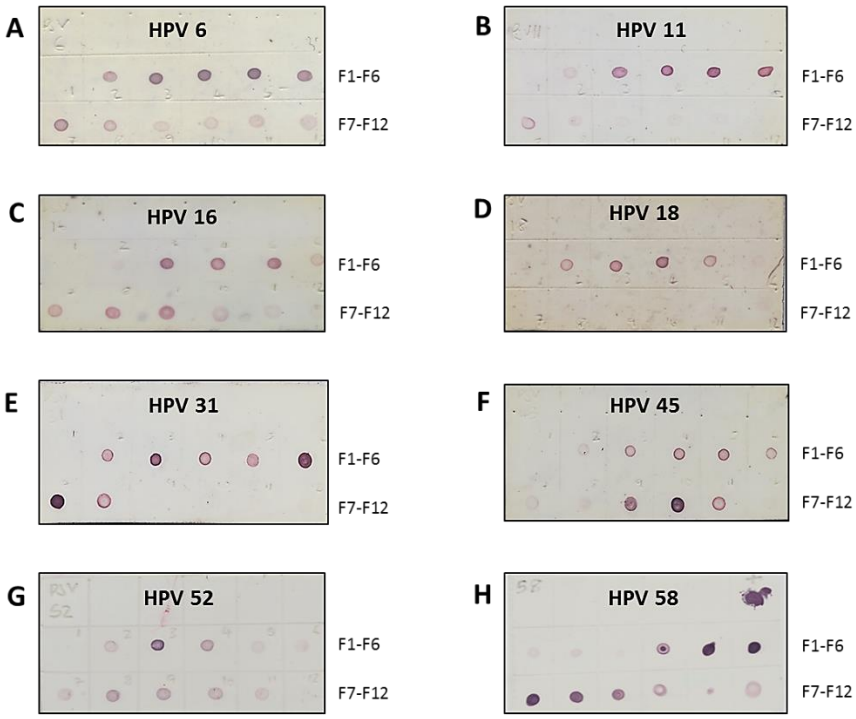

Figure 4.4: Dot blot detection of purified HPV PsVs. Purified fractions (1-12) were spotted onto nitrocellulose membranes and probed with anti-L1 monoclonal antibodies H6.C6, H11.B2, H16.V5, H18.J4, H31.A6, H45.N5, H52.D11, H58.J6.3 to detect HPV-6 **(A)**, -11 **(B)**, -16 **(C)**, -18 **(D)**, -31 **(E)**, -45 **(F)**, -52 **(G)** and -58 **(H),** respectively.

4.3.3.2. TEM analysis of purified PsVs

Pooled PsV fractions were analysed by TEM to determine their assembly and morphology (Figure 4.5). PsVs for all HPV types showed structures ranging from capsomeres (~10 nm in size) to fully assembled PsVs measuring 55-60 nm in size. HPV-6, -11, and -45 PsVs fully assembled into icosahedral structures measuring 55 nm in diameter (Figure 4.5 A, B and F, respectively). Both HPV-16 and -18 PsVs showed fully assembled 55 nm particles, however smaller particles of 25-30 nm and capsomeres were also observed for HPV-16 and -18, respectively (Figure 4.5C and D). HPV-31 (Figure 4.5E) and -52 PsVs (Figure 4.5G) measured 50-60 nm is size, but were however few in number. In addition, the HPV-52 PsV preparation mainly consisted of capsomeres, this could be attributed to low levels of protein expressed in 293TT cells – these results were similar to PsVs previously purified in our group (Pineo, 2011). HPV-58 PsVs (Figure 4.5H) were fully assembled into particles measuring 60 nm, with a few smaller particles of 40-50 nm observed.

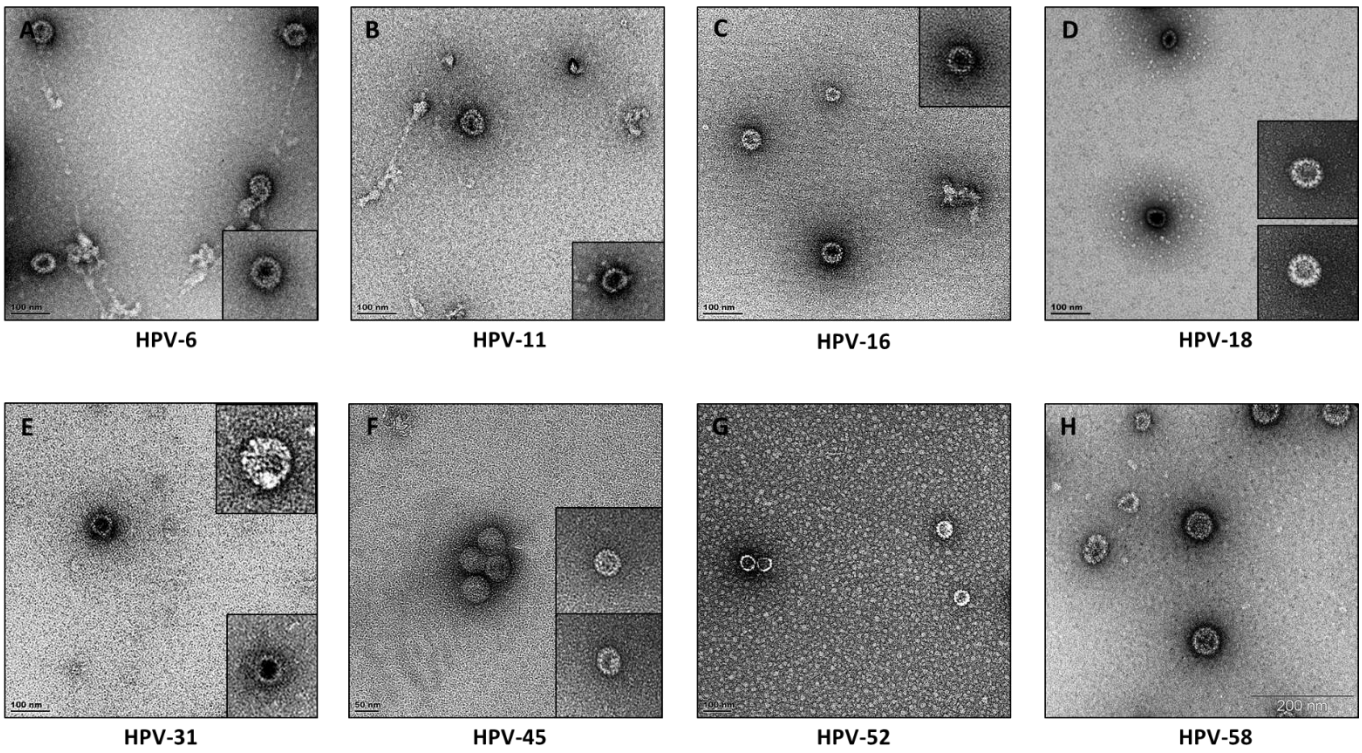

Figure 4.5: Transmission electron micrographs of purified HPV PsVs. PsVs were harvested from 293TT cells, matured and purified on a discontinuous iodixanol gradients. PsVs were diluted 1:10 in 1x DPBS prior to viewing. **A)** HPV-6, **B)** HPV-11, **C)** HPV-16, **D)** HPV-18, E**)** HPV-31, **F)** HPV-45, **G)** HPV-52, **H)** HPV-58. Mag 53 000 x. PsVs are 45-60 nm. Scale bar at bottom of each image – 100 nm, except HPV-45 at 50 nm and HPV-58 at 200 nm.

4.3.4. Titration of PsVs and monoclonal antibodies

Titrations assays of HPV PsVs were carried out to determine the best dilution at which to use the PsVs in neutralisation assays. The reciprocal values of PsV dilutions are presented in Figure 4.6. Four-fold dilutions ranging from 1:250-1:16000 were performed for HPV-6/11/16/18/45 and -58 PsVs (Figure 4.6 A and B). The linear range in the titration was used to determine the dilution of PsV to be used in L1 PBNAs. The linear range for HPV-6 and -11 was 1:250-1:1000; HPV-45 was 1:250-1:4000; HPV-16 and -18 was 1:250-1:1000; and HPV-58 was 1:250-1:4000. Based on these data, in subsequent L1 neutralisation assays, the PsVs were used at dilutions of 1:500 for HPV-6 and -11, 1:250 for HPV-45, and 1:1000 for HPV-16/18 and -58.

HPV-31 and -52 PsVs were titrated using a dilution range of 1:50-1:400 (Figure 4.6C), since previous dilutions ranging from 1:250-1:16000 showed low SEAP signal that was similar to the cell only control (data not shown). The linear range for HPV-31 and -52 1:50-1:200 and 1:50-1:100, respectively. Dilutions of 1:100 and 1:75 for HPV-31 and -52, respectively were used in the L1 PBNAs. Low titres obtained for HPV-52 PsVs were not surprising as it had previously been shown by the

Laboratory of Cellular Oncology (https://home.ccr.cancer.gov/Lco/packaging.htm) that these PsVs consistently had low titre yields.

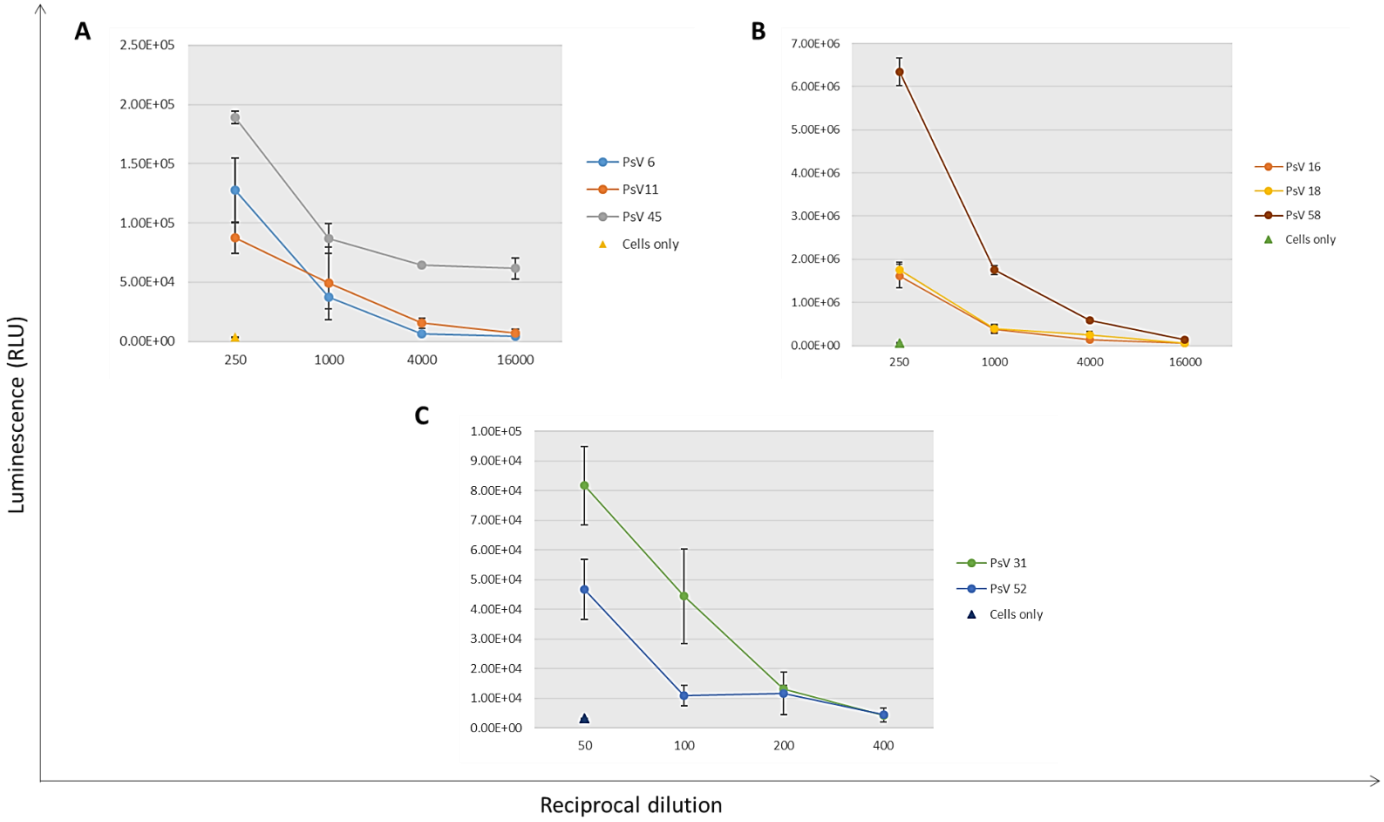

Figure 4.6: Titration curves of purified PsVs. A) HPV -6, -11 and -45 at dilution range of 1:250-1:16000. **B)** HPV -16, -18 and -58 at dilution range of 1:250 – 1:16000. **C)** HPV -31 and -52 at dilution range of 1:50-1:400. Error bars indicate standard deviation obtained from triplicate readings.

MAbs for each HPV PsV (Table 4.3) were titrated to determine the range where 0-100% neutralisation of PsVs occurred. Figure 4.7 shows titration curves for each MAb/PsV combination, and reciprocal values of MAb dilutions are presented. The dashed horizontal line in all the titration curves indicates luminescence readings obtained for PsVs incubated in the absence of MAbs, thereby representing 0% neutralisation. Therefore, the area underneath the dashed line represents 0-100% neutralisation and was used to show that PsVs could be neutralised by MAbs, and that neutralisation levels were antibody concentration dependent. A cell only control was included to determine the background luminescence readings.

HPV-6 PsVs were 100% neutralised with MAb H6.C6 at a dilution of $2x10^2$-$2x10^4$ after which neutralisation decreased to ~50% at a dilution of $2x10^4$. SEAP readings at a dilution of $2x10^6$ were very high (greater than PsV only control) and this was due great variations in readings (indicated by a

large error bar) (Figure 4.7A). HPV-11 PsVs were 100% neutralised with MAb H11.B2 at a dilution of $2x10^2$ after which neutralisation decreased to ~50% at a dilution of $2x10^6$ (Figure 4.7B). HPV-16 (Figure 4.7C) and -18 (Figure 4.7D) PsVs were 100% neutralised with MAb H16.V5 and H18.J4, respectively, at a dilution of $2x10^2$-$2x10^4$, with ~20% neutralisation of HPV-16 and 50% neutralisation of HPV-18 at a dilution of $2x10^6$. HPV-31 PsVs were 100% neutralised with MAb H31.A6 at a dilution of $2x10^2$-$2x10^4$ after which neutralisation decreased to ~75% at a dilution of $2x10^6$ (Figure 4.7E). Neutralisation of HPV-45 (Figure 4.7F) and -52 (Figure 4.7G) PsVs required a higher concentration of MAbs H45.N5 and H52.D11, respectively, and were 100% neutralised at a dilution of $1x10^2$ after which neutralisation decreased to ~10% and 0% at a dilution of $1x10^6$. HPV-58 PsVs were 100% neutralised with MAb H58.J6.3 at dilutions of $2x10^4$-$2x10^5$, with 0% neutralisation observed at a dilution of $2x10^8$ (Figure 4.7H). All MAbs successfully neutralised PsVs and were subsequently used as positive controls in L1-PBNAs for the analysis of G1-G8 sera.

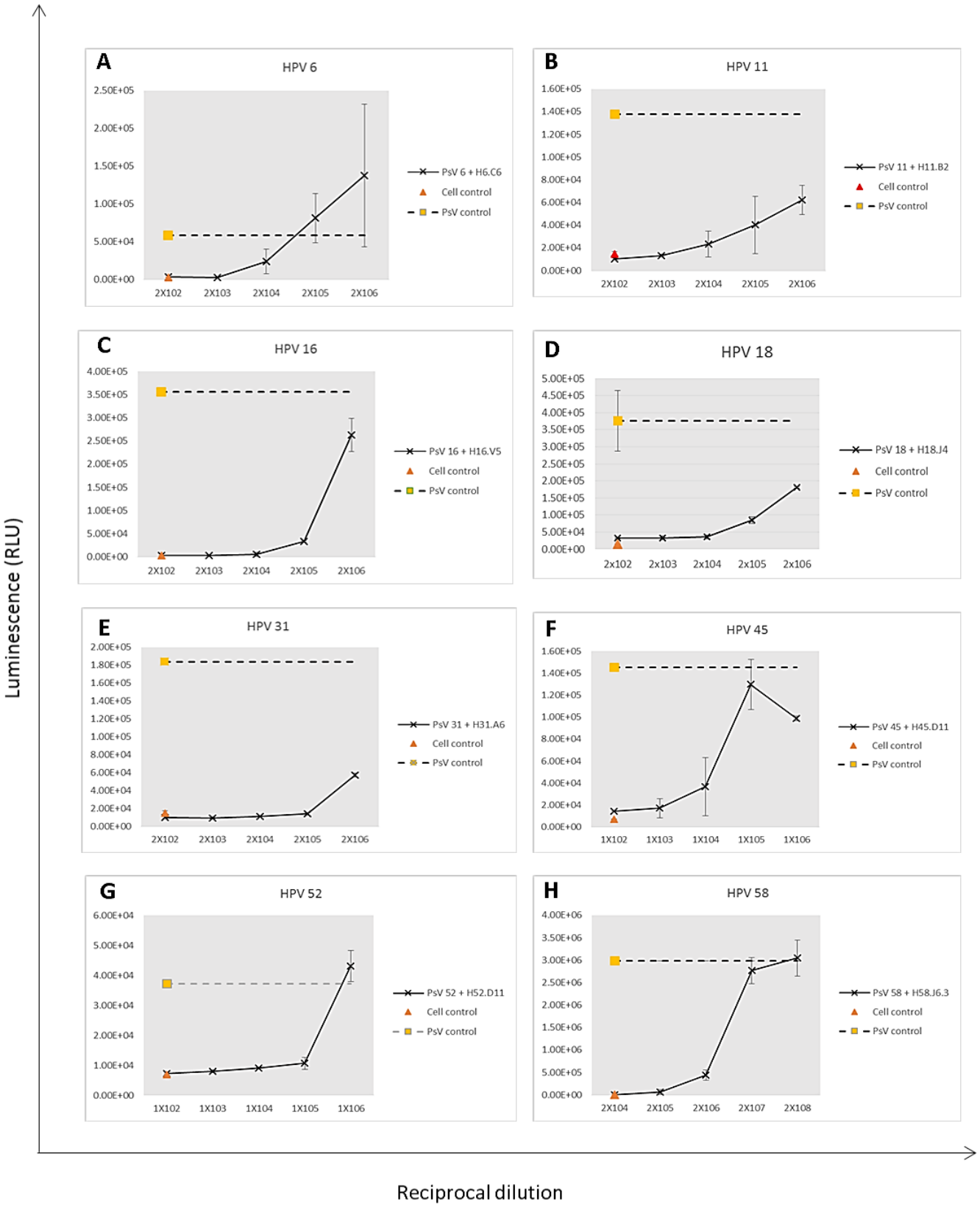

Figure 4.7: Positive control MAb titration. MAbs were titrated to determine the range for neutralisation of PsVs from 0-100%. HPV-6 **(A)**, -11 **(B)**, -16 **(C)**, -18 **(D)** and -31 **(E)** antibody titre range: $2x10^2$-$2x10^6$; HPV-45 **(F)** and -52 **(G)** antibody titre range: $2x10^2$-$2x10^6$; and HPV-58 **(H)** antibody titre range: $2x10^4$-$2x10^8$. Cell control indicates background readings. PsV control, dashed horizontal line indicating PsVs incubated without MAb. Error bars indicate standard deviation from triplicate readings.

4.3.5. L1 PBNA

Purified PsVs of HPV types 6/11/16/18/31/45/52 and 58 were used in L1-PBNAs to detect L1-specific neutralising antibodies in sera obtained from vaccinated mice. These HPV types were chosen based on the HPVs the L2 epitopes are known to cross-neutralise, the HPV types included in the commercial vaccines , as well their phylogenetic relation to each other – HPV-31, -52 and -58 are related to HPV-16 (HPV species α9); HPV-18 and -45 are closely related (HPV species α7)(de Villiers et al., 2004). Pooled mouse sera were initially tested for neutralisation at dilutions of 1:50 and 1:200 prior to titration (data not shown), and only sera that showed at least 50% neutralisation of PsVs was titrated further due to costs associated with the SEAP assay. PB sera was only tested at a 1:50 dilution due to limited sample volume, and no neutralisation was observed with PB sera. MAbs were included in each assay as positive neutralisation controls. Each MAb was used at dilutions which resulted in 100% neutralisation as shown in Figure 4.7. Neutralisation titres are stated as the reciprocal of the maximum serum dilution which reduced SEAP activity by >50% in comparison to the PsV only control sample, which was not treated with serum/MAb.

Table 4.4 shows a summary of the average percentage neutralisation of PsVs by sera from vaccine candidates (G1-G8) at 1:50 dilution. The low-risk HPV-6 PsVs were only weakly neutralised by hL1 serum (G6) (33%), while HPV-11 PsVs were neutralised by SAC 17-36 (G4) and SAE 65-81 (G5) (45% and 54%, respectively). HPV-16 PsVs were strongly neutralised at 83% by hL1 serum (G6), followed by SAE 65-81 (G5) serum with 54% neutralisation. No neutralising activity was observed from G1-G4 sera suggesting that L2 epitope substitution may have affected L1 immunogenicity. HPV-18 PsVs were neutralised by SAC 108-120 (G1), SAC 65-81 (G2), SAC 56-81 (G3) and SAE 65-81 (G5) sera, by 83%, 69%, 43% and 50%, respectively, with no neutralisation from SAC 17-36 (G4) and hL1 (G6) sera. However, phylogenetically related HPV-45 showed poor neutralisation by SAC 65-81 (G3) and SAE 65-81 (G5) by 21% and 20%, respectively. The empty vector serum (G7) also showed neutralisation of HPV-45 PsVs by 19% but this may be due to large error bars observed from the replicates (data not shown). HPV-31 and -45 PsVs, phylogenetically related to HPV-16, were poorly neutralised, with HPV-31 showing neutralisation by SAC 56-81 (G2), SAC 65-81 (G3) and hL1 (G6) at 40%, 12% and 32%, respectively; HPV-52 PsVs were not neutralised by any sera. HPV-58 PsVs however, were neutralised by SAC 108-120 (G1) and SAC 65-81 (G2) sera at 45% and 51%, respectively, and weakly neutralised by SAC 56-81 (G1) (39%) and SAC 17-36 (G4) (29%), with no neutralisation by SAE 65-81 (G5) and hL1 (G6) serum. Sera from mice vaccinated with the empty vector (G7) and PBS (G8) did not contain NAbs as none of the PsVs tested (with the exception of G7 serum for HPV-45) showed any neutralisation.

Table 4.4: Summary of average percentage neutralisation of PsVs obtained with immune sera.

	% Neutralisation of PsVs							
Vaccine candidate	**HPV-6**	**HPV-11**	**HPV-16**	**HPV-18**	**HPV-31**	**HPV-45**	**HPV-52**	**HPV-58**
G1	0	7	0	83	0	2	0	45
G2	0	0	0	69	40	0	0	51
G3	0	0	0	43	12	21	0	39
G4	0	45	0	0	0	6	0	29
G5	0	54	54	50	0	20	0	0
G6	33	3	83	0	32	0	2	0
G7	0	0	0	0	0	19	0	0
G8	0	0	0	0	0	0	0	0

Assays were performed in duplicate due to limited sample volume.

Based on these results, sera that neutralised PsVs by at least 50% were titrated to determine end-point titres (Table 4.5). Sera was serially diluted 2-fold starting at 1:50. Neutralisation <50% was assigned a titre of <50. Overall, the neutralisation titres for all sera tested was very low, except for hL1 (G6) neutralisation of HPV-16 PsVs, with a titre ≥6400. This was expected as no structural modifications were made to L1 VLPs in comparison to the chimaeras tested. SAE 65-81 (G5) serum neutralised HPV-11 PsVs at a titre of 50. HPV-18 PsVs were neutralised with sera from SAC 108-120 (G1), SAC 65-81 (G2) and SAC 56-81 (G3) at titres of 200, and with SAE 65-81 (G5) serum at a titre of 50. HPV-58 PsVs had a neutralisation titre of 50 for SAC 65-81 (G2) sera. Although all assays were successful, demonstrated by neutralisation of PsVs with MAbs (data not shown), it appeared that most sera obtained from vaccination with cVLPs did not contain potent anti-L1 NAbs.

Table 4.5: Summary of *in vitro* PsV neutralisation titres in L1 PBNA

	Neutralisation titres			
Vaccine candidate	**HPV-11**	**HPV-16**	**HPV-18**	**HPV-58**
G1	<50	<50	200	<50
G2	<50	<50	200	50
G3	<50	<50	200	<50
G4	<50	<50	<50	<50
G5	100	50	50	<50
G6	<50	≥6400	<50	<50
G7	<50	<50	<50	<50
G8	<50	<50	<50	<50

Assays were performed in duplicate due to limited sample volume.

4.3.6. L2-specific PBNA

The L2 PBNA is more sensitive than the L1-based neutralisation assay for the detection of L2 NAbs due to the exposure of L2 epitopes after cleavage with furin (Day et al., 2012). Although the L1 PBNA did not show the presence of potent NAbs (Table 4.4 and 4.5), all sera were tested in the L2-specific PBNA. Sera from all vaccinated groups showed no neutralisation of any of the HPV PsVs tested (data not shown), suggesting that the sera did not contain anti-L2 NAbs. Positive control K18L2 MAb showed neutralisation of PsVs (40-60%) and negative control sera showed no neutralisation (data not shown). Surprisingly, G6 sera did not neutralise homologous HPV-16 PsVs. Therefore, no statistical comparisons of the L2 assay versus the standard L1 PBNA could be made.

4.3.7. Summary of vaccine immunogenicity

Table 4.6 summarises the anti-L1 humoral responses and cross-neutralising potential of the L1:L2 cVLP vaccine candidates tested in this study. Although TEM analysis (Figure 3.3, Chapter 3, section 3.3.1.2) showed that small cVLPs and cVLPs were obtained after purification of these vaccine candidates, they elicited only weakly neutralising anti-L1 antibodies that showed limited neutralisation of heterologous HPV types. No anti-L2 NAb responses were detected in any of the sera tested, suggesting that presentation of L2 peptides on the surface of the L1 capsid may have been insufficient to generate NAb.

Table 4.6: Summary of antibody responses of HPV-16 L1:L2 chimaeras

Vaccine candidate	Antigen	Anti-L1 response	Anti-L1 titre	HPV neutralisation	
				L1 PBNA	L2 PBNA
G1	L1:L2 SAC 108-120	Y	1350	18,58	-
G2	L1:L2 SAC 65-81	Y	450	18	-
G3	L1:L2 SAC 56-81	Y	450	18	-
G4	L1:L2 SAC 17-36	Y	1350	-	-
G5	L1:L2 SAE 65-81	Y	150	11,16,18	-
G6	HPV-16 L1 (+)	Y	6400	16	-
G7	Empty vector (-)	N	0-50	-	-
G8	PBS (-)	N	0	-	-

4.4. Discussion

VLPs have been shown to be strongly immunising due to the repetitive display of epitopes on their surfaces, their interaction with APCs, and their activation of B cells (Chackerian et al., 2008). HPV

VLPs have 360 copies of L1 (Modis et al., 2002) and are the basis for the current prophylactic vaccines that exhibit long-lasting, high titre NAbs. However, the type specificity of L1 is concentrated within exposed L1 surface loops and determines the type-specific nature of L1 NAb (Carter et al., 2003; Chen et al., 2000c). In addition, L1 VLPs have shown limited or no cross-neutralisation to heterologous types not included in the vaccines (Brown et al., 2009; Joura et al., 2015; Toft et al., 2014; Wheeler et al., 2012). The N-terminus of L2 can induce neutralisation of homologous and heterologous PV types (Pastrana et al., 2005); however, L1:L2 VLPs do not show cross-neutralising activity to L2, as L2 is buried within the capsid (Buck et al., 2008; Gambhira et al., 2007b) and is subdominant to L1 (Roden et al., 2000). The display of highly conserved L2 epitopes in L1 surface loops to generate cVLPs is a step towards the production of next-generation vaccines that are broadly protective against multiple HPV types.

Several L2 aa epitopes have been inserted by others into L1 loops for the generation of cVLPs. Insertion of L2 aa 17-36, 28-31, 35-75, 69-81, 108-120 and 115-154 into BPV-1 L1 DE loop at position 133/134, elicited anti-L1 and anti-L2 responses in mice (Schellenbacher et al., 2009; Slupetzky et al., 2007). Additionally, substitution (from position 131) of L2 aa 108-120 into the HPV-16 DE loop of insect-cell produced chimaeras (Varsani et al., 2003a), or L2 aa 108-120, 56-81 and 17-36 in the h4 helix (from position 414) of plant produced (Pineo et al., 2013) or insect cell produced (McGrath et al., 2013) chimaeras, also elicited anti-L1 and anti-L2 responses in mice. In this study, L2 aa 108-120, 65-81, 56-81 and 17-36 were substituted into the DE loop (from position 131 - SAC) or between the h4 and β-J structural regions (from position 431 - SAE). These regions were selected based on preliminary studies done by Varsani et al. (2003a) where L2 aa 108-120 was substituted into 5 HPV-16 L1 loops. Chimaeras were subsequently expressed and purified from plants, and anti-L1 and -L2 humoral responses in mice evaluated by western blotting (Figure 4.1) and indirect ELISA (Figure 4.2). L1 protein (56 kDa) was detected in western blots by sera from SAC 108-120, SAC 56-81 and positive control hL1 VLPs (G1, G3 and G6, respectively) (Figure 4.1). Interestingly, sera from SAC 65-81 and SAC 17-36 (G2 and G4, respectively) detected a protein smaller than the expected 56 kDa L1 protein. This may be due to proteolytic degradation during antigen purification, and could be C-terminal truncated L1 proteins that are incorporated into assembled VLPs (Deschuyteneer et al., 2010; Huber et al., 2017). These data suggest that insertion of the L2 epitopes did not completely compromise the immunogenicity of L1 (confirmed in indirect ELISAs, Figure 3.6, Chapter 3, section 3.3.3). However, no L2 protein was detected by immune sera, suggesting that anti-L2 antibodies present may have been at too low a concentration or that presentation of the epitopes was not sufficient to generate an immune response.

Anti-L1 responses in indirect ELISAs (Figure 4.2) of SAC 108-120 (G1) and SAC 65-81 (G3) were significant (p=0.043 and 0.047, respectively) in comparison to the PBS control group, and hL1 (G6) responses were significant in comparison to empty vector (p=0.029) and PBS (p=0.0002) controls. These groups correspond to the vaccine candidates with the highest antigen doses ~5 µg (Table 4.1) indicating a dose-dependent anti-L1 response. An anti-L1 titre of ≥6400 was observed for hL1 (G6) sera (Figure 4.3). In previous studies, titres of plant-produced L1 VLPs have been in the range of 12800-40960 (Fernandez-San et al., 2008; Maclean et al., 2007; Pineo et al., 2013). Although these titres are higher, mice were vaccinated with higher antigen doses of 10-30 µg compared to 5 µg in this study, in addition to the use of adjuvant. Adjuvant was not used in this study as a previous report by Maclean et al. (2007), showed identical end-point titres with/without adjuvant, but more interestingly a 4x greater neutralisation titre of HPV-16 in the absence of adjuvant, implying the use of Freund's adjuvant may be deleterious. Furthermore, Freund's complete or incomplete adjuvant was used in these studies, yet the current vaccines are formulated in monophosphoryl lipid A or amorphous aluminum hydroxyphosphate sulfate. These adjuvants would be better suited for future animal experiments to elucidate the effect on the immune response. Anti-L1 titres for all chimaeric vaccine candidates (G1-G5) were lower than the anti-L1 titres for hL1 VLPs (G6) (Figure 4.3), suggesting assembly of chimaeras may have compromised the exposure of L1 epitopes. The empty vector and PBS negative controls (G7 and G8, respectively), and PB sera of all groups did not show any anti-L1 responses (Figure 4.3); however, the detection of smears in anti-L2 western blots (Figure 4.1B), suggests that plant proteins may have been co-purified with vaccine antigens, which then reacted with plant proteins in the L2 extract used in western blotting. Unfortunately, purified L2 peptides were not available to perform ELISAs to determine the titres of any anti-L2 antibodies present in collected sera.

The ability of antibodies obtained from sera to neutralise PsVs was investigated in PBNAs – the gold standard to test the efficacy of HPV vaccines. HPV PsVs were selected based primarily on the cross-neutralising potential of the L2 peptides. The HPV-16 L2 aa 108-120, 65-81, 56-81 and 17-36 peptides have all been shown to elicit NAbs to homologous HPV-16 and cross-neutralise several heterologous types e.g. HPV-18/31/45/52/58 (Jagu et al., 2013; Kondo et al., 2007; Nieto et al., 2012; Rubio et al., 2009; Schellenbacher et al., 2013; Seitz et al., 2014; Tumban et al., 2012). Specifically, cVLPs where L2 aa 17-36 was inserted into the DE loop of L1 have shown protection from challenge for over 15 high-risk HPVs (Schellenbacher et al., 2013).

In L1 PBNAs, neutralisation of homologous HPV-16 was only observed with SAE 65-81 (G5) at a titre of 50 and hL1 VLPs (G6) at titre ≥6400 (Table 4.5). The titre observed for hL1 (G6) was similar to NAb titres obtained in other studies testing plant produced chimaeras or VLPs: 500-5000 (Pineo et al., 2013); 6400 (Maclean et al., 2007); 400 (Paz De la et al., 2009). The low titre obtained by SAE 65-81 (G5), in addition to the low vaccination dose, may be due to partially formed cVLPs (Figure 3.3E, Chapter 3, section 3.3.1.2) and the presentation of L2 on the capsid. Position 431 is located in the C-terminal arm of L1 and is not directly involved the correct folding of VLPs, but it is close to the h4 helix region where residues 414-426 play a role in VLP assembly (Bishop et al., 2007a; Varsani et al., 2003a). Steric hindrance due to the substitution of residues with different charges may therefore affect correct folding. No detectable NAbs to HPV-16 were elicited by SAC 108-120 (G1), SAC 65-81 (G2), SAC 56-81 (G3) and SAC 17-36 (G4) despite forming cVLPs, suggesting that antigen bound by sera in western blots and indirect ELISA (Figure 4.1 and 4.2) were detected by non-neutralising antibodies. Cross-neutralisation of heterologous HPV types was observed with neutralisation of HPV-18 by SAC 108-120 (G1), SAC 65-81 (G2), SAC 56-81 (G3) and SAE 65-81 (G5) antisera (Table 4.6). HPV-58 was cross-neutralised by SAC 108-120 (G1), and HPV-11 by SAE 65-81 (G5) (Table 4.6). All neutralising titres observed were between 50-200 (Table 4.5). Similar titres were observed by Kondo et al. (2007) with antisera for L2 epitopes 18-38, 56-75, and 64-81. Surprisingly, SAC 17-36 (G4) did not show any cross-neutralisation despite being shown by Schellenbacher et al. (2013) to elicit robust anti-L2 antibodies and cross-neutralise up to 16 high-risk HPVs.

Moreover, in L2-specific PBNAs, no NAb titres were observed for all sera tested (Table 4.6). L2 PBNAs previously performed in our lab with sera from plant-produced HPV-16 L1:L2 chimaeras (L2 substituted in the h4 helix (Pineo et al., 2013)) showed low cross-neutralisation, with NAb titres of 50 for HPV-11 (L1:L2 56-81) and -18 (L1:L2 17-36), but no neutralisation to homologous HPV-16 PsVs (Megan Hendrikse et al., personal communication). Surprisingly, no L2 NAb titres were detected for L1:L2 108-120, even though it was found to be the best candidate vaccine as it elicited NAbs to HPV-16 and -52 in L1 PBNAs (Pineo et al., 2013). These same chimaeras produced in insect cells (McGrath et al., 2013) elicited sera that showed neutralisation of HPV-16/18/31/52 (Megan Hendrikse et al., personal communication), suggesting that plant-produced chimaeras may not assemble as efficiently and thus not display L2 epitopes as well. It is possible this may be the case as it has been suggested that VLP assembly is sensitive to cell type (Li et al., 1997). Overall these data show that presentation of L2 epitopes on the plant-made L1 chimaera surface was not sufficient to produce potent anti-L2 NAbs that are protective against multiple oncogenic HPV types, and this could be because the L2 protein was not released from host APCs for activation of B cells in the right quantity or frequency (Hitzeroth et al., 2009).

There are several possible explanations for why the anti-L1 responses and L1 and L2 NAb titres were lower or not observed than has previously been reported. The display of L2 epitopes in L1 loops should preserve the L1 epitopes critical for binding by MAbs. The MAb H16.V5 binding site is a major immunodominant epitope used for the assessment of integrity and antigenicity of VLPs. It has been shown to block the binding of >70% human sera (Roden et al., 1997; Wang et al., 2003) and recognizes aa 266-297 in the FG loop and aa 339-365 in the HI loop (Christensen et al., 2001). MAbs H16:V5 and H16:E70 have been extensively mapped and aa Phe^{50}, Ala^{266} and Ser^{282} of L1 are vital for binding and the generation of potent NAbs (White et al., 1999). The residues of the DE loop (aa 110-149) are not predicted to have any impact on Phe^{50}, Ala^{266} and Ser^{282} residues suggesting that substitution of L2 epitopes in this region should not affect H16:V5 epitope display. However, Lee et al. (2015) have recently shown that the BC (aa 181 and 184) and DE (aa 138-141) loops contribute to binding by H16.V5, with a few contact residues in the EF and HI loops. Furthermore, Bissett et al. (2016) showed that L1 epitopes necessary for the generation of cross neutralising antibodies are present in the DE and FG loops. The type-specific nature of L1 NAbs is due to the variation found within the L1 surface loops of different HPV genotypes (Carter et al., 2003; Chen et al., 2000b). The exposed surface loops e.g. BC and EF, show more sequence heterogeneity than the core loops, seen from analysis of intra- and inter-genotype amino acid variability (Bissett et al., 2014). This variability is thought to be a mechanism in which the virus can avoid NAbs.

Through crystallographic homology models, Bissett et al. (2016) predicted structural differences in the L1 surface loops between HPV-16 and the loops of the non-vaccine alpha-9 genotypes (HPV-31/33/35/52/58). The DE loop is centrally positioned within L1 and encircles the lumen of the capsomere and is predicted to be structurally similar for non-vaccine types. The authors determined specific L1 domains of vaccine (Cervarix® and Gardasil®) induced cross-neutralising antibodies by generating PsVs with intergenotype loops swaps (DE, FG and HI), and demonstrated that the FG loop is necessary for recognition of cross-neutralising antibodies, and that the DE loop enhances this recognition. Tyr^{135} and Val^{141} have also been shown to be critical for binding by MAb 26D1 (Xia et al., 2016) further supporting the importance of the DE loop as a cross-neutralising epitope.

In this study, L2 epitopes between 13 and 26 codons were substituted into L1 from position 131 (DE loop) or 431 (between h4 and β-J structural regions), based on anti-L1 and anti-L2 responses reported by Varsani et al. (2003a); however, the neutralising potential of these antibodies was not evaluated. Substitution at position 131 thereby replaced regions of L1 in the DE loop that have been shown to be critical epitopes for binding by MAbs and cross-neutralising antibodies. Huber et al.

(2017) have recently shown that sequence replacement of HPV-5 L1 (aa 132-145) with HPV-17 RG1 (L2 aa 14-33) resulted in low type-specific neutralising titres to HPV-5 and antiserum was not protective against PsV challenge *in vivo*. The authors postulated that replacement of the DE loop resulted in steric hindrance of the major HPV-5 L1 neutralisation epitope(s). The 4 SAC chimaeras (G1-G4) assembled into cVLPs (Figure 3.3, Chapter 3, section 3.3.1.2) but showed low anti-L1 titres and low NAb titres in PBNAs (Table 4.6), potentially as a result of the disruption of the abovementioned residues in the DE loop. Additionally, although SAE 65-81 (G5) was the only candidate vaccine that neutralised homologous HPV-16 (Table 4.6), due to the disulphide bonds between Cys^{175} and Cys^{428}, residues 433-443 are less accessible (Varsani et al., 2003a) and therefore presentation of the L2 peptides may not have been efficient to elicit anti-L2 antibodies.

In conclusion, all chimaeric candidate vaccines were immunogenic and elicited anti-L1 immune responses, with antisera showing PsV neutralisation of HPV-11 (SAE 65-81), HPV-16 (SAE 65-81), HPV-18 (SAC 108-120, SAC 65-81, SAC 56-81 and SAE 65-81) and HPV-58 (SAC 108-120) in L1 PBNAs. Unexpectedly, antisera did not neutralise PsVs in L2 PBNAs, despite L2 being displayed on the L1 capsid. It is important to consider L1 neutralising epitopes when determining the display position of L2 peptides. Although L2 substitutions did not seem to drastically affect the assembly of cVLPs, misassembled or disrupted VLPs expose epitopes with limited HPV type-specificity (Christensen et al., 1996a; Christensen et al., 1994). Thus, structural analysis of cVLPs is required to achieve success with second-generation HPV vaccines, that elicit potent anti-L1 and anti-L2 NAbs against oncogenic HPV types.

Chapter 5: Encapsidation of a Zera®E7SH-encoding gene in plant-made HPV PsVs as a potential prophylactic and therapeutic DNA vaccine

5.1. Introduction

HPVs cause neoplasms that range from benign warts to carcinomas. The current HPV VLP-based vaccines Cervarix®, Gardasil® and Gardasil®9 are very effective and elicit strong humoral immune responses with an efficacy of >90% in preventing cervical infection (Cuzick, 2015; Kjaer et al., 2009; Paavonen et al., 2009a; Schiller et al., 2008). However, these are prophylactic vaccines based on the L1 major capsid protein alone, and do not appear to induce the regression of established infections (Markowitz, 2007; Schiller et al., 2008). The late L1 and L2 structural protein genes are not expressed in pre-cancerous or cancerous tissue, but only in the upper epithelial layers (Doorbar, 2005; zur Hausen, 2002) and thus immunity to them cannot provide protection for individuals with pre-existing infections or HPV-associated lesions (Hildesheim et al., 2016; Hildesheim et al., 2007). The current treatment for HPV associated lesions such as CIN, VIN and cervical cancer involves tissue destruction procedures such as laser treatment, radiation, chemotherapy, cryotherapy and surgical incisions, and in more aggressive cancers, hysterectomy (American Cancer Society, 2017).

The HPV early genes E1, E2, E4, E5, E6 and E7 are associated with replication, transcription, viral release, virus maintenance and potential progression to invasive cancer. Of particular interest are the E6 and E7 genes as they induce keratinocyte immortalisation, maintain growth and are expressed constitutively in the basal epithelium (Münger et al., 1989; zur Hausen, 2000; zur Hausen, 2002). E6 and E7 are therefore ideal targets for vaccine therapy due to their role in the disruption of the cell cycle, their constitutive expression and their involvement in the multistep progression to cervical cancer (zur Hausen, 2002).

There is an urgent need for therapeutic HPV vaccines to reduce the burden of cervical cancer and to treat current infections. Viral clearance is essential for vaccine therapy to be effective. Cell-mediated T cell responses that activate T lymphocytes after antigen presentation to CD8+ or CD4+ effector T cells via the MHC I and MHC II complexes respectively, are essential for the regression of neoplasia (van der Burg and Melief, 2011). No HPV therapeutic vaccine has been approved for use in humans; however, several studies in animal models and clinical trials based on E6, E7 or chimaeras of these

are being investigated. Candidate vaccines have been produced that are in phase I or II studies, treating CIN or VIN 1-3 and advanced cervical cancer using peptides, protein-based subunits, DNA/RNA based vaccines, live vector vaccines, DC-based vaccines and differential prime-boost regimens (Hung et al., 2008; Lee et al., 2016; Vici et al., 2016; Yang et al., 2016). HPV-specific anti-tumour immune responses have been induced; however, there has been limited success in their progression to phase III trials. The most promising candidate is VGX-3100 from Inovio Pharmaceuticals, Inc., a HPV-16 and -18 E6 and E7-based DNA phase IIb vaccine that elicited regression of CIN 2/3 (Trimble et al., 2015), and is expected to enter phase III trials in 2017 (Kim, 2017).

DNA vaccines promote MHC I antigen presentation which mimics what happens in natural infections; however, they have been shown to have limited potency due to their lack of cell type specificity, non-replicative ability and lack of spread *in vivo* (Lin et al., 2010). Therefore, improvements of DNA vaccine potency by improving antigen presentation and processing, and the DC and T cell interaction have been considered (Hung and Wu, 2003; Tsen et al., 2007). Most recently, Wu et al. (2017) have described the use of a chimaeric gene producing B cell-activating factor (BAFF) fused to E7 as a therapeutic DNA vaccine. BAFF is a type II membrane protein secreted through the ER-Golgi pathway (Schneider et al., 1999). BAFF was postulated to enhance antigen presentation to MHC I molecules, and results showed that E7-specific CD8+ T cell responses were increased, anti-tumour effects against E7-expressing tumours were observed, and there was prolonged survival of immunised mice. Additionally, ER-targeting of the fusion protein by BAFF improved the potency of the DNA vaccine (Wu et al., 2017).

DNA plasmids can be packaged into L1+L2 PV particles to generate PsVs and have been shown to efficiently deliver DNA into multiple cell lines (Gordon et al., 2012; Peng et al., 2011; Peng et al., 2010) and present the opportunity to develop combination vaccines that generate robust systemic and local antigen-specific immune responses (Ma et al., 2011). PsVs could be used to improve DNA vaccine delivery, as they can target DNA to specific cells, in addition to protecting it from nucleases. HPV PsVs can additionally potentially act as adjuvants to facilitate the activation and maturation of APCs (Lenz et al., 2001; Rudolf et al., 2001). HPV PsVs have been used in gene therapy experiments for ovarian cancer (Hung et al., 2012; Peng et al., 2011; Peng et al., 2010), in SIV Gag DNA delivery to the vaginal tract of macaques (Gordon et al., 2012) and in the delivery of encapsidated M and M2 genes of RSV (Graham et al., 2010), and showed gene specific T cell responses.

High vaccine costs have limited their use in developing countries. Plants provide an alternative platform to produce affordable vaccines (Fischer et al., 2004; Rybicki, 2010), given the very flexible scalability of production, rapid production and low risk of contamination. HPV therapeutic vaccine candidates based on E7 have been produced in plants, and were able to elicit NAbs, cell-mediated immune responses, and reduce tumour growth in mice (Franconi et al., 2002; Franconi et al., 2006); however, poor immunogenicity of specific HPV antigens has been an issue. Several strategies have been developed to improve immunogenicity of these vaccines, such as the use of adjuvants (Gérard et al., 2001), fusion proteins (Massa et al., 2007; Venuti et al., 2009) and protein targeting to the secretory pathway (Franconi et al., 2006; Torrent et al., 2009). Fusion of proteins to the Zera® protein body-forming peptide has shown high accumulation of proteins in the ER, and an increase in protein yields. In addition, Zera® forms membrane-bounded protein bodies up to 10 µm in diameter that can protect proteins against degradation (Geli et al., 1994; Torrent et al., 2009). Whitehead et al. (2014) tested a plant-made HPV-16 E7SH protein fused to Zera® and found that this fusion protein induced a potent cellular immune response, was prophylactic, and furthermore resulted in the reduction of established tumours in mice. These results were at least comparable to a HPV-16 E7SH DNA vaccine previously shown to be highly promising in the same model system. Furthermore, Zera® protein bodies were found to have adjuvanting activity, contributing to an enhanced immune response (Whitehead et al., 2014).

Combination prophylactic and therapeutic vaccine candidates have also been produced in plants. Chimaeric VLPs made of L1 fused to epitopes of E6 and E7 expressed in transgenic tomato have shown potential as a combination vaccine candidate as their use resulted in the development of NAbs and cytotoxic T-cell responses in mice (Paz De la et al., 2009), elicited long-lasting (over 12 months) anti-L1 antibodies and provided long-term protection from tumour growth (Monroy-Garcia et al., 2014).

Our group has recently developed the first completely plant-made PsVs (Lamprecht et al., 2016): these consist of L1+L2 proteins encapsidating a self-replicating circular dsDNA vector based on the genome of the geminivirus BeYDV. BeYDV has a single-stranded (ss) circular DNA genome that can replicate to high copy number by rolling circle replication, and which accumulates as plasmid-like dsDNA in plant cell nuclei in the absence of capsid protein (Liu et al., 1997; Zhang and Mason, 2006). Huang et al. (2009) showed an increase in the transient expression of HBc antigen (0.8 mg/g FW) and NV capsid protein (0.34 mg/g FW) with the co-delivery of BeYDV derived vectors and a Rep/RepA-supplying vector, compared to transgenic expression (<0.1 mg/g FW) in previous studies (Tacket et al., 2000; Thanavala et al., 2005). Regnard et al. (2010) developed a novel vector series (pRIC) based

on a mild strain of BeYDV (Halley-Stott et al., 2007), that resulted in an increase in transgene copy number of HPV-16 L1, HIV-1C p24 and eGFP-encoding vectors by at least two orders of magnitude, compared to a non-replicating vector. pRIC was used to develop expression cassettes and pseudogenomes for the encapsidation of SEAP- or luciferase-encoding reporter plasmids in HPV-16 PsVs (Lamprecht et al., 2016). The authors showed that these PsVs could be neutralised by several anti-L1 HPV MAbs in PBNAs, that plant production could be used as a cheaper alternative to current PsV production methods that involve mammalian cell culture and expensive transfection reagents, and could potentially be used as plant-made DNA vaccine delivery vehicles. Therefore, the development of E6 and/or E7 DNA vaccines delivered in plant-made HPV capsids gives the opportunity to provide a highly novel and affordable combination prophylactic and therapeutic vaccine.

Furthermore, changes to the tumour microenvironment have been shown to result in a reduction in antitumour activity and an acceleration to tumour progression. Current tumour immunotherapies such as checkpoint inhibitors have shown promising results in early stage trials (Pardoll, 2012); however, clinical benefits have been limited and combination therapies will probably be required for optimal efficacy. To overcome the highly immunosuppressive tumour microenvironment, plant-produced viral vectors have been developed (Saunders et al., 2009) for use in bio-nanotechnology. *In situ* vaccination of tumours with eCPMV VLPs showed the induction of potent antitumour immune responses in melanoma, ovarian, colon and breast cancer models and generated effective local and systemic antitumour immunity (Lizotte et al., 2016). The responses induced by *in situ* vaccination therefore have the potential to enhance the antitumour response of therapeutic vaccines by direct modulation of the local microenvironment. Moreover, HPV PsVs have been reported to bind and infect several tumours *in vivo* (Kines et al., 2016) therefore plant-made PsVs have the potential to deliver DNA vaccines directly to tumours.

Based on the success of the plant-made PsVs in PBNAs, I investigated using the geminivirus-derived replicating pRIC to make a vector encoding Zera®E7SH, in order for a replicon form of this vector to be encapsidated in plant-made PsVs. In this chapter, I describe how a unique cloning strategy based on type IIS restriction sites was used to assemble a pRIC-like plasmid. Additionally, I describe the first use of plant-made HPV-16 and -35 PsVs to encapsidate a therapeutic DNA vaccine candidate, and the ability of PsVs containing Zera®E7SH-encoding replicons to infect and produce the protein in mammalian cells.

5.2. Materials and methods

5.2.1. GoldenBraid cloning technology

GoldenBraid (GB) technology provides a tool that can be used to assemble an indefinite number of reusable gene modules (Sarrion-Perdigones et al., 2011). Assembly of standardized basic parts are incorporated into a double loop (braid), and this allows binary assembly of several constructs. It is an improvement on the Golden Gate technique (Engler et al., 2008; Engler et al., 2014) which does not allow a transcriptional unit (promoter – coding sequence – terminator) to be used further once constructed. GB technology makes use of type IIS REs which recognise and cut at unique sequences outside of their recognition sites (Engler et al., 2009; Engler et al., 2008). This generates fragments with a unique 4 bp overhang, which can be used as identification markers for fragment assembly. GB has unique 4 bp barcodes that determine the order for the placement of each sequence for correct assembly (Figure 5.1). Sarrion-Perdigones et al. (2013) describe the words and phrases that define GB grammar.

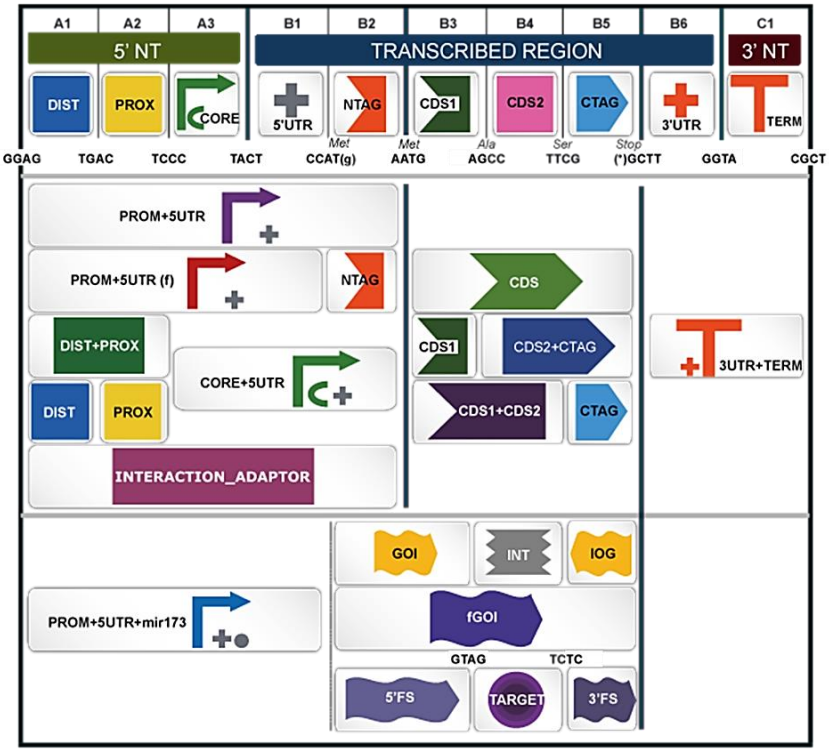

Figure 5.1: GB convention for 4 bp barcode for various DNA sequence parts. Barcodes determine the position of each DNA fragment to ensure correct assembly. Multiple arrangements are possible depending on required experimental design. E.g. defined functional regions: complete promoter region or full coding region. Image from GB cloning website - https://gbcloning.upv.es/search/

The GB toolbox consists of 4 destination plasmids called pDGBs: these are 2 level alpha and 2 level omega plasmids, which are used for the assembly of multiple transcriptional units (Sarrion-Perdigones et al., 2013). The positioning of recognition and RE digestion sites in opposite directions in entry and destination vectors is therefore essential for assembly. Once unique overhangs for specific sequences have been selected, the sequences must be domesticated. This is the process of PCR mutagenesis followed by type IIS-based assembly of mutated fragments. The PCR reaction eliminates type IIS restriction sites (e.g. *BsmB*I and *Bsa*I) in the sequence(s) of interest and adds the appropriate 4 bp flanking overhangs. Cyclical RE digestion at 37°C and ligation at 16°C of template DNA, results in recombination of multiple sequences in the same PCR reaction (Engler et al., 2008). A second type IIS restriction site in the destination plasmid enables the assembly of several transcriptional units.

5.2.2. GB assembly of Zera®E7SH into a pRIC-like backbone

5.2.2.1. Plasmid isolation, RE digestion and ligation reactions

DNA from all plasmids in this study was isolated using the QIAprep® Spin Miniprep kit (Qiagen) as per the manufacturer's instructions. All RE digests were performed using REs from Fermentas (ThermoFisher Scientific). Ligation of DNA fragments was performed using T4 DNA Ligase (ThermoFisher Scientific) as per the manufacturer's instructions.

5.2.2.2. Sequence domestication

The CMV-Zera®E7SH-BGHpolyA (Promoter-CDS-Terminator) region of pTH Zera®E7SH (Whitehead et al., 2014) was domesticated using the gbcloning program software found at https://gbcloning.upv.es/tools/domestication/. The custom prefix GGAG and the custom suffix CGCT were selected as these flank the promoter and terminator regions respectively (Figure 5.1). The program generated 4 primer sets, P1-P4 (Table 5.1) and PCR was performed with pTH Zera®E7SH DNA as the template, generating 4 patches. PCR was performed using the Kapa Hifi HotStart ReadyMix PCR Kit (Kapa Biosystems) as per the manufacturer's instructions. Forty nanograms template DNA was used per reaction. The patches were purified using the QIAquick® PCR Purification kit (Qiagen) as per the manufacturer's instructions.

Table 5.1: Primers used in domestication, PCR and sequencing of CMV-Zera®E7SH-BGHpolyA

Primer name	5' – 3' sequence	Tm (°C)	Size (bp)	Used for
P1 For	GCGCCGTCTCGCTCGGGAGTAGTAATCAATTACGGGGTCATTA	56-58	619	Domestication
P1 Rev	GCGCCGTCTCGGCCTCCAGGCGATCTGACGG	56-58		
P2 For	GCGCCGTCTCGAGGCGCCATCCACGCTGTTT	56-58	425	Domestication and sequencing
P2 Rev	GCGCCGTCTCGTAAGACCCCATCCTGTAAAAATAC	56-58		
P3 For	GCGCCGTCTCGCTTATTTATTATTTACAAATTCACATATACAA	56-58	997	
P3 Rev	GCGCCGTCTCGTGGCCTCGGGCTGCAGGTCC	56-58		
P4 For	GCGCCGTCTCGGCCACCGACCTGTACTGCAT	56-58	763	Domestication
P4 Rev	GCGCCGTCTCGCTCAAGCGGCCATAGAGCCCACCGCATC	56-58		
pUPD2 F2	CCCGATCAACTCGAGTGCCA	52	2987	Colony PCR and sequencing
pUPD2 R2	GAGGAAGCCTGCATAACG	52		
ZeraInt For	GCCACTACCCTACTCAAC	52	n/a	Colony PCR and sequencing

The 4 patches were subsequently assembled by cyclical RE digestion and ligation into the domestication entry vector, pUPD2 (Table 5.2), in a PCR reaction. The domestication reaction was performed as follows: in a final volume of 10 µL, 40 ng of each fragment, 75 ng pUPD2, 10u *BsmB*I, 5u T4 ligase and 1 µL of ligase buffer were combined. The PCR cycling conditions were as follows: 37°C for 20 min and 45x cycles of: 37°C for 2 min, 16°C for 5 min, followed by 37°C for 30 min, 80°C for 20 min and 4°C for 30 min. Competent *E. coli* (E. cloni™, Lucigen) cells were transformed with 1 µL of the PCR reaction and positive clones were selected on LB agar plates containing chloramphenicol (25 µg/mL), isopropyl β-D-1-thiogalactopyranoside (0.5 mM IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 µg/mL X-gal). White colonies were screened via PCR using pUPD2 primers (Table 5.1) and confirmed by sequencing.

5.2.2.3. Assembly of GB-pRIC- Zera®E7SH

The pRIC-like backbone is based on the replication-associated DNA elements of the genome of the geminivirus BeYDV. The vector contains two copies of the long intergenic region (LIR) (essential for the formation of a dsDNA replicon), the short intergenic region (SIR) and the Rep/RepA replication-associated protein complex gene with its own viral promoter, that is responsible for the recircularisation and replication of the replicons *in planta* (Regnard et al., 2010). The Rep/RepA proteins are responsible for replication initiation; the LIR has promoter elements and contains the viral double-stranded ori; the SIR contains the transcription termination signal and binding site for a primer for antigenome-sense DNA synthesis.

Table 5.2 shows the GB units required for assembly of GB-pRIC-Zera®E7SH. A positive pUPD2 CMV-Zera®E7SH-BGHpolyA clone was digested and ligated into pDBalpha14 (destination plasmid) using 80 ng of each plasmid, 10u *Bsa*I, 5u T4 ligase, 1 µL ligase buffer in a final volume of 10 µL. PCR conditions used were the same as described above and 1.5 µL of the reaction was transformed into *E. coli*. Positive clones were selected on kanamycin (50 µg/mL), IPTG (0.5 mM) and X-gal (40 µg/mL) LB agar plates. White colonies were screened by PCR using P1 For and P4 Rev, and ZeraIntFor and P4 Rev primers (Table 5.1).

Table 5.2: GB units used in assembly of GB-pRIC-Zera®E7SH

GB units	Property	Antibiotic resistance
pUPD2	GB domestication entry vector	Chloramphenicol (25 µg/mL)
pDBalpha11 empty	Empty	Kanamycin (50 µg/mL)
pDBalpha12_SF1-35	Stuffer fragment	Kanamycin (50 µg/mL)
pDBalpha13_LIR	LIR1, Long intergenic region of BeYDV	Kanamycin (50 µg/mL)
pDBalpha14 empty	Position to add gene of interest	Kanamycin (50 µg/mL)
pDBalpha2R-LIR_REP_SIR	LIR2_REP_SIR in reverse orientation	Kanamycin (50 µg/mL)
pDGB3omega2	Final left and right border backbone	Spectinomycin (50 µg/mL)

For assembly of the final plasmid (GB-pRIC- Zera®E7SH), pDBalpha14 CMV-Zera®E7SH-BGHpolyA was digested and ligated with pDBalpha11, pDBalpha12_SF1-35, pDBalpha13_LIR, pDBalpha2R-LIR_REP_SIR and pDGB3omega2 (obtained from our lab GB database). These plasmids contain the elements necessary for recircularisation and amplification of the replicons containing the expression cassette. Eighty nanograms of each plasmid, 10u *BsmB*I, 5u T4 ligase, 1 µL ligase buffer were added together in a final volume of 10 µL. PCR conditions are the same as those described above and 1.5 µL of the reaction was transformed into *E. coli*. Positive clones were selected on spectinomycin (50 µg/mL), IPTG (0.5 mM) and X-gal (40 µg/mL) LB agar plates. White colonies were screened via RE digest with *Bsa*I and *Hind*III. Figure 5.2 shows the final plasmid and its associated replicon.

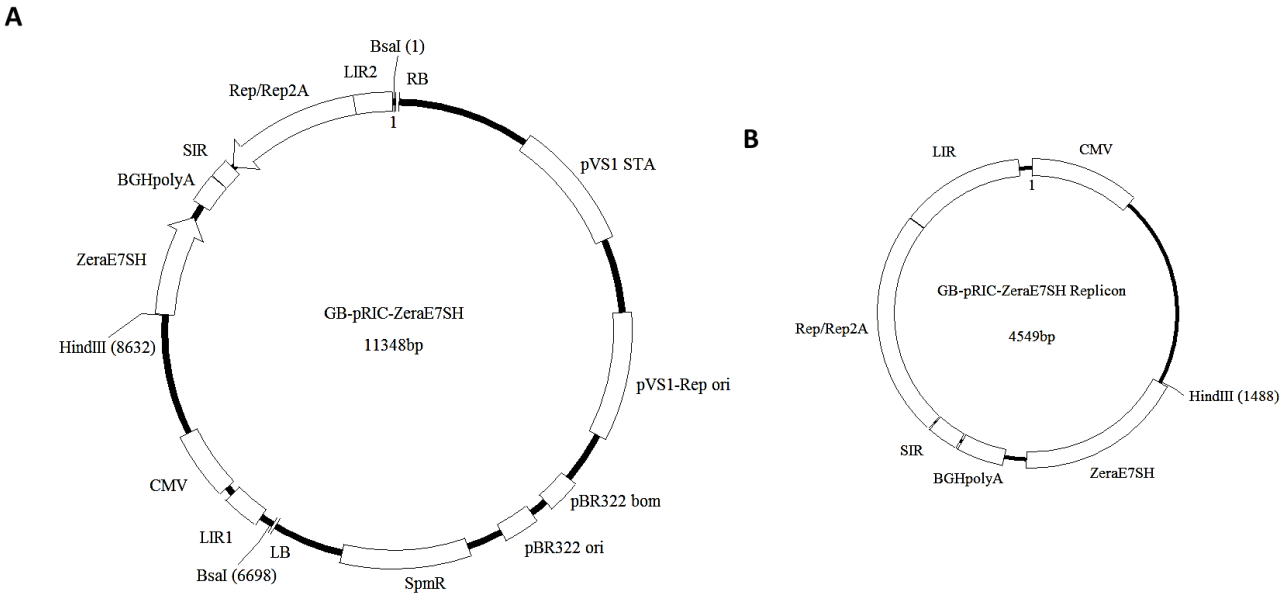

Figure 5.2: The final assembled GB-pRIC-Zera®E7SH plasmid. The vector elements for **A)** the plasmid and **B)** the derived replicon, are shown. The plasmid and replicon contain: LIR, BeYDV long intergenic region; SIR, BeYDV short intergenic region; and Rep/RepA, BeYDV rep gene (Regnard et al., 2010); CMV, cytomegalovirus intron/enhancer/promoter element; Zera®E7SH, gene of interest; and BGHpolyA, bovine growth hormone polyadenylation signal. The plasmid also contains: pBR322ori*, E. coli* origin of replication; pBR322 bom, basis of mobility region; pSV1-Rep ori, *Agrobacterium* origin of replication; PVS1 STA, replicon stability region; SpmR, spectinomycin-resistance gene; and LB/RB, left and right borders for T-DNA integration.

5.2.3. Transformation into *A. tumefaciens*

A. tumefaciens EHA105 cells were made competent by the method described (Shen and Forde, 1989). Transformation of EHA105 was carried out by adding 1 µL of GB-pRIC-like Zera®E7SH to 40 µL competent cells. The tube was placed in liquid nitrogen for 5 min, followed by 5 min at 37°C to thaw. The freeze/ thaw cycle was repeated, after which the tube was incubated on ice for 5 min. Five hundred microliters of LB media was added to the cells and incubated with shaking at 28°C for 30-50 min. The cells were pelleted by centrifugation and resuspended in 100 µL LB media, after which they were plated on spectinomycin (50 µg/mL) and rifampicin (50 µg/mL) LB agar plates and incubated at 27°C for 2 days.

5.2.4. Encapsidation of Zera®E7SH into HPV-16 and -35 L1:L2 VLPs

Twenty to twenty-five 6-week old *N. benthamiana* plants were vacuum infiltrated as described in Chapter 3, section 3.2.1 with recombinant *A. tumefaciens* HPV-16 pTRAc hL1 and hL2 or HPV-35 pTRAc hL1 and hL2, and co-infiltrated with GB-pRIC-Zera®E7SH. Cell suspensions were mixed as

follows: L1 at OD_{600} 0.25, L2 at OD_{600} 0.05 and GB-pRIC- Zera®E7SH OD_{600} 0.5. These parameters were chosen based on work by Lamprecht et al. (2016) and A.R. van Zyl (personal communication). Plants were grown at 22°C under 16 h/ 8 h light/ dark cycles and harvested 4 dpi.

5.2.5. Purification, western blot detection and TEM of HPV-16 and -35 PsVs

Harvested leaf material was homogenised in HSNaOAc (pH 5.2) and clarified as described in Chapter 3, Figure 3.1. The clarified extracts were loaded onto a 30% (5 mL) and 50% (1 mL) sucrose cushion and centrifuged for 1 h, 15°C at 174 500 *x g*. The 30% cushion was collected and dialysed overnight in HSPBS (pH 7.4). The dialysate was loaded onto a discontinuous Optiprep™ gradient (as described in Chapter 3, section 3.2.2.2, after which 1 mL fractions were collected from the bottom of the tube. Western blot detection of L1 and L2 protein in collected fractions was performed as described in Chapter 2, section 2.2.9. HPV-16 L1 and L2 were probed with Camvir-1 (1:20000) and anti-L2 rabbit-raised sera (1:1000) respectively. HPV-35 L1 was probed with the H16.J4 MAb (1:5000) (provided by Dr Neil Christensen) and HPV-35 L2 was not detected as no anti-L2 antibody was available. PsVs were prepared and viewed by TEM as described in Chapter 2, section 2.2.10.

5.2.6. Rolling circle amplification and RE digestion of Zera®E7SH replicons

Rolling circle amplification (RCA) of purified PsV fractions F2-F6 (selection based on western blot analysis), was performed as per the illustra™ Templiphi 100 Amplification Kit (GE Healthcare) instructions. Briefly, 0.5 µL of HPV-16 and -35 PsV fractions 2-6 were added separately to 5 µL sample buffer. The samples were denatured at 95°C for 3 min and cooled to 4°C. In a separate tube, 5 µL reaction buffer and 0.2 µL enzyme mix (per reaction) were combined (premix), and 5 µL of the premix was transferred to the sample buffer tubes. The reactions were incubated at 30°C for 18 h, after which the enzyme was heat inactivated at 65°C for 10 min and cooled to 4°C. Two microlitres of each sample was digested with *Hind*III RE to produce a linear product of 4.5 kb.

5.2.7. Transfection and infection of 293TT cells

293TT cells were maintained as described in Chapter 4, section 4.2.5. One 25-well plate was seeded with $1.25x10^5$ cells/well and incubated overnight. RCA samples of F2-F6 were prepared for transfection by adding 7 µL RCA DNA to 493 µL DMEM (A); Lipofectamine® 2000 was diluted 1:100 (500 µL) in DMEM (B), and both incubated at room temperature for 5 min. Preparations (A) and (B) were mixed together gently and incubated at room temperature for 20 min. The DNA/Lipofectamine

complex was added to the cells and incubated for 72 h. One microgram of pTH Zera®E7SH (Whitehead et al., 2014) and GB-pRIC-Zera®E7SH plasmid DNA were transfected as described, and used as positive expression controls. For cell infection with purified PsV fractions, F2-F6 were diluted 1:10 in DMEM (final volume 1 mL), and the total volume added to the cells and incubated for 72 h.

5.2.8. Cell harvesting and western blot detection of Zera® and E7 proteins

Culture supernatant of each sample was aspirated and cells washed in 1x PBS, pH 7.4, followed by lysis in 200 µL PBP3 buffer (100 mM Tris, pH 8, 50 mM KCl, 6 mM $MgCl_2$, 10 mM EDTA, 0.4 M NaCl) supplemented with 0.5% SDS and 200 mM DTT (Torrent et al., 2009). The cells were transferred to a microfuge tube and 20 µL lysozyme (1mg/mL, Roche) and 0.5 µL benzonase (250 units/µL, Sigma Aldrich) added to each sample. The tubes were incubated for 1 h at room temperature with shaking. The samples were centrifuged for 30 min at 10 000 *x g* to remove cellular debris and the supernatant loaded onto 10% SDS-PAGE gels. Western blotting was performed as described in Chapter 2, section 2.2.9. Zera® and E7 proteins were probed with anti-Zera® (1:100) or anti-E7 (1:500) rabbit-raised sera as the primary antibodies respectively. Alkaline phosphatase-conjugated anti-rabbit IgG (Sigma Aldrich) (1:5000) was used as the secondary antibody.

5.3. Results

5.3.1. GB Assembly of Zera®E7SH into pRIC-like backbone

To domesticate the CMV-Zera®E7SH-BGHpolyA sequence without the internal type IIS REs *BsmB*I and *Bsa*I (required for GB assembly), 4 primers sets, P1-P4 (Table 5.1) were synthesised and used in 4 separate PCR reactions. Successful PCR amplification was confirmed by expected size fragments of 619 bp (P1), 425 bp (P2), 997 bp (P3) and 763 bp (P4) observed after agarose gel electrophoresis (black arrows, Figure 5.3A). The remaining PCR reaction volumes were used in a digestion/ligation reaction with *BsmB*I RE into the domestication vector, pUPD2, via PCR, which resulted in destruction of the *BsmB*I site. Positive white colonies selected by blue/white screening were screened by colony PCR using pUPD2 primers (Table 5.1). Clones 8 and 10 were PCR positive (Figure 5.3B, black arrow) and sequenced, after which clone 8 was selected for use in subsequent assembly experiments.

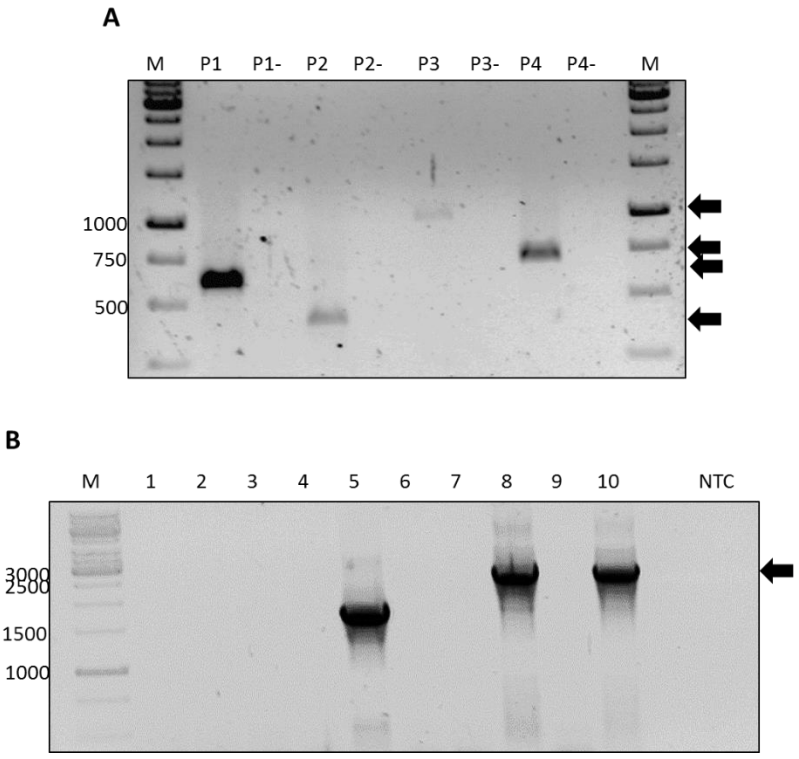

Figure 5.3: Domestication of DNA for GB assembly. A) Four primer sets (P1 – P4) were used to generate 4 patches by PCR. P1 – P4, experimental reactions; P1- to P4-, no template controls (NTC). Four fragments of 619 bp (P1), 425 bp (P2), 997 bp (P3) and 763 bp (P4) were generated and used in the domestication reaction. **B)** PCR screening of white colonies after digest/ligation domestication reaction with pUPD2 primers. Colonies 8 and 10 show expected product of 2987 bp. Labels: M, molecular weight marker (bp); black arrows, bands of interest.

Table 5.2 describes the GB parts used in the construction of the GB-pRIC-like vector. The CMV-Zera®E7SH-BGHpolyA transcriptional unit of pUPD2 was first cloned into the alpha plasmid, pDBalpha14 empty, and digested/ligated with *Bsa*I (site is now destroyed) to insert it into the correct position for assembly. pDBalpha14 CMV-Zera®E7SH-BGHpolyA was transformed into *E. coli*, and 5 white colonies were selected by blue/white selection via colony PCR. Using primers P1 For and P4 Rev or an internal Zera® primer and P4 Rev (Table 5.1) products of ~2700 bp or ~900 bp were observed respectively (Figure 5.4, black arrows). DNA of a PCR positive clone was prepared and final assembly into the GB-pRIC-like vector with plasmids containing elements for replication and replicon formation (Table 5.2) was performed with digestion/ligation using *BsmB*I RE. The ligation product was transformed into *E. coli*, after which positive clones were confirmed by RE digestion with *Bsa*I and *Hind*III. Positive clones showed the expected banding pattern of 4.5 kb and 6.7 kb (*Bsa*I) and 11.3 kb (*Hind*III) (data not shown) and was selected for transformation into *A. tumefaciens* EHA105.

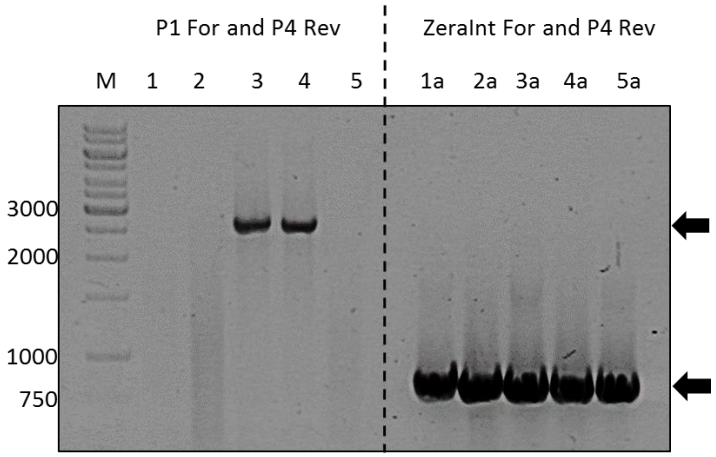

Figure 5.4: Colony PCR of CMVZera®E7SHBGHpolyA into pDBalpha14 empty using P1 For or ZeraInt For and P4 Rev primers. P1 For and P4 Rev produced fragment of ~2700 bp; ZeraInt For and P4 Rev produced fragment of ~900 bp. Labels: M, molecular weight marker (bp); black arrows, bands of interest.

5.3.2. Purification and TEM analysis of HPV-16 and -35 PsVs

N. benthamiana plants were vacuum infiltrated with recombinant *Agrobacterium* cultures containing pTRAc hL1 and pTRAc hL2 plasmids for HPV-16 (selected due to its high prevalence in cervical cancer) or -35 (selected as it is found more commonly in Africa), and GB-pRIC-Zera®E7SH. Leaves were harvested 4 dpi and purified by centrifugation in discontinuous Optiprep™ gradients. Collected fractions were resolved on 10% SDS-PAGE gels and HPV-16 probed by western blotting for L1 and L2 with anti-L1 MAb Camvir-1 and anti-HPV-16 L2 sera respectively. HPV-35 L1 was probed with anti-L1 H16.J4 MAb (Figure 5.5). An expected band of 56 kDa (black arrows) was observed in F2-F5 for HPV-16 L1 (Figure 5.5A) and F3-F6 for HPV-35 L1 (Figure 5.5C), which corresponds to the 33%-39% range in the Optiprep™ density gradient. Detection of L2 for HPV-16 was shown by a band in F2-F6 at ~80 kDa (Figure 5.5B, black arrow). Although L2 is a ~50 kDa protein, it has been demonstrated to migrate at ~80 kDa in SDS-PAGE gels (Doorbar and Gallimore, 1987; Tomita et al., 1987). The strongest signal for HPV-16 L1 and L2 was seen in F2-F5, suggesting these fractions contain L1:L2 VLPs or PsVs. L2 of HPV-35 was not detected as no antibody was available. For both HPV-16 and -35, degradation products were observed (white arrows) in both anti-L1 (~48 kDa, Figure 5.5A and C) and anti-L2 (~65 kDa, Figure 5.5B) western blots.

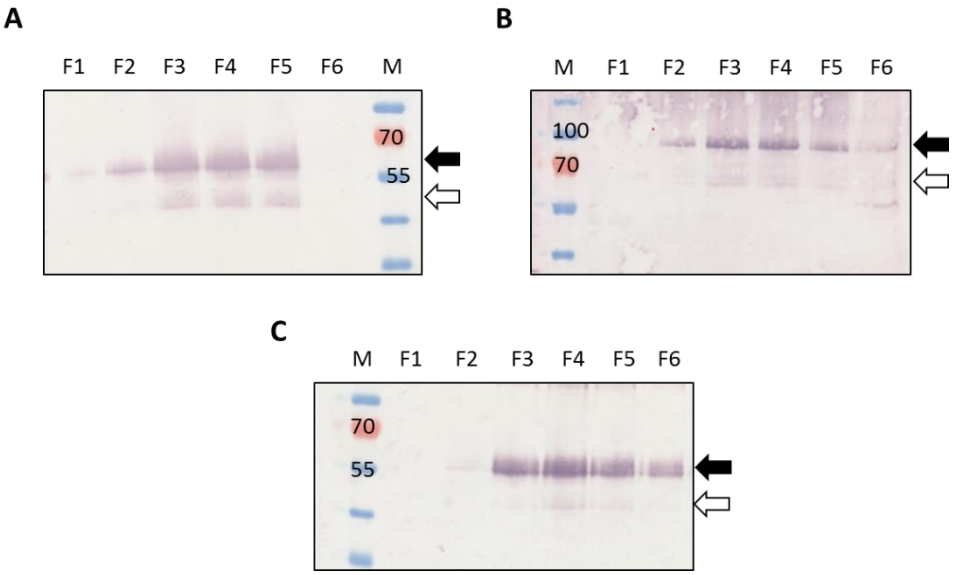

Figure 5.5: Anti-L1 and anti-L2 western blots of HPV 16 and 35 PsVs. Detection of L1 and L2 protein in PsVs. **A)** HPV-16 L1 protein (56 kDa) probed with anti-L1 antibody MAb Camvir-1 (1:20000). **B)** HPV-16 L2 protein (~80 kDa) protein probed with rabbit anti-L2 antibody (1:1000). **C)** HPV-35 L1 protein (56 kDa) probed with H16.J4 MAb (1:5000). Labels: F1-F6, fractions 1 to 6 collected from isopycnic gradient; M, molecular weight marker (kDa); black arrow, band of interest; white arrow, degradation products.

Following confirmation of the presence of L1 and L2 in purified fractions by western blotting, the assembly of L1 and L2 into capsids was assessed by TEM (Figure 5.6), and the encapsidation of DNA required for PsV formation was confirmed by RCA (Figure 5.7, section 5.3.3).

Both the L1 and L2 proteins of HPV-16 were detected in F2-F5 (Figure 5.5 A and B), and TEM of these fractions (Figure 5.6, blue boxes) showed structures that resembled L1 VLPs and PsVs seen in literature (Lamprecht et al., 2016; Maclean et al., 2007; Spoden et al., 2013). The density of empty capsids in Optiprep™ is 1.25 g/mL and the density of full capsids is 1.20 g/mL (Buck et al., 2005a). PsVs should therefore be found at Optiprep™ concentrations of 32-36%, which correspond to F3-F5. F2 showed particles of assorted sizes, from capsomeres (~10 nm) to particles ranging from 30-60 nm. F3 and F4 showed a homogenous sample of particles measuring 50-60 nm, which is the size of genuine HPV virions. F5 showed particles that were 'loosely' assembled (i.e. not as round as particles observed in F3 and F4), as well as capsomeres that seemed to form chains. Detection of L1 and L2 protein was strongest in F3-F5 (Figure 5.5 A and B). L1 protein of HPV-35 was detected in F3-F6 by western blotting (Figure 5.5C) and TEM of HPV-35 particles (Figure 5.6, green boxes) demonstrated a homogenous population in F3-F5, with particles 50-60 nm in size. Particles in F6 were heterogenous ranging in size from 20-50nm. F6 was collected from higher up in the density gradient (~27% fraction), where - based on buoyant density - PsVs are not expected.

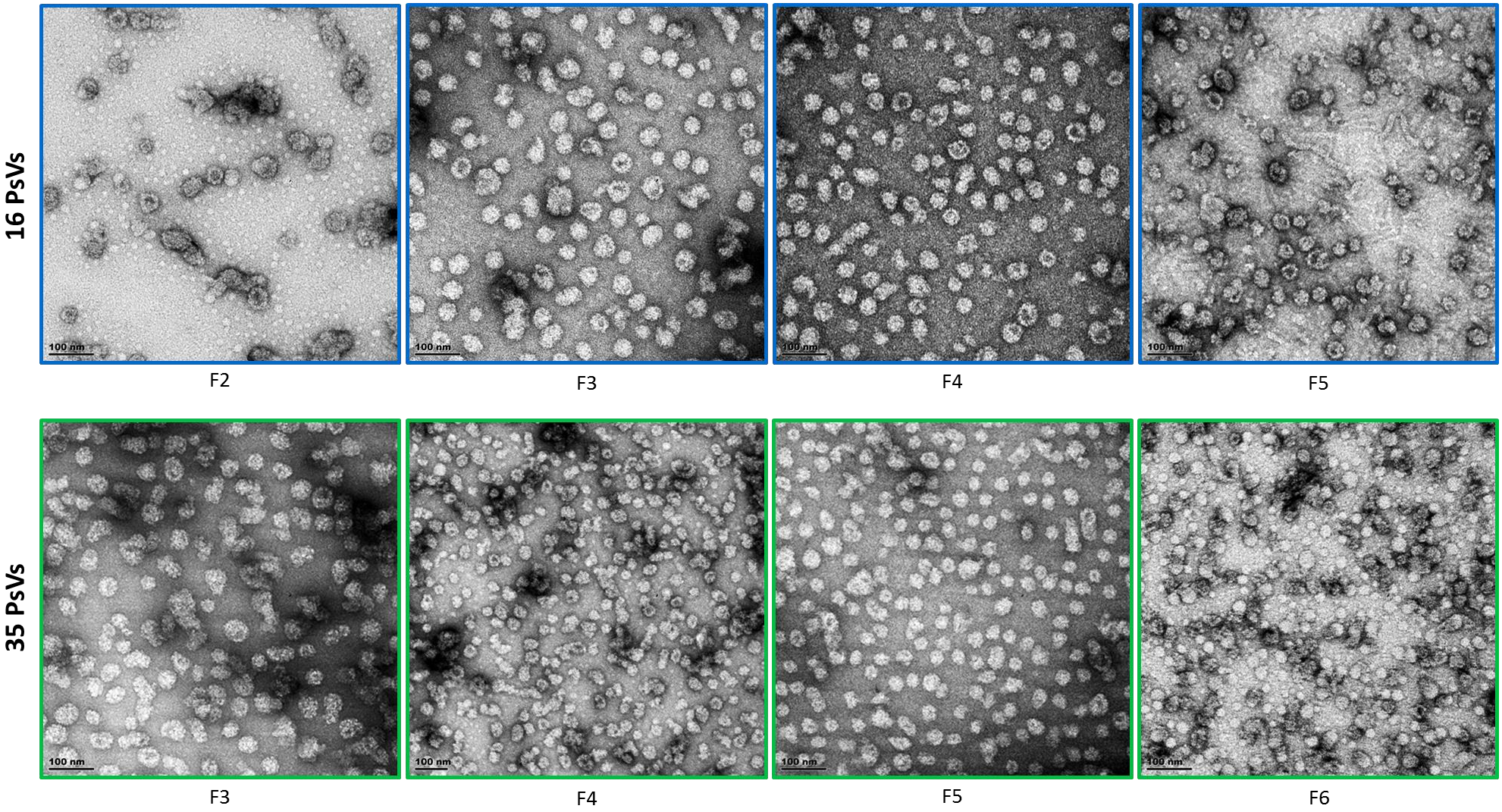

Figure 5.6: Transmission electron micrographs of purified HPV 16 and 35 PsVs. Fractions of GB-pRIC-Zera®E7SH crude plant extract purified by isopycnic gradient centrifugation. Samples were negatively stained with 2% uranyl acetate. Labels: F2-F6, purified fractions of HPV-16 and -35 PsVs; Scale bar 100 nm. Mag 34000x or 53000 x.

5.3.3. Confirmation of encapsidated Zera®E7SH replicons

VLPs are empty capsids that resemble the native virion, whereas PsVs encapsidate plasmids that express a gene of interest. Plant-made PsVs were first described by Lamprecht et al. (2016), and the authors confirmed the presence of encapsidated SEAP or luciferase replicons by inverse PCR. In the present study, fractions containing HPV-16 and -35 PsVs were screened for encapsidated Zera®E7SH replicons by RCA (Figure 5.7) which is an efficient method for amplifying circular DNA templates (Dean et al., 2001; Lizardi et al., 1998). The GB-pRIC-Zera®E7SH replicon is 4.5 kb in size. After RCA on purified fractions, F2-F6 for both HPV 16 and 35 PsVs were digested with *Hind*III and a linear fragment of 4.5 kb (black arrows) was present in all fractions, except F2 for HPV-16 PsV. pUC19 DNA was used a positive control for the RCA reaction and after digestion with *Hind*III showed an expected band of 2.6 kb (red box). A band >10 kb was observed (white arrow), and represents undigested plasmid. RCA was also performed on VLPs of HPV-16 and-35 that were purified as negative controls. After digestion with *Hind*III no bands were observed (data not shown). Inverse PCR (as described by Lamprecht et al. (2016)) was also performed on purified PsV fractions and the expected band of 0.9 kb observed (data not shown).

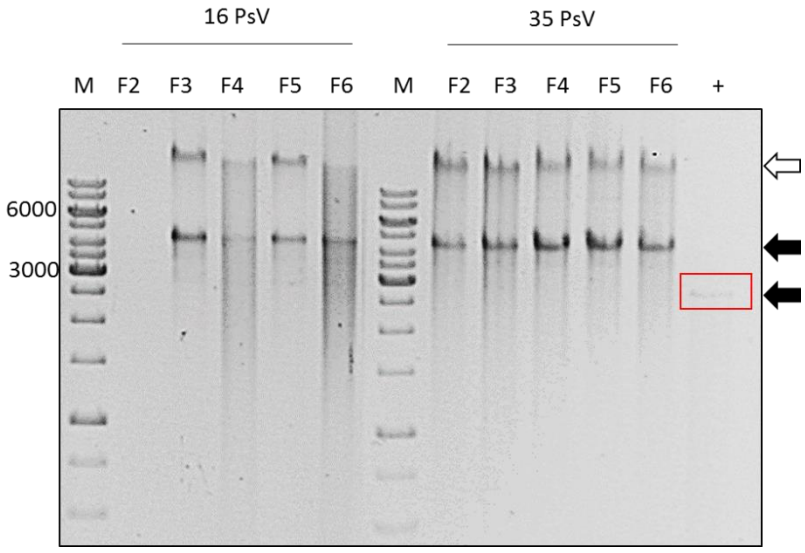

Figure 5.7: Rolling circle amplification of purified HPV-16 and HPV-35 PsV fractions. Fractions 2 – 6 of purified fractions run on 0.8% agarose gel after digesting with Hind*III*. Expected band sizes of 4.5 kb and 2.6 kb for GB-pRIC-Zera®E7SH and pUC19 respectively were observed. Labels: M, molecular weight marker (bp); +, pUC19 control cut with *Hind*III; black arrows, band of interest; white arrow, circular plasmid.

5.3.4. Expression of Zera®E7SH PsVs in mammalian cells

293TT cells were transfected with undigested RCA samples of F2-F6 – which would deliver linear concatamers of the pseudogenomes – for both HPV-16 and -35, to test the ability of these replicons to be transcribed in a cell system. Zera®E7SH expression is under control of the mammalian CMV promoter and the BGH polyadenylation signal. As transfection and expression controls, pTH Zera®E7SH and the original GB-pRIC- Zera®E7SH plasmids were used, and untransfected cells were used as a negative control. Transfected cells were lysed 72 h post-transfection, with lysis buffer containing SDS and DTT, to solubilise Zera® protein bodies. After lysis, the supernatants of F2-F6 were resolved on 10% SDS-PAGE gels and protein detected with anti-E7 or anti-Zera® sera on western blots (Figure 5.8). GB-pRIC-Zera®E7SH and pTH Zera®E7SH plasmid DNA were transfected as positive controls. Zera®E7SH protein is ~29 kDa (Whitehead et al., 2014); however, it migrated to ~37 kDa. Cells transfected with RCA products of HPV-16 F2-F6 and probed with anti-E7 (Figure 5.8A) or anti-Zera® (Figure 5.8B) sera showed the presence of Zera®E7SH in all fractions, with F2 of HPV-16 showing the weakest band intensity suggesting fewer replicons may have been packaged than in other samples. Detection of HPV-35 RCA products with anti-E7 (Figure 5.8C) or anti-Zera® (Figure 5.8D) showed low levels of detection of Zera®E7SH in F2 with an increase in protein concentration observed in F3-F5 as evidenced by the more intense protein bands. No band was observed in the cell only control.

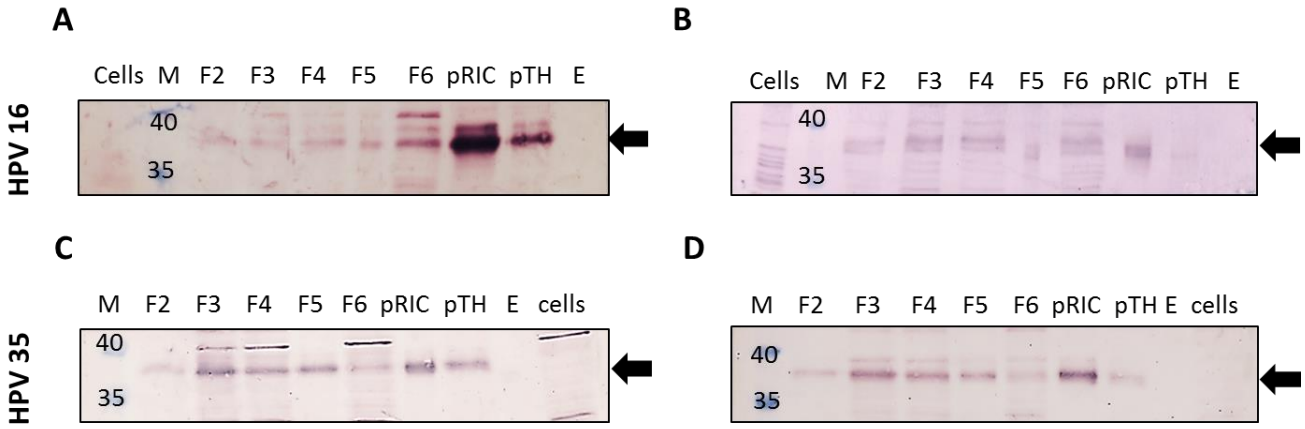

Figure 5.8: Anti-E7 and anti-Zera® western blots of 293T cells transfected with RCA samples of HPV-16 and 35 PsVs. Cells were harvested 72 h post transfection of RCA DNA **A)** HPV-16 RCA samples (37 kDa) probed with anti-E7 antibody (1:500). **B)** HPV-16 RCA samples (37 kDa) probed with anti-Zera® antibody (1:100). **C)** HPV-35 RCA samples (37 kDa) probed with anti-E7 antibody (1:500). **D)** HPV-35 RCA samples (37 kDa) probed with anti-Zera® antibody (1:100). Labels: F2-F6, fractions 2 to 6; M, molecular weight marker (kDa); pRIC, GB-pRIC-Zera®E7SH; pTH, pTH Zera®E7SH; Cells, cell only control; E, empty lane; black arrow, band of interest.

In addition to transfection of RCA products, the purified PsV fractions were also directly added to 293TT cells. The cells were lysed after which the supernatant was run on 10% SDS-PAGE gels and probed with anti-E7 serum for detection of Zera®E7SH. Zera®E7SH protein was detected in HPV-16 PsVs in F3 and F4 (Figure 5.9A), with no bands observed in F2 and F5. HPV-35 PsVs (Figure 5.9B) showed bands in F2-F4, with no detection observed in F5. No band was observed in the cells only control as expected. The detection of Zera®E7SH protein after PsVs infection (Figure 5.9) versus RCA products transfection (Figure 5.8) was lower as observed by weaker band intensity.

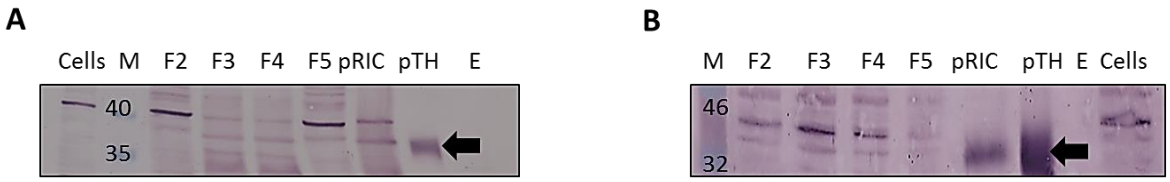

Figure 5.9: Anti-E7 western blots of PsV fractions added to 293TT cells. Detection of E7 protein in PsVs. **A)** HPV-16 PsVs and **B)** HPV-35 PsVs. PsVs were diluted in cell media at a ratio of 1:5. Cells were harvested 72 h post addition of PsVs. Band of 37 kDa was expected. Labels: F2-F5, PsV fractions 2 to 5; M, molecular weight marker; pRIC, GB-pRIC-Zera®E7SH; pTH, pTH Zera®E7SH; Cells, cell only control; E, empty lane; black arrow, band of interest.

5.4. Discussion

Persistent infection with high-risk HPVs is the main cause of invasive cervical cancer (zur Hausen, 2002). Despite the high efficacy of current commercial prophylactic HPV vaccines, they confer type-specific immunity and are not effective against pre-existing infections (Hildesheim et al., 2016; Hildesheim et al., 2007). Additionally, the combination of vaccine cost and poor healthcare resources in developing countries means the burden of cervical cancer remains high (Bruni et al., 2016). Treating early disease with therapeutics is thus the best option for an immediate reduction in cervical cancer incidence.

Although HPV-16 and -18 account for >70% of cervical cancers, HPV-35 is important particularly in an African context, as while it is the 8th most prevalent type worldwide, in Africa it is the 5th most common type, and accounts for 3-5% of all cervical cancer cases (Li et al., 2011). In some regions, it has been shown to have a prevalence of up to 17% (de Sanjose et al., 2010; Smith et al., 2007). HPV-35 is also not included in the most recent nonavalent vaccine, Gardasil®9. HPV-16 and -35 were therefore selected as the focus of this study, to investigate their potential use for the delivery of a therapeutic DNA vaccine.

Plants offer an alternative and attractive platform to reduce vaccine costs (Biemelt et al., 2003; Fischer et al., 2004; Rybicki, 2010). The transgenic production of VLPs of various kinds in plants has been successful; however, yields are generally low (Biemelt et al., 2003; Kohl et al., 2007; Tacket et al., 2000; Varsani et al., 2003b; Warzecha et al., 2003). To increase antigen expression levels in plants, DNA replicon systems have been explored. Vectors based on BeYDV have been shown by several groups to significantly increase protein expression levels (Hefferon and Fan, 2004; Huang et al., 2009; Regnard et al., 2010; Zhang and Mason, 2006). The pRIC vector (Regnard et al., 2010) is the backbone on which GB technology was applied to assemble a plasmid with re-usable parts.

Recombination technology for the generation and assembly of large DNA sequences has improved over the last several years (Ellis et al., 2011; Gibson et al., 2009). However, the single-use of genetic modules and the requirement of re-design for alternative use, is time consuming and inefficient. GB technology addresses this problem through the standardisation of basic sequences or parts that allow their reusability through simple standard rules of assembly, in addition to multipartite assembly (Sarrion-Perdigones et al., 2011; Sarrion-Perdigones et al., 2013). In this study, a transcriptional unit of a promoter – coding sequence – terminator (CMV-Zera®E7SH-BGHpolyA), was domesticated and cloned into the pUPD2 entry vector. The promoter is the cytomegalovirus intron/enhancer/promoter element, and the terminator is the bovine growth hormone polyadenylation signal. Both these elements have been extensively utilised for constitutive, high-level expression in mammalian cells (Buck et al., 2005a; Burgers et al., 2006; Tanzer et al., 2011). Several promoter and terminator regions are available on the online GB database; however, these are for gene expression in plants. Although the PsVs are plant-made, the encapsidated DNA must be able to replicate in mammalian cells. The generation of a GB database with several mammalian promoter or terminator regions can be used to rapidly generate several plasmids and compare expression levels of the gene of interest. Our research group is in the process of domesticating several mammalian promoter and terminator sequences. While the initial domestication of all parts may be deemed time consuming, once a database is established, the construction of several plasmids can be performed on the same day.

Figures 5.3 and 5.4 show the successful amplification of 4 patches to domesticate the CMV-Zera®E7SH-BGHpolyA sequence and its subsequent cloning into a destination vector. The fully assembled GB-pRIC- Zera®E7SH plasmid is ~11.3 kb and produces a replicon size of ~4.5 kb (Figure 5.2). The size of encapsidated DNA has been shown to influence its packaging. Genuine HPV virions package a genome of 8 kb. Touze and Coursaget (1998) demonstrated that HPV VLPs preferentially packaged plasmid DNA of 5-8 kb and Buck et al. (2004) showed that the size of the plasmid to be

packaged influenced vector production efficiency. Therefore, plasmid size is important. The GB-pRIC replicon is smaller than the 5kb 'cut off' described by Touze and Coursaget (1998); however, cell infections with mSEAP (4.8 kb)-containing plant-made HPV-16 PsVs showed consistently higher SEAP readings than for iSEAP PsVs (6.6 kb) (Lamprecht et al., 2016). This suggests that packaging of different size replicons requires further study. The reusabilty of GB parts opens the door to simple manipluation of the size of the encapsidated DNA by the addition of stuffer fragments. The abovementined advantages of GB cloning therefore make it an attractive tool for the simple and efficient construction of complex biological modules.

L1 produced in a variety of expression systems can spontaneously assemble into VLPs (Hagensee et al., 1993; Kirnbauer et al., 1992; Rose et al., 1993). However, several studies have shown that both L1 and L2 are required for the efficient packaging of DNA. The viral genome of BPV-1 was encapsidated when BPV-1 L1 and L2 were co-expressed (Okun et al., 2001), and packaging of DNA plasmids into BPV-1 L1:L2 VLPs was at least 50 times more effective than into BPV-1 L1-only VLPs (Zhao et al., 1998). Additionally, Holmgren et al. (2005) showed that raft cultures transfected with wild-type HPV-31 or a HPV-31 with a mutated L2, showed a ~10-fold reduction in encapsidation of viral DNA in the L2 mutants. To this end, PsVs were produced with L1 and L2 of HPV-16 and -35 respectively. The expression of L1 and L2 in plants has previously been described by Maclean et al. (2007) and Pereira (2008) respectively, where expression using human codon optimised genes was greater than expression with the wild type genes. To make PsVs that encapsidated a Zera®E7SH-encoding DNA, plants were co-infiltrated with human codon optimised L1, L2 and the BeYDV-derived replicon-generating GB-pRIC-Zera®E7SH. Expression of the capsid proteins was confirmed by western blotting of purified PsV fractions (Figure 5.5). For HPV-16, bands of 56 kDa for L1 (Figure 5.5A) and ~80 kDa for L2 (Figure 5.5B) were observed on western blots. For HPV-35 hL1, a band at 56 kDa (Figure 5.5C) confirmed expression and successful purification. Confirmation of the presence of L2 for HPV-35 was not performed because HPV-35 anti-L2 antibodies were not available. Despite the unconfirmed presence of L2 for HPV-35, further analysis of capsid formation and encapsidation were performed.

The assembly of HPV-16 and -35 L1 and L2 into PsVs was determined by TEM (Figure 5.6). Infiltrated leaves were purified on discontinuous Optiprep™ gradients and fractions were trapped on grids. PsVs observed were similar to particles that have been purified in mammalian cells, plants and insect cells (Buck et al., 2005b; Lamprecht et al., 2016; Maclean et al., 2007; Touze and Coursaget, 1998). Empty capsids (VLPs) have a density of 1.25 g/mL (38% - >40% Optiprep™), while full capsids (PsVs) have a density of 1.20 g/mL (32% - 36% Optiprep™) (Axis-Shield PoC AS, 2016; Buck et al., 2005a).

Empty capsids are therefore found towards the denser layers (bottom) of the discontinuous gradient. HPV-16 PsVs (Figure 5.6, blue boxes) showed a heterogeneous population of particles in F2 and F5; F3 and F4 showed a homogenous population. F2 corresponds to ~39% Optiprep™ and contained a mixture of capsomeres and particles ranging from 30-60 nm which were most likely VLPs. This was confirmed by RCA analysis (Figure 5.7), where no DNA band was present after RE digestion. F3-F4 (~33% Optiprep™) showed a homogenous population with particles of 50-60 nm, and these fractions are where PsVs were expected. Similarly, for HPV-35 PsVs (Figure 5.6, green boxes) F3-F5 showed a homogenous sample of particles 50-60 nm. These PsVs are structurally similar to plant PsVs produced by Lamprecht et al. (2016), and therefore showed promise for use in *in vitro* studies. However, PsV concentration and the ratio of VLPs versus PsVs in each fraction was not determined. Several groups have described PsV quantitation via L1 ELISA (Fleury et al., 2008; Touze and Coursaget, 1998), PCR (Unckell et al., 1997), qPCR (Cerqueira et al., 2017; Kwak et al., 2014) or the estimation of transducing units in reporter assays (Buck et al., 2004). As Zera®E7SH is not a reporter gene, quantitation methods by ELISA or PCR will be more suited for future experiments.

The assessment of packaged DNA in PsVs has previously been performed by DNase treatment followed by PCR. This demonstrated that DNA was within the virion and not associated on the outside of the capsid shell (Rossi et al., 2000; Unckell et al., 1997). Proteinase K was used by Lamprecht et al. (2016) to digest HPV-16 plant-made PsVs as PCR denaturing conditions did not degrade the protein shell of the PsVs, and minimal amplification of pseudogenome DNA was observed without it. Proteinase K digestion followed by inverse PCR resulted in a dramatic increase in amplification of encapsidated DNA. The authors indicated that this lack of denaturation showed that PsVs were thermostable, which is an advantage over PsVs made in other production systems, as these seem more thermolabile. In this study, we investigated the use of RCA in addition to inverse PCR to confirm the presence of pseudogenome DNA. RCA is a simple method to amplify single- or double-stranded circular DNA templates (Dean et al., 2001; Lizardi et al., 1998). Figure 5.7 shows F2-F6 of purified HPV-16 and -35 PsV samples on which RCA was performed. Digestion of replicons with *Hind*III revealed a linear fragment of 4.5 kb, confirming the presence of packaged DNA. Inverse PCR of these purified PsV samples also showed the amplification of circularised pseudogenome DNA (data not shown). These data indicate that L2 was probably present in HPV-35 PsVs, as virions encapsidated circular DNA of Zera®E7SH replicons. Furthermore, purified L1:L2 VLPs of HPV-16 and -35 did not show any bands after inverse PCR or RCA (data not shown). Although packaging in the absence of L2 was not investigated, the data presented is sufficient to conclude that packaging of Zera®E7SH replicons into particles in plants is reasonably efficient.

Nevertheless, it has been postulated that not all packaged DNA may be the DNA of interest, as L1 and L2 can bind DNA non-specifically *in vitro* (Mallon et al., 1987; Zhou et al., 1994). Roden et al. (1996) suggested that VLPs produced in mammalian systems can encapsidate miscellaneous cellular DNA. Therefore, it is possible that pTRAc plasmids may also have been packaged. RE digests of these plasmids with *Hind*III would not result in a band of 4.5 kb, as pTRAc hL1 and hL2 plasmids are 7.7 kb and 7.5 kb respectively, showing that the bands observed after RCA are predominantly pseudogenome DNA specific. Sequencing of RCA samples from purified PsVs could confirm the identity of the packaged DNA, but this was however not deemed necessary for this project.

Co-expression of L1 and L2 (Figure 5.5), PsV formation (Figure 5.6) and detection of encapsidated Zera®E7SH DNA replicons (Figure 5.7) was observed, suggesting that these plant-made particles are genuine PsVs that could be used to infect mammalian cells. *In vitro* expression of Zera®E7SH was confirmed by transfection of DNA replicons into 293TT cells (Figure 5.8). Detection of Zera® or E7 protein expressed from the replicons with anti-Zera® and anti-E7 sera showed a band in western blots at 37 kDa. This band was also observed for purified plasmid DNA of pTH and GB-pRIC-Zera®E7SH, showing that plant-made replicons were successfully assembled, transfected and transcribed in mammalian cells. Additionally, PsVs of HPV-16 and -35 were capable of infecting 293TT cells (Figure 5.9), with detection of a band at 37 kDa. These data suggest that these candidate vaccines have the potential to deliver DNA into cells. L2 is required for the binding and delivery of DNA into the cells (Day et al., 2004; Kämper et al., 2006), therefore supporting our conclusion that L2 was probably present in HPV-35 PsVs. The faint bands detected after PsV infection suggest low infectivity. Low PsV infectivity may be attributable to the cell type used, or the absence of mammalian cell nuclear extract which has recently been shown to be necessary for cell infection, in addition to reassembly of infectious PsVs (Cerqueira et al., 2016). PsVs in this study were produced in plants therefore lack factors which may be necessary for optimal mammalian cell infection. Disassembly/ reassembly experiments using plant-made PsVs in the presence of nuclear extract could improve cell infection and therefore should be explored. *In vivo* testing of the HPV-16 and-35 PsV vaccines will be the next step to elucidate their ability to induce humoral and cellular immune responses, as well as tumour protection and regression in challenge experiments. *In vivo* delivery of DNA vaccines with HPV PsVs has been demonstrated by Gordon et al. (2012), where PsVs delivering SIV Gag DNA to the vaginal tract of macaques induced Gag specific antibodies and T-cell responses in blood, the vaginal mucosa and draining lymph nodes. Furthermore, Lizotte et al. (2016) have recently shown that VLPs of plant-produced eCPMV VLPs stimulate potent anti-tumour immune responses to metastatic ovarian cancer, breast cancer and melanoma via *in situ* vaccination, which involves delivery of VLPs directly to the tumour. Therefore, routes of vaccine delivery and the

comparison of immune responses between Zera®E7SH naked DNA and protein vaccines (Whitehead et al., 2014) and PsV vaccines will need to be investigated.

This study is the first report of plant-made PsVs encapsidating a self-replicating plasmid for the delivery of a candidate DNA vaccine. In this proof-of-concept study, I demonstrated that the PsVs are able to infect mammalian cells and express Zera®E7SH. Future work to investigate the potential of this candidate vaccine as a combination prophylactic and therapeutic vaccine is required. Optimal size of encapsidated DNA and concentration of PsVs can be investigated to further optimise PsVs production in plants.

Chapter 6: General discussion and conclusions

6.1. General discussion

There is a relatively high incidence of cervical cancer in developing countries, as well as high mortality rates (World Health Organisation, 2015), largely due to limited healthcare resources and lack of appropriate screening and therapy. In addition, the high rate of HIV infection particularly in sub-Saharan Africa has resulted in an increase in HPV prevalence, as HIV infection has been shown to influence acquisition, persistence and carcinogenicity of HPV (Clifford et al., 2017; Massad et al., 2015). Three prophylactic HPV vaccines are currently on the market; however, they are expensive due to production in yeast and mammalian cell culture systems, are type-specific and lack therapeutic efficacy. The most recent vaccine (Merck's Gardasil®9) has addressed some of these concerns as an additional 5 HPV L1 VLPs are included in the vaccine; however, this has not had an impact on bringing down vaccine cost. A next-generation broadly protective vaccine could potentially address the type-specificity and cost of these vaccines. Furthermore, plant-based expression could facilitate a further reduction in cost due to its rapid scalability in comparison to other systems (Fischer et al., 2004; Merlin et al., 2014; Rybicki, 2010).

L2-based vaccines are a promising target for broad protection against HPV, as aa 1-120 in the N-terminus of the L2 minor capsid protein are highly conserved among different HPV types (Lowe et al., 2008). Many researchers have shown that a few L2 peptides within this region elicit cross-neutralising antibodies to several heterologous HPV types (Alphs et al., 2008; Gambhira et al., 2007b; Jagu et al., 2009; Kawana et al., 2003; Kawana et al., 1999; Kondo et al., 2007; Pastrana et al., 2005; Schellenbacher et al., 2009; Slupetzky et al., 2007). However, there is still an urgent need for therapeutic vaccines to clear established infections, given the large number of people who are infected. DNA vaccines have shown therapeutic efficacy as they promote MHC I antigen presentation and induction of CTL responses (Lee et al., 2016). Therefore, one of the aims of this study was to produce plant-made L1:L2 chimaeras, test their immunogenicity in mice and analyse the cross-neutralising potential of sera in PBNAs. Additionally, a novel E7-based DNA vaccine was constructed, encapsidated in plant-made PsVs, and the ability of PsVs to infect and deliver the DNA vaccine tested *in vitro*. This vaccine has the potential to be used as a combination prophylactic and therapeutic vaccine.

Four HPV-16 L2 peptides – aa 108-120, 65-81, 56-81 and 17-36 – were substituted into the HPV-16 L1 surface DE loop from position 131 (SAC) or in the C-terminal between the h4 and β-J structural region from position 431 (SAE), to generate 8 HPV-16 L1:L2 chimaeras. Substitution positions were selected as they were previously shown to successfully display L2 epitopes, and the chimaeras elicited anti-L1 and -L2 humoral immune responses to insect cell-produced cVLPs (Varsani et al., 2003a). Expression of the chimaeras in *N. benthamiana* was optimized using 4 plant expression vectors that had characteristics that could potentially increase protein expression levels. Targeting of expressed protein to the cytoplasm or chloroplast (Maclean et al., 2007), the use of a self-replicating viral vector (Regnard et al., 2010), and a vector that contains a hypertranslational region and also encodes a silencing suppressor on the same plasmid (Sainsbury et al., 2009) were used to compare chimaera expression levels. All chimaeras were transiently expressed in *N. benthamiana* via *Agrobacterium*-mediated transfer. Chloroplast targeting and the use of the self-replicating vector showed the highest chimaera yields (90-470 mg/kg); however, assembly of the SAC chimaeras targeted to the chloroplast appeared to favour the formation of higher order structures compared to the cytoplasm targeted ones, potentially due to reduced proteolysis in the chloroplast. Therefore, 4 chloroplast targeted SAC chimaeras and 1 SAE chimaera with L2 aa 65-81 which had not previously been tested in chimaeras, were used in large-scale expression and subsequent purification experiments.

Two extraction buffers and the use of continuous and discontinuous Optiprep™ density gradient centrifugation were investigated for the purification of cVLPs. Extraction in HSNaOAc buffer was beneficial in the partial removal of host cell proteins, with the low pH and high salt concentration of the buffer aiding in maintaining cVLP integrity during extraction and purification. Purification on discontinuous gradients was better suited than continuous gradients for purification of structurally intact cVLPs. cVLPs were concentrated in fewer fractions, and showed better separation from contaminating plant proteins. TEM analysis showed cVLPs measuring 25-55 nm in size, consistent with VLP populations seen in other studies (Kim et al., 2012a; Maclean et al., 2007; Matic et al., 2011). Plant expressed L1-only VLPs mainly assembled into particles that were 50-60 nm in size, with SAC chimaeras 108-120, 65-81, 56-81 and 17-36 assembling into cVLPs 50-60 nm as well as small cVLP 25-40 nm, with a few capsomeres and aggregates also observed. SAE 65-81 showed the poorest assembly into cVLPs, where mainly aggregates were observed. This may be due to the substitution location (from position 431) in the C-terminal arm of L1 which is critical for VLP assembly (Bishop et al., 2007a; Chen et al., 2001; Modis et al., 2002). Although the substitution position of these chimaeras did not affect the Cys^{175} and Cys^{428} residues which have been shown to be essential for the formation of disulphide bonds between neighbouring L1 capsomeres to form

VLPs (Fligge et al., 2001; Li et al., 1998; McCarthy et al., 1998; Sapp et al., 1998; Varsani et al., 2006a), changes in aa composition (size and charge) may have led to steric hindrance preventing correct particle assembly. cVLP assembly may be L2 peptide sequence-dependent and it is therefore important that it is determined on a case by case basis. The display of L1 and L2 epitopes on L1 capsids was characterised by analysing the binding of MAbs in indirect ELISAs. The MAbs bind epitopes that are required for the induction of the humoral immune response and production of NAbs. Diminished binding by HPV-16 L1 neutralising MAbs H16.V5 and H16.E70 was observed for all chimaeras and indicated that these epitopes were disrupted by L2 substitutions; however, binding by neutralising MAbs H16.U4, H16.9A and H16.J4 was observed showing there wasn't a total loss in L1 neutralising epitopes on the capsids. L2 epitope presentation was characterised by binding by anti-L2 polyclonal serum and L2 4B4 MAb to SAC 108-120, confirming the display of L2 peptides on the L1 surface. Based on these results, the immunogenicity of these chimaeras was tested in mice.

Previous studies have shown that plant-produced L1 VLPs and cVLPs were immunogenic and elicited the production of NAbs (Fernandez-San et al., 2008; Maclean et al., 2007; Paz De la et al., 2009; Pineo et al., 2013). Therefore, the 5 cVLP candidate vaccines and an L1 VLP positive control were tested for immunogenicity in this study. All chimaeras elicited an anti-L1 response, with L1 VLPs showing the highest anti-L1 titres followed by SAC 108-120 and SAC 17-36. SAE 65-81 showed the lowest anti-L1 titres. The neutralising potential of sera was investigated in L1 and L2-based PBNAs. In L1 PBNAs, antisera from SAC 108-120, SAC 65-81 and SAC 56-81 neutralised HPV-18 PsVs, in addition to SAC 108-120 neutralising HPV-58. However, none of the SAC chimaeras' antisera neutralised homologous HPV-16 PsVs. Only SAE 65-81 antisera neutralised homologous HPV-16 PsVs in addition to cross-neutralising HPV-11 and -18, despite having the lowest anti-L1 titre of all vaccine candidates. SAC chimaeras have L2 peptides substituted in the DE loop which contains residues recently shown to be bound by H16.V5 MAb (Bissett et al., 2016; Lee et al., 2015). H16.V5 binds an immunodominant epitope known to generate potent NAbs (Roden et al., 1997; Wang et al., 2003), therefore, the lack of HPV-16 neutralisation is probably as a result of epitope disruption. These results correspond to the ELISA data that showed a lack of binding by H16.V5. However, homologous HPV-16 neutralisation by SAE 65-81 antisera was observed, even though the chimaera did not show binding by H16.V5. It is possible that the low yield and poor assembly of SAE 65-81 was not sufficient for MAb binding in ELISAs, as the SAE region has not been shown contain epitopes for H16.V5 binding. Unexpectedly, all antisera tested failed to neutralise any PsVs in L2 PBNAs, suggesting that display of L2 peptides was insufficient for the induction of NAbs, possibly as a result of poor antigen presentation on APCs, as well as low antibody titres from immunogenicity studies. Interestingly, other L2 PBNA studies in our lab have shown that sera from other plant-produced chimaeras (Pineo

et al., 2013) did not neutralise HPV-16 PsVs or heterologous HPVs, while the same chimaeras produced in insect cells (McGrath et al., 2013) showed homologous and heterologous HPV PsV neutralisation (Megan Hendrikse et al., personal communication). This suggests that plant-produced chimaeras may not be assembling as efficiently as cVLPs produced in other systems. The large-scale production of plant-made L1 VLPs in our lab has shown inconsistent preparations, with co-purification of RuBisCo and capsomeres observed with VLPs (A. R. van Zyl, personal communication). Therefore, the true potential of cVLPs generated in this study may require their production in other systems (e.g. insect cells or yeast) to determine if there is an effect on cVLP assembly and epitope presentation due to the host expression system, which may ultimately affect display and immunogenicity of epitopes.

Lastly, a proof of concept study to address the lack of therapeutic efficacy of current vaccines, using a DNA vaccine delivered by plant-made PsVs, was investigated. DNA vaccines are attractive for therapeutic application as they are safe, easy to manufacture and promote MHC I antigen presentation. A Zera®E7SH DNA plasmid was constructed using GB technology (Sarrion-Perdigones et al., 2011). This system allowed for assembly of a self-replicating plasmid using several parts, and is advantageous in that it can allow for the control of plasmid size due to the ability to control the number of parts used. HPV-16 and -35 PsVs were produced for the encapsidation of the Zera®E7SH pseudogenome. HPV-16 was selected for PsV production as it is detected in ~61% of ICCs (de Sanjose et al., 2010; Li et al., 2011). HPV-35 was selected as it holds a significant importance in Africa due to its prevalence (~5% of ICCs) (Li et al., 2011) and specifically because it is not included in current HPV vaccines. PsVs were successfully produced in plants and resembled PsVs produced in other systems. These PsVs successfully encapsidated the Zera®E7SH pseudogenome, which formed replicons capable of infecting mammalian cells. Furthermore, the PsVs delivered the DNA vaccine into mammalian cells where expression of Zera®E7SH was observed. To our knowledge, this is the first report of plant-made HPV-16 and -35 PsVs encapsidating and delivering a potential DNA vaccine in mammalian cells.

6.2. Conclusions and future work

Eight HPV-16 L1:L2 chimaeras were transiently expressed in plants, with targeting of protein to the chloroplast and L2 peptide substitution in the DE loop (SAC chimaeras) favouring the assembly of cVLPs. These cVLPs were shown to display L1 and L2 epitopes on their surface through binding of MAbs or polyclonal serum. Immunisation of mice with partially purified cVLPs elicited anti-L1

immune responses for all chimaeras with SAC 108-120 and SAC 17-36 showing the highest anti-L1 titres. However, limited neutralisation and cross-neutralisation of HPV PsVs was observed with all chimaera antisera, suggesting antibodies generated from immunogenicity studies were mainly non-neutralising. It is evident from these data that the substitution position of L2 peptides needs to consider L1 neutralising epitopes in order to maintain their display, in addition to its effect on VLP assembly. Future work to improve vaccine purity is required and methods that favour the purification of functional cVLPs also requires investigation. A strategy to improve the stability of plant-made cVLPs could be the encapsidation of a genome or plasmid (plant-made PsVs in this study were shown to assemble into structures that resemble VLPs and PsVs). Immunogenicity of the cVLPs could also be improved by the co-administration of cVLPs with HPV-16/18 L1 VLPs, which have been shown to enhance protection against PsV and quasivirion challenge (Boxus et al., 2016).

Plant-produced HPV-16 and -35 PsVs encapsidating a Zera®E7SH DNA vaccine were shown to infect mammalian cells and to deliver the therapeutic vaccine-encoding pseudogenome in this proof of concept study. These PsVs show great promise for the development of a combination prophylactic and therapeutic vaccine to provide immediate and long-term protection. Future work includes a comparison between naked Zera®E7SH DNA and subunit vaccines to determine if PsVs have adjuvanting properties, and can deliver a DNA plasmid that can stimulate both a humoral and cellular immune response as has been reported in other studies (Gurunathan et al., 2000; Lenz et al., 2001; Peng et al., 2010; Rudolf et al., 2001; Yang et al., 2004b). Recent studies by the Steinmetz group have described the role of the tumour microenvironment as a barrier to successful cancer therapy. Gene delivery by *in situ* vaccination has been proposed as a strategy to overcome this barrier and plant VLPs have been shown to induce antitumour immune responses when introduced to the tumour microenvironment (Lizotte et al., 2016). In conjunction with recent findings that HPV PsVs have a strong tropism for cancer cells (Kines et al., 2016), the therapeutic efficacy of our PsVs could be further characterised using this *in situ* vaccination strategy.

In conclusion, it is expected that it will take decades before the preventive HPV vaccines can generate impact on the incidence of cervical cancer. Ultimately, an affordable vaccine that shows both therapeutic and prophylactic efficacy, is single-dose and can be used in low-resource settings, is the way forward for the eradication of cervical cancer.

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