Optimization and characterisation of plant produced Human Papillomavirus pseudovirions in *Nicotiana benthamiana*

by

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Plagiarism declaration

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List of Abbreviations:

BeYDV	**Bean Yellow Dwarf Virus**
BGH	**Bovine growth hormone**
BPV	**Bovine papillomavirus**
BRU	**Biopharming research unit**
CaMV	**Cauliflower mosaic virus**
CMV	**Cytomegalovirus**
CTL	**Cytotoxic T-lymphocyte**
DMEM	**Dulbecco's Modified Eagle's medium**
DPBS	**Dulbecco's Phosphate-Buffered Saline**
dsDNA	**Double stranded DNA**
EGF	**Epidermal growth factor**
EGFP	**Enhanced Green Fluorescent Protein**
ELISA	**Enzyme-linked Immunosorbent Assay**
GAVI	**Global Alliance for Vaccines and Immunisation**
HEK293TT	**Human Embryonic Kidney cells**
HIV	**Human immunodeficiency virus**
HR-HPVs	**High-risk human papillomavirus types**
HS-PBS	**High salt phosphate-Buffered Saline**
HSPG	**Heparan sulphate proteoglycan**
HSV-tk	**Herpes simplex virus thymidine kinase**
HPV	**Human papillomavirus**
LB	**Luria-Bertani medium**
LIR	**Long intergenic region**
LR	**Low-risk human papillomavirus types**
ND-10	**Nuclear domain 10**

NIKs	**Near-diploid Immortalised Keratinocyte Skin cells**
PBNA	**Pseudovirion-based neutralisation assay**
PCR	**Polymerase chain reaction**
PDGF	**Platelet-derived growth factor**
PolyA	**Poly adenylation signal**
Pro	**Promoter**
PsV	**Pseudovirion**
PV	**Papillomavirus**
qPCR	**Quantitative PCR**
SDS-PAGE	**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis)**
shRNA	**Short hairpin RNA**
SIR	**Short intergenic region**
SV40	**simian vacuolating virus 40**
TMV	**Tobacco Mosaic virus**
T-DNA	**Transfer DNA**
TEM	**Transmission electron microscopy**
TSP	**Total soluble protein**
VLP	**Virus-like particle**
WPRE	**Woodchuck Hepatitis posttranscriptional regulatory element**

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Abstract

Human papillomavirus (HPV) is known to be the cause of anogenital and oropharyngeal cancers as well as genital and common warts. There are currently three prophylactic virus-like particle (VLP) based vaccines. These vaccines, however, do not offer protection against all HPV strains and cannot act therapeutically and so further vaccine development is still needed. The burden of HPV is also highest in low-income countries for which the vaccine costs are still quite high, and therefore alternative methods of vaccine production and testing are needed. HPV pseudovirions (PsVs) are synthetic viral particles that are made up of the L1 major and L2 minor HPV capsid proteins and encapsidate up to 8Kb of pseudogenome DNA without the need of an encapsidation signal. HPV PsVs are used to test neutralising antibodies elicited by vaccines, for studying the virus life cycle, and potentially for delivery of therapeutic DNA vaccines. HPV PsVs are typically produced in mammalian cells; however, it has recently been shown that HPV PsVs can be produced in plants, a potentially safer, cheaper and more easily scalable means of production. While, a current problem with plant HPV PsV production is low yields, research has shown that using pseudogenome DNAs between 5-7Kb increases yields of papillomavirus PsVs in mammalian cells. Therefore, the objective of this study was to determine the optimal pseudogenome size for encapsidation by plant produced PsVs, in order to increase the amount of PsVs in a sample as opposed to VLPs.

Pseudogenome constructs encoding Enhanced Green Fluorescent Protein (EGFP)and ranging in size from 4.8Kb – 7.8kb were cloned into a geminivirus-derived replicating vector, transformed into *Agrobacterium tumefaciens* and then infiltrated into *Nicotiana benthamiana* along with plant expression vectors encoding the HPV 35 L1 and L2 capsid proteins. Particles were purified by iodixanol density gradient ultracentrifugation and the 27% and 33% fractions of this gradient analysed. Transmission electron microscopy (TEM) was used to confirm particle assembly and L1 expression was quantified by ELISA. Particles were disrupted with proteinase K and quantitative PCR was used to quantify the encapsidated DNA. Ratios of encapsidated DNA to L1 capsid protein were calculated for each of the PsV samples with different sized pseudogenomes, to account for batch-to-batch variation and as an approximation of which size pseudogenome is better encapsidated. Infective ability of the particles was analysed by incubating the PsVs onto HEK293TT cells and then checking for DNA delivery and protein expression by measuring EGFP expression by Western blots. The results showed that PsVs are found predominantly in the 27% fraction of the iodixanol gradient whereas the 33% fraction of the gradient appears to only contain VLPs. The data also indicated that the smaller pseudogenomes, were packaged more efficiently into PsVs as higher concentrations of encapsidated DNA and higher levels of EGFP expression were obtained when the 4.8Kb pseudogenome was used, compared to when the

larger 5.8 - 7.8Kb pseudogenomes were used. Thus, the results showed that smaller pseudogenomes, around 4.8Kb, should be used for the plant production of HPV 35 PsVs as they are better packaged than larger pseudogenomes and thereby produce higher yields of functional PsVs.

Chapter 1: Literature review

1.1. Introduction

In 2015, the World Health Organization (WHO) estimated cancer to be one of the top two leading causes of death in persons younger than 70 years old in 91 out of 172 countries. Human papillomavirus (HPV) is a sexually transmitted virus that was estimated to be the most common sexually transmitted disease in the USA (Hamborsky *et al.*, 2015). HPV causes genital and common warts, as well as anogenital and oropharyngeal cancers, with 5% of all human cancers being caused by HPV (De Martel *et al.*, 2012). HPV affects both men and women with more women developing cancers, particularly cervical cancer, and warts than men (Bray *et al.*, 2018). High-risk (HR) HPV strains like HPV 16 and 18 often cause anogenital and oropharyngeal cancers whereas low-risk (LR) strains, such as HPV 6 and 11, often result in genital and common warts.

1.1.1. Human papillomavirus (HPV) statistics and demographics

Cervical cancer is the most common HPV induced cancer and is the second most frequent cancer in women aged 15 to 44 years (Bruni *et al.*, 2015a, Bruni *et al.*, 2015b, Ferlay *et al.*, 2013). In 2018, it was estimated that 570 000 cases of cervical cancer arose annually globally, of which 311 000 were fatal (Bray *et al.*, 2018). Furthermore, studies show that low-income countries have the highest burden of incidences and fatalities, particularly in Southern, Western and Eastern Africa (Ferlay *et al.*, 2013, Bray *et al.*, 2018) whereas high income countries are close to eradicating HPV, with North America, Australia/New Zealand, and Western Asia having 7-10 times lower incidence and mortality rates than developing countries, with Australia being close to completely eradicating HPV (Bralsford and Jamieson, 2019, Bray *et al.*, 2018, Serrano *et al.*, 2018). Lower incidence and mortality in high-income countries can be attributed to better sex education, better genital hygiene and most importantly better accessibility and affordability of healthcare including HPV screening and vaccination programmes (Bray *et al.*, 2005a, Bray *et al.*, 2018, Bray *et al.*, 2005b, Bray *et al.*, 2013, W.H.O., 2005).

1.1.2. HPV mitigation

HPV involvement in female genital lesions is currently screened for by Pap smear and PCR genotyping, with surgery being the main treatment for HPV related cancers and warts. There are three prophylactic vaccines on the market. These vaccines are L1 virus-like particle (VLP) based vaccines. VLPs are synthetic, non-replicative, self-assembling viral capsids that stimulate protective neutralising antibody responses. The first vaccine to be released was the Gardasil® quadrivalent vaccine, in 2006. This vaccine is licenced for use in both males and females and protects against the globally common HR HPV strains, 16 and 18, which cause of 70% cervical cancers (Winer *et al.*, 2006), and the LR HPV

strains, 6 and 11 which are known to cause genital warts. GlaxoSmithKline (GSK) then released Cervarix ® in 2007: this protects against HPV 16 and 18 and is currently only licenced for use in women. Finally, the Gardasil® nonavalent vaccine, first released in the USA in 2014, protects against HPV 6, 11, 16, 18, 31, 33, 45, 52, 58 strains and is licenced for use in both males and females. These vaccines have been shown to be highly efficacious with the HPV incidence decreasing by 56% in the USA in the first four years following vaccine implementation (Markowitz *et al.*, 2013).

While great success has been achieved with these vaccines, they are, however, unable to function therapeutically and are therefore unable to treat already infected individuals, offer limited cross-protectivity against non-vaccine strains, and finally, they are expensive (Schiller and Müller, 2015, Petrosky *et al.*, 2015) which makes them less accessible to low income countries where incidence and mortality are between 20% (Africa) and 50% (Asia) (Bray *et al.*, 2018, W.H.O., 2018). Furthermore, the prevalence of HPV strains varies geographically, with the vaccine strains being the most globally prevalent strains. There are other strains however, such as HPV 35 which is not included in any of the vaccines but is prevalent in Africa and not necessarily in other parts of the world (Smith *et al.*, 2007, De Sanjose *et al.*, 2010). Thus, more work is needed to broaden the spectrum of HPV prophylactic vaccines, develop therapeutic vaccines and decrease the cost of the vaccine production to increase affordability for low-income countries where the burden of HPV is the highest.

1.2 HPV Characterisation

HPV belongs to the *Papillomaviridae* family of viruses that are known to infect cutaneous and mucosal epithelia. There are 16 genera in the *Papillomaviridae* family, 5 of which, the α, β, γ, μ, and ν genera, are known as HPVs. To date, these 5 genera contain over 207 different HPV types. These HPV types have been identified and separated into genera based on their L1 capsid protein gene sequence homology. The α HPVs are the largest genus, containing mostly mucosal infecting viruses, 40 of which infect the anogenital tract but only 15 of which are HR, oncogenic types (HPVs 16, 18, 31, 33, 35, 39, 45, 51, 56, 58, 59, 68, 73, 82) (Stanley, 2010, Van Doorslaer *et al.*, 2012, Van Doorslaer *et al.*, 2016) . The β HPVs are the second largest group of HPVs, infecting cutaneous epithelia of patients who are immunosuppressed and patients with genomic mutations that cause epidermodysplasia verruciformis disease (Pass *et al.*, 1977, Pfister, 2003). These β HPVs have also been known to be associated with non-melanoma squamous cell carcinomas when combined with UV, one of the most common cancers in humans (Tommasino, 2017, Van Doorslaer, 2013).

1.2.1. HPV particle structure

HPV is a double-stranded DNA (dsDNA) virus, with a circular 8Kb genome. The virion has a T=7 icosahedral structure, composed mainly of the L1 major capsid protein (360 copies) with the L2 minor capsid proteins (12-72 copies) to form a 55nm particle (Conway and Meyers, 2009, Cerqueira and Schiller, 2017). To form an HPV capsid, L1 molecules bind to each other to form pentameric capsomeres. These capsomeres bind to each other, via disulphide bridges, to form the T=7 icosahedral capsid (Fligge *et al.*, 2001, Ishii *et al.*, 2005, Wolf *et al.*, 2010). Approximately one L2 molecule binds to one capsomere, resulting in a L1 capsid containing 12 L2 molecules (Buck *et al.*, 2008). However, some research has shown that L2 can actually bind up to 72 molecules per capsid (Buck *et al.*, 2008, Finnen *et al.*, 2003, Pereira *et al.*, 2009, Cardone *et al.*, 2014).

1.2.2. *HPV genome structure*

The HPV genome is divided into three regions (Figure 1). The first region encodes the early expressed regulatory proteins, the second encodes the late expressed regulatory proteins and the third region contains non-coding regions/long control regions (LCR) that contain regulatory sequences for DNA replication and transcription (Harden and Munger, 2017). The early expressed regulatory genes include the E1, E2, E4, E5, E6 and E7 genes and a E2^E8 fusion. These genes are responsible for DNA replication, transcription and carcinogenesis (Harden and Munger, 2017, Graham, 2017b, Brentjens *et al.*, 2002, Münger and Howley, 2002). E1 and E2 are the first genes to be expressed. E1 is a helicase enzyme responsible for initiating replication of the DNA by binding to the origin of replication along with E2 to stabilise it (Bergvall *et al.*, 2013). The E4 gene is located within the E2 gene and is expressed as an E2^E4 fusion protein which is involved in aiding viral release and spread in later stages (Doorbar, 2013). The E5 protein is a transmembrane protein that facilitates the evasion of immune responses and apoptosis by activating epidermal growth factor (EGF) and Platelet-derived growth factor (PDGF) receptors (Dimaio and Petti, 2013). Finally, E6 and E7 are the oncogenes responsible for cancer formation after infection with HR HPVs. E6 targets the p53 tumour suppressor for degradation and E7 targets retinoblastoma (Rb) tumour suppressor proteins for degradation and therefore, they are essential for carcinogenesis as they disrupt the cell cycle and cause genomic instability and ultimately carcinogenesis (Münger *et al.*, 1989, Roman and Munger, 2013, Mittal and Banks, 2017). The HPV genome also encodes for the L1 major and the L2 minor capsid proteins (Figure 1), however, these proteins are only expressed during the late stages of the HPV life cycle when the newly synthesised virions are assembled (Graham, 2017b, Harden and Munger, 2017).

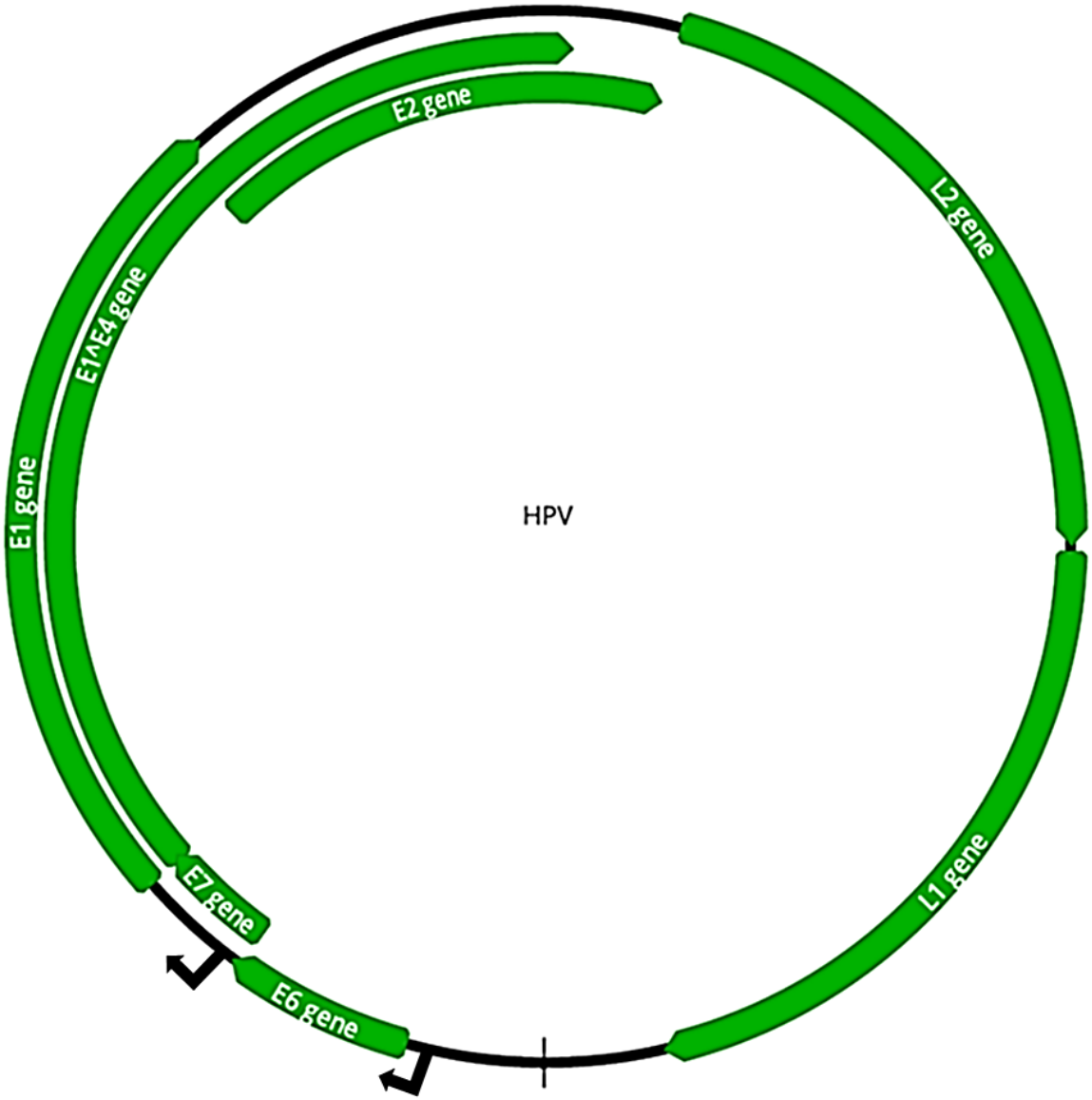

Figure 1: HPV genome structure. Three genome regions shown: Early region encoding DNA replication and transcription regulatory proteins as well as the E6 and E7 oncogenes, Late region encoding the capsid proteins and the Long control regions (arrows) containing the DNA replication and transcription regulatory sequences. Image drawn using Geneious version 2019.1 created by Biomatters, available from https://www.geneious.com, using the Human papillomavirus isolate KC8 complete genome (Accession JX413110) as a backbone.

1.3. HPV Life Cycle

1.3.1. *HPV infectivity*

HPV infects through microabrasions in the skin and binds to heparin sulphate proteoglycan receptors (HSPGs) on either the basement membrane or the basal epithelial cells (Figure 2) (Johnson *et al.*, 2009, Joyce *et al.*, 1999, Kines *et al.*, 2009). This binding causes a cyclophin-B-mediated conformational change in the particle, exposing the L2 protein to furin and proprotein convertase cleavage (Kines *et*

al., 2009, Day *et al.*, 2008) which in turn allows for a conformational change in L1 to allow it to bind to a secondary receptor on the basal keratinocytes (Kines *et al.*, 2009, Day *et al.*, 2008, Day *et al.*, 2010, Aksoy *et al.*, 2017). The virus enters the cell via clathrin independent micropinocytosis (Digiuseppe *et al.*, 2017b, Pyeon *et al.*, 2009). Cyclin B then dissociates L1 from the L2/genome complex and sequesters it for degradation in the lysosome (Raff *et al.*, 2013). L2 is responsible for trafficking the -genome, within the endosome, through the trans-Golgi network and into the nucleus (Day *et al.*, 1998, Wang and Roden, 2013), however, the viral genome can only enter the nucleus during mitosis (Pyeon *et al.*, 2009). The L2 and genome then localise at nuclear domain 10 where transcription can take place (Day *et al.*, 2004). Most HPV infections are able to be cleared by the immune system (Goodman *et al.*, 2008, Rosa *et al.*, 2008), however, in the case of a compromised immune system and infection with an HR HPV, disease progression occurs (Zur Hausen, 2002).

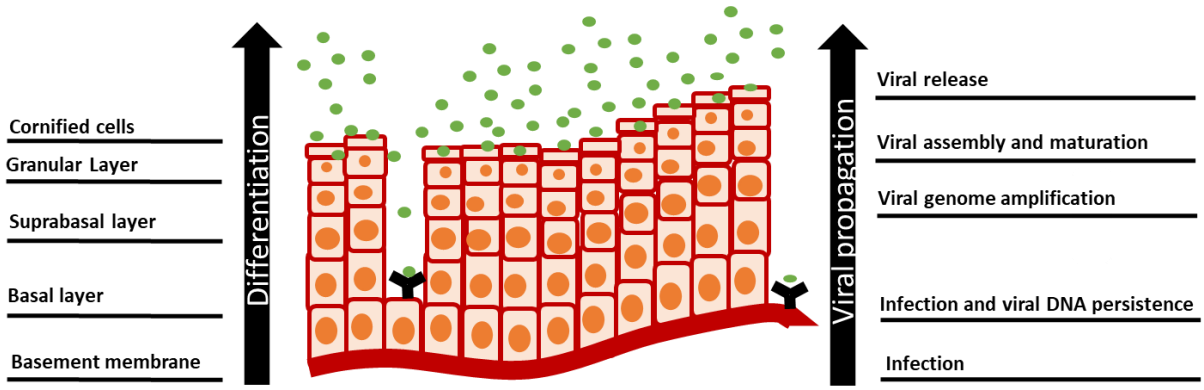

Figure 2: HPV viral life cycle. HPV enters through a microscopic cut in the skin and binds to heparin sulphate proteoglycan receptors on the basement membrane or the basal keratinocytes of the epithelia. This causes a conformational change and opens L2 up for furin cleavage. L2 is then able to bind a secondary receptor on the basal cells and enter the cell. The viral life cycle, DNA replication and gene expression, develops as cell differentiation and proliferation occurs. Infection of the basal cells and viral persistence in the basal cell layer progresses to viral genome amplification as the cells differentiate into suprabasal cells. Progression to viral assembly and maturation occurs when the suprabasal cells differentiate into the granular cells. Following differentiation of the granular cells into cornified cells, on the skin surface, the viral particles are released. Image drawn in Microsoft Powerpoint ® with guidance from Graham *et. al.*, 2017 and Harden and Munger 2017 (Graham, 2017a, Harden and Munger, 2017).

1.3.2. HPV latent phase

HPV requires actively dividing cells in order for new viruses to be generated and so the HPV viral life cycle is closely linked to the differentiation of the basal epithelial cells that they infect (Egawa *et al.*, 2015, Pyeon *et al.*, 2009). In the basal layer of the cells, E2 acts as a linker between the viral episome and the host chromosome allowing it to be replicated with the host DNA, while also repressing replication, keeping the viral load low (Mcbride, 2013). The viral load is maintained at 50-100 copies

by E1 and E2 binding to the origin of replication on the episome and this keeps the infection latent, potentially for decades (Maglennon *et al.*, 2011). Low copy number maintenance ensures immune evasion (Westrich *et al.*, 2017) and it also ensures that the genome is transferred into the new basal cells as they divide, thereby moving up through the layers of the epithelium (Peh *et al.*, 2002, Oldak *et al.*, 2004).

1.3.3. HPV vegetative phase

The vegetative stage is where the viral genome is amplified, and the rest of the early genes are expressed. Here, the E1 and E2 proteins regulate the expression of the E6 and E7 oncogenes, which bind the p53 and pRB tumour suppressors, respectively, and target them for degradation. This causes disruptions in the cell cycle and the apoptotic process, causing the cells to continue replicating and accumulating mutations which ultimately lead to cancer (Münger *et al.*, 1989, Roman and Munger, 2013, Mittal and Banks, 2017). The HPV genome is known to often linearize and integrate into the host genome. This causes further genetic instability in the host genome and can disrupt the E2 gene and thereby inhibits the repression of E6 and E7 oncogene transcription (Doorbar *et al.*, 2012) which causes upregulation of these oncogenes and thereby cancer progression.

1.3.4. HPV late phase

In the late phase of the viral life cycle the L1 major and L2 minor capsid proteins are expressed and the virus is assembled and released. L2 is the first to accumulate in the nucleus at the nuclear domain 10 site (ND-10, also known as PML bodies) with the help of the Hsc70 chaperone protein (Day *et al.*, 1998, Florin *et al.*, 2002, Becker *et al.*, 2004). The L1 proteins assemble into capsomeres in the cytoplasm and then translocate into the nucleus with the help of karyopherin chaperones (Bird *et al.*, 2008, Merle *et al.*, 1999, Nelson *et al.*, 2002). Once in the nucleus the L1/L2 capsid can form and bind the genome to encapsidate it (Nelson *et al.*, 2000, Mallon *et al.*, 1987, Li *et al.*, 1997). Once assembled, the virions mature in the oxidising environment of the granular layer, with the L1 disulphide bonds crosslinking and becoming more resistant to proteolysis (Conway and Meyers, 2009, Conway *et al.*, 2009, Cardone *et al.*, 2014). The virus is then spread when the skin cells die naturally, and the nuclear membrane and cell walls break down.

1.4. Synthetic HPVs

HPV naturally infects and replicates in terminally differentiated basal keratinocytes (Barksdale and Baker, 1993). However, these cells are hard to maintain in a laboratory and this has, in the past,

hindered the study of HPV particles and vaccine development (Conway and Meyers, 2009, Kang *et al.*, 2000). Therefore, different model systems had to be derived to propagate HPV particles.

One of the first models used to generate HPV were xenografts. Xenografts are made by grafting infectious patient tissue onto immunodeficient mice. This results in the formation of a tumour from which the virus can be extracted (Christensen *et al.*, 1997, Howett *et al.*, 1990, Bonnez, 2005). The alternative to xenografts, are raft cultures, a 3T3 fibroblast matrix simulating the stratified nature of epithelia which enables researchers to mimic the viral life cycle throughout basal keratinocyte differentiation (Dollard *et al.*, 1992, Meyers *et al.*, 1992). However, these methods are both expensive and require highly specialised skills and so alternative ways of studying and producing HPV particles had to be developed. Thus, synthetic HPV particles - VLPs, virions and pseudovirions that can be produced in mammalian, insect or yeast cells, were developed.

1.4.1. Virus-like-particles

VLPs are synthetic, non-replicating viral particles that self-assemble and do not contain viral DNA. These particles have been used with much success in the current HPV vaccines inducing high titres of neutralising antibodies (Handisurya *et al.*, 2016, Schiller and Lowy, 2018). Clinical trials and follow-up studies have found these L1 VLP-based vaccines to be safe and effective (Handisurya *et al.*, 2016, Schiller and Lowy, 2018, Schiller *et al.*, 2012), and resulting in no new HPV-16/18 infections 9 years after vaccination (Naud *et al.*, 2014, Markowitz *et al.*, 2013). The success of the HPV VLP vaccine can be attributed to a variety of characteristics associated with VLPs (Mohsen *et al.*, 2017). The first reason for this is that VLPs are small (HPV = 55nm) and are able to diffuse through the pores of the lymph vessels, allowing them to be circulated throughout the body. Secondly, the repetitive structure of the VLP is also ideal for stimulation of the immune response by recognition by pathogen-associated receptors and it provides stable crosslinking to B-cell receptors which could explain the high levels of neutralising antibodies induced by these vaccines (Bachmann *et al.*, 1997, Bachmann and Jennings, 2010, Schiller and Lowy, 2018). Furthermore, the innate immune system components are also multimeric in structure which makes for strong binding to the VLP and thereby increases the efficiency of opsonisation uptake by antigen presenting cells (Gomes *et al.*, 2017). Thus, the VLP vaccines are very effective.

There is, however, still room for improvement on these vaccines. The first problem is that they are very expensive to produce and deliver (cold chain) and low-income countries struggle to afford them, even with funding from the Global Alliance for Vaccines and Immunisation (GAVI) keeping the cost down to 5 USD/dose (Rep, 2013). Secondly, these vaccines are unable to act therapeutically as L1 is only expressed in the late stages of infection (Schiller and Lowy, 2012). Thirdly, the vaccines have very

little cross-protectivity and don't cover the full spectrum of HR HPVs, with some of the HR non-vaccine types, such as HPV-35, being more prevalent in low-income countries than in high-income countries (Smith *et al.*, 2007, De Sanjose *et al.*, 2010, Malagón *et al.*, 2012). However, there are a few strategies in the making to overcome these challenges. In order to decrease the cost of the vaccine, VLPs could be made using different expression systems. Currently, the Gardasil® vaccines are produced in yeast (*Saccharomyces cerevisiae*) (Inglis *et al.*, 2006) with L1 being produced in the cell and then the VLPs being assembled using *in vitro* disassembly-re-assembly methods (Shi *et al.*, 2007). The VLPs in the Cervarix® (GlaxoSmithKline) vaccine are produced similarly using recombinant baculoviral vectors in insect cells (*Trichoplusinia)* (Shi *et al.*, 2007). While these systems have successfully produced HPV vaccines for years, studies have shown that producing vaccines in plants could decrease the cost of raw product by up to 31% (Rybicki, 2009) and that HPV VLP vaccine candidates have been successfully produced in plants before (Maclean *et al.*, 2007). Moreover, several different second generation vaccines are currently being developed to broaden the spectrum of protectivity by e.g. using L2 within the VLPs to increase cross-protectivity (Schellenbacher *et al.*, 2013) and by e.g. targeting the early expressed genes/proteins to treat already infected individuals (Almajhdi *et al.*, 2014, Öhlschläger *et al.*, 2006).

1.4.2. HPV pseudovirions (PsVs)

Pseudovirions (PsVs) are L1/L2 VLPs that encapsidate up to 8Kb of DNA of the researcher's choosing. They are used as infectious challenge material, for studying the viral life cycle, testing neutralising antibody producing vaccines and for delivering DNA into foreign cells.

PsVs differ in their make-up to VLPs in that they contain specific DNA (of the researcher's choosing) and require L2 as well as L1 in order to be functional and deliver the DNA (Wang and Roden, 2013). This is because L2 has been implicated in facilitating endosome escape (Florin et al., 2006, Richards et al., 2006), DNA trafficking into the nucleus (Day et al., 1998, Kondo et al., 2009, Florin et al., 2006), aiding in capsid assembly (Chen et al., 2011, Day et al., 1998, Ishii et al., 2005, Kirnbauer et al., 1993), DNA packaging in certain PV strains (Holmgren et al., 2005, Zhao et al., 1998) and enhancing infectivity of PsVs (Buck et al., 2008, Guan et al., 2017). Furthermore, a functional PsV encapsidates a pseudogenome of up to 8Kb and it does so without the use of an encapsidation signal (Stauffer *et al.*, 1998, Touze, 1998, Touze and Coursaget, 1998, Cerqueira *et al.*, 2016, Cerqueira *et al.*, 2017, Buck *et al.*, 2004), meaning literally any sequence can be encapsidated.

The first successful methods to produce DNA-encapsidating papillomavirus (PV) particles initially used *in vitro* chemical disassembly and reassembly. These studies were able to successfully produce PV particles and were also able to show that, unlike with intracellular assembly, extracellularly assembled

PV particles are able to encapsidate DNA in the absence of L2 (Touze and Coursaget, 1998, Zhao et al., 1998, Unckell et al., 1997, Müller et al., 1995, Yeager et al., 2000). However, yields of PsVs obtained from these methods were quite low. Alternative intracellular methods of PV production were also developed. Early versions of the intracellular methods included the use of viral vectors, such as Semiliki Forest virus (SFV) and vaccinia virus for mammalian cells and baculoviral vectors for insect cells, to enhance replication of the DNA. However, PsV and virion production from these systems was not very efficient which led to the development of the current gold standard for producing HPV PsVs, an intracellular mammalian expression system designed by Buck *et al.* (2004). This method is a product of over 10 years' worth of research (1993-2004) (Touze and Coursaget, 1998, Zhao *et al.*, 1998, Unckell *et al.*, 1997, Müller *et al.*, 1995, Yeager *et al.*, 2000) and was able to increase particle production efficiency by 10 million-fold compared to the previous Semiliki Forest viral vector-based methods (Buck *et al.*, 2004). This protocol expresses and assembles PV PsVs in Human Embryonic Kidney (HEK293TT)cells which contain a plasmid constitutively expressing the large T-antigen which enhances transgene DNA replication by binding to the SV40 origin of replication on the plasmids (Buck *et al.*, 2004). Moreover, this method has shown to produce approximately 10^9 PV transducing units/ml and PsVs in these cells have further been shown to also be effective for use in pseudovirion-based neutralisation assays (PBNAs) (Pastrana *et al.*, 2004a). However, though this method is well thought-out and results in high titres of PsVs, it is an expensive process and requires specialised equipment and skills as well as carries the risk of contamination by mammalian pathogens and possibly oncogenes. Thus, given the importance of HPV PsVs, it may be worthwhile to try and develop alternative methods for producing HPV PsVs cheaply and safely.

1.5. HPV PsV applications

HPV PsVs are important for the development of broader ranged prophylactic vaccines as they are used in pseudovirion-based neutralisation assays (PBNAs) to test for neutralising antibodies in response to new HPV vaccines (Pastrana *et al.*, 2004b) and HPV PsVs have the potential to deliver therapeutic DNA (Lin *et al.*, 2010, Chabeda *et al.*, 2018). PsVs are also a safer alternative for use in these applications, compared to live or attenuated virions, as they do not contain any viral DNA.

1.5.1. PsV-based neutralisation assays

PBNAs have become the preferred method of testing neutralising antibody producing vaccines. An HPV PBNA generally comprises of L1/L2 PsVs encapsidating a reporter gene and these PsVs are incubated with antisera on mammalian cells. In the event that the antiserum (taken from vaccinated individual) is neutralising, the antibodies bind the PsVs, preventing them from infecting the cell and

so prevent the DNA of interest from entering the cell and being expressed and so no signal would be obtained (Pastrana *et al.*, 2004b). PBNAs were first shown to be more successful than the Enzyme-linked Immunosorbent Assay (ELISA)-based methods by Pastrana *et al.* in 2004. The old ELISA methods used labelled antibodies to outcompete the antibodies in the serum for binding to the VLP (Yeager *et al.*, 2000, Dillner, 1999), and so the more signal obtained, the less antibody was present in the sera. These ELISAs, though, are unable to differentiate between cross-reactive and type-specific antibodies and are also unable to distinguish between neutralising and non-neutralising antibodies (Pastrana *et al.*, 2004b). Thus, PBNAs are necessary to determine the efficiency of neutralising antibody producing vaccines.

1.5.2. PsVs in viral life cycle studies

The development of HPV PsVs have been seminal in advancing viral life cycle studies. An example of this is a study done by Day *et al.* in 2008 which used *in vitro* neutralization assays to determine the mechanism of HPV-16 infection. This was done using HPV 16 PsVs encapsidating fluorescent marker genes. Neutralization with L1 and L2 antibodies revealed that the virus undergoes conformational changes to allow for infectivity, with reporter gene signalling patterns being absent when the antibody bound an exposed epitope. Thus, it was shown that L2 is needed to be exposed and undergo furin cleavage before it can infect the cell (Day *et al.*, 2008).

1.5.3. PsV DNA delivery

HPV infects epithelial cells, with different strains infecting either the cutaneous or mucosal epithelial tissue. PsVs can also specifically target cancer cells, such as melanomas and carcinomas, that evolve to display the form of HSPG usually only found on the basement membrane and basal cells of the skin (Kines *et al.*, 2009). These traits make PV PsVs valuable assets for the delivery of heterologous DNA into foreign cells, specifically for targeted gene and cancer therapies.

There are currently no therapeutic HPV vaccines on the market, but considerable research has been done on potential candidates. The current prophylactic vaccines are not effective against established infections. This is due to the fact that when HPV persists, L1 and L2 are only expressed in the late stages of infection and so would not be available to stimulate an immune response (Schiller and Lowy, 2012). DNA vaccines appear to be one of the best candidates for an HPV therapeutic vaccine. This is because they are easily produced and generate cytotoxic lymphocyte (CTL) responses whereas most vaccines only produce antibody responses and weak CTL responses (Pachuk *et al.*, 2000). However, naked DNA vaccines have low infectivity of target cells, particularly mucosal cells, and are unable to produce strong immunogenic effects (Ma *et al.*, 2011, Graham *et al.*, 2010).

HPV PsVs are potentially effective vectors for targeted delivery of therapeutic DNA as they can target cutaneous and mucosal epithelia as well as cancer cells specifically. Thus far, there have only been a few studies that have successfully shown PsV-mediated therapeutic DNA delivery in mammals. Two studies, one by Peng *et al.* (2010), and the other by Graham *et al.* (2010), used HPV PsVs to deliver antigens. These studies showed that the PsVs were able to target the correct epithelial cells and produced a more intense cytotoxic response to the antigen than the naked DNA (Peng *et al.*, 2010, Graham *et al.*, 2010). A study by Gordon *et. al.,* (2012) indicated that HPV PsVs could be used to deliver SIV Gag genes into the genital mucosa. This PsV in combination with the SIV Gag were able stimulate both local and systemic effects (Gordon *et al.*, 2012). Another study by *Hung et al.,* (2012), utilised HPV-16 PsVs encapsidating the HSV-tk (herpes simplex virus thymidine kinase) gene to treat ovarian cancer (Hung *et al.*, 2012). The PsVs were used to infect the ovarian tumour cells, allowing them to express the HSV-tk protein which activates the prodrug, ganciclovir, causing a halt in DNA replication and eventually apoptosis of the cells. The results of this study showed that the PsVs containing the HSV-tk had significantly higher cytotoxic and antitumour effects compared to the PsV control and that HPV PsVs preferentially infected the tumour cells as opposed to the normal, healthy cells. Finally, a study by Bousarghin *et. al.* (2009) was able to deliver DNA encoded short hairpin RNA (shRNA) into CaSki and TCI HPV positive cells which caused E6/E7 degradation and thereby allowed for cell death to occur and block tumour progression (Bousarghin *et al.*, 2009). Thus, these studies suggest that HPV PsVs are an effective vehicle for targeted therapeutic DNA delivery.

HPV PsVs, however, are currently produced in mammalian cells using the Buck *et al.,* 2004 protocol. This method is expensive, requires specialised equipment and expertise and also carries the risk of contamination by mammalian pathogens and oncogenes. An alternative option to mammalian cell production is *in planta* HPV PsV production. While HPV PsV production in plants is still relatively new, there being only one published study to date (Lamprecht *et al.*, 2016), HPV VLPs have been produced in plants for several years (Santi *et al.*, 2006, Zahin *et al.*, 2016, Maclean *et al.*, 2007) and so there is still considerable potential for plant-made HPV PsVs.

1.6. Plant Expression Systems

Plants have been used as expression systems for heterologous proteins for many years (Giddings, 2001). They are known to be a potentially safer, more easily handled and highly scalable expression system compared to mammalian, insect and yeast cell expression systems (Ma *et al.*, 2003, Lico *et al.*, 2008, Rybicki, 2010). Plants also carry little risk of contamination with mammalian pathogens and

endotoxins (Rybicki, 2010, Rybicki, 2014). Furthermore, plants have the advantage over other expression systems in that they post-translationally modify proteins similar to humans (Margolin *et al.*, 2018, Ma *et al.*, 2003, Faye *et al.*, 2005), whereas insect and yeast cells' are limited in their glycosylation abilities (Chen and Lai, 2013). Expression in plants can also be easily upscaled with little cost when doing *Agrobacterium*-mediated transient expression in *Nicotiana benthamiana* (Giddings, 2001, Goodin *et al.*, 2008). Various plants have been used so far for recombinant protein expression, including potato, alfalfa and *Arabidopsis thaliana*. However, *N. benthamiana* appears to be superior as they produce the most biomass (Giorgi *et al.*, 2010, Rybicki, 2009, Rybicki, 2010, Rybicki, 2014) and are classified as a non-food stuff, thereby ensuring that the transgenic plants cannot contaminate food stock. Moreover, producing PsVs in plants has shown to be more cost effective, as studies have shown that the cost of production could be significantly reduced given that no transfection reagents and no cell culture media are required. Several companies are starting to invest in plant-made vaccines and therapeutics. Medicago Inc. is the first company to successfully get a plant-made product close to market, after having produced a candidate H7N9 avian influenza vaccine in 1 month instead of 6. Medicago Inc. also have many ongoing projects and clinical trials for plant-made human therapeutics (Lomonossoff and D'aoust, 2016). Another notable advancement in plant-made therapeutics was the production of ZMapp antibodies in plants against the Ebola virus (Qiu *et al.*, 2014). Thus, it may be more feasible to produce VLPs and PsVs in plants as it is potentially safer and could potentially decrease the cost of production and make vaccine research less expensive.

The current HPV L1 VLP vaccines are made in yeast (Gardasil®, Merck) and insect cells (Cervarix™, GlaxoSmithKline), however, research has shown that HPV particles can be made in plants (Rybicki, 2010, Rybicki, 2014, Giorgi *et al.*, 2010, Hefferon, 2014). HPV VLPs have been made by both stable transformation and transient expression, with the transient production system being faster and resulting in higher yields and better immunogenicity (Rybicki, 2010, Rybicki, 2014, Giorgi *et al.*, 2010, Hefferon, 2014). HPV 16 and 11 L1 VLPs were first made in transgenic tobacco and potato tubers. Although L1 was able to be expressed and VLPs were formed in these studies, yields were found to be below the 1% total soluble protein (TSP) threshold for commercial value and the VLPs produced were either very weakly immunogenic or not immunogenic at all (Biemelt *et al.*, 2003, Warzecha *et al.*, 2003, Varsani *et al.*, 2003). Thus, transient expression was then used to try increase yields and the speed of production (Rybicki, 2010, Rybicki, 2014, Giorgi *et al.*, 2010, Hefferon, 2014). Transient expression by a full viral vector, was the first method to be used (Varsani *et al.*, 2006). These vectors transfer nucleic acid by either inoculating the plant with the viral nucleic acid, edited to contain the transgene, or by infecting the plant with the full viral particle to facilitate the transfer of the transgene into the plant cells' nucleus (Komarova *et al.*, 2010, Santi *et al.*, 2006). The viral particles expressed and assembled

in the plants from the nucleic acid, are used to transfer the recombinant nucleic acid from plant cell to plant cell (Gleba *et al.*, 2004). Moreover, the viral replication of the nucleic acid generates high copy numbers and thereby enhances gene expression. Successful transient expression of HPV 16 L1 was first achieved using this method, by Varsani *et al. 2006* , where recombinant TMV viral vectors were used to infect tobacco plants with L1 containing viral RNA. Though, this study achieved ten-fold higher yields of L1 protein than the transgenic models, the L1 generally only formed capsomeres and only stimulated a weak immune response (Varsani *et al.*, 2006). *A. tumefaciens* mediated transient expression was attempted next.

Expression by *A. tumefaciens* involves the stimulation of the *vir* genes, on the *Agrobacterial* Ti plasmid, to release the T-DNA from the plasmid and facilitate the translocation of the T-DNA into the plant host and its nucleus (Zupan *et al.*, 2000, Tzfira *et al.*, 2004, Komarova *et al.*, 2010). This method of production has been shown to be highly scalable compared to previous methods and is faster (Komarova *et al.*, 2010). Using this method, Maclean *et al.* demonstrated the first highly successful production of HPV VLPs in plants, achieving up to 14.9% TSP, which was even better when human codon optimised L1 was targeted to the chloroplast (17% TSP, Maclean *et al.,* 2007). Furthermore, deconstructed viral vectors in combination with *A. tumefaciens*-mediated transfection have been used to try and further increase the replication and expression of transgenes in plants. These deconstructed viral vectors lack unnecessary and limiting genes, such as the capsid protein gene and they include vectors such as the magniCON vector (Gleba *et al.*, 2004) and the pRIC 3.0 vector (Regnard *et al.*, 2010). The magniCON vector consists of a deconstructed TMV genome that contains a transgene. This vector enhances gene expression by generating high levels of RNA replicons in plants and uses the TMV movement protein for cell-to-cell movement of the RNA replicons. This method has been used in several studies, the most recent of which reports L1 protein yields of up to 2.5% TSP (Zahin *et al.*, 2016). Moreover, pRIC 3.0 has also been shown to increase L1 protein yield by increasing L1 dsDNA replicon copy numbers by 100-fold, compared to that produced by the non-replicating pTRAc vector (Regnard *et al.*, 2010). Additionally, the pRIC vector has been successfully used as a vector for plant produced HPV PsV pseudogenomes.

Production of HPV PsVs in plants have been reported once previously (Lamprecht *et al.*, 2016). This study indicated that HPV L1 and L2 and a pseudogenome based on the replicating vector pRIC could be expressed in plants and that the PsV particles could assemble correctly in the plant. It also demonstrated that plant-made HPV PsVs functioned similarly in PBNAs to mammalian cell-produced HPV PsVs. However, one current issue with *in planta* production of HPV PsVs is the low yield of PsV vs VLPs obtained. One way in which yields of stable HPV PsVs were shown to improve, in other expression systems, was to determine the ideal size of pseudogenome that can be encapsidated by

an HPV particle. HPV is known to encapsidate linear or circular DNAs by size, with the theory being that PV PsVs are in constant state of disassembly and reassembly but once they encounter DNA of an appropriate size below 8Kb, they form a stable particle that is resistant to disassembly (Stauffer *et al.*, 1998, Zhao *et al.*, 1998, Buck *et al.*, 2004, Touze and Coursaget, 1998, Cerqueira *et al.*, 2016). The study by Lamprecht *et al.* used two different sized pseudogenomes in their experiments, the smaller of the two resulting in better transduction. This was the first indication that plant-made HPV PsVs may encapsidate DNA similarly to mammalian HPV PsVs and that, by altering the size of the pseudogenome, yields of PsVs vs VLPs could potentially be improved. Thus, by improving yields of stable HPV PsVs produced from plants, the cost of production of PsVs and all its downstream applications could decrease, and the safety could increase.

1.7. Project Aims and Objectives

HPV is known to be the cause of anogenital and oropharyngeal cancers as well as genital and common warts. The burden of HPV however is highest in low-income countries, for whom the vaccine costs are still high, and so alternative methods of vaccine production and testing are needed. HPV PsVs are important for studying, protecting against and treating HPV infections. Plants represent a potentially safer, cheaper and more easily scalable means of production of PsVs. However, plant PsVs studies are relatively new and still require some optimisation, particularly where yield is concerned. Nevertheless, research has shown that using pseudogenome DNAs between 5-7Kb increases yields of stable papillomavirus PsVs in mammalian cells. Therefore, this study aimed to optimise the production HPV PsVs in *Nicotiana benthamiana* by determining the optimal size of DNA required for *in planta* HPV PsV encapsidation.

The objectives set out to achieve this goal were as follows:

1. **To clone the different sized pseudogenomes:**
   The first objective of this project was to clone pseudogenomes of 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb in size, using the EGFP and luciferase reporter genes in a geminivirus-derived replicating vector to allow for enhanced DNA replication.

2. **To express and purify the PsVs with the different sized pseudogenomes in tobacco plants:**
   The second objective was to express and purify the PsVs by infiltrating the different sized pseudogenomes into *Nicotiana benthamiana* along with plant expression vectors encoding the HPV 35 L1 and L2 capsid proteins and then purifying the particles.

3. **To characterise the PsVs containing the different sized pseudogenomes:**

   The third objective was to characterise the PsVs produced with the different sized pseudogenomes and to determine which size pseudogenome was better encapsidated.

Chapter 2: Cloning of different sized pseudogenomes

2.1 Introduction

HPV is a dsDNA virus whose virions are composed of the L1 major capsid protein and the L2 minor capsid protein encapsidating a circular genome of 8Kb, complexed with host histones so as to form compact "minichromosomes". HPV pseudovirions (PsVs) are L1/L2 VLPs that have encapsidated up to 8kb of dsDNA, without the obvious use of DNA encapsidation signals (Stauffer *et al.*, 1998).

2.1.1. Encapsidation by size discrimination

Lamprecht *et. al* (2016) showed that HPV 16 PsVs can be produced in plants, and that these PsVs can be used in *in vitro* neutralization assays with mammalian cells (Lamprecht *et al.*, 2016). This is advantageous over mammalian cell produced PsVs as it reduces the possibility of contamination with mammalian pathogens which are not able to infect plant cells, and mammalian oncogenes which are not present in plant genomes. Plant production could also significantly reduce the cost of PsV production (Rybicki, 2009). However, a current limitation with PsV production in plants is low yields. While it is known that any type of DNA less than 8Kb can be encapsidated, studies have found that encapsidation efficiencies do vary according to the size of the pseudogenome (Stauffer *et al.*, 1998, Touze and Coursaget, 1998, Zhao *et al.*, 1998, Roden *et al.*, 1996, Cerqueira *et al.*, 2016, Buck *et al.*, 2004). Buck *et al.* showed that using pseudogenome DNA between 5-7Kb could increase the yield of bovine papillomavirus (BPV1) PsVs produced in mammalian cells: higher yields of PsVs were obtained when DNA of 5.9kb was encapsidated compared to when 7.9Kb was encapsidated. With this in mind, the authors hypothesized that the L1/L2 VLPs may be in a constant state of assembly and disassembly until they are able to encapsidate DNA smaller than 8kb, and only then are they able to produce a stable VLP structure (Buck *et al.*, 2004). Cerqueira *et al,* (2016) provided more evidence for this theory by showing that the HPV PsVs encapsidating DNA smaller than 8Kb were stable, and did not reassemble even in the presence of DNA of the correct size (Cerqueira *et al.*, 2016). Furthermore, Lamprecht *et al.* (2016) found that DNA encapsidation size might be important for plant-made HPV PsV formation as a 4.8Kb pseudogenome was found to produce more signal in infectivity assays than the 6.6Kb pseudogenome, indicating that the 4.8Kb pseudogenome may have been more efficiently packaged than the 6.6Kb pseudogenome. Thus, by experimenting with different sized pseudogenomes, the optimal encapsidation size for plant-made HPV PsVs could be elucidated and potentially result in greater yields of stable, functional PsVs.

2.1.2. Use of reporter genes in pseudogenomes

PsVs can be used in PsV-based neutralisation assays (PBNAs) to test a vaccine candidate's ability to elicit neutralising antibodies. An HPV PBNA utilises PsVs encapsidating a reporter gene; these PsVs are incubated with antisera before being put on mammalian cells. If the antiserum is neutralising, the antibodies bind the PsVs, which prevents them from infecting the cell, which in turn prevents the reporter plasmid from entering the cell, being expressed, and producing a signal. These PBNAs can become expensive depending on the reporter gene used. For example, the SEAP detection kit is 490 USD/1000 rxns; this could make the testing of neutralising antibody vaccines very expensive. The cost of PBNAs could be kept to a minimum by using fluorescent protein reporter genes, which don't require substrates, or luciferase reporter genes which have a detection kit that is approximately one third of the price of the SEAP kit. Thus, by using cost effective reporter genes, the cost of PBNAs could be kept at a minimum and thereby the cost of testing new, neutralising antibody producing vaccines could be kept down.

2.1.3. Plant viral vectors for encapsidation of pseudogenomes

A strategy to optimize encapsidation efficiency in plants is to create an overabundance of the plasmi-like pseudogenome in the plant cell. This is potentially achieved by using autonomously replicating plasmid-like DNA plant virus-derived expression vectors to replicate the pseudogenome DNA in the plant. Geminiviruses are one such family of viruses. They are small, ssDNA viruses that use rolling circle replication to replicate continuously and autonomously in plants. Geminiviruses, and particularly bean yellow dwarf virus (BeYDV), have been successfully used in several studies to enhance expression of transgenes in plants (Mor *et al.*, 2003, Hefferon and Fan, 2004, Zhang and Mason, 2006, Regnard *et al.*, 2010, Huang *et al.*, 2009). BeYDV has an ambisense genome that replicates autonomously in plants. The mechanisms by which it does this were exploited by Regnard *et. al.,* in 2010 to make the pRIC3.0 vector (Figure1). The pRIC 3.0 vector design was based on a mild strain of BeYDV (Halley-Stott *et al.*, 2007) and was designed for *Agrobacterium tumefaciens* mediated transient expression in plants. The vector contains the left and right border sequences which are cleaved by the *A. tumefaciens vir* genes in order to release the T-DNA into the plant cell nucleus (Komarova *et al.*, 2010, Zupan *et al.*, 2000). This T-DNA is the component of the pRIC 3.0 vector that contains the self-excising BeYDV 5'LIR-SIR-*rep*-3'LIR element, and it is in between the 5'LIR and the SIR that the desired pseudogenome elements need to be cloned into.

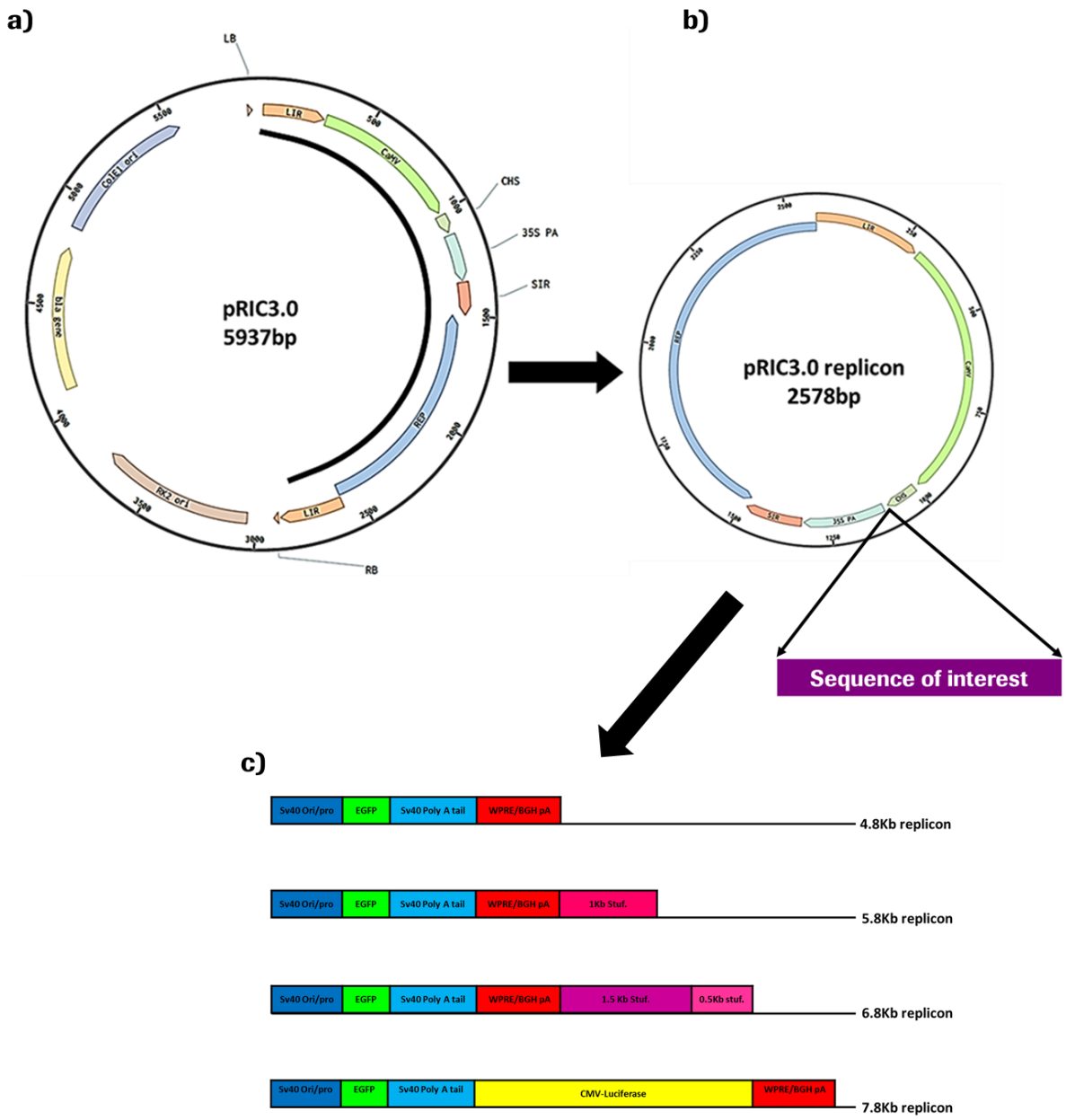

Figure 1: Schematic representation of the pRIC 3.0 vector and the resulting replicons containing the sequences of interest. a) The pRIC 3.0 vector. Indigo, origin of replication for *E. coli*; lilac, RK2 ori, origin of replication for *Agrobacterium tumefaciens*; peach, *bla* gene for ampicillin⁄carbenicillin resistance; orange arrows, left (LB) and right borders (RB) for T-DNA integration; light green, P35SS CaMV 35S promoter with duplicated transcriptional enhancer; dark green, CHS, chalcone synthase 5`-untranslated region; light blue, pA35S, CaMV 35S polyadenylation signal; orange, LIR, BeYDV long intergenic region; red, SIR, BeYDV short intergenic region; dark blue, *rep*, BeYDV *rep* gene. The black bar within the vector indicates the components of the vector that become the replicon. b) the pRIC replicon, replicated in the plant from the pRIC 3.0 vector. The pRIC replicon contains the rep gene, SIR, one LIR, CHS, P35SS CaMV 35S promoter and the pA35S polyadenylation signal. The gene/sequence of interest is cloned into the multiple cloning site in between the CaMV 35S promoter and the CaMV 35S polyadenylation signal of the pRIC 3.0 vector and is therefore present in the pRIC 3.0 replicon. c) Diagram of all the pseudogenome replicons formed from the pRIC 3.0 constructs cloned in this study. The schematic of pRIC 3.0 and it's replicon was created using Benchling (Benchling [Biology Software]. 2019. Retrieved from https://benchling.com) and information obtained from Regnard *et. al.,* (2010).

BeYDV elements were added to the pRIC vector. This allows for the production of small replicons in plants, which can replicate continuously and autonomously. The vector retains the BeYDV *rep* gene

and the short intergenic region (SIR) but has an extra copy of the long intergenic region (LIR, Figure 1). The LIRs are stem-loop structures that contain bidirectional promoters and origins of replications. Their duplication is essential for the formation of the small replicons in the plant nucleus as the Rep protein initiates replication by binding to them and nicking them. The sense and complementary strands of the vector are separated by the SIR with the region between the SIR and the 3'LIR being the complimentary strand of the vector. The SIR contains a primer binding site that allows for the initiation of replication of the complimentary strand and the Rep/RepA proteins are transcribed from the *rep* gene on this strand, using the 3' LIR promoter and plant host machinery. The *rep* transcript is then alternatively spliced to create the Rep and RepA proteins (Liu *et al.*, 1998, Gutierrez, 1999) . The RepA protein is responsible for keeping the plant cell cycle in S-phase (Hefferon and Dugdale, 2003, Liu *et al.*, 1999, Liu *et al.*, 1998), while the Rep protein is responsible for initiating replication of the sense strand. The Rep protein initiates replication by nicking the LIR stem loops, which stimulates DNA repair of the sense strand, and a new strand replaces the old one. Rep remains bound to the 5' end of the original, displaced sense strand and allows for the recircularization of the strand and thus the formation of a small replicon (Laufs, 1995b, Laufs, 1995a, Gutierrez, 1999). From this replicon, a dsDNA intermediate is made and more Rep/RepA is expressed. Consequently, an increased production of Rep results in an increase in the number of replicons produced. This triggers more Rep to be expressed and thus creates a continuous cycle of autonomous replication. In the absence of coat protein, the replicons are rapidly and efficiently converted to dsDNA.

This pRIC vector was used by Lamprecht *et al.* (2016) to construct the mSEAP (mammalian SEAP expression cassette, 4.8Kb) pseudogenome, which was used to successfully produce the first published HPV PsVs produced *in planta*. Replicon copy number has been shown to be amplified up to 1000-fold in plants (Regnard *et al.,* 2010), and the dsDNA intermediate form of these replicons is encapsidated to form the PsVs. Thus, pRIC allows for replication of recombinant pseudogenome DNA and therefore increases the likelihood of encapsidation of the pseudogenome replicons.

2.1.4. Pseudogenome replication and expression

To make functional HPV PsVs, L1/L2 particles must encapsidate DNA in plants and deliver this DNA into mammalian cells where it can be expressed. Plant-made HPV pseudogenomes replicate in the form of the parent plasmid in *E. coli* and *Agrobacterium tumefaciens,* and in the form of the derived replicon in plants. However, expression cassettes need to be designed so that the gene(s) of interest is only expressed in mammalian cells to avoid oversaturating the plant cell with expressed protein. In this study, the cytomegalovirus (CMV) enhancer and promoter was used for the luciferase gene expression cassette of the 7.8Kb pseudogenome. The CMV promoter is known to be a very strong promoter for mammalian cell gene expression (Brewer, 1994, Boshart *et al.*, 1985, Khan, 2013),

especially when combined with the strong transcription termination signal used in this study, WPRE/BGH. The pSHELL vectors, commonly used to produce high yields of mammalian HPV PsVs, make use of the bovine growth hormone (BGH) poly-A-tail and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) to create a strong transcription termination signal, when using the CMV promoter (Buck *et al.*, 2006b, Real *et al.*, 2011). The EGFP reporter gene, present in all the pseudogenomes used in this study, is under the control of the SV40 promoter/enhancer and the SV40 polyadenylation signal. These transcription regulatory elements have been known produce high levels of recombinant gene expression in mammalian cells, specifically in HEK293TT cells. This is due to the SV40 poly-A-tail, which allows for efficient post-transcriptional processing (Van Den Hoff *et al.*, 1993), and the SV40 origin of replication, which overlaps the SV40 promoter and allows for increased DNA replication in HEK293TT cells. HEK293TT cells have been modified so that they produce the SV40 small t-antigen and the large T-antigen. The large T-antigen enhances replication by binding to the SV40 origin of replication and increasing copy number and thereby gene expression in HEK293TT cells (Buck *et al.*, 2005a, Buck *et al.*, 2004). These cells are essential for the production of HPV PsVs in mammalian cells, for testing PsV infectivity and for PBNAs (Buck *et al.*, 2006a). Thus, all pseudogenome constructs used in this study were created with an SV40 origin of replication to ensure high copy number generation in HEK293TT cells, and strong mammalian expression cassettes to ensure high levels of gene expression.

This chapter describes the cloning of HPV pseudogenomes sized 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb using the pRIC 3.0 vector to generate high copy numbers for encapsidation, and strong mammalian expression cassettes are used to produce high levels of EGFP or luciferase reporter gene expression. These constructs provide the pseudogenomes that will be used to determine optimal pseudogenome encapsidation size for plant-made HPV PsVs.

2.2. Methods

2.2.2. Plasmid isolation and restriction enzyme digestion

Plasmids were isolated from *E. coli* and *Agrobacterium tumefaciens* using the QIAprep Spin Miniprep kit (QIAGEN, Germantown, USA) as per the manufacturer's instructions. All restriction enzymes used were procured from ThermoFischer Scientific, except for *Afl*III which was obtained from New England Biolabs (NEB, Ipswich, USA). All restriction enzyme digestions were performed as per the manufacturer's instructions. Some sequential digestions were performed and cleaned up using the QIAquick Nucleotide Removal Kit between digests (QIAGEN, Germantown, USA). DNA bands from PCR and restriction enzyme digests, which were used for cloning, were gel extracted using the QIAquick gel extraction kit (QIAGEN, Germantown, USA) as per the manufacturer's instructions.

2.2.3. Ligation and transformation into E. coli

Blunt end cloning into the pJET intermediate vector was performed, using the CloneJET PCR Cloning Kit (ThermoFischer Scientific, Waltham, USA), when restriction sites were added to insert DNA by HI-FI PCR. The inserts were digested, and gel extracted from pJET using the appropriate enzymes and ligated into the final vector. Vector DNA was always dephosphorylated with rAPid Alkaline Phosphatase (Roche, Basel, Switzerland) when cloning with compatible ends, as per the manufacturer's instructions. T4 DNA ligase (ThermoFischer Scientific, Waltham, USA) was used, as per the manufacturer's instructions, in 16hr overnight ligations, to ligate all the inserts into their vectors. Clones were transformed by adding 4µl of ligation mixture to 15µl NEB® 10-beta chemically Competent *E. coli* (NEB, Ipswich, USA) and then performing the transformation as per the manufacturer's instructions. All pRIC 3.0 constructs, pJET, pCLucf and mSEAP contained the *bla* ampicillin/carbenicillin resistance gene and thus the DH10β cells were grown overnight at 37˚C in Luria Broth (LB: 10g/L Bacto-tryptone, 5g/L yeast extract, 10g/L NaCl, up to 1L water) or Luria Agar (LB, 15g/L agar) supplemented with 100µg/ml of Ampicillin.

2.2.4. Polymerase chain reaction (PCR)

The EGFP, CMV-Luciferase and all the stuffer fragments (random DNA fragments used to increase replicon size) were all cloned into the vectors using PCR. Primers were designed to add restriction enzyme recognition sites onto the 5' and 3' ends of the desired sequences (Table. 1). The pCLucf plasmid, a gift from Dr. John Schiller (Addgene, Watertown, Massachusetts, USA, plasmid # 37328; http://n2t.net/addgene:37328 ; RRID:Addgene_37328) was used as a template for the EGFP and CMV-Luciferase sequences. The 1Kb and 1.5Kb stuffer fragments were amplified from the luciferase gene in the pCLucf plasmid and the 0.5Kb stuffer was amplified from the E1a sequence of the mSEAP plasmid (Lamprecht *et al.*, 2016). The Q5® High-Fidelity DNA Polymerase (NEB, Ipswich, USA) was used to amplify the desired sequences as per the manufacturer's instructions. Annealing temperatures used were 65°C for the EGFP gene amplification, 60°C for the stuffer fragment PCRs and 70°C for the luciferase gene PCR. Amplicons were purified by agarose gel electrophoresis and gel extraction and then ligated into pJET (CloneJET PCR Cloning Kit, Thermo Fisher Scientific, Waltham, USA). Final inserts were obtained by restriction enzyme digestion of pJET and purification of the DNA by agarose gel electrophoresis and gel extraction.

Colony PCR (Mirhendi *et al.*, 2007) was used to screen for recombinant colonies, using insert specific primers and vector specific primers where possible. Colonies were resuspended in 10µl of water and 2µl of this was used in the PCR. Screening PCRs were carried out using the Taq DNA polymerase 2X

Master mix RED (AMPLIQON III, Odense, Denmark), following the manufacturer's recommendations. Positive colonies were additionally confirmed by restriction enzyme digestion.

Table 1: Primers used for cloning, screening and sequencing of putatively positive clones.

PRIMER	SEQUENCE (5'- 3')	AMPLIFIED FROM	RE SITE ADDED	TM (˚C)	PURPOSE
STUFFER 1000BP FOR	ATTGCTAGCTTCAACGAGTACGACTTCGTG	pCLucf	5' *Nhe*I	62	Cloning, screening
STUFFER 1500BP FOR	TATGCTAGCCATTCTACCCACTCGAAGACG	pCLucf	5' *Nhe*I	63	Cloning, screening
STUFFER 1000_1500BP REV	ATAAAGCTTAGCTTCTTGGCGGTTGTAACCTG	pCLucf	3' *Hind*III	62	Cloning, screening
STUFFER 500BP FOR	TTAAAGCTTAATACCAGTGTGCAGATCTTGGC	mSEAp	5' *Hind*III	61	Cloning, screening
STUFFER 500BP REV	ATTGAGCTCGTAAACTGGGAAAGTGATGTCGTG	mSEAp	3' *Sac*I	63	Cloning, screening
EGFP 5' SPEI FOR	TTAACTAGTATGGTGAGCAAGGGCGAGGAGCTG	pCLucf	5' *Spe*I	69	Cloning, screening, sequencing
EGFP 3' XHOI REV	TATTCTCGAGTTACTTGTACAGCTCGTCCATGCC	pCLucf	3' *Xho*I	68	Cloning, screening, sequencing
CMV-LUC 5' PACI FOR	ATATTAATTAACGCCACCTCTGACTTGAGCGTCGA	pCLucf	5' *Pac*I	69	Cloning, sequencing
CMV-LUC REV	CCAAACTCATCAATGTATCTTATCATGTCTGCTCG	pCLucf	-	65	Cloning, sequencing
CMVLUC INNER FOR	ATAGCTTGCAGTTCTTCATGCC	pCLucf	-	60	Sequencing
CMVLUC INNER REV	CTCGTTGTAGATGTCGTTAGCT	pCLucf	-	59	Sequencing

2.2.5. *Generation of multiple sized pseudogenome constructs*

Different sized pseudogenome constructs were cloned such that their replicons would be 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb in size.

2.2.5.1. *Synthesis of mammalian expression cassette*

A mammalian expression cassette was designed for the cloning of EGFP and CMV-Luciferase into the different sized pseudogenomes and this expression cassette was synthesised by GenScript USA Inc. (Piscataway, USA). The expression cassette contains an SV40 origin of replication and an SV40 enhancer-promoter and poly-A-tail between which the EGFP gene can be cloned. A WPRE transcription terminator and a BGH poly-A tail were also added so that the CMV promoter and luciferase gene could be cloned in downstream of the EGFP cassette. This fragment was received in the pUC57 vector and is therefore known as pUC57-synth (Figure 2).

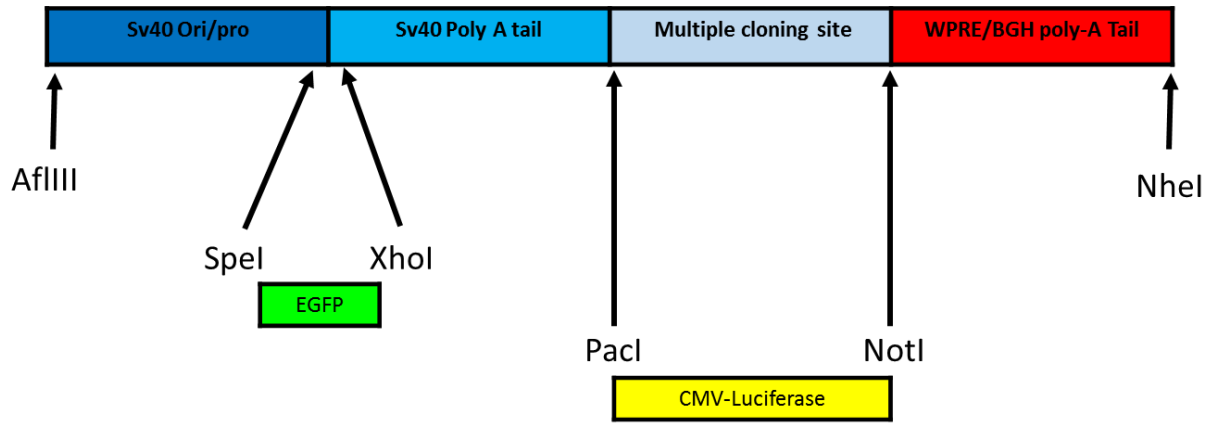

Figure 2: Schematic of pUC57-synthesised mammalian cassette. Fragment contains sites for cloning of the EGFP gene between the SV40 promoter and poly-A tail via the 5' *Spe*I and 3' *Xho*I restriction sites. Fragment also contains the *Pac*I and *Not*I restriction sites upstream of the WPRE/BGH Poly-A-tail for the introduction of a CMV promoter-Luciferase cassette, downstream of the EGFP cassette. The synthesised mammalian cassette was received in pUC57, pUC57-synth.

2.2.5.2. *EGFP mammalian expression cassette*

The EGFP gene was modified by PCR to add 5'*Spe*I and 3' *Xho*I restriction enzyme sites. This EGFP gene was then blunt end cloned into pJET and extracted and ligated into the pUC57-synth vector using the 5'*Spe*I and 3' *Xho*I restriction enzyme sites (Figure 3).

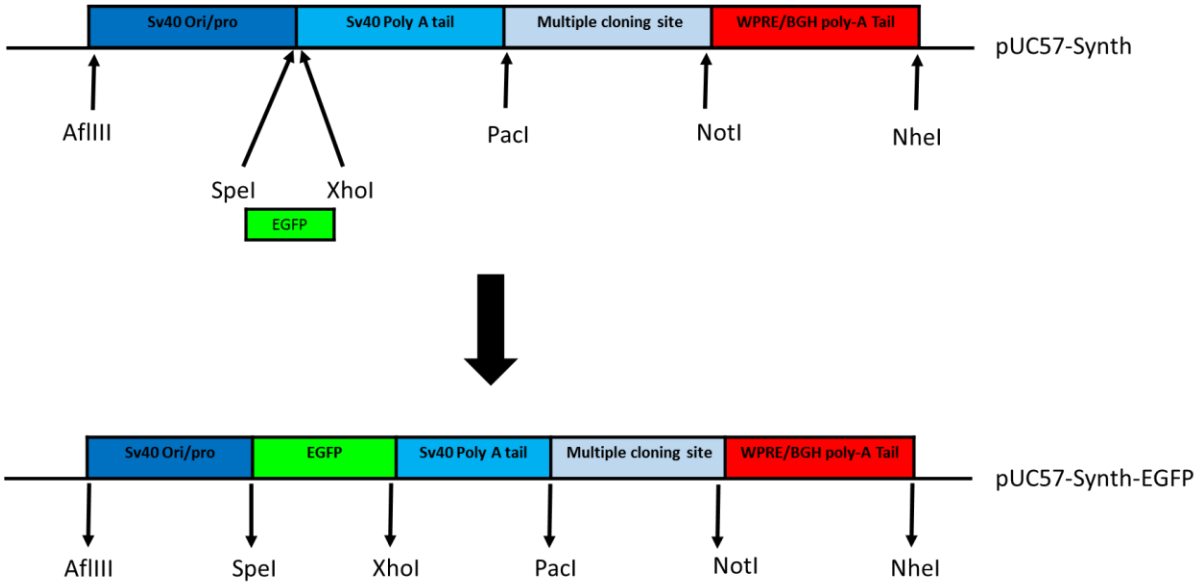

Figure 3: Schematic representation of the cloning of pUC57-Synth-EGFP, the EGFP mammalian expression cassette. The EGFP gene was cloned in between the SV40 promoter and poly-A tail using the 5' *Spe*I and 3' *Xho*I restriction sites.

2.2.6. Generation of different sized pRIC 3.0 backbones

Different sized stuffer fragments were cloned into the pRIC 3.0 vector to enable the production of various sized replicons. The pRIC 3.0 vector, without any modifications, produces a replicon of 3.3Kb. The 1Kb, 1.5Kb and 0.5Kb stuffers were created by amplifying up different sections of the luciferase gene in the pCLucf plasmid (1Kb and 1.5Kb stuffers) or sections of the E1a promoter of the mSEAP plasmid (0.5Kb stuffer). Restriction enzyme sites were also added to these stuffers by PCR. The 1Kb stuffer was cloned into the pRIC 3.0 vector using the 5' *Nhe*I and 3' *Hind*III sites to make a construct that would produce a 4.3Kb replicon. The 1.5Kb and 0.5Kb stuffers were cloned into the pRIC 3.0 vector sequentially, using the 5'*Nhe*I/3'*Hind*III sites and the 5'*Hind*III/3'*Sac*I restriction enzyme sites, respectively, making a stuffer fragment of 2Kb. This new 2Kb stuffer construct therefore produces a replicon of 5.3Kb (Figure 4).

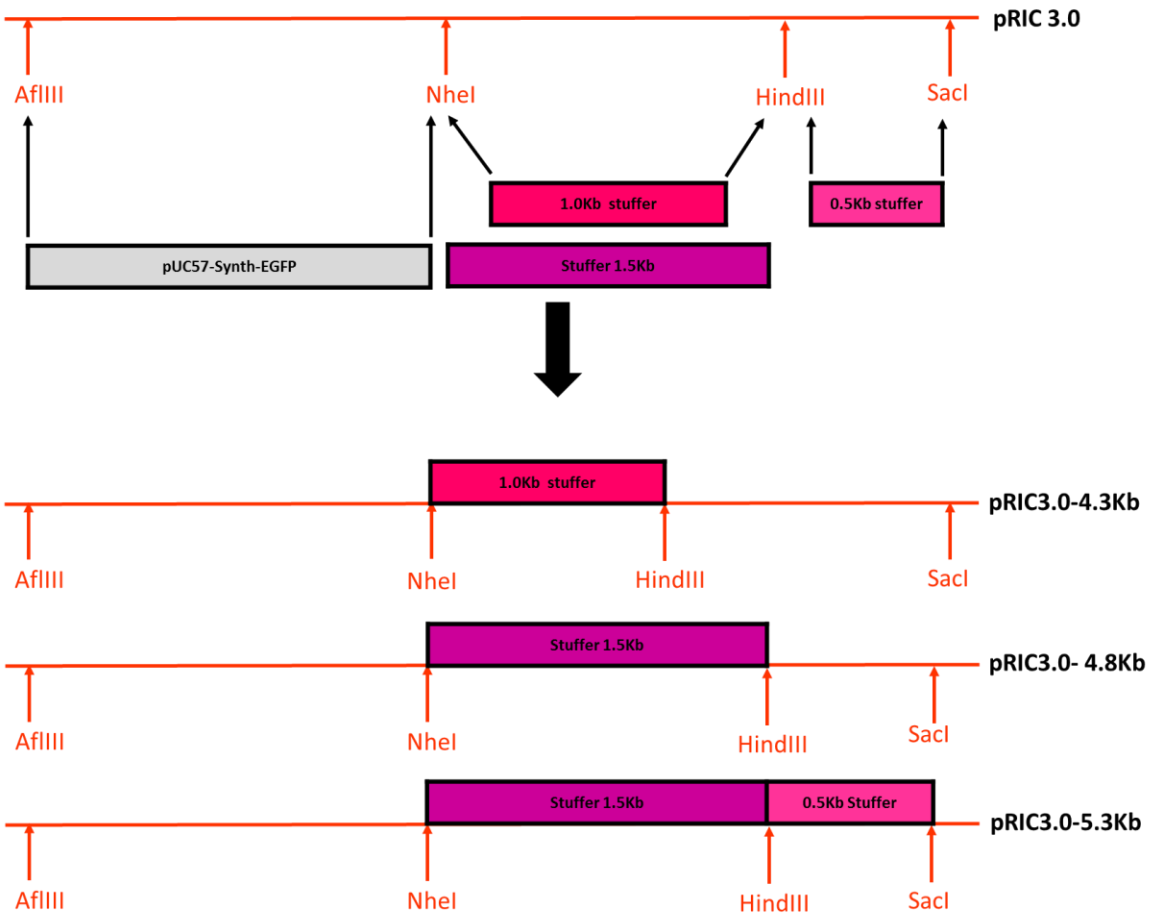

Figure 4: Schematic representation of the cloning of the different sized pRIC3.0 vectors. The 1Kb stuffer fragment was cloned into pRIC3.0 using the 5' *Nhe*I and 3' *Hind*III sites. The 1.5Kb and 0.5Kb were cloned into pRIC3.0 sequentially using the 5'NheI/3'HindIII sites and the 5'*Hind*III/3'SacI restriction enzyme sites, respectively.

2.2.7. Cloning of different sized EGFP pseudogenomes

The 4.8Kb, 5.8Kb and 6.8Kb pseudogenomes were created by digesting the EGFP mammalian expression cassette from the pUC57-Synth-EGFP vector and ligating it into the different sized pRIC3.0 backbones, using the 5' *Afl*III and 3' *Nhe*I restriction enzymes. The backbones used for these constructs contained either no stuffer fragments, a 1Kb stuffer fragment or a 2kb stuffer (1.5Kb + 0.5Kb) fragment.

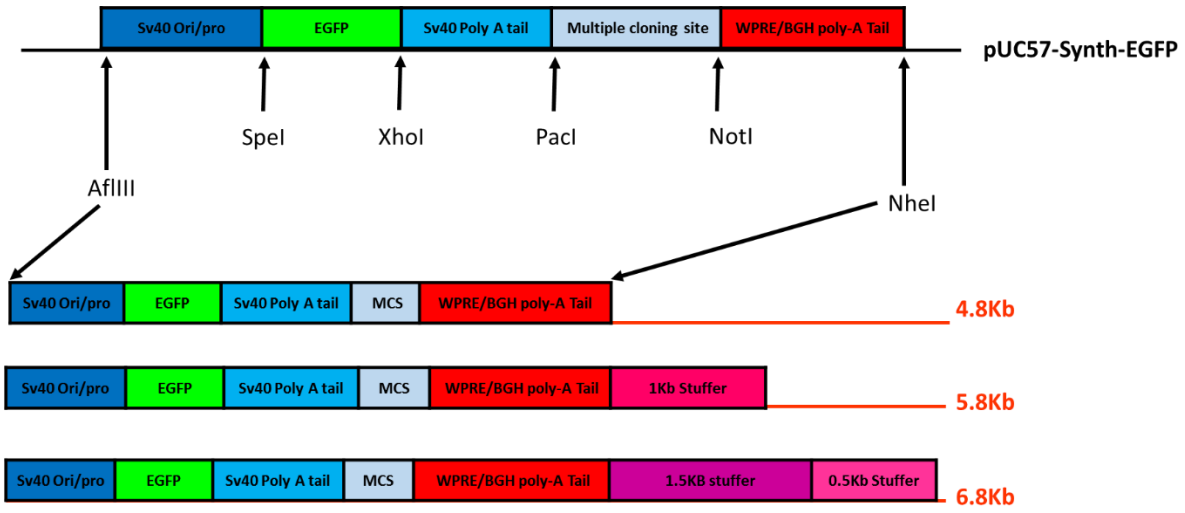

Figure 5: Schematic representation of the cloning of the different EGFP pseudogenomes. The EGFP mammalian expression cassette was digested out of pUC57-Synth-EGFP and ligated into the different sized pRIC 3.0 backbones, using the 5'*Afl*III and 3'*Nhe*I restriction enzymes, to make the 4.8Kb, 5.8Kb and 6.8Kb pseudogenome constructs. Orange labels represent the final pseudogenome construct sizes.

The EGFP cassette was cloned into the pRIC 3.0 empty backbone which results in a 4.8Kb replicon. The cloning of the EGFP cassette into the pRIC3.0 backbones containing the 1Kb and 2Kb (1.5Kb+0.5Kb) stuffers, result in pseudogenome replicons of 5.8Kb and 6.8Kb (Figure 5).

The 4.8Kb pseudogenome construct was used to create the 7.8Kb pseudogenome. The CMV promoter and luciferase were amplified as one unit out from the pCLucf plasmid. The CMV-Luciferase fragment was cloned into pJET and then digested, extracted and ligated into the 4.8Kb pseudogenome construct, upstream of the WPRE/BGHA poly-A-tail, using the 5'*Pac*I and 3' *Not*I restriction enzymes. This addition created a luciferase mammalian expression cassette and results in a 7.8Kb replicon (Figure 6).

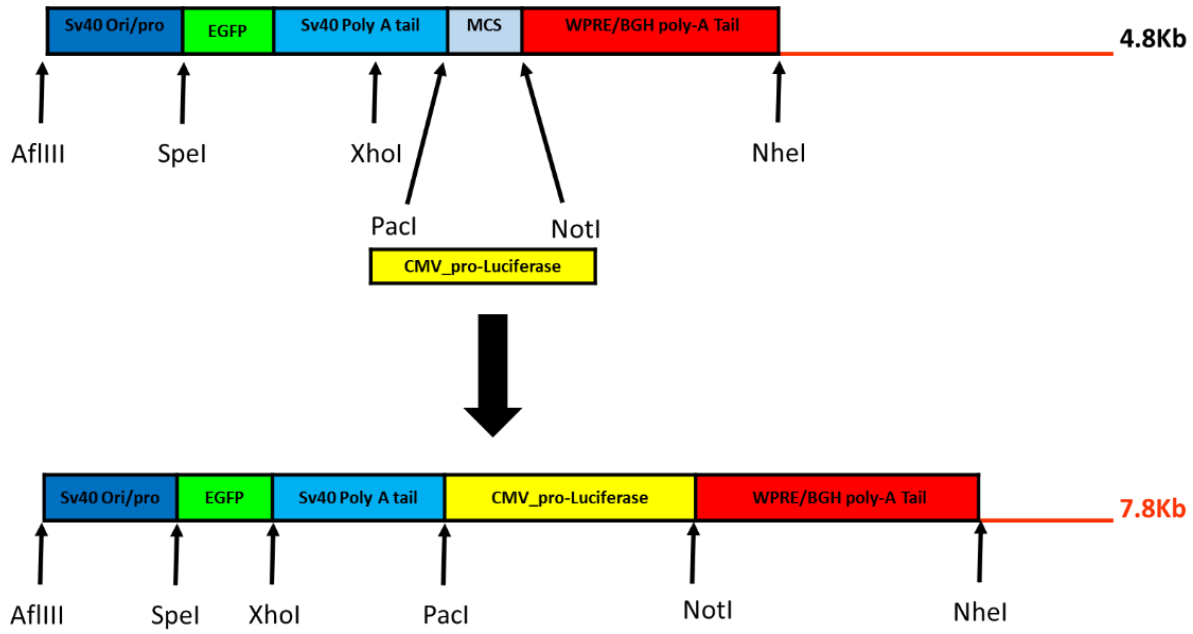

Figure 6: Diagram of the cloning strategy for the 7.8Kb pseudogenome construct. The CMV_promoter-Luciferase was cloned into the 4.8Kb pseudogenome construct using 5' *Pac*I/3'*Not*I. Orange labels represent the final pseudogenome construct.

2.2.8. Agrobacterium tumefaciens GV3101::pMP90RK

Electrocompetent *Agrobacterium tumefaciens* GV3101::pMP90RK was used for infiltration of the plants, for all the constructs in this study. The *Agrobacterium* was made electro-competent using the methods described (Shen and Forde, 1989). Briefly, 100µl of competent cells and 300ng of DNA were added to 0.1cm electroporation cuvette (BioRad, Hercules, USA) and electroporated at 1.8kV, 25µF, 200Ω using a GenePulser (BioRad, Hercules, USA). After the addition of 0.9ml of LB media, cells were incubated at 27 ˚C for 2 hours to allow for recovery and growth. The pMP90RK, helper plasmid, carries the antibiotic Kanamycin resistance gene, which allows for positive selection of the recombinant cells. The rifampicin resistance genes are carried within the *Agrobacterium* genome and the pRIC 3.0 vectors contain the Carbenicillin resistance gene. Therefore, the culture was plated and grown up on LB agar containing 50µg/mL, Kanamycin, 30µg/mL Rifampicin and 50µg/mL of Carbenicillin.

Agrobacterium colonies were screened with PCR, back-transformed into *E. coli* and confirmed with restriction enzyme digestion.

2.2.9. Sequencing

The EGFP and CMV promoter-luciferase clones that were positive for both the PCR and the restriction enzyme digestions, were sequenced, in pJET, to ensure that no errors had occurred during the PCR. Sanger sequencing was performed by Macrogen Inc. (Amsterdam, Netherlands) using insert specific

and vector primers (table 1). Sequences were analysed and aligned to reference sequences using CLC Main Workbench 6 (QIAGEN, Germantown USA).

2.2.10. HEK293TT cell maintenance

HEK293TT stock cells were grown in Dulbecco's Modified Eagle's medium (DMEM) with 10% bovine foetal calf serum (v/v), 1% non-essential amino acids (v/v), 1% penicillin-streptomycin (v/v) and 250µg/mL hygromycin, in Corning® T75 cell culture flasks (Merck, Kenilworth, USA). Cells were grown in an incubator at 37˚C with 5% $C0_2$ and 95% humidity. Cells were passaged at approximately 80% density.

2.2.11. Confirmation of reporter gene expression in HEK293TT cells

EGFP reporter gene expression was tested using fluorescent microscopy and luciferase gene expression was tested using a luciferase assay.

2.2.12. Fluorescent microscopy for confirmation of EGFP expression

Square, 0.1mm thick, 22x22mm coverslips (Marienfeld Superior, Lauda-Königshofen, Germany) were prepared by acid washing for 4hrs at 60˚C in 1M HCl. The slides were then washed with dH_2O and 96% ethanol and autoclaved. Next, the slides were the coated, in a 6 well Corning® Costar® TC-Treated plate, with 2ml of 0.1mg/ml poly-D-Lysine (10% (v/v) 1mg/ml Poly-D-Lysine solution (Merck, Kenilworth, USA) in 1x Gibco™ DPBS) for 2 hours at 37˚C and then dried for 1hr -1.5hrs at 37˚C. Following this, HEK293TT cells were seeded onto the coverslips at 150 000 cells/well. Cells were transfected approximately 24hrs after seeding, using Lipofectamine® 2000 Reagent (Invitrogen ™, Carlsbad, USA). The transfection procedure was carried out as per the manufacturer's instructions, using 2µg of DNA and 12.5µl Lipofectamine per well of a 6 well plate. After 24 hours, the cells were washed thrice with 1x Gibco™ DPBS and then fixed with 4% formaldehyde for 20 mins. Cells were washed thrice again, with 1x Gibco™ DPBS, and stained with 2mg/ml Hoechst (Merck, Kenilworth, USA) for 10 mins before washing and mounting onto glass slides (Marienfeld Superior, Lauda-Königshofen, Germany) using Mowiol mounting media with propyl gallate (anti-fade) (Confocal and Light Microscope Imaging Facility, University of Cape Town, South Africa). The slides were air dried at 37˚C for 10mins. Finally, the slides were viewed under an inverted fluorescent microscope (Zeiss Axiovert 200M) using the 40X objective lense and the DAPI filter to view the Hoechst stained nuclei and the ALEXA 488 filter to view EGFP fluorescence. Images were captured and analysed with a monochrome Zeiss AxioCam HRm and the AxioVision 4.8 software.

2.2.12.1. Confirmation of luciferase expression

A luciferase assay was used to test the 7.8Kb pseudogenome construct for luciferase expression. The luciferase assay was carried out using the Luciferase Assay System kit (Promega, Madison, USA) as per manufacturer's instruction. Luminescence was read in a Corning® 96 Well White Polystyrene Microplate (Merck, Kenilworth, USA) using the GloMax Multi Plus luminometer (Promega, Madison, USA).

Luminescence results were normalised to the amount of total soluble protein in the sample. Total soluble protein was calculated using a Bradford Protein Assay (Bio-Rad, Hercules, USA) as per the manufacturer's instructions. Absorbance was read at 750nm in a Multiwell immuno polysorb plate (ThermoFischer Scientific, Waltham, USA).

2.3. Results

Four different sized constructs were designed so that the pseudogenome replicons being encapsidated would be 4.8Kb, 5.8Kb, 6.8Kb or 7.8Kb in size (Figure 7). These constructs contain the EGFP and luciferase reporter genes instead of SEAP and can replicate in *E. coli, A. tumefaciens*, plants and mammalian cells. However, reporter gene expression can only occur in mammalian cells.

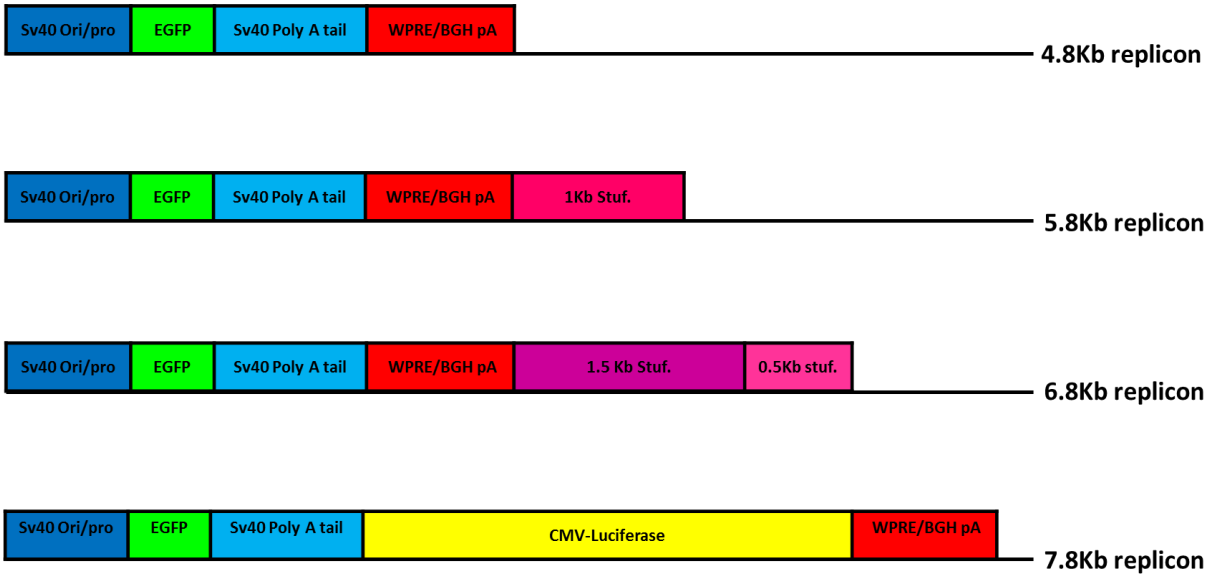

Figure 7: Schematic representation of the different sized pseudogenomes. EGFP, Luciferase and stuffer (Stuf.) fragments cloned into pRIC 3.0, so that a 4.8Kb replicon, 5.8Kb replicon, 6.8Kb replicon and a 7.8Kb replicon would be made in plants.

2.3.2. Cloning of pseudogenomes

A mammalian expression cassette was synthesised for the production of the different sized pseudogenomes (Figure 2). EGFP was then cloned into this cassette to create an EGFP mammalian expression cassette (Figure 3). Next, different sized pRIC3.0 vector backbones were made by cloning

different sized stuffers into pRIC 3.0 (Figure 4) and from this the 4.8Kb, 5.8Kb and 6.8Kb pseudogenome constructs were made by cloning the EGFP mammalian cassette into these various sized pRIC3.0 vectors (Figure 5). The final, 7.8Kb construct was made by cloning CMV promoter-Luciferase DNA fragment into the 4.8Kb construct (Figure 6).

The cloning of the constructs was confirmed in *E. coli* with PCR and then by restriction enzyme digest (Figure 8). A double digest with *Spe*I/*Xho*I was used to confirm the cloning of the 4.8Kb (17 ran off, 726, 1149 and 6323bp) and the 5.8Kb (726bp, 2170bp, 6323bp) pseudogenome constructs. The 6.8Kb pseudogenome construct sequence was confirmed by digesting with *Hind*III/*Xho*I (733, 2647, 6799bp), and the 7.8Kb pseudogenome sequence was confirmed by digesting with *Age*I/*Nde*I (1885, 1727, 7523bp). All the expected band sizes were obtained and the cloning of all four constructs were confirmed (Figure 8).

The DNA was then electroporated into *Agrobacterium tumefaciens* GV3101::pMP90RK for infiltration into plants. The *Agrobacterium* colonies were screened for the constructs by PCR and then confirmed by digestion by back cloning into *E. coli*.

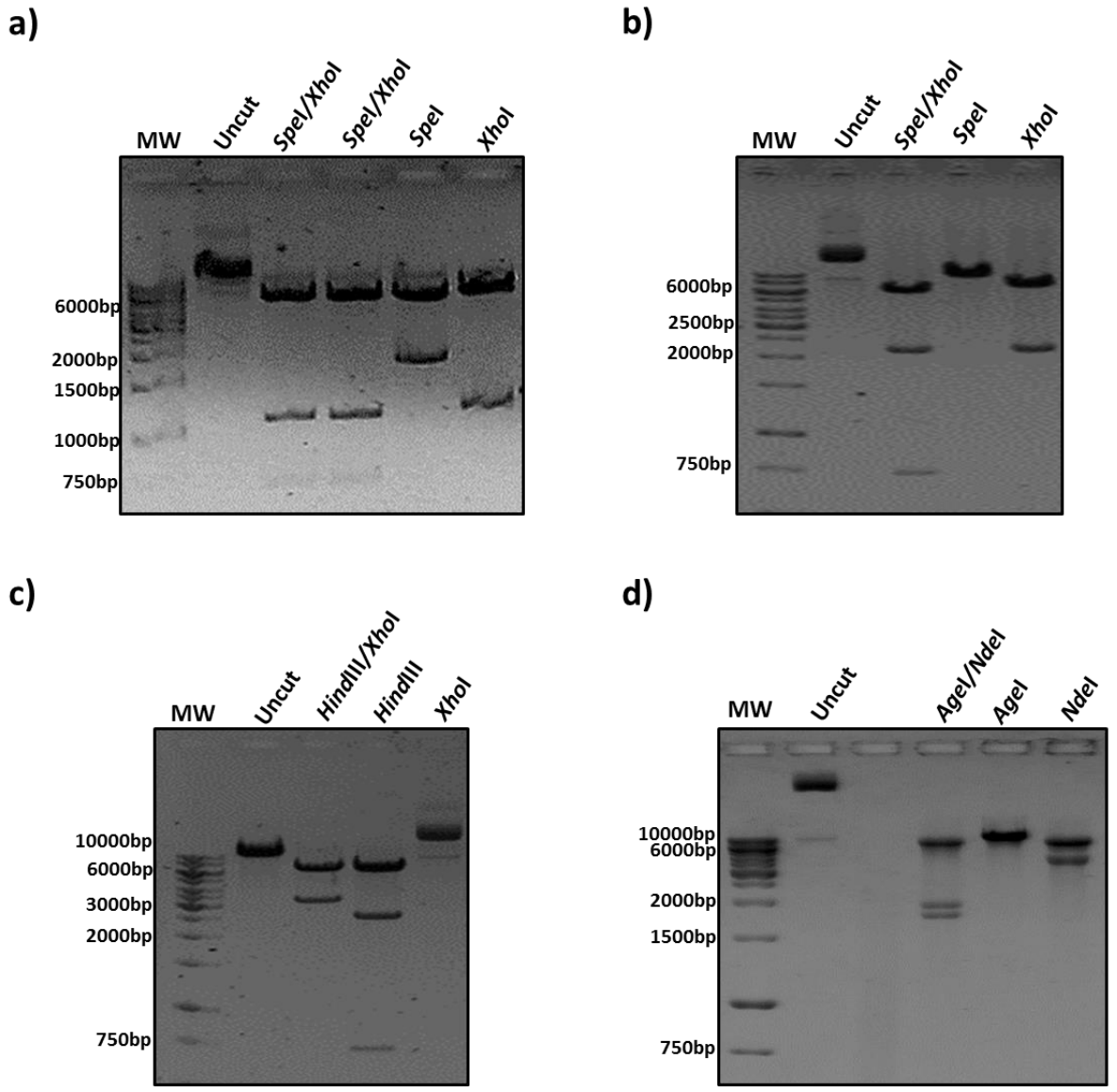

Figure 8: Confirmation of the pseudogenome construct sequences. Diagnostic digests were done on the pseudogenome DNA from PCR positive *E. coli* colonies. DNA in the digestions were separated by 1% agarose gel electrophoresis, using 0.5ug/ml EtBr to visualise the DNA bands. Uncut, undigested template DNA; MW, molecular weight marker, O'GeneRuler. a) Two colonies of pRIC3.0-E-4.8Kb DNA digested with *Spe*I and *Xho*I; b) pRIC3.0-E-5.8Kb DNA digested with *Spe*I and *Xho*I. c) pRIC3.0-E-6.8Kb digested with *Hind*III and *Xho*I. d) pRIC3.0-EL-7.8Kb DNA digested with *Age*I/*Nde*I.

2.3.3. Confirmation of pseudogenome reporter gene expression

DNA isolated from the positive colonies were also used to test the functionality of the pseudogenome expression cassettes. To confirm EGFP expression, all construct DNA was transfected onto HEK293TT cells and after 48hours, the cells were viewed under the fluorescent microscope to check for EGFP fluorescence (Figure 9). Cells fluorescing green were observed in all the pseudogenome construct images, indicating that EGFP was able to be expressed. (Figure 9).

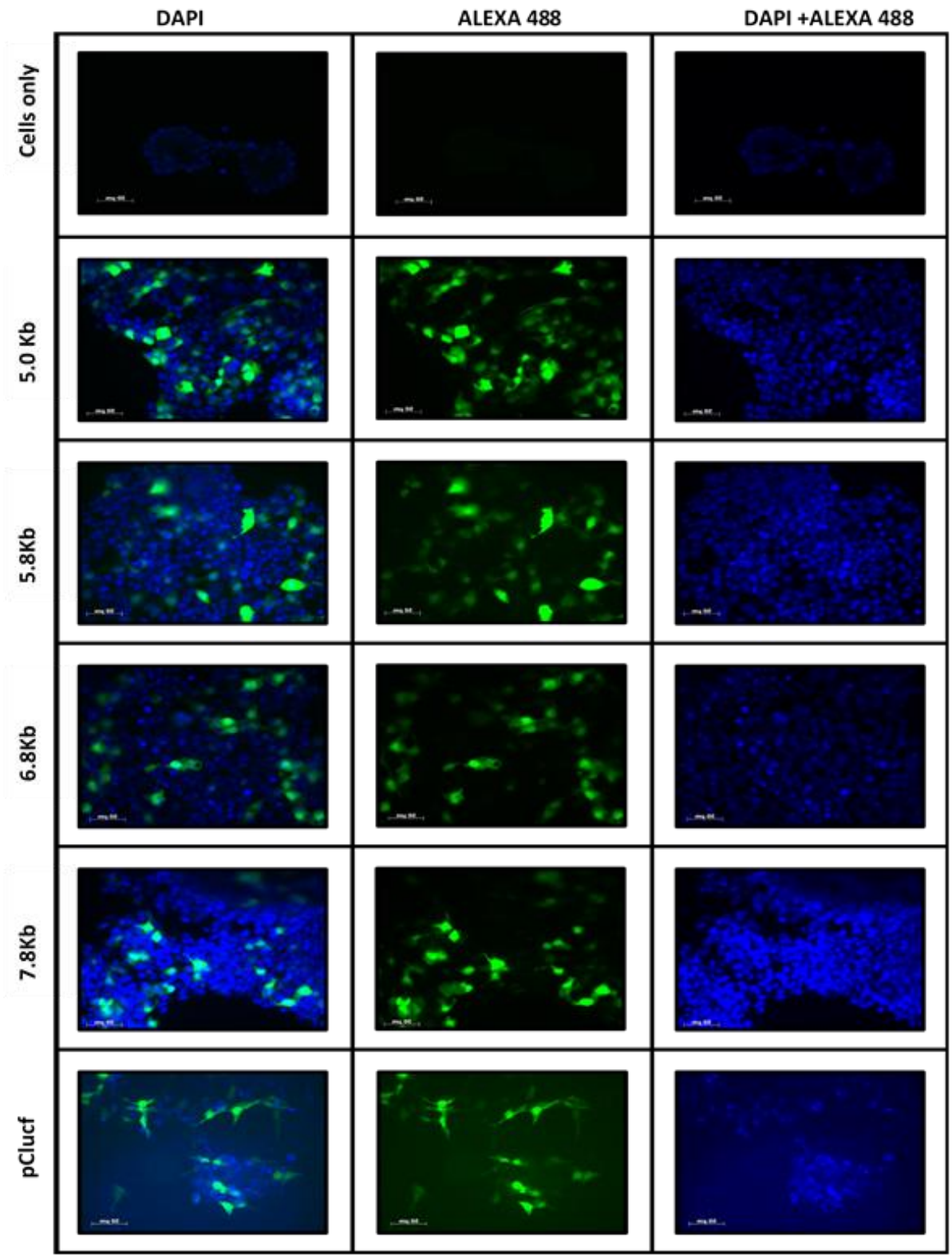

Figure 9: Pseudogenome constructs expressing EGFP. Cells were grown on glass slides and transfected with the pseudogenome construct DNA using Lipofectamine® 2000. After 48 hours, the cells were fixed with 4% formaldehyde and the cell nuclei were stained with Hoechst (Excitation⁄Emission (nm): 352⁄455). Coverslips were mounted onto glass slides using Mowiol. The DAPI (Excitation/Emission (nm): 325-375/440-480) filters were used to visualise the nuclei (left-hand panel) and the ALEXA 488 filters (Excitation/Emission (nm): 460-490/510-560) were used to visualise EGFP fluorescence (middle panel). The images of the DAPI and ALEXA 488 filters were merged in the right-hand panel. Positive control consisted of similarly transfected pCLucf DNA.

The 7.8Kb pseudogenome construct was also tested for luciferase expression in HEK293TT cells. HEK293TT cells were transfected with 7.8Kb pseudogenome construct DNA. These cells were lysed after 48 hours and a luciferase assay was performed on the cell lysate (Figure 10). Luciferase expression levels from the 7.8Kb pseudogenome construct ($1.08x10^{10}$ RLU/mg tsp) were found to be comparable to that of the positive control ($2.78x10^{9}$ RLU/mg tsp) and a thousand-fold higher than that of the cells only control ($2.78x10^{7}$ RLU/mg tsp). This indicates that luciferase is expressed from the 7.8Kb pseudogenome construct in HEK293TT cells.

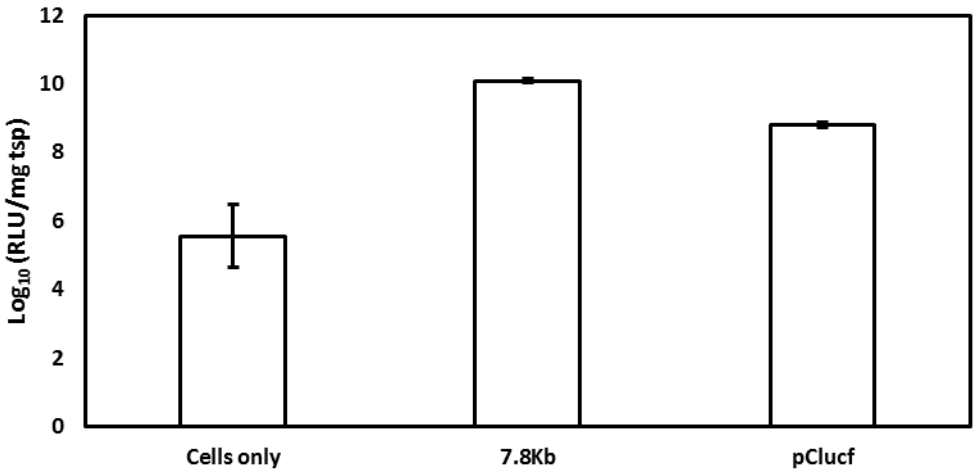

Figure 10: Luciferase gene expression from the 7.8Kb pseudogenome construct. HEK293TT cells were transfected with 7.8Kb pseudogenome DNA using Lipofectamine® 2000 and lysed after 48hours. Luciferase assay was carried out on the cell lysate and luminescence was read using a GloMax Multi Plus luminometer. Luminescence readings were corrected for background and normalised to total soluble protein (tsp). Error bars represent standard error between technical repeats.

2.4. Discussion

Four pseudogenome constructs with sizes of 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb were successfully cloned (Figure 8). These sizes were chosen for testing DNA encapsidation in plants by HPV L1 and L2 proteins, as literature shows that pseudogenomes 5-7Kb in size are best suited for papillomavirus PsV production (Buck *et al.*, 2004, Cerqueira *et al.*, 2016, Touze and Coursaget, 1998). Furthermore, it was shown in a study by Lamprecht *et al.* (2016) that PsVs containing the 4.8Kb pseudogenome produced significantly more signal in infectivity assays than the PsVs containing the 6.6Kb pseudogenome, which indicates that more 4.8Kb DNA was being encapsidated than 6.6Kb DNA. This is evidence that DNA size may play an important role in governing plant-made HPV PsV DNA encapsidation. Therefore, by using pseudogenomes differing by 1Kb to each other, a narrow size range for optimal DNA encapsidation can be obtained.

These pseudogenome constructs were made by cloning into the pRIC 3.0 vector (Figure 5, 6). The pRIC 3.0 vector has previously been successfully used in a study by Lamprecht *et al.* (2016) as a pseudogenome vector for plant-made HPV PsVs. These PsVs were found to be able to encapsidate DNA and to be efficient in PBNAs. Moreover, the pRIC 3.0 vector is important for *in planta* PsV production, in that it allows for the continuous and autonomous generation of small replicons in plants. The rolling circle mechanism of replication that pRIC 3.0 undergoes in plant cells, has been shown to be able to increase the copy number of pRIC 3.0 vectors, carrying the HIV p24 antigen or the EGFP gene, from 10^6 to 10^{10} copies after only three days post infiltration (Regnard *et al.*, 2010). This is promising because increased pseudogenome copy numbers, increases the chance of pseudogenome encapsidation and therefore PsV formation. Thus, lack of pseudogenome DNA availability will not affect encapsidation, making pseudogenome size the biggest factor affecting encapsidation.

The pseudogenome expression cassettes were also found to be able to successfully express EGFP and luciferase (Figure 10). These EGFP and luciferase constructs could therefore successfully replace the commonly used mSEAP plant-made HPV pseudogenome and this could aid in keeping the cost of PBNAs to a minimum. Support for this is that the luciferase gene detection kit is three times cheaper than the SEAP detection kit and in that EGFP requires no substrate to be detected, only the fixing reagents and access to a fluorescent microscope or lysis buffer and western blot reagents and apparatus. Furthermore, EGFP and luciferase have previously been used as pseudogenomes for papillomavirus PsVs (Cerqueira *et al.*, 2017, Johnson *et al.*, 2009, Bayer *et al.*, 2018, Touze and Coursaget, 1998) which allows for these new EGFP and luciferase pseudogenomes that are replicated in plants to be comparable to those in literature.

However, some problems were encountered while working with the EGFP pseudogenome. The HEK293TT cells used in study were often clumped and overlapping in certain areas, and so were not ideal for visualising individual fluorescing cells and quantifying transfection or transduction efficiency (Figure 9). Most studies done using EGFP as pseudogenomes have used flow cytometry to quantify EGFP expression (Cerqueira *et al.*, 2017, Bayer *et al.*, 2018). However, this was not possible for this study due to monetary and time constraints. The EGFP expressed by the pseudogenomes in HEK293TT cells, can be detected and semi-quantified by western blot. Therefore, for this study western blots were used to analysed EGFP expression in HEK23TT cells.

Luciferase expressed from the 7.8Kb pseudogenome construct was easily detected and quantified (Figure 10). Luciferase is an ideal reporter gene as the protein is the cheapest and easiest to detect; however, in this study, creating different sized replicons using luciferase proved difficult due to its length and the abundance of restriction sites between the gene and promoter. In future, a luciferase

pseudogenome can be cloned to make a pseudogenome of 5.3Kb, by cloning the synthesised mammalian expression cassette, without the EGFP, into pRIC 3.0 and then cloning the CMV-Luciferase fragment into pRIC.

2.5. Conclusion

Four pseudogenome constructs of sizes 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb were cloned. These constructs could replicate autonomously and continuously in plants to generate high copy numbers of pseudogenome replicons for L1/L2 encapsidation. These pseudogenomes also contain EGFP and luciferase instead of the SEAP reporter gene and therefore, could be used to decrease the cost of PBNAs and thereby reduce the cost of testing new neutralising antibody producing vaccines. Therefore, in future, these pseudogenome constructs will be used to for *Agrobacterium* infiltration into plants, wherein they will replicate and be encapsidated by L1/L2. The efficiencies at which these different sized replicons are encapsidated will then be determined to ascertain the optimal DNA encapsidation size for plant HPV PsVs

Chapter 3: Production of HPV 35 PsVs *in planta*, using different sized pseudogenomes

3.1. Introduction

The current HPV prophylactic vaccines consist of L1 VLPs that are made in either insect (Cervarix™) or yeast (Gardasil®) cells. However, these vaccines do not confer protection against all high-risk strains of HPV, such as HPV 35 which has a high prevalence in Africa (Smith *et al.*, 2007, De Sanjose *et al.*, 2010), and so further vaccine development is still needed. HPV PsVs are used as challenge material, to study the viral life cycle, to test new neutralising antibody vaccines and are potential delivery vehicles for therapeutic DNA vaccines. Thus, development of an efficient, low cost and sustainable means of HPV PsV production would be an important step towards understanding, preventing and treating HPV infections and cervical cancer.

3.1.1. Assembly of papillomavirus particles

HPV naturally infects and replicates in terminally differentiated basal keratinocytes (Barksdale and Baker, 1993). These cells have been hard to maintain in cell culture and produce very low yields of virions which has limited the study of HPV particles and vaccine development (Conway and Meyers, 2009, Kang *et al.*, 2000, Meyers *et al.*, 1992). This has led to the development of several methods to produce synthetic HPV particles *in vitro*.

Various intracellular and cell-free assembly protocols were created to try and increase PV particle yields. These methods were either chemical disassembly-reassembly based methods or intracellular methods which made use of autonomously replicating viral vectors in mammalian (Semiliki virus, Vaccinia virus) and insect cells (Baculovirus). While these methods were able to increase yields, they also led to the discovery that L2 is not needed for DNA encapsidation when assembling particles extracellularly (Touze and Coursaget, 1998, Zhao *et al.*, 1998, Unckell *et al.*, 1997, Müller *et al.*, 1995, Yeager *et al.*, 2000) but that the presence of L2 does enhance DNA encapsidation and/or infectivity of the particles when assembled intracellularly (Roden *et al.*, 1996, Zhao *et al.*, 1998, Zhou *et al.*, 1993, Unckell *et al.*, 1997, Stauffer *et al.*, 1998). This may be because PV particle assembly occurs in the nucleus, with L2 being responsible for the co-localization of all the elements required (Day *et al.*, 1998, Wang and Roden, 2013). Furthermore, L2 is also known to be essential for functional PsV production as it has been implicated in facilitating endosome escape (Florin *et al.*, 2006, Richards *et al.*, 2006), DNA trafficking into the nucleus (Day *et al.*, 1998, Kondo *et al.*, 2009, Florin *et al.*, 2006), aiding in capsid assembly (Chen *et al.*, 2011, Day *et al.*, 1998, Ishii *et al.*, 2005, Kirnbauer *et al.*, 1993), DNA

packaging in certain PV strains (Holmgren *et al.*, 2005, Zhao *et al.*, 1998) and enhancing infectivity of PsVs (Buck *et al.*, 2008, Guan *et al.*, 2017). Thus, L2 is needed for efficient production of functional PsVs intracellularly.

Additionally, in 2004, Buck *et al.* produced an intracellular assembly method for PV L1/L2 PsVs that was able to increase particle production efficiency by 10 million-fold compared to the previous Semliki Forest virus-derived vector methods. This protocol uses HEK293TT cells which constitutively express the large T-antigen, allowing for enhanced transgene DNA replication (Buck *et al.*, 2004). Moreover, this method has shown to produce approximately 10^9 PV transducing units/ ml and PsVs in these cells have further been shown to also be effective for use in PBNAs (Pastrana *et al.*, 2004a). The current plant PsV production and purification protocols were based on this method, along with the plant HPV VLP protocols of Varsani *et al.,* (2003) and Maclean *et al.* (2007).

HPV PsV production *in planta* has since been performed using the L1 and L2 capsid proteins and pseudogenomes with the Sv40 origin of replication to ensure high levels of replication and gene expression when doing infectivity assays and PBNAs in HEK293TT cells.

3.1.2. Papillomavirus particle purification

While initial chemical disassembly-reassembly of PV PsVs did not generate high enough yields, these methods did provide insight into what conditions are needed for PV particles to assemble. These chemical disassembly-reassembly studies were based on those designed for polyomaviruses, like the Sv40, which also uses disulphide bonds for capsid assembly. These methods indicated that factors such as ionic strength, redox environments and pH all affect capsid assembly (Colomar *et al.*, 1993, Brady *et al.*, 1977, Mukherjee *et al.*, 2008). Several studies have shown that PV particles require reducing reagents such as DTT for disassembly and that an increase in ionic strength with NaCl can inhibit disassembly. Therefore, in these methods, particles are disassembled with reducing agents at physiological ionic strength (0.15M NaCl) and then reassembled by diluting out the reducing agent and increasing the ionic strength to approximately 0.5M NaCl at a low pH (Mccarthy *et al.*, 1998, Touze and Coursaget, 1998, Chen *et al.*, 2001, Mukherjee *et al.*, 2008). This indicates that particles need to be purified in a non-reducing, low pH environment at high salt concentrations. Moreover, all the studies seem to indicate that high salt concentration is important for particle assembly and several plant HPV VLP and PsV studies have since used 0.5M NaCl buffers for HPV particle purification (Buck *et al.*, 2004, Varsani *et al.*, 2003, Maclean *et al.*, 2007, Lamprecht *et al.*, 2016).

The PV particle purification method that is most commonly used is isopycnic density gradient ultracentrifugation. CsCl is a common density gradient medium for purifying virus particles and has been used frequently for PV particle purification. Purification of PVs produced intracellularly often uses a sucrose cushion to remove the cell debris and pellet the sample, followed by ultracentrifugation on a discontinuous CsCl gradient to separate the VLPs from the PsVs (Zhou *et al.*, 1993, Roden *et al.*, 1996, Stauffer *et al.*, 1998, Unckell *et al.*, 1997, Varsani *et al.*, 2003, Maclean *et al.*, 2007). However, the Buck *et al*. HEK293TT cell method was the first to make use of an iodixanol (OptiPrep™) density gradient to purify HPV PsVs. The use of the OptiPrep™ medium has several advantages over CsCl gradients. CsCl samples need to be further purified before they can be used in animals, usually by chromatography, and sample is often lost here whereas OptiPep™ does not require additional steps. OptiPrep™ is sterile, non-ionic, non-toxic to cells and has been known to preserve the infectivity of the purified virus particles (Zolotukhin *et al.*, 1999, Andersen *et al.*, 1995). Furthermore, the researchers found that using OptiPrep™ actually increased the yields of PsVs compared to VLPs after purification and that in contrast to CsCl the VLPs were more dense in OptiPrep™ than the PsVs (Buck *et al.*, 2004). Thus, the OptiPrep™ gradient has since been used for plant PsV production (Lamprecht *et al.*, 2016).

In summary, purification of HPV particles that have been assembled intracellularly should ideally involve low pH, high salt buffers for particle formation, a sucrose cushion to remove debris and an OptiPrep™ density gradient to separate the empty particles from the DNA encapsidating ones.

3.1.3. Production of PsVs using different sized pseudogenomes

Various studies have shown that pseudogenomes up to 8Kb can be encapsidated by a PV VLP. However, while particles were always produced with these pseudogenomes, the efficiency of DNA encapsidation varied with size of the pseudogenome (Stauffer *et al.*, 1998, Touze and Coursaget, 1998, Zhao *et al.*, 1998, Roden *et al.*, 1996, Cerqueira *et al.*, 2016, Buck *et al.*, 2004). A study by Zhao *et al.* indicated that BPV particles efficiently packaged a maximum of 8.8kb DNA and a study by Lamprecht *et al.* demonstrated that plant HPV PsVs can efficiently encapsidate DNA as small as 4.8Kb (Zhao *et al.*, 1998, Lamprecht *et al.*, 2016). Therefore, it should be possible to produce HPV PsVs with the 4.8Kb, 5.8Kb, 6.8Kb, and 7.8Kb pseudogenomes used in this study.

3.1.4. In planta expression of HPV Particles

Transient expression is known to be a fast means of production HPV VLPs in plants and one that often results in higher yields and better immunogenicity than stable transformation. (Varsani *et al.*, 2006, Maclean *et al.*, 2007, Regnard *et al.*, 2010). Maclean *et al.* demonstrated the first highly successful

production of HPV VLPs in plants using *Agrobacterium*-mediated transient expression. The results of this study demonstrated that HPV L1 yield is best when using human codon optimised L1 (pTRAc-hL1), achieving up to 14.9% TSP, and is even better when hL1 is targeted to the chloroplast (17% TSP, Maclean *et al.,* 2007). HPV PsVs however, cannot be made in the chloroplast and this is because the T-DNA of the L1/L2 genes and the pseudogenomes are transported by the *A. tumefaciens* virulence genes into the nucleus (Zupan *et al.*, 2000) where the pseudogenomes are made into dsDNA replicons. If the L1/L2 proteins were chloroplast targeted, they would be post-translationally transported from the cytoplasm to the chloroplast (Li and Chiu, 2010) and so would not be able to encapsidate the pseudogenomes in the nucleus. Therefore, L1/L2 need to be targeted to the cytoplasm for transport into the nucleus (Day *et al.*, 1998, Florin *et al.*, 2002, Becker *et al.*, 2004, Bird *et al.*, 2008, Merle *et al.*, 1999, Nelson *et al.*, 2002), to allow for encapsidation of the pseudogenome. Therefore, HPV PsVs should be made be *in planta* and should be done by transient expression using human codon optimised L1/L2 genes in the pTRAc, non-replicating, high copy, cytoplasm targeting vector (Figure 1).

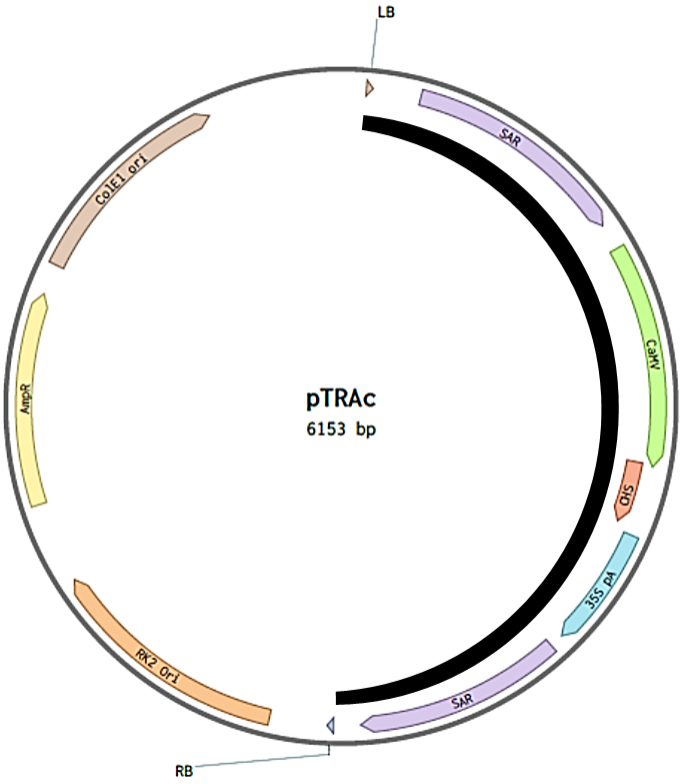

Figure 1: Diagram of the high copy number, non-replicating pTRAc vector. Cytoplasm targeted vector used for hL1 and hL2 expression in this study. ColE1 ori (brown), *Escherichia coli* origin of replication; RK2 ori (orange), origin of replication for *Agrobacterium tumefaciens*; AmpR (yellow), ampicillin / carbenicillin-resistance gene; LB and RB, left and right borders for T-DNA integration; SAR (purple), scaffold attachment region of the tobacco Rb7 gene; CaMV (green), CaMV 35S promoter with duplicated transcriptional enhancer; CHS (red), chalcone synthase 5'-untranslated region; 35S pA (blue), CaMV 35S polyadenylation signal. Black bar represents the T-DNA that will be excised and transported into the plant cell nucleus by the agrobacterial *vir* genes. Image recreated from Maclean *et al.* and Regnard *et al.* using CLC WORKBENCH 6 (Maclean *et al.*, 2007, Regnard *et al.*, 2010).

3.1.5. *In planta production of HPV PsVs*

In 2016, a study by Lamprecht *et al.* in our group, demonstrated the successful production of HPV 16 PsVs in *N. benthamiana*, using *Agrobacterium*-mediated expression. This study employed the various strategies, described above, to ensure efficient production of HPV PsVs. Firstly, the hL1 and hL2 sequences were used in pTRAc vectors for high levels of protein expression. Next, the pseudogenome was cloned with the pRIC 3.0 vector (described in chapter 2.1.3) and was infiltrated at a very high OD_{600} to potentially increase the rate of encapsidation. A high salt, low pH NaOAc buffer was used for protein extraction as it has been shown that HPV particles form best under low pH and high salt conditions (Mccarthy *et al.*, 1998, Chen *et al.*, 2001, Mukherjee *et al.*, 2008) and that plant proteins, such as Rubisco, precipitate out of solution at ≤ pH7.4 (De Jong, 2011, Prevot-D'alvise *et al.*, 2004). When purifying the particles, a two-layer sucrose density gradient of 30% and 50% was first used to concentrate all particles in the sample into a small 5ml 30%-layer (HPV 1.34g/mL) and to remove residual plant proteins from the sample before separating the particles out by iodixanol (Optiprep™) density gradient ultracentrifugation. Thus, using all these strategies, a protocol was devised for efficient HPV PsV production *in planta*.

In addition, further research has been done in our lab since, using Lamprecht *et al.*'s protocol to produce HPV 18 and 35 PsVs. However, the results of these studies indicated that, while both strains of HPV PsVs could be produced, HPV 35 appeared to produce more regularly shaped and sized particles as well as higher yields of these particles (data not shown). Thus, going forward, the optimal DNA size for encapsidation will be determined using HPV 35 and subsequently DNA of this size will be used to obtain higher yields of HPV 16 and HPV 18 PsVs.

This chapter describes the *in planta* production of HPV 35 PsVs using the 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb pseudogenomes and employing the optimised production and purification strategies described above. These PsVs will be used in subsequent experiments to determine the optimal DNA size for plant HPV PsV encapsidation.

3.2. Methods

3.2.1. *Agrobacterium* infiltration

Agrobacterium tumefaciens and its *vir* genes and the T-DNA on its Ti plasmid, were used to transport the L1, L2 and pseudogenome constructs into the plant cell where it can be expressed, form particles and encapsidate DNA.

3.2.1. *Preparation Agrobacterial cultures*

Agrobacterium tumefaciens GV3101::pMP90RK colonies containing the human codon optimised HPV 35 pTRAc-hL1, pTRAc-hL2 and the 4.8Kb 5.8Kb, 6.8Kb and 7.8Kb pseudogenomes were inoculated, separately, into 10mL of LB, containing 50µg/mL Kanamycin, 30µg/mL Rifampicin and 50µg/mL of Carbenicillin, and were incubated at 27˚C, for 48 hrs. Next, these cultures were inoculated into 50mL LBB (tryptone 2.5g/L, yeast extract 12.5g/L, NaCl 5g/L, MES 1.95g/L, pH5.6), containing the same antibiotics as before, and were incubated overnight at 27˚C. Thereafter, these 50mL cultures were inoculated into 500ml LBB supplemented with 50µg/mL kanamycin, 50µg/ml of carbenicillin and 200µm acetosyringone and were then incubated overnight at 27˚C. The infiltration media was made by diluting the cultures in resuspension media, as in Lamprecht *et al.* (2016), and was then left to incubate for an hour, to allow for the stimulation of the *vir* genes before infiltration (Figure 2a).

3.2.2. *Infiltration of plants to make PsVs*

Four to six-week old plants, grown under 16hr light/ 8 hr dark cycles, were used for infiltration. The plants were pressure-infiltrated using a vacuum pump. This was done by creating a vacuum of ±100 kPa to remove air/liquid from the leaf abaxial spaces and then releasing the vacuum to ±0 kPa to allow the infiltration media to fill the empty abaxial spaces (Figure 2b). Fifteen plants were infiltrated for each experimental and control sample.

3.2.3. *Harvesting and protein extraction*

Leaves were harvested 4 days post infiltration (dpi), homogenised in high salt NaOAc (HS-NaOAc: 0.1M NaOAc and 0.5M NaCl, pH 5.2) at 2x the volume (ml) of the leaf weight (mg, Figure 2c). Homogenate was then incubated at 4˚C for 1 hr to allow for protein extraction (Figure 2d). Thereafter, the homogenate was clarified using Miracloth (Sigma-Aldrich, St. Louis, USA, Figure 2e) and centrifuged for 20 mins at 10 000 x *g* at 4˚C (Figure 2f).

3.2.4. *Purification of HPV particles*

The clarified samples were loaded onto a two layered sucrose cushion. The sucrose cushion steps were prepared in 1x HS-NaOAc to 50% and 30%. The cushions were then spun at 17 4587 x *g* (Optima™ L-100 XP centrifuge, Beckman Coulter SW32Ti rotor, Brea California) for 50 mins, after which the 30% fraction was removed, using a needle and syringe (Figure 2g), and dialysed overnight (Figure 2h), at 4˚C into 1X high salt PBS (6x HS-PBS: 223.3g/L NaCl, 1.2g/L Na_2HPO_4, 1.44g/L KH_2PO_4 pH 7.4).

An OptiPrep™ (Merck, Kenilworth, USA) density gradient was used to separate the particles. The 60% OptiPrep™ solution was diluted in 6x high salt PBS to make a 50% solution which was then further diluted with 1X HS-PBS to make the 39%, 33% and the 27% layers of the OptiPrep™ gradient. Samples were then ultracentrifuged at 17 4587 x *g* for 3.5hrs (Figure 2h). Subsequently, the gradient was fractionated into 1 ml aliquots, from the bottom of the gradient, into siliconized tubes.

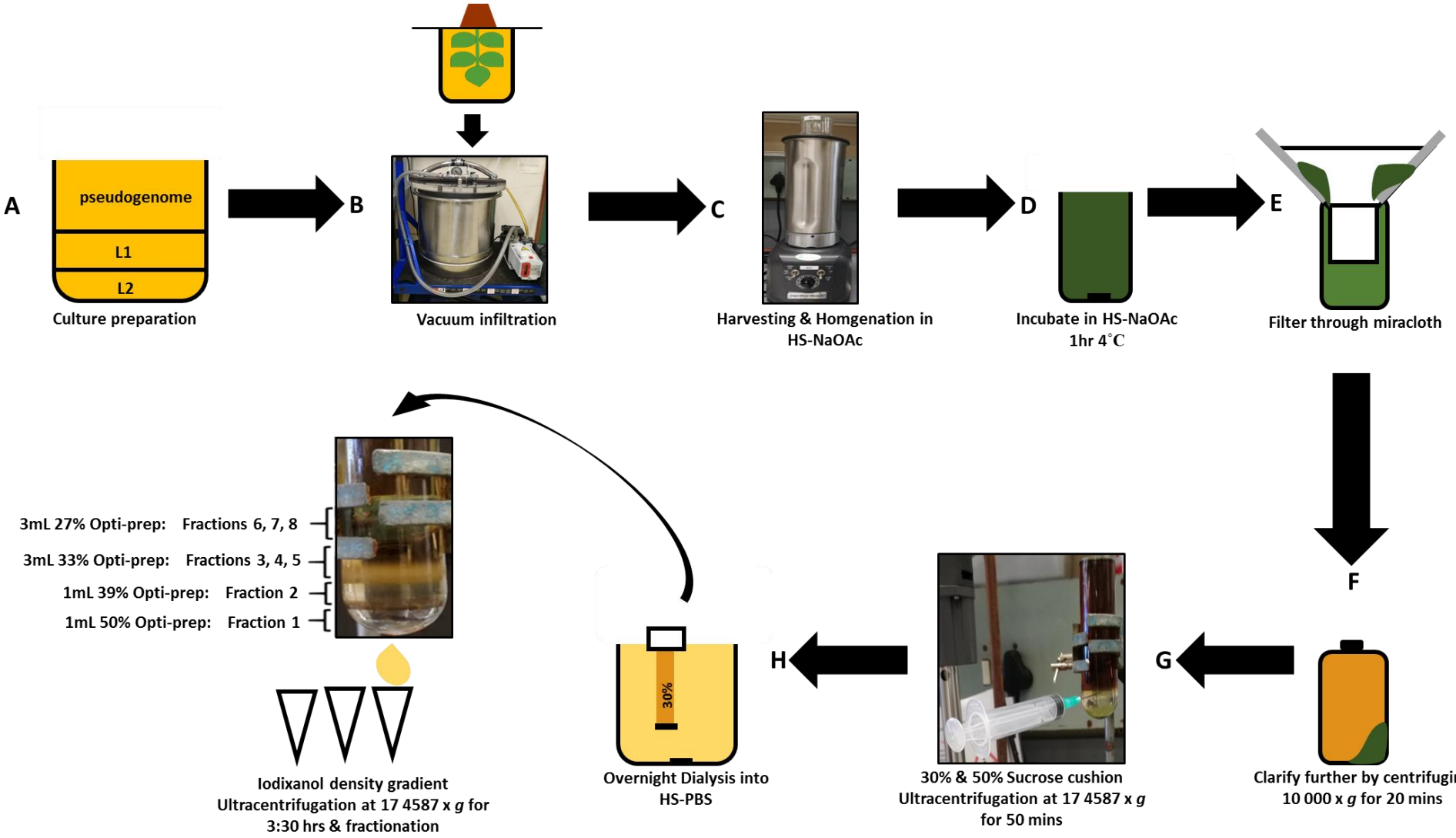

Figure 2: Purification process of HPV PsVs and L1/L2 VLPs. a) *Agrobacterial* cultures were prepared to L1:L2:DNA infiltration medium and b) vacuum infiltrated into the plant. c) The leaves were harvested 4 dpi, homogenised in a blender with HS-NaOAc, d) incubated at 4˚C, e) filtered through Miracloth and f) clarified by centrifugation. g) Ultracentrifugation was used to purify and concentrate the particles into a 30% layer of a two-layered sucrose cushion. The 30% fraction was then extracted, h) dialysed into HS-PBS and ultracentrifuged on an iodixanol gradient with, 50%, 39%, 33% and 27% layers, and then fractionated into 1mL aliquots.

3.2.5. Confirmation of L1 protein expression

The L1 protein content in the sample fractions were analysed by western blotting. In preparation for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the samples were denatured with 1x sample application buffer (5x SAB: SDS loading 2% SDS, 100mM TrisCl pH 7.5, 2mM EDTA, 52% glycerol, 4.3% B-mercaptoethanol) and heated for 10 minutes at 95˚C. Samples were then loaded in equal volume onto 10% SDS-PAGE gels and the proteins were separated, by electrophoresis, at 120V for ±2hrs using a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, USA). Proteins were transferred onto the nitrocellulose membranes at 15V for 1.5 hrs using a Trans-blot® semi-dry transfer cell (Bio-Rad, Hercules, USA). Following this, the open protein binding sites on the membranes were blocked for 30mins (Blocking buffer: 1xPBS, 5% non-fat dairy skim milk, 1% of 10%TWEEN 20) after which the membranes were incubated overnight at 4˚C with in-house rabbit anti-Gardasil polyclonal antisera at a 1:5000 dilution in blocking buffer (as determined in-house by Dr Alta Van Zyl, BRU, University of Cape town, South Africa)The blots were washed 3x for 10mins in blocking buffer and incubated for 1hr at 37˚C with goat anti-rabbit IgG alkaline phosphatase conjugated antibody (Merck, Kenilworth, USA) at a 1:5000 dilution in blocking buffer. Subsequently, the blots were washed 3x for 10mins using PBS-T and detection was carried out using the BCIP/NBT chromogenic reagent (Thermo Scientific, Waltham, USA). The molecular weight maker used on these blots was the Colour Protein Standard, Broad Range (NEB, Ipswich, USA).

3.2.6. Confirmation of particle assembly

Particle assembly was confirmed using transmission electron microscopy. Carbon-coated, copper grids were hydrophilized by glow discharging at 25mA for 30s using a Model 900 SmartSet Cold Stage Controller (Electron Microscopy Sciences, Montgomery County, USA). Samples were fixed onto the grids at a 1:5 dilution by incubating the grid on a 20µL drop of sample for 4 minutes. Following this, the grid was washed in 3 drops of dH_2O, for 1 minute each and then negatively stained for another minute using 2% (w/v) Uranyl acetate. Grids were viewed at between 27 000 – 53 000X magnification using a FEI Tecnai 20 electron microscope, equipped with a 200kV LaB6 emitter. Ten fields of view were collected for all the samples for statistical significance when counting and measuring the particles by eye.

3.3. Results

In planta production of HPV PsVs, using different sized pseudogenomes was investigated. The PsV and VLP sample gradients were fractionated into 1mL aliquots with Fraction (F) 1 corresponding to the 50% layer of OptiPrep™, F2 the 39% layer, F3-5 the 33% layer and F6-8 the 27% layer of the OptiPrep™ gradient (Figure 3). The negative control was fractionated with pRIC 3.0 F1 representing the 50% layer of OptiPrep™, F2-3 the 39% layer, F4-8 the 33% layer and F9-12 the 27% layer of the OptiPrep™

gradient. Previous work showed that the 33% layer of the gradient usually contains empty HPV particles which have a density of 1.24g/mL in OptiPrep™ and the 27% fractions usually consists of DNA encapsidated particles that have a density of 1.20g/mL in OptiPrep™ (Buck *et al.*, 2004). Thus, the 33% layer and 27% layers of these fractions were analysed for L1 protein expression and the L1 positive fractions were pooled and then examined for HPV particles that resemble 30-120nm particles found in previous studies on *in planta* production of HPV PsVs (Aves, 2016, Lamprecht *et al.*, 2016).

3.3.1. Confirmation of L1 capsid protein expression

Samples from each fraction were analysed for protein expression by Western blot. The 55KDa L1 protein bands were observed in the 33% and 27% OptiPrep™ fractions of all the different sized pseudogenome PsV samples and the VLP samples. L1 protein was found most concentrated in the 33% fractions and decreased in concentration as the density of the gradient decreased (Figure 3). Furthermore, the 50% and 39% OptiPrep™ fractions do not appear to contain L1 protein, except for in the 4.8Kb PsV sample which appears to contain a light 55KDa L1 protein band in the 39% fraction. However, this is could be due to human error while fractionating (Figure 3a). No L1 protein was observed in the negative control, confirming that the 55 KDa L1 bands detected in the experimental samples were not due to non-specific binding of the antibodies (Figure 3f). Subsequently, the sample fractions were pooled based on L1 concentration and density as it is known that PV PsVs are usually found in the 27% layer of the OptiPrep ™ gradient and VLPs in the 33% layer of the OptiPrep ™ gradient (Buck *et al.*, 2004, Bayer *et al.*, 2018, Lamprecht *et al.*, 2016). Thus, the F3-5 of the 33% and F6 and F7 of the 27% fractions were pooled for all the PsV and VLP samples. These pooled samples were then used in downstream analyses.

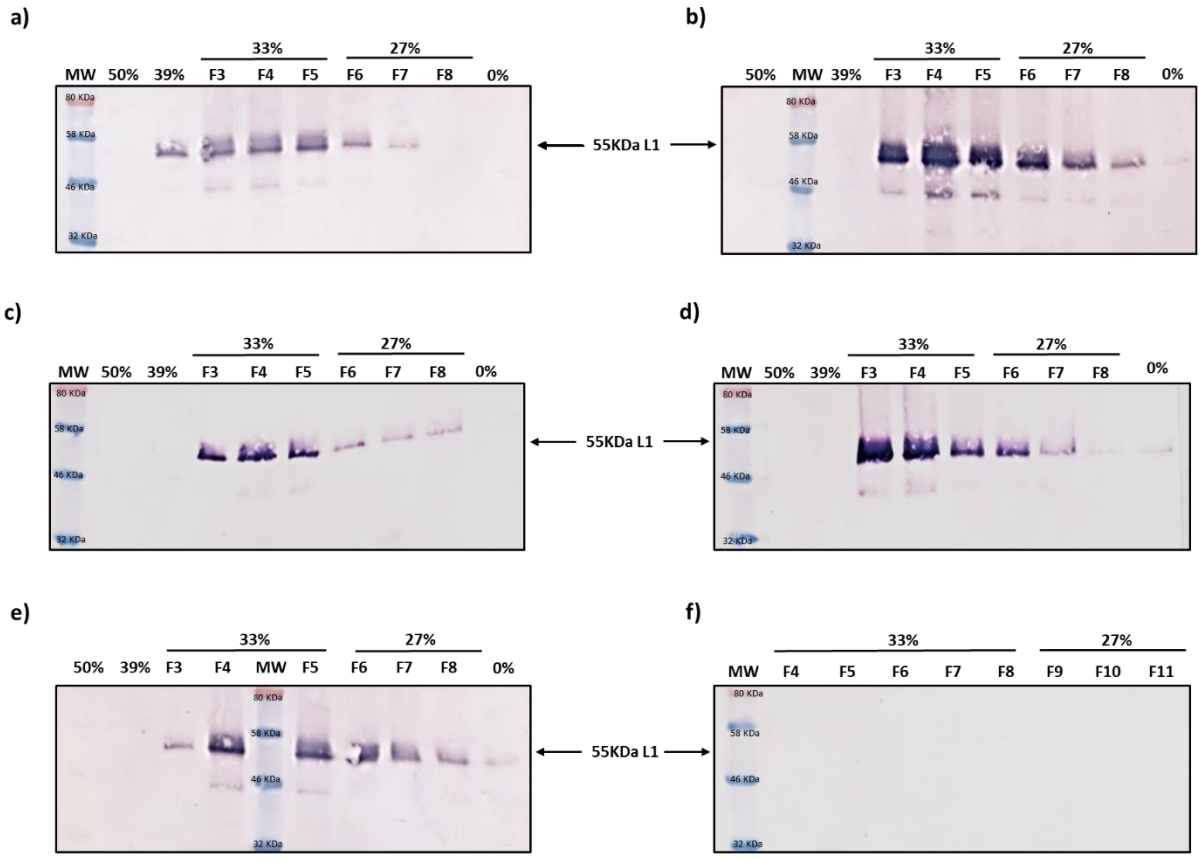

Figure 3: Confirmation of L1 protein expression. Representative western blots of the purified PsVs and VLPs 50%, 39%, 33% and 27% iodixanol fractions probed with the anti-Gardasil, polyclonal, primary antibody (1:5000) and the anti-rabbit IgG alkaline phosphatase conjugated secondary antibody (1:5000). Samples were loaded at equal volume (30µL) and blots were detected with BCIP for 1hr. MW, molecular weight marker; F, fraction; a), 4.8Kb PsVs; b), 5.8Kb PsVs; c), 6.8Kb PsVs; d), 7.8Kb PsVs; e) L1/L2 VLPs; f) pRIC 3.0 empty vector control. Western blots were repeated for 5 biological repeats.

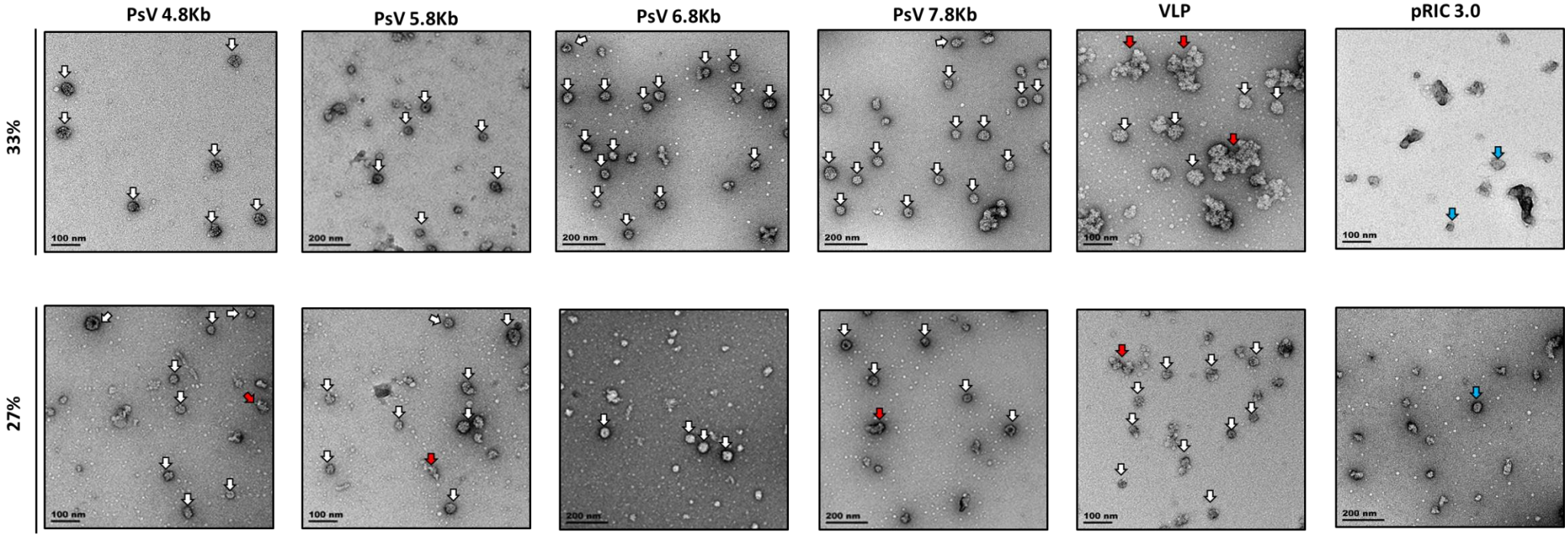

Figure 4: Confirmation of particle assembly. Representative transmission electron microscopy images of the L1/L2 VLPs and the PsVs with different sized pseudogenomes. Grids were fixed with the 27% and 33% fractions of each sample, at a 1:5 dilution. The grids were then washed and negatively stained with 2% (w/v) uranyl acetate. White arrows, potential HPV particles; red arrows, potential L1 aggregates; blue arrows, purification artefacts. Scale bars range between 100nm and 200nm and samples were viewed between 27 000 - 53 000X magnification. Four out of five biological repeats were analysed for particle assembly, and ten fields of view, of the PsV and VLP samples, were used to investigate differences in particle distribution across the gradient, particle size and particle number.

3.3.2. Confirmation of particle assembly

Transmission electron microscopy was used to confirm particle assembly in the 33% and 27% fractions of all the samples. HPV VLPs are known to have a density of 1.24g/mL in OptiPrep™. HPV PsVs are slightly less dense than the VLPs, with the particles appearing at the interface of, or within the 27% layer of the iodixanol gradient (Buck *et al.*, 2006a, Kirnbauer *et al.*, 1992, Bayer *et al.*, 2018). The TEM results show particles in both the 33% and the 27% fractions of all the different sized pseudogenome PsV samples and the VLP samples, ranging in size from 30-55nm. Furthermore, the PsV and VLP particles were compared across biological repeats and across different sized pseudogenomes and no difference in size, particle distribution across the gradient or number of particles was observed (data not shown). Moreover, the negative control, pRIC 3.0, does not appear to contain any HPV particles, with only a few artefacts appearing at below 25nm (Figure 4, blue arrows). All the samples, across biological repeats, also appear to contain amorphous pentameric structures that resemble the L1 aggregates shown by Varsani *et al*. (2006). These structures were not visible in the negative, pRIC 3.0 control and are therefore likely to be L1 aggregates and not plant or vector induced proteins. Hence, the VLP and PsV samples contain 30-55nm HPV particles and some L1 aggregates.

In summation, L1 expression was confirmed and 30-55nm HPV particles were assembled for all the different sized pseudogenome PsV samples and the VLP samples. No significant difference in L1 expression or particle assembly was found between the different sized pseudogenome PsVs in both the 33% and 27% fractions.

3.4. Discussion

Many studies have investigated PV assembly, but many questions remain unanswered. The assembly of PVs is usually studied using PsVs, and by these methods, a few basics of PV assembly have been agreed upon. The first is that L1 is able to form VLPs on its own and encapsidate DNA on its own extracellularly (Touze and Coursaget, 1998, Zhao *et al.*, 1998, Unckell *et al.*, 1997, Müller *et al.*, 1995, Yeager *et al.*, 2000). However, studies have shown that L2 is essential for the formation of functional PsVs intracellularly (Roden *et al.*, 1996, Zhao *et al.*, 1998, Zhou *et al.*, 1993, Unckell *et al.*, 1997, Stauffer *et al.*, 1998). More specifically, L2 is known to be necessary for HPV virion assembly as it is needed for efficient encapsidation in some PV types, assembly of a more stable particles and the co-localisation of L1 and DNA in the nucleus, which allows for the assembly of PsVs. L1 VLPs assemble differently to L1/L2 particles, in that L1 VLPs can form in the cytoplasm whereas L1/L2 particles are assembled, with the DNA and the L2, in the nucleus (Wang and Roden, 2013). Thus, for L1/L2 particles to be made, all elements of assembly must localise in the nucleus. L2 is the first to accumulate in the nucleus at the nuclear domain 10 site (ND-10, also known as PML bodies) with the help of the Hsc70

chaperone protein (Day *et al.*, 1998, Florin *et al.*, 2002, Becker *et al.*, 2004). The L1 proteins have been found to assemble into capsomeres in the cytoplasm and then translocate into the nucleus with the help of karyopherin chaperones (Bird *et al.*, 2008, Merle *et al.*, 1999, Nelson *et al.*, 2002). Moreover, these karyopherin proteins, along with the Hsp70 heat shock protein, have been shown to prevent the formation of L1 VLPs in the cytoplasm (Florin *et al.*, 2004, Darshan *et al.*, 2004, Bird *et al.*, 2008). Once in the nucleus the L1/L2 capsid can form and bind DNA to encapsidate it (Nelson *et al.*, 2000, Mallon *et al.*, 1987, Li *et al.*, 1997). Furthermore, these chaperone proteins are common to most eukaryotic cells, plants included. In fact, Hsc70 and karyopherins have been known to be part of the replication and assembly mechanisms of several plant viruses (Byth *et al.*, 2001, Gorovits *et al.*, 2013, Kunik *et al.*, 1999, Gudleski *et al.*, 2010, Sullivan and Pipas, 2001, Kennedy, 2013) and this indicates that these proteins could be readily available for transporting the L1 and L2 proteins into the plant nucleus for assembly into PsVs. However, this has yet to be confirmed and so further studies are still needed to test the localisation of the L1/L2 proteins in plants by either fusing them to fluorescent proteins or visualising the L1/L2 particles in immunogold-labelled leaf sections using TEM.

HPV VLPs have been successfully produced in plants and *in planta* production of HPV PsVs has recently been achieved (Lamprecht *et al.*, 2016, Kennedy, 2013). The most notable successes that have led to the production of the first HPV PsVs in plants was a study by Maclean *et al.* which showed that up to 14.9% L1/TSP could be obtained when human-codon optimised L1 was used in the pTRAc vector (Maclean *et al.*, 2007). Furthermore, another study that contributed, was that done by Regnard *et al.,* using the deconstructed BeYDV viral vector, pRIC. This study illustrated the ability to replicate DNA constructs in plants by obtaining at least 100-fold increase in DNA, in the form of dsDNA replicons (Regnard *et al.*, 2010). Finally, in 2016, the first study on HPV PsVs produced *in planta* was published. This study combined the work from Maclean *et al.* and Regnard *et al.* and illustrated that it is possible to make HPV PsVs in plants by expressing human codon optimised L1 and L2 from the pTRAc non-replicating plasmids and encapsidating pseudogenome dsDNA replicons between 4.8Kb and 6.6Kb. Researchers were also able to show that these PsVs can be effectively used in PBNAs (Lamprecht *et al.*, 2016).

The data represented in this chapter confirms the first aspect of HPV PsV production in plants, L1 capsid protein expression (Figure 3). HPV L1 protein expression patterns were found to be similar across all the different sized pseudogenome PsV samples as well as between the PsV and VLP samples. More specifically, the pattern of L1 expression obtained indicates that more L1 is present in the 33% fractions as opposed to the 27% fractions. This pattern of L1 accumulation was found to be consistent with that of other PsV studies and, notably, these studies also found that the 33% fraction of

OptiPrep™ (1.24g/mL) is more likely to contain VLPs and the 27% fraction of OptiPrep™ (1.20g/mL) is most likely to contain PsVs (Buck *et al.*, 2004, Bayer *et al.*, 2018). Therefore, more protein is found in the putative VLP fractions as opposed to the putative PsV fractions. Overall, the results show that L1 was expressed in all PsV and VLP samples and that the addition of DNA, of any type or size, does not affect L1 expression.

In planta particle assembly was confirmed with TEM. Particles were formed in all the PsV and VLP samples, across the 33% and 27% fractions. Particle morphology resembled those of native virions and of other HPV PsVs previously made *in planta* (Lamprecht *et al.*, 2016, Kennedy, 2013, Aves, 2016). The particles seen in these samples usually ranged between 30-55nm and this corresponds to that produced in previous HPV *in planta* PsV studies, with the study by Lamprecht *et al*. (2016), producing HPV 16 particles of between 30-120nm and a study done in our lab (Aves, 2016) resulting in HPV 35 particles ranging between 35-55nm. The negative control was found to be empty and free of any L1 protein (Figure 3f) or HPV particles, with only a few artefacts (Figure 4, blue arrows) appearing at ≤25nm. The size of these artefacts and the fact that they do not bear resemblance to the pentameric structure of capsomeres ensures that they cannot be mistaken for HPV particles (Figure 4, white arrows) or L1 aggregates (Figure 4, red arrows). The PsV and VLP samples were also analysed for difference in particle size and number when using the different sized DNAs but no visible, reliable difference was observed (data not shown). This indicates that there is no visible marker to distinguish DNA encapsidating particles from empty particles, and therefore further testing would be required to establish this. Thus, HPV particles resembling HPV virions, found to range in size between 30-55nm, were observed in all the experimental and VLP samples.

Furthermore, TEM indicated that L1 aggregates as well as particles are present in all the experimental samples. Varsani *et al*. (2006), showed that L1 aggregates can be the result of unstable or misassembled particles. When L2, which is required for assembly (Chen *et al.*, 2011, Day *et al.*, 1998, Ishii *et al.*, 2005, Kirnbauer *et al.*, 1993) was mutated, these aggregate structures formed and were unable to bind to conformational antibodies. Additionally, studies carried out in our lab have found that, while high yields of L1 protein can sometimes be obtained in plants, only a small percentage of these proteins form particles and even less form PsVs (personal communication with Megan Hendrikse, Alta Van Zyl and Renate Lamprecht, BRU, University of Cape Town, South Africa) and this is evident in the appearance of L1 aggregates in these samples (Figure 4). These L1 aggregates are seen in all the samples of all the biological repeats, suggesting that some of the particles are not particularly stable even though high salt, low pH, particle stabilising buffers were used (Volkin *et al.*, 2002, Mccarthy *et al.*, 1998). Nevertheless, a possible solution to this problem may lie in the findings

of studies done by Cerqueira *et al.* These studies show that by performing disassembly-reassembly with HEK293H nuclear extract and ATP or by using blunt end linear pseudogenomes, HPV PsV expression can be increased (Cerqueira *et al.*, 2017, Cerqueira *et al.*, 2016). These studies were able to achieve efficient assembly of HPV PsVs and have reported up to 10^{11} infectious units/mg L1 (Cerqueira *et al.*, 2017) which is higher than that achieved by the Buck *et al.* SV40, intracellular assembly method which quotes 10^{9} infectious PV units/ml (Buck *et al.*, 2004). Furthermore, Cerqueira *et al.,* also used L-glutathione oxidized (GSSG) to improve the maturation of the PsV capsids (Buck *et al.*, 2005b). Papillomavirus virion maturation is redox dependent and studies have shown that the suprabasal layer of Keratinocytes (terminally differentiated), that these particles naturally mature in, are in an oxidative state as opposed to the reductive state of the basal layer which these particles infect (Conway *et al.*, 2009). GSSG is an oxidised molecule that when added to the assembly mixtures, creates an oxidising environment which allows for the crosslinking of the L1 disulphide bonds which improves the regularity and stability of the capsids and enhances DNA encapsidation and infectivity (Buck *et al.*, 2005b, Cardone *et al.*, 2014). Thus, this method, designed by Cerquiera *et al.,* could potentially be optimised for use with plant made L1/L2 VLPs to reduce the amount of L1 aggregates and increase the number of stable PsVs being formed.

While it can be said that L1 was expressed and particles were formed, the data presented in this chapter is unable indicate whether the particles are encapsidating the EGFP/Luciferase pseudogenome DNA. This is firstly, because only L1 expression could be confirmed and not the expression of L2 due to the lack of availability of an HPV 35 L2 antibody. It is possible that L1 alone encapsidated DNA extracellularly to form a PsV (Touze and Coursaget, 1998, Zhao *et al.*, 1998, Unckell *et al.*, 1997, Müller *et al.*, 1995, Yeager *et al.*, 2000). However, L2 is known to be essential for the efficient production of infectious PsVs (Roden *et al.*, 1996, Zhao *et al.*, 1998, Zhou *et al.*, 1993, Unckell *et al.*, 1997, Stauffer *et al.*, 1998, Wang and Roden, 2013) and therefore it is important that a distinction is made between the L1 only PsVs and the L1/L2 PsVs by doing infectivity assays. Secondly, there are no differences in the L1 accumulation patterns or particle morphology, size, number and distribution across the 33% and 27% OptiPrep™ densities of the PsV and VLP samples. Thus, there are no visual markers by which to distinguish a VLP from a PsV or by which to determine whether one pseudogenome size is more frequently encapsidated than another. However, many studies have shown that particles can be made, using different sized pseudogenomes of any type, provided that they are below 8Kb in size (Stauffer *et al.*, 1998, Touze and Coursaget, 1998, Cerqueira *et al.*, 2016, Cerqueira *et al.*, 2017, Buck *et al.*, 2006a, Bayer *et al.*, 2018, Zhao *et al.*, 1998). Therefore, HPV particles used in this study should be able to encapsidate the pseudogenomes, which range between 4.8Kb and

7.8Kb. However, encapsidation and infectivity assays are still needed to confirm that these particles are indeed functional L1/L2 PsVs encapsidating these various sized pseudogenomes.

3.5. Conclusion

HPV L1 was expressed *in planta* and particles were formed using the 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb pseudogenomes. However, further downstream testing is needed to determine whether these particles are indeed PsVs and to determine which size pseudogenome is best for encapsidation by plant-produced HPV PsVs.

Chapter 4: Characterisation of Pseudovirions

4.1. Introduction

4.1.1.PsV infectivity:

HPV infects epithelial cells, with different strains infecting either the cutaneous or mucosal epithelial tissue. HPVs infect healthy basal keratinocytes by entering through micro-abrasions in the skin. The virus enters the cell through the binding of the L1 capsid protein to the HSPGs on the basement membrane or the basal keratinoctyes of the skin (Johnson *et al.*, 2009, Joyce *et al.*, 1999, Kines *et al.*, 2009) and this causes a conformational change in the particle, exposing the L2 protein for furin and proprotein convertase cleavage (Kines *et al.*, 2009, Day *et al.*, 2008). This in turn allows for a conformational change in L1 to allow it to bind to a secondary receptor on the basal keratinocytes and enter into the cell via endocytosis (Kines *et al.*, 2009, Day *et al.*, 2008, Day *et al.*, 2010, Aksoy *et al.*, 2017). HPV PsVs are known to be able to specifically target cancer cells, such as melanomas and carcinomas, that evolve to display the form of HSPG usually only found on the basement membrane and basal keratinocytes of the skin (Kines *et al.*, 2009). These traits make PV PsVs valuable assets for the delivery of heterologous DNA into foreign cells, specifically for targeted gene and cancer therapies.

HPV PsVs are also important for many other applications. Culturing HPV is difficult, thus PsVs can serve as infectious challenge material and as a tool for monitoring the viral life cycle (Conway *et al.*, 2009, Kang *et al.*, 2000, Meyers *et al.*, 1992, Ma *et al.*, 2011). Furthermore, HPV PsVs are also important for the development of broader ranged prophylactic vaccines as they are used in PBNAs to test for neutralising antibodies in response to new HPV vaccines (Pastrana *et al.*, 2004b) and potentially for the production of therapeutic vaccines (Lin *et al.*, 2010, Chabeda *et al.*, 2018).

4.1.2.PsV DNA encapsidation

HPV is a dsDNA virus composed of the L1 major and the L2 minor capsid protein encapsidating a circular genome of up to 8Kb. The mechanisms of this DNA encapsidation have yet to be fully elucidated, although studies have shown that L1 and L2 both contain DNA binding sites (Li *et al.*, 1997, Zhou *et al.*, 1994, Zhao *et al.*, 1999, Schäfer *et al.*, 2002, Digiuseppe *et al.*, 2017a, Fay *et al.*, 2004) which play important roles in PV DNA encapsidation and gene transfer. The L1 c-terminal domain is known to contain an overlapping DNA-binding domain and DNA nuclear localisation signal (NLS). This domain has been found to be essential for efficient DNA packaging (Schäfer *et al.*, 2002) and gene transfer (Touzé *et al.*, 2000, Digiuseppe *et al.*, 2017a), with L1 DNA binding allowing for the stabilisation of the PV capsid (Digiuseppe *et al.*, 2017a). While the L2 DNA-binding site has been found to be insufficient for DNA encapsidation by an L1/L2 PV particle (Schäfer *et al.*, 2002, Unckell *et al.*, 1997), it has been shown to enhance the encapsidation efficiencies of some intracellularly produced

PV PsVs (Zhao *et al.*, 1998, Zhou *et al.*, 1993, Roden *et al.*, 1996). It has also been shown that the L2 DNA-binding site and NLS allow for the L2 mediated transfer of the DNA into the nucleus after endosome release (Unckell *et al.*, 1997, Roden *et al.*, 2001, Kawana *et al.*, 1998) and for the introduction of the DNA to the assembled L1 VLP (Zhou *et al.*, 1994). Thus, PV PsVs and virions produced using various expression systems, have shown that both the L1 and L2 DNA binding sites and NLS's are important for PV encapsidation of DNA and gene transfer from the particle into the nucleus (transduction).

While the L1 DNA binding domain is known to play a role in pseudogenome encapsidation, HPV has been shown to encapsidate DNA based on size rather than with the use of encapsidation signals. HPV PsVs were first successfully produced in 1996 (Roden *et al.*, 1996) and by 1998, it was discovered that HPV does not require a DNA encapsidation signal and rather encapsidates based on size. This discovery was made using the intracellular assembly of PV PsVs in mammalian HEK293T cells and was later confirmed, by many different groups, using mammalian intracellular assembly methods (Zhao *et al.*, 1998, Buck *et al.*, 2004) and extracellular disassembly-reassembly methods (Touze, 1998, Cerqueira *et al.*, 2016). All these studies indicated that PV PsVs, no matter what the expression system, encapsidated best between 5-8Kb, with the smaller DNA resulting in the better encapsidation and/or transduction. These findings resulted in the HPV size discrimination theory, which was first applied in Buck *et al.* 2004 and then refined by the Cerqueira *et al.,* in 2016. This theory states that PV PsVs are in constant state of disassembly and reassembly but once they encounter DNA of an appropriate size, below 8Kb, they form a stable particle that is resistant to disassembly. However, most of the studies leading up to this theory were done on non-human PVs and on either extracellularly produced PsVs or PsVs produced within in mammalian cells. To date, there is only one published study on the production of HPV PsVs *in planta* (Lamprecht *et al.*, 2016). This study used two pseudogenomes of 4.8Kb and 6.6Kb, with the results showing that a higher transduction signal was obtained when the 4.8Kb pseudogenome was used as opposed to when the 6.6Kb pseudogenome was used. This result indicates that there may be a DNA size range that is optimally encapsidated by plant-made HPV PsVs and one way to ensure optimal plant-made HPV PsV yields, would be to ensure that the optimal size of pseudogenome is being used every time. Therefore, in this study, 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb pseudogenomes were used to make PsVs *in planta* and these PsVs were analysed and compared for DNA encapsidation and transduction efficiencies.

4.1.3.Characterisation of HPV PsVs

4.1.3.1. DNA encapsidation

The PsVs with the 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb pseudogenome, produced *in planta* need to be characterised for frequency of pseudogenome encapsidation. Some of the early methods of testing for DNA encapsidation include treating the sample with benzonase nuclease and semi-quantifying PCRs by agarose gel densitometry (Buck *et al.*, 2004, Touze and Coursaget, 1998, Stauffer *et al.*, 1998), semi-quantifying Southern blots (Stauffer *et al.*, 1998) and the use of pseudogenomes containing resistance genes, and the quantitation thereof by transformation of *E.coli* (Zhao *et al.*, 1998). One of the most recent methods allows for the detection of the DNA while in the particle. The virions are resolved on a native agarose gel strained with GelRed and Coomasie® and the encapsidated DNA band appears at the same position as the protein band (Masarapu *et al.*, 2017). However, this method does not allow for the determination of the exact amount of DNA in the particle, the size of the DNA and what DNA is encapsidated in the particle. Moreover, yields of plant produced HPV PsVs are currently too low to be able to visualize the encapsidated DNA on an agarose gel (data not shown). Alternatively, plant HPV PsV studies, have used inverse PCR to determine whether the pRIC-pseudogenome replicon was encapsidated as opposed to the full constructs (Lamprecht *et al.*, 2016, Kennedy, 2013), however, this method is not quantitative. The most recent and most effective method currently is the use of qPCR to quantify and detect DNA purified out of the particles. PsV samples are treated with benzonase nuclease and then lysed with proteinase K and amplified and quantified by qPCR (Cerqueira *et al.*, 2016, Cerqueira *et al.*, 2017, Bayer *et al.*, 2018). Thus, this current study used proteinase K to break open the capsids and qPCR as an accurate and efficient way to quantify encapsidated DNA.

DNA to L1 ratios are used to determine the frequency of encapsidation of the DNA. L1 concentration provides an indication of how many PsVs there are in the sample. However, it should be noted that the formation of functional HPV PsVs is also dependent on L2 incorporation and so the DNA to L1 ratio is only an estimation of PsV concentration in the sample. However, normalising to L1 concentration also accounts for any batch-to-batch variation in expression and purification, as one batch of sample may have replicated and expressed protein better than another batch and/or have been purified better than another batch of sample. If there was more protein expressed in and/or extracted in one batch of plants compared to the other, there would most likely be more PsVs in that sample. Thus, the DNA to L1 ratio also normalises against batch-to-batch variation making the DNA concentrations comparable.

4.1.3.2. Transduction efficiency

Transduction efficiency is necessary to determine PsV functionality since DNA encapsidation does not necessarily equate to increased transduction (Bayer *et al.*, 2018). Thus, the transduction efficiencies and the encapsidation frequencies need to be compared. Using different sized DNAs may not affect the structure of the particle but a study by Cerqueira *et al.,* (2016) has indicated that when DNA close to or above 8Kb is used, the particle is less stable due to DNA protruding from it. Thus, DNA may be encapsidated, but the size of the DNA could affect the particle's ability to infect the cell and deliver the DNA into the cell. So far research has shown that HPV 16 PsVs can be produced up to 1x 10^{11} transducing units (Buck *et al.*, 2004, Cerqueira *et al.*, 2016, Cerqueira *et al.*, 2017). However, plant produced HPV PsVs have yet to be fully characterised for transduction efficiency.

HPV is known to infect basal keratinocytes, however, HEK293T and HEK293H cells have been known to be able to be infected by HPV PsVs (Buck *et al.*, 2004, Stauffer *et al.*, 1998). The mechanism of PV PsV infection of HEK293TT cells has yet to be fully elucidated; however, HEK293TT cells are able to act as an affordable, low risk model system for PsV production and testing. These cells overexpress the large T antigen that enhances replication of DNA by binding to its SV40 origin of replication. Therefore, HEK293TT cells allow for enhanced replication and expression of the PsV genes, making them useful for PsV expression, PBNAs and infectivity assays. Alternatives to this are NIKS (immortalised human foreskin keratinocytes) which have been shown to be able to support the life cycle of several different types of HPVs (Allen-Hoffmann *et al.*, 2000), thus making them a valid model for testing infectivity and viral replication. Other cells that can be used are primary cells, mostly W12 (Stanley *et al.*, 1989) and CIN-612 (De Geest *et al.*, 1993, Doorbar *et al.*, 2015), which originate from infected individuals. However, these cells are more difficult and expensive to maintain and pose a greater health risk and therefore require specialised skills and facilities. Thus, HEK293TT cells will be used in this study to assay infectivity.

This chapter describes the characterisation of the different sized pseudogenome PsVs by encapsidation and transduction efficiencies whereby the optimal size of pseudogenome for plant PsVs encapsidation can be determined.

4.2. Methods

4.2.1.Quantitative PCR (qPCR) sample preparation

The PsV and VLP samples, made as described in section 3.2, were digested with proteinase K (Merck, Kenilworth, USA) to lyse the capsid and release the encapsidated DNA (20µl of sample, 1µl of a 1/10 dilution of proteinase K, 30µl of 1xPBS). The reaction was carried out at 55˚C for 3hrs, thereafter the

proteinase K was denatured at 95˚C for 10 mins. Dilutions of 1/1000 were then made of each sample for analysis by qPCR.

4.2.2.Quantitative PCR

An EGFP DNA standard curve was used to quantify the amount of encapsidated DNA. Standard curves were tested in various buffers, dH_20, 1xPBS and an Optiprep™-PBS mixture (proteinase K reaction). No significant difference was found between the standard curves for the different buffers and so the OptiPrep™ - PBS buffer was chosen (1/10 dilution of a 2:3 ratio of 33% OptiPrep™ in HS-PBS and 1xPBS) as it was representative of the sample conditions (Figure 1a). Five standards were used for the standard curve, 1ng – 0.001ng/ul (131 000 000 – 13 100 copies/rxn) of EGFP, and the samples were diluted to 1/1000 (Figure 1b).

The qPCR reaction was optimised for the EGFP primers. The primers, qPCR EGFP For2: ACCACTACCAGCAGAACACC and qPCR EGFP Rev2: CCATGTGATCGCGCTTCTC, were designed to generate an amplicon of 117bp. The primers were checked theoretically for primer dimer formation and amplicon secondary structures using CLC workbench 6 and UMELT. Reaction mixtures were optimised for primer concentration, annealing temperature and elongation times (Figure 1c). The final reaction mixtures used consisted of 1x LuminoCT SYBR Green qPCR Ready Mix (Merck, Kenilworth, USA) and 200nM of each primer. The PCR profile was as follows: 95˚C hold for 20s, 30 cycles of annealing at 58˚C for 8s and elongation at 62˚C for 12s and a Melt analysis (72-95˚C). Reactions were carried out in a Rotor-Gene 3000A (QIAGEN, Venlo, Netherlands) and the data was analysed by Rotor-Gene 6000 Series Software 1.7 (QIAGEN, Venlo, Netherlands).

Data was then further analysed and normalised in excel. The graphs were drawn using GraphPad Prism® and Mann-Whitney U non-parametric tests were used to test for significance between the PsV samples and a Wilcoxon test (one sample test) was used to test for significances differences between the PsV and the VLP samples.

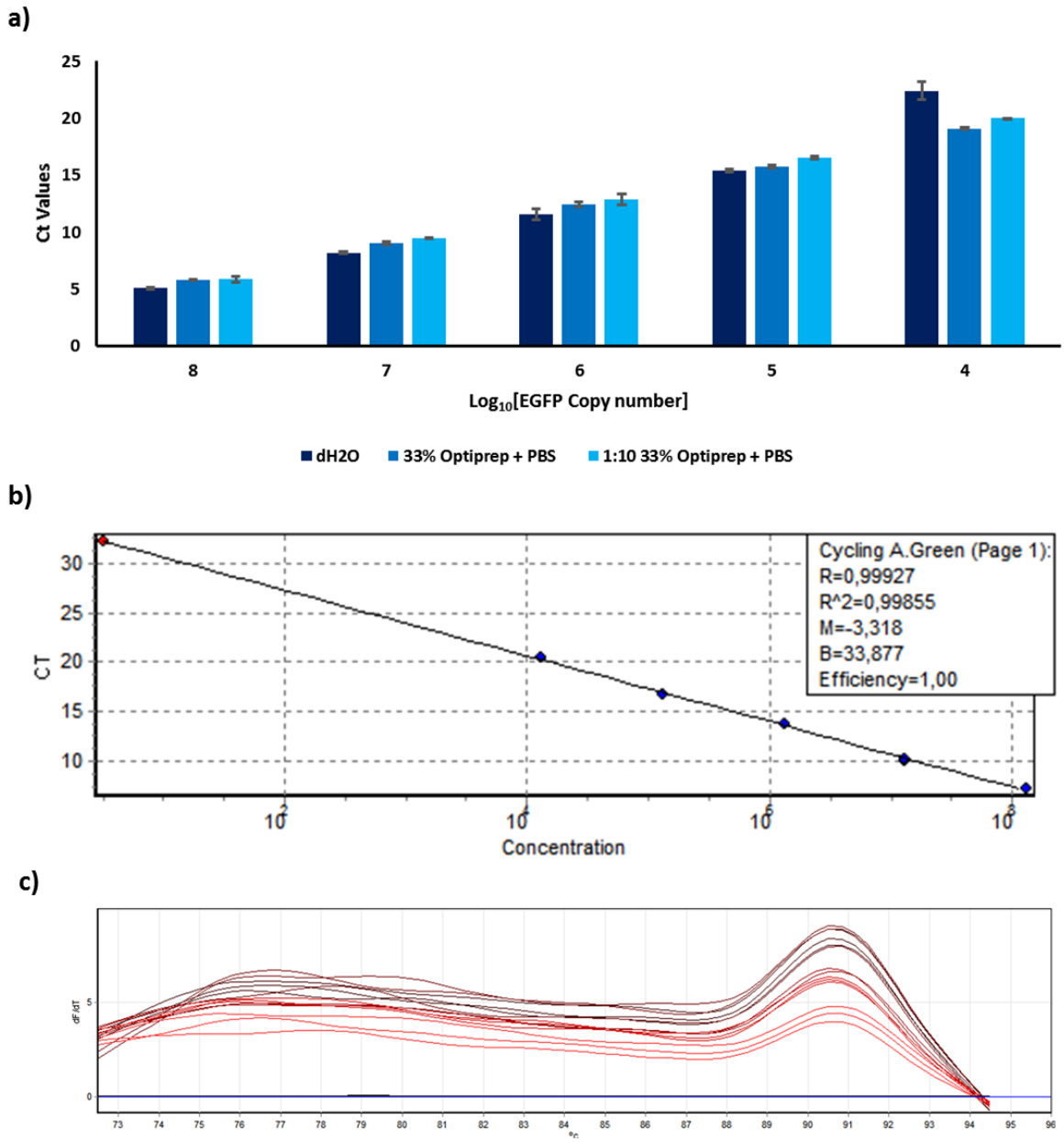

Figure 1: Optimisation of the standard curve and reaction conditions. a) Standard curve buffer optimisation. b) Standard curve analysis in 1:10 33% Optiprep+PBS buffer. R^2 value of 0.99927 indicates standard curve is statistically accurate and the efficiency of 1 indicates 100% reaction efficiency – the DNA input matches the DNA predicted values. c) Melt curve analysis of standard curve and the no template control (NTC). dCt/dt, rate of change in the change in cycle time.

4.2.3. Protein quantification

4.2.3.1. Production of L1 VLP standard curve

An L1 VLP standard curve was used to calculate the L1 concentration in the PsV samples. The standard was made by producing VLPs in plants and purifying them as described in Chapter 2. Following Western blot analysis, L1 VLP fractions 5 (33% OptiPrep) and 6 (27% OptiPrep), were selected for quantification. The VLP fractions were resolved on SDS-PAGE gels along with a five-point BSA standard,

ranging from 0.09 - 1.5 µg. Gels were stained with Coomassie Brilliant Blue ®(Merck, Kenilworth, USA) for an hour at 37˚C and then de-stained, first for an hour at 37˚ in de-stain solution and then overnight at room temperature, shaking. Gels were washed and the L1 content quantified by densitometry using the Syngene Gene Tools software and the L1 concentration for F5 and F6 were calculated in Excel.

4.2.3.2. Enzyme-linked Immunosorbent Assay quantification of PsV L1 concentration

The quantified L1 VLP fraction, was used to generate an L1 VLP Enzyme-linked Immunosorbent Assay (ELISA) standard curve. A five-point standard, ranging from 3.75-60ng of L1 was used. Standards and samples were diluted in coating buffer (10mM Tris pH 8.5) and a 100µL of each sample was added to an Immuno polysorb plate (ThermoFischer Scientific, Waltham, USA) and adsorbed overnight at 4˚C, shaking. Plates were blocked with 200µL of TBS-M (1xTBS pH7.5 and 5% non-fat dairy milk) for an hour at 37˚C and washed, four times, in TST-T (1xTBS p7.5, 0.05% Tween). Next, the plates were incubated with 100µl/well of rabbit anti-Gardasil polyclonal antisera at a 1:5000 dilution in TBS-M for 1hr at 37˚C and washed again, four times, with TST-T. Following this, the plate was incubated with 100µL of goat anti-rabbit IgG alkaline phosphatase conjugated antibody (Merck, Kenilworth, USA) at a 1:5000 dilution in TBS-M for 1hr at 37˚C and washed with 1xTBS (pH 9.0), four times. Finally, the wells were incubated with 200µL of SIGMAFAST™ p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, USA) for 30 mins in the dark and then luminescence was read at 405nm. PsVs were produced and analysed five times to account for variance. However, while 5 infiltration repeats were carried out, only the last two were purified in exactly the same way. The first three repeats were purified more dilute, with larger volume OptiPrep gradient layers. This resulted in low concentrations of particles and so the particles were concentrated for the subsequent repeats by lowering the 33% and 27% gradient layer volumes to 3ml instead of 5ml. This could not be done for a third repeat due to time and financial constraints. Data was analysed on Excel and the DNA:L1 ratios were calculated. GraphPad Prism® was used to plot graphs and carryout statistical analyses on the samples. A Mann-Whitney U non-parametric test was used to test for significance between the PsV samples and Wilcoxon test was used to test for significance between the PsV and the VLP samples across the two biological repeats.

4.2.4.Infectivity assay

HEK293TT cells were maintained as described in Section 2.2.7. Corning® Costar® TC-Treated 6 well plates (Sigma-Aldrich, Merck, Kenilworth, USA) were treated with 2ml of 0.1mg/ml poly-D-Lysine (10% (v/v) 1mg/ml Poly-D-Lysine solution (Sigma-Aldrich, Merck, Kenilworth, USA) in 1x Gibco™ DPBS) for 2 hours at 37˚C and dried for 1hr -1.5hrs at 37˚C. Thereafter, HEK293TT cells were seeded on coverslips at 150 000 cells/well. Next, 300µl of the pooled OptiPrep fractions corresponding to the 27% (fractions 3-5) and 33% densities (fractions 6 and 7) of the purified PsVs and the VLPS were added onto the cells at 1/10 dilution in DMEM. After 72 hours. The cells were lysed with NP-40 buffer (20mM Tris-HCl

pH7.4, 150mM NaCl, 1mM $MgCl_2$, 1% Nonidet-40 substitute and 10% glycerol). Bradford Assays were carried out to determine the concentration of total soluble protein (TSP) (as described in section 2.2.8.2) and equivalent TSP was loaded onto SDS-PAGE gels. SDS-PAGE gels and Western blots were carried out as described in section 2.2.8.1, using a mouse anti-GFP primary antibody (Merck, Kenilworth, USA) at 1:1000 dilution in blocking buffer and a goat anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (Abcam, Cambridge, UK) at 1:5000 dilution in blocking buffer. Differences in band intensity were detected by densitometry using the Syngene Gene Tools software. Five biological repeats were carried out but only the final two were purified in exactly the same way and so only the data of the last two repeats was used.

4.3. Results

The plant-made HPV PsVs, with 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb pseudogenomes were expressed and purified by Optiprep density gradient ultracentrifugation. HPV VLPs are known to have a density of 1.24g/mL (33% OptiPrep™). HPV PsVs, however, are slightly less dense than the VLPs, with the particles appearing at the interface of, and within, the 27% layer of the iodixanol gradient (Buck *et al.*, 2006a, Kirnbauer *et al.*, 1992, Bayer *et al.*, 2018). The iodixanol gradients were fractionated and pooled as described in section 3.2.3, to obtain one 33% pooled fraction and one 27% pooled fraction for each PsV sample. The L1 protein concentrations were quantified by ELISA (Figure 2a) and the particles in the 33% and 27% pooled fractions were analysed for DNA encapsidation by qPCR (Figure 2b). The L1 protein concentrations were used to normalise the DNA concentrations to account for batch-to-batch variation in expression and purification and to obtain an approximation of the frequencies of DNA encapsidation for each PsV sample (Figure 2c). Finally, particle infectivity was analysed by adding the PsVs onto HEK293TT cells and confirming DNA delivery by measuring EGFP expression by Western blot (Figure 3).

4.3.1.Pseudogenome encapsidation frequencies

The L1 concentration of the putative VLP (33% fractions) and PsV (27% fractions) containing fractions of the PsV samples, were quantified, by ELISA. The L1 concentrations obtained were 8-fold higher in the 33% PsV fractions than in the 27% PsV fractions ($p=0.001$). Across biological repeats, the 33% of the 5.8Kb PsV sample contained significantly more L1 compared to the 33% PsV fractions of the other PsVs.

The PsVs, with the different sized pseudogenomes, were lysed with proteinase K and the encapsidated DNA released and quantified by qPCR. The DNA concentrations (Figure 2b) were normalised using the corresponding L1 concentrations (Figure 2c) to account for batch-to-batch variation and to give an

indication of the frequency of DNA encapsidation. These DNA: L1 ratios indicated that the PsV samples contained more DNA than the VLP samples, across all biological repeats, with the VLP samples containing 10^4- 10^6 - fold lower concentrations of DNA than the PsV samples (p=0.0002, Figure 2c). Moreover, a significantly higher concentration of pseudogenome DNA, approximately 100-fold, was found in the 27% fractions compared to the 33% fractions (p=0.001, Figure 2c). A higher concentration of encapsidated pseudogenome was found in the 4.8Kb (1.5 x10^{10} copies/ng L1) and 5.8Kb (3.2 x10^9 copies/ng L1) PsVs, however this difference was not found to be significant (p=0.298). A significant difference was however found between the 4.8Kb PsVs and the 6.8Kb and 7.8Kb PsVs (p=0.012) with the 4.8Kb PsVs containing approximately 100-fold higher concentrations of DNA.

4.3.2. Particle infectivity

Particle infectivity was investigated by infecting HEK293TT cells with PsVs. Cells were lysed 72 hours post infection and probed for EGFP expression via Western blot. Overall, the infectivity data indicated that EGFP was only expressed, in HEK293TT cells, after incubation with the 27% fractions and not when the cells were incubated with the 33% fractions. The 26kDa EGFP bands were only detected in the 27% samples (Figure 3b) and not in the 33% samples (Figure 3a). The data also indicated that EGFP expression decreased as the size of the pseudogenome increased. This is clear in that the 4.8Kb PsV samples produced bands 3.8 times more intense than that of the 5.8Kb PsV samples, 6.6 times more intense than the 6.8Kb sample and 10.8 times more intense than the 7.8Kb sample (Figure 3b). Thus, overall, the samples with more encapsidated DNA were found to express more EGFP.

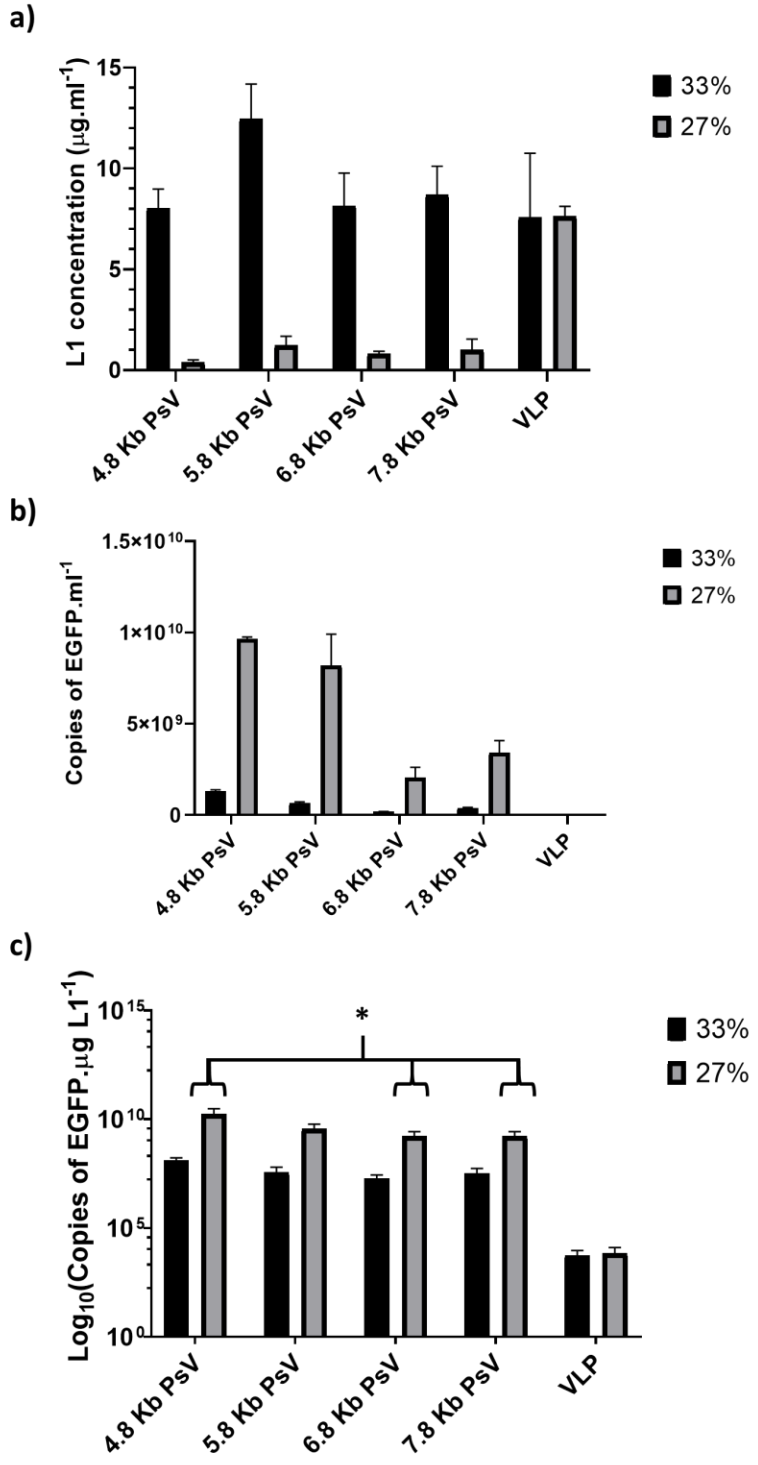

Figure 2: Quantification of encapsidated DNA. a) Quantification of L1 protein in the PsVs with different sized pseudogenome samples and the VLP control sample by ELISA. Samples were probed with rabbit anti-Gardasil polyclonal antisera at a 1:5000 dilution and a goat anti-rabbit IgG alkaline phosphatase conjugated secondary antibody at a 1:5000 dilution. b) Graph of the quantification of encapsidated DNA. Particles were broken open with proteinase K and quantified by qPCR using EGFP primers. c) Graph of DNA:L1 ratios, an indicator of the frequency DNA encapsidation. Graphs were generated using GraphPad Prism®. Error bars represent the standard deviation from the mean between two biological repeats. A Mann-Whitney U non-parametric test was used to test for significance between the PsV samples across the last two biological repeats and Wilcoxon test to test for significance between the PsV and the VLP samples across the last two biological repeats.

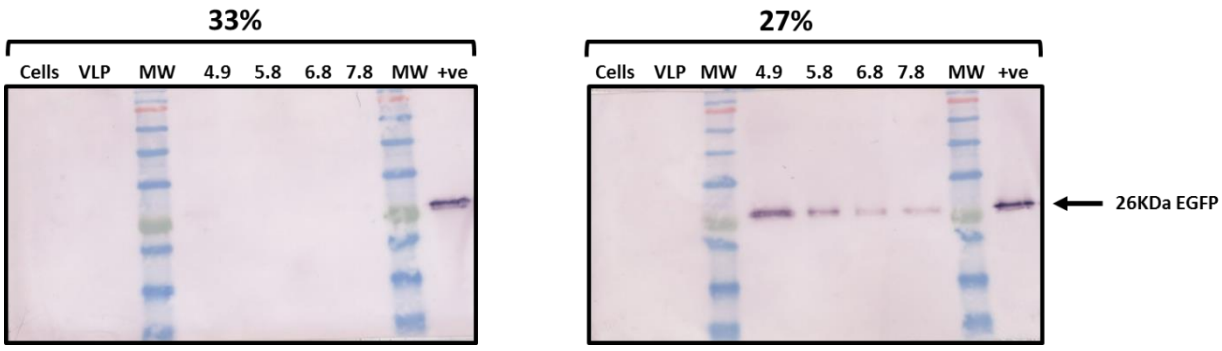

Figure 3: Confirmation of PsV infectivity. Representative images of western blot analyses of EGFP expression and DNA delivery from the PsVs containing the 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb target DNAs. Two-hundred microlitres of the 33% (a) and 27% (b) of each of the PsVs were incubated HEK293TT cells for 4 days. Cells were then lysed with NP40 buffer and equal total soluble protein was loaded onto the SDS-PAGE gel. Primary antibody; 1:1000 anti-GFP (preabsorbed with HEK293TT cells lysate), secondary antibody, 1:5000 anti-mouse antibody conjugated to alkaline phosphatase. Blot was left to detect for 1 hour.

4.4. Discussion

While HPV L1 DNA binding is essential for encapsidation, the efficiency of encapsidation still relies on the size of the pseudogenome being optimal. Several studies have confirmed that HPV does not require an encapsidation signal but rather that encapsidation efficiency is based on DNA size (Stauffer *et al.*, 1998, Touze and Coursaget, 1998, Zhao *et al.*, 1998, Roden *et al.*, 1996, Cerqueira *et al.*, 2016). The only PV type that does not fully align to this model is BPV1, as a region of its genome has been found to enhance the encapsidation of DNA by an L1/L2 particle (Zhao *et al.*, 1999). Multiple studies have shown that extracellularly produced, mammalian, and yeast cell- produced PV PsVs encapsidate DNA between 5-8Kb, with the smaller pseudogenomes resulting in the better encapsidation and/or transduction (Stauffer *et al.*, 1998, Touze and Coursaget, 1998, Zhao *et al.*, 1998, Roden *et al.*, 1996, Cerqueira *et al.*, 2016, Buck *et al.*, 2004). The smallest sized pseudogenome ever successfully encapsidated appears to be 4.7 Kb (De Los Pinos, 2013, Bayer *et al.*, 2018). Although one patent does state that PV PsVs can encapsidate DNA as low as 2Kb, this was directly contradicted by Zhao *et al.,* (1998), who show that DNA below 3.5Kb could not be encapsidated by BPV 1. The largest pseudogenome ever encapsidated was 8.6Kb (Zhao *et al.*, 1998); however, most studies suggest that plasmids close to 8Kb in size are encapsidated less efficiently than those below 7Kb (Cerqueira *et al.*, 2016, Buck *et al.*, 2004, Touze and Coursaget, 1998, Stauffer *et al.*, 1998). Thus far studies have shown that DNA encapsidates most optimally around 5Kb in size, with Touze and Coursaget (1998) and Buck *et al*. (2004) indicating that 5Kb and 5.9Kb pseudogenomes, respectively, are more frequently encapsidated by PV particles than those above 6Kb. However, the optimal size ranges for plant-made PV PsVs have not yet been investigated. Therefore, this study aimed to determine the optimal pseudogenome size for the production of functional plant-made HPV PsVs, using pseudogenomes of 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb.

Mammalian cell produced HPV VLPs are known to have a density of 1.24g/mL in OptiPrep™ (33%). HPV PsVs are slightly less dense than the VLPs, with the particles appearing at the interface of and/or within the 27% layer of the iodixanol gradient (Buck *et al.*, 2006a, Kirnbauer *et al.*, 1992, Bayer *et al.*, 2018). Clear differences in the amount of protein and encapsidated DNA was observed between the 33% and 27% fractions. L1 concentrations were approximately 8-fold higher in the 33% fractions compared to the 27% fractions (Figure 2a) but the corresponding DNA concentrations were approximately 100-fold higher in the 27% fractions compared to the 33% fractions (Figure 2b and c). This result was confirmed after Western blot analyses indicated that EGFP was only expressed, in HEK293TT cells, after incubation with the 27% fractions and not when the cells were incubated with the 33% fractions (Figure 3). Thus, only the particles in the 27% fractions were able to deliver DNA and therefore the particles found in the 27% fractions are, mostly, functional PsVs whereas those in the 33% fractions are most likely VLPs. This result is consistent with other literature on mammalian cell produced HPV PsVs (Bayer *et al.*, 2018, Lamprecht *et al.*, 2016, Buck *et al.*, 2004).

Results from this study indicated that smaller pseudogenomes are more frequently encapsidated than larger pseudogenomes. DNA concentrations were normalised against the L1 protein concentrations in each fraction, to account for batch-to-batch variation in expression and purification and to give an indication of the frequency of DNA encapsidation. PsV samples contained significantly more DNA than the VLP samples, with the VLP samples containing 10^4- 10^6 - fold lower concentrations of DNA than the PsV samples (Figure 2b), across all biological repeats. VLPs often contain random DNA, as PV particles are able to encapsidate DNA, less than 8Kb, without a DNA encapsidation signal. VLPs differ from PsVs which contain DNA of the researcher's choosing. Therefore, the presence of DNA signal in the VLP samples is most likely an artefact of the purification process, as agarose gel electrophoresis analyses show no EGFP expression bands and the pRIC-3.0 vector only samples contained similar levels DNA signal as the VLP samples (data not shown). No significant difference was found between the 6.8Kb PsVs and the 7.8Kb PsVs. A higher concentration of encapsidated pseudogenomes was found in the 4.8Kb PsVs (1.5 x10^{10} copies/ng L1) compared to 5.8Kb PsVs (3.2 x10^9 copies/ng L1), however, statistical analyses revealed that this difference was not significant across the last two biological repeats, even though this difference occurred in 3 out of 4 total biological repeats. The lack of significant difference between the 4.8Kb PsVs and the 5.8Kb PsVs in the last biological repeat may be due to the contribution of free DNA in the gradient. Note, while the data for the concentrated and dilute biological repeats could not be collated together, the data can still be compared separately and were found to follow the same trend of DNA encapsidation. Empty vector controls (data not shown) indicate that there is some free DNA in both the 33% and 27% OptiPrep fractions. DNase was not used in this experiment as the DNAse I activity was found to be inefficient in the OptiPrep buffer high-salt

PBS buffer. Another reason DNAse was avoided was that it may degrade DNA stuck to the outside or protruding from the particle, as this DNA can still be transduced although most likely not very efficiently. The overall encapsidated DNA concentrations for these plant-made PsVs, were found to be approximately 1000-fold higher than those produced in mammalian cells (2x 10^8 DNA copies/ng L1) and with cell-free assembly methods (6x 10^8 DNA copies/μg L1, Cerqueira *et al.,* in 2016). This is unexpected as plant HPV PsV transduction is usually lower than that of mammalian PsVs (personal communication with Megan Hendrikse, Alta Van Zyl and Renate Lamprecht, BRU, University of Cape Town, South Africa). Therefore, this result may indicate that plant-made HPV PsVs do not infect HEK293TT cells as well as mammalian cell produced HPV PsVs. This may be because the mechanisms of pseudogenome encapsidation and assembly may be slightly different and/or less efficient *in planta,* possibly allowing DNA to protrude from the particle or bind to its outside.

Although DNA encapsidation is a good indicator of a functional PsV, it is insufficient to determine PsV functionality. The transduction efficiency of an HPV PsV is the best indicator of its functionality. The data indicated that EGFP expression decreased as the size of the pseudogenome increased. This is clear in that the 4.8Kb PsVs produced bands 3.8 times more intense than that of the 5.8Kb PsVs, 6.6 times more intense than the 6.8Kb PsVs and 10.8 times more intense than the 7.8Kb PsVs. Additionally, these data indicated that packaging of different sized DNA does not affect the infectivity of the PsV. This is evident in that the DNA content is highest in the 4.8Kb sample, even though this difference was found to be statistically insignificant, and consistently decreases as the size of the target DNA increases (Figure 2b,c) and that this pattern is identical to the pattern of EGFP expression (Figure 3b). Thus, the more DNA that was packaged, the more DNA was delivered and therefore more EGFP expression was obtained. This data therefore also shows that the 4.8Kb pseudogenome, across all biological repeats, was able to transfer the most DNA into the HEK293TT cells.

Altogether, the data indicates that 4.8Kb is the most optimal DNA size for HPV PsV production *in planta.* This is clear in that the DNA encapsidation as well as the transduction efficiency of these PsVs was much higher than that of the PsVs with larger pseudogenomes (Figure 3). There is a small and statistically insignificant difference in encapsidated DNA between the 4.8Kb and 5.8Kb PsVs (Figure 2c) but a marked difference in the transduction between these two PsV samples (Figure 3b). The reason for this could be that a similar amount of DNA is encapsidated for both PsV samples but that the 4.8Kb pseudogenome is better encapsidated than the 5.8Kb pseudogenome, with less protrusion from the capsid and therefore less instability (Fligge *et al.*, 2001, Cerqueira *et al.*, 2016). Thus, this data suggests that the 4.8Kb pseudogenome is more frequently and possibly more stably encapsidated and is better transduced than other, larger pseudogenome PsVs. These results are consistent with the literature on mammalian cell produced and cell-free assembled PV PsVs which suggest that smaller

DNA, closer to 5Kb in size, encapsidate and/or transduce better than PsVs with pseudogenomes closer to 8Kb (Stauffer *et al.*, 1998, Touze and Coursaget, 1998, Zhao *et al.*, 1998, Roden *et al.*, 1996, Cerqueira *et al.*, 2016). The size of the pseudogenome currently being used for plant-made HPV PsVs is 4.8Kb. This data therefore validates that the size of the pseudogenome size currently being used for plant-made HPV PsVs produces the optimal amount functional PsVs, in terms of pseudogenome size (Lamprecht *et al.*, 2016), and that size needs to be taken into consideration when designing future pseudogenomes for *in planta* production of HPV PsVs.

This study could be taken further, by fully quantifying the encapsidation and transduction efficiencies of mammalian and plant-made HPV PsVs containing the 4.8Kb pseudogenomes. This comparison is important as this study, and others done in our lab, have shown varying yields of plant HPV PsV L1,encapsidated DNA and PsV to VLP ratios, compared to mammalian HPV PsVs (personal communication with Megan Hendrikse and Alta Van Zyl, BRU, University of Cape Town, South Africa). Future work could involve using alternative reporter genes and more quantitative downstream assays such as flow cytometry or FACS. Previous studies (personal communication with Megan Hendrikse and Alta Van Zyl, BRU, University of Cape Town, South Africa also show that mammalian HPV PsVs, generally, have a higher transduction efficiency than plant-made PsVs. Thus, there is still a need to properly quantify and compare mammalian HPV PsVs to plant HPV PsVs, data which could aid in identifying areas of HPV PsV *in planta* production that still require optimisation.

Moreover, investigations into the stability of mammalian HPV PsVs compared to plant-made HPV PsVs could also aid in the optimisation of the *in planta* production of HPV PsVs. Various studies seem to suggest that viral particles are stabilised by encapsidation of DNA (Fligge *et al.*, 2001, Digiuseppe *et al.*, 2017a). However, research in BRU (University of Cape Town, unpublished) has shown that plant-made HPV PsVs become non-functional after a few weeks whereas the plant-made HPV VLPs and mammalian HPV PsVs remain stable at -80°C for several months. Thus, the plant-made HPV PsVs may not keep their particle structure so their ability to infect and deliver DNA is greatly diminished with time. The reason for this could be that the PsVs were not efficiently assembled and thereby the DNA was not completely encapsidated inside the particle but is rather stuck to the outside or may be protruding from the plant-made HPV particle. DNA exposed on the outside of the particle would not be protected and is therefore open to sheering by ice crystals, after storing at low temperatures and freeze thawing, and to degradation over time by plant DNAses in the sample. This theory is supported by the TEM data obtained in section 3.3.2 which shows that plant-made HPV particles, unlike mammalian PsVs, are often irregular and vary in size, which could indicate that the particles have not assembled efficiently and therefore the pseudogenomes are not being fully encapsidated. This would indicate that plant PsVs do not “stably” encapsidate as well as the mammalian PsVs and therefore

further, in depth, comparison of encapsidation and transduction between mammalian and plant-made HPV PsVs is necessary. To confirm this theory, a qPCR would need to be done on the same sample of mammalian and plant HPV PsVs, over a course of a few months, to determine differences in DNA degradation and therefore encapsidation efficiencies between them. Another way to investigate whether the mammalian and plant-made HPV PsVs may be assembling differently and/or with different efficiencies would be to visualise leaf sections to ensure that the particles are being made in the leaf and upon confirmation of this, optimise the maturation and regularity of the particles by adding oxidising reagents to the purification buffers (Buck *et al.*, 2005a, Cardone *et al.*, 2014) and then comparing particle formation, DNA encapsidation and transduction between mammalian and plant-made HPV PsVs. Therefore, the next step in plant-made PsV optimisation would be to compare mammalian and plant-made PsVs containing the 4.8Kb pseudogenomes' for particle formation, DNA encapsidation frequency and transduction efficiency in order to highlight the areas of *in planta* HPV expression that still require optimisation.

4.5. Conclusion

In conclusion, the 4.8Kb pseudogenome was found to be more efficient for producing plant-made HPV PsVs compared to the 5.8Kb – 7.8Kb pseudogenomes, as the 4.8Kb pseudogenome DNA was more frequently encapsidated and resulted in higher transduction efficiencies. Going forward, mammalian and plant-made HPV PsVs, containing 4.8Kb pseudogenomes, need to be compared in order to identify areas of improvement for HPV PsV production *in planta*.

Chapter 5: Conclusions and future work

Cervical cancers are the second most frequent cancers in women aged 15 to 44 years (Bruni *et al.*, 2015b, Ferlay J, 2013). In 2018, it was estimated that 570 000 cases of cervical cancer arose globally, of which 311 000 were fatal (Bray *et al.*, 2018). Studies show that HPV has the highest burden and the most fatalities in low income countries (Ferlay J, 2013), whereas high income countries, such as Australia, are close to eradicating HPV (Bralsford and Jamieson, 2019, Bray *et al.*, 2018, Serrano *et al.*, 2018, Albeck-Ripka, 2018). There are currently three L1 VLP-based prophylactic vaccines on the market, Gardasil™, Gardasil 9™and Cervarix®. However, these vaccines do not cover the full spectrum of high-risk HPVs (16, 18, 31,35,39,45,51,52,56,58,66, and 68) and do not function therapeutically. Thus, more research is still needed to broaden the spectrum of protection against HPV infection.

HPV PsVs have various applications. They are used in PBNAs to test neutralising antibody responses of VLP vaccines and this makes them important for the testing of new prophylactic vaccines (Pastrana *et al.*, 2004b). Currently, there are no therapeutic vaccines on the market. HPV PsVs are able to deliver DNA to cutaneious and mucosal epithelia as well as target specific cancer cells (Kines *et al.*, 2009, Kines *et al.*, 2016) and have been shown to be able to deliver DNA into cells for targeted DNA vaccination and gene therapy (Graham *et al.*, 2010, Peng *et al.*, 2010). Thus, HPV PsVs are important for the development of both prophylactic and therapeutic HPV vaccines.

Lamprecht *et al.* (2016) showed that HPV 16 PsVs can be produced in plants and these PsVs were found to function similarly to mammalian PsVs in PBNAs. This is advantageous as plant expression systems are less likely to be contaminated with mammalian pathogens and are a potentially cheaper and more easily scalable means of PsV production (Nandi *et al.*, 2016, Mir-Artigues *et al.*, 2019, Rybicki, 2009). The use of plant expression systems could therefore decrease the cost of testing new prophylactic VLP vaccines and the cost of production of HPV PsV-based therapeutic vaccines.

Several studies have shown that PV particles encapsidate pseudogenomes between 5-8Kb, with the smaller sizes encapsidating better than the larger sizes (Stauffer *et al.*, 1998, Touze and Coursaget, 1998, Zhao *et al.*, 1998, Roden *et al.*, 1996, Cerqueira *et al.*, 2016). Therefore, in order to optimise the amount of functional plant-made HPV PsVs obtained, this study investigated what size of pseudogenome is optimal for plant-made HPV PsV encapsidation.

This study used EGFP containing pseudogenomes of 4.8Kb, 5.8Kb, 6.8Kb and 7.8Kb (Chapter 2). These pseudogenomes were expressed and purified (Chapter 3) and analysed for encapsidation and transduction efficiencies (Chapter 4). The results of this study showed that the 4.8Kb pseudogenome was encapsidated and transduced, by the plant-made HPV particles, more efficiently compared to all the other pseudogenome sizes tested, with the efficiencies decreasing as the size of the

pseudogenomes increased. This result is consistent with literature on mammalian cell and extracellular produced PV PsVs (Stauffer *et al.*, 1998, Touze and Coursaget, 1998, Zhao *et al.*, 1998, Roden *et al.*, 1996, Cerqueira *et al.*, 2016). Thus, pseudogenome size should be considered during the design of a plant-made HPV PsVs.

Future work could focus on the comparison between mammalian and plant-made HPV PsVs. This could allow for the identification of areas of optimization for the *in planta* production of HPV PsVs. These PsVs could also be deep sequenced to ascertain what non-target DNA's are being encapsidated. Mammalian cell produced HPV PsVs are more likely to contain harmful DNA (mammalian oncogenes) than the plant-PsVs, as plant genes, encoding plant proteins, will most likely not affect mammalian cells. This could therefore provide further evidence as to whether plant PsVs are indeed a safer alternative to mammalian cell produced HPV PsVs.

Future work could also focus on the optimisation of the plant-made HPV PsV purification process. The process could be optimised by adding a maturation step containing oxidising reagents to improve PsV stability (Buck *et al.*, 2005a, Cardone *et al.*, 2014). Moreover, the purification process is not entirely sterile, as the plants are not sterile, and so contamination of the plant HPV PsV sample is always a risk. The likelihood of purification sterility could be increased by adding low doses of antibiotics to the purification buffers that will not negatively affect the downstream applications e.g. Anti-myotic/Anti-mycotic. Following expression and purification, the next step to optimisation would be to investigate the particle more thoroughly. This could include testing the PsVs in PBNAs and testing infectivity in NIKs or primary cell lines as more relevant tests of functionality.

HPV PsVs are a promising candidate for gene therapy and delivery of DNA vaccines. However, one caveat to using a high-risk strain like HPV 16 or 18 to deliver the vaccine is pre-existing immunity due to vaccine intake or cleared infections. Thus, alternative strains like HPV 35 could be used, although, this may soon form part of a broader range prophylactic vaccine. The other option is to use non-human HPV strains. Research by Bayer *et al.,* (2018) shows that it is possible to make several types of non-human PsVs in HEK293TT cells, with the Puma concolor papillomavirus-1 strain showing particular promise in terms of *in vivo* transduction efficiencies. Thus, future work could also focus on the production of non-human HPV PsVs *in planta* for possible use as DNA delivery vehicles for targeted vaccine and gene therapies.

Finally, this research could contribute towards cheaper testing of new HPV VLP vaccines and virus life cycle research, making them more affordable and accessible to low income countries. It could also lead to the production of cost-effective plant-made therapeutic DNA vaccines.

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