HPV Pseudovirion Production in Plants

Paul Kennedy

Thesis presented for the degree of Master of Science

in the department of Molecular Biology under the supervision of

Ed Rybicki, Inga Hitzeroth and Ann Meyers

University of Cape Town

February 2013

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Plagiarism Declaration

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is my own.
2. I have used the Plant Biotechnology Journal referencing style for citation and referencing. Each contribution to, and quotation in this dissertation from the work(s) of other people has been contributed, and has been cited and referenced.
3. This dissertation is my own work.
4. I have not allowed, and will not allow, anyone to copy my work.

Signature:_____

Date:_____

Acknowledgements

I would like to formally acknowledge the following people and institutions:

My supervisors, Prof. Ed Rybicki, Dr. Inga Hitzeroth and Dr. Ann Meyers, for their tireless advice, support and guidance for the last two and a half years, as well as financial support.

Mark Whitehead, Guy Regnard, Aleyo Chabeda, Alta van Zyl, Suzanne Huddy, and Sandiswa Mbewana for endless support and technical advice.

Prof. Anna-Lise Williamson for the use of her group's facilities.

Cathy Pineo and Bruce Allan for technical assistance with PBNA work.

Mohammed Jaffer for assistance with the electron microscopy work.

Dr John Schiller for supplying the HEK293TT cells and pYSEAP vector, Dr. Rainer Fischer for supplying the pTRAc vector, and Dr. Neil Christensen for supplying monoclonal antibodies.

Jessica Hitchcock and my family for seeing only the best in me, when I couldn't.

UCT, the National Research Foundation and the Poliomyelitis Research Foundation for financial support.

Medical Research Council for funding the project.

Abstract

Human papilloma virus (HPV) infection is the most common etiological agent of cervical cancer, the most common cancer in women in Africa. The lifecycle of HPV has historically made the virus difficult to culture *in vitro*, and this has hindered the study of the virus, as well as development of vaccines. The development of synthetic HPV particles, such as virus-like particles (VLPs) and more recently pseudovirions (PsVs), has allowed for unprecedented insights into the lifecycle and immunology of this virus. This has led to the development of two currently available vaccines, namely Cervarix™ and Gardasil®. Cervarix offers protection against high-risk HPV types 16 and 18, while Gardasil offers further protection against types 6 and 11. Both of these vaccines are based on major capsid protein L1 Virus-like particles (VLPs). While these vaccines show no loss of efficacy, further work is underway to develop a second generation HPV vaccine that is cheap, stable and displays cross-neutralising activity across a broader range of HPV types.

The recent efficient methods for intracellular production of HPV PsVs encapsidating non-papillomaviral DNA (pseudogenomes) has allowed for development of a robust and sensitive pseudovirion-based neutralisation assay (PBNA), which has become the gold standard neutralisation assay for the testing of candidate HPV vaccines. The currently accepted PsV production method utilises mammalian cell culture to produce HPV PsVs, encapsidating a SEAP reporter plasmid, at high titres. While this is an effective method of PsV production, mammalian cell culture is expensive and time-consuming.

Transient recombinant protein expression in plants offers a rapid and cost-effective alternative to mammalian cell culture. Here, we developed a method of high-titre HPV PsV production in plants. The autonomously replicating plant vector, pRIC3, was modified to include mammalian reporter cassettes encoding luc or SEAP, for the production of reporter pseudogenomes DNA in plants by *Agrobacterium*-mediated transient expression. The SEAP and luc cassettes were introduced into pRIC3 upstream of the plant cassette, which was included only to increase the final pseudogenome size for efficient packaging into PsVs. The SEAP cassette was also introduced into pRIC3 in place of the plant cassette, to form a smaller pseudogenome. Thus three vectors were created, namely pRIC3-mSEAP+ (6.4Kbp pseudogenome), pRIC3-mluc+ (7.4Kbp pseudogenome), and pRIC3-mSEAP (4.8Kbp pseudogenome), which would produce pseudogenomes that covered the full range of plasmid sizes incorporated by assembling HPV capsid proteins *in vivo*. All three replicating vectors demonstrated the formation of a replicon, and autonomous replication, in *Nicotiana benthamiana* plants. Each of these vectors were co-infiltrated with the non-replicating transient plant expression

constructs pTRAc-hL1 and pTRAc-hL2, which encode human-codon optimised forms of HPV-16 major and minor capsid proteins, respectively. It was expected that encapsidation of replicon DNA as a pseudogenome into assembling HPV particles would result in the production of HPV PsVs *in planta*. In addition, L1 and L2 were expressed in the absence of replicon DNA to form L1/L2 VLPs. Particles were extracted from plant material at four days post-infiltration, using a modified VLP extraction protocol. HPV particles were separated on the basis of isopycnic caesium chloride density gradient ultracentrifugation, dialysed against high-salt PBS and identified by fractionation and probing with an anti-L1 antibody. Particles corresponding to the buoyant density of pseudovirions were seen in samples with or without replicon DNA. Western blotting showed that all particles had incorporated both L1 and L2 proteins. Particles were digested with proteinase K to release encapsidated pseudogenome DNA and PCR confirmed the presence of replicon-specific DNA in each PsV. Electron microscopy confirmed the presence of HPV-16 PsVs in all samples.

To test whether plant-produced HPV-16 PsVs could be used in pseudovirion-based neutralisation assays, mammalian cells were pseudoinfected with purified mSEAP, mSEAP+ or mluc+ PsVs. mSEAP and mluc+ PsVs elicited a reporter gene response in mammalian cells 72 hours post-infection using SEAP and luciferase assays, respectively, while mSEAP+ PsVs showed no reporter gene expression in mammalian cells. PsVs incubated with a known HPV-16 neutralising antibody showed partial neutralisation of mSEAP PsVs and complete neutralisation of mluc+ PsVs

To our knowledge, this is the first demonstration of production of HPV PsVs in plants, and their use in a PBNA. Further, it is the first demonstration of production of HPV L1/L2 VLPs in plants. While much work remains to improve plant production and purification methods of PsVs, as well as mammalian expression following PsV pseudoinfection, this is an important step towards a new method of PsV production.

Table of Contents

Abbreviations

PsV	Pseudovirion
VLP	Virus-like Particle
QV	Quasivirion
SEAP	Secreted Alkaline Phosphatase
luc	Luciferase
BLAM	B-lactamase
HPV	Human Papillomavirus
CIN	Cervical Intraepithelial Neoplasia
NLS	Nuclear Localisation Signal
ORF	Open Reading Frame
DC	Dendritic Cell
MHC	Major Histocompatibility Complex
TLR	Toll-Like Receptor
GDV	Gene Delivery Vehicle
shRNA	Short hairpin RNA
HIV	Humun Immunodeficiency Virus
RSV	Respiratory Syncytial Virus
SIV	Simian Immunodeficiency Virus
OVA	Ovalbumin
CTL	Cytotoxic T-lymphocyte
PBNA	Pseudovirion-based Neutralisation Assay
GMP	Good Manufacturing Practice
TMV	Tobacco Mosaic Virus
PVX	Potato Virus X
CP	Coat Protein
MP	Movement Protein
Rep/RepA	Replication Associated Proteins
HBsAg	Hepatits B Surface Antigen
NDV	Newcastle Disease Virus
HBV	Hepatitis B Virus
TSP	Total Soluble Protein
BeYDV	Bean Yellow Dwarf Virus
ssDNA	Single-stranded DNA
RCR	Rolling Circle Replication
SIR	Small Intergenic Region
LIR	Long Intergenic Region
CaMV	Cauliflower Mosaic Virus
MCS	Multiple Cloning Site
CMV	Cytomegalovirus
LB	Left Border
RB	Right Border
T-DNA	Transfer-DNA

EGFP	Enhanced Green Fluorescent Protein
Dpi	Days Post-Infiltration
PCV	Porcine Circovirus
CLE	Conserved Late Element
Pac	Puromycin resistance gene
BGH	Bovine Growth Hormone
polyA	Polyadenylation signal
SV40	Simian Virus 40
DMEM	Dulbecco's Modified Eagles Medium
FCS	Foetal Calf Serum
NEAA	Non-Essential Amino Acids
cIAP	Calf Intestinal Alkaline Phosphatase
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
OE-PCR	Overlap Extension Polymerase Chain Reaction
Bla	Ampicillin resistance gene
ELISA	Enzyme-linked Immunosorbent Assay
IgG	Immunoglobulin G
BPV	Bovine Papillomavirus
CPMV	Cowpea Mosaic Virus
OD	Optical Density
TEM	Transmission Electron Microscope
BiP	Binding Protein

Chapter 1: Literature Review

1.1. Introduction

Human Papillomaviruses (HPV) are a group of viruses in the family *Papillomaviridae.* They are non-enveloped, double-stranded DNA viruses, with a genome of approximately 8Kb (de Villiers et al., 2004). The genome contains 8 open reading frames (ORFs) encoding six early genes E1, E2, E4, E5, E6, and E7, and two late genes L1 and L2 (Figure 1.1). L1 and L2 are the major and minor capsid proteins, respectively. L1 forms pentamers, or capsomeres, that assemble along with minor capsid protein L2 to form a T=7 icosahedral lattice (See Fig. 1.1B) (Conway and Meyers, 2009).

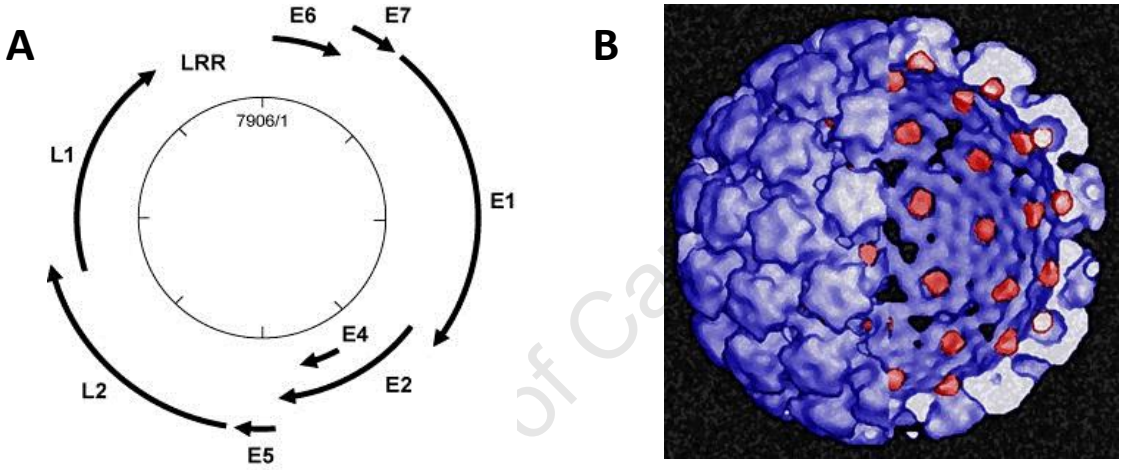


Figure 1.1| HPV structure and genome organisation (A) genome map of HPV-16. E1, E2, E4 E5 E6 and E7 are early genes, L1, the major capsid protein, and L2, the minor capsid protein, are late genes. LRR, long regulatory region (Reproduced from Gerard, 2006). (B) Model of outer surface structure of HPV-16 (L1 in blue), with cut-away showing L2 (red). Adapted from Buck et al, (2008).

There are 16 genera in the family *Papillomaviridae*; viruses in five of these genera – Alpha, Beta, Gamma, Mu and Nu – infect the squamous and cutaneous epithelia of humans and are referred to as HPVs (de Villiers et al., 2004; zur Hausen, 2000). Within these genera there are over 200 types identified to date, which are classified based on L1 major capsid protein sequence homology (Conway and Meyers, 2009; de Villiers et al., 2004). These can be divided into those that cause benign lesions, known as "low-risk" types, and those that cause malignant lesions, known as "high-risk" types. HPV-16 and HPV-18 are two high-risk types responsible for the vast majority of cervical cancers (Bosch et al., 2008). Infection with a high-risk HPV type is now considered to play a causative role in the progression to cervical neoplasia (Walboomers et al., 1999). Cervical cancer is the second most common cancer in women worldwide; it is of particular concern in developing countries, where

the lack of regular cervical screening results in a far higher burden of disease and mortality than in developed countries (Ngoma, 2006).

The prevalence and persistence of HPV, as well as cervical cancer, worldwide, has been widely documented (Burchell et al., 2006; Crow, 2012; Kawana et al., 2009; Walboomers et al., 1999). Recent estimates suggest that approximately 10.4% of women with normal cervical cytology harbour an HPV infection, while the overall estimate of infected males stands at approximately 7.9% (Burchell et al., 2006; Partridge and Koutsky, 2006). The majority of cervical HPV infections that reach the stage of low-grade lesions, or Cervical Intraepithelial Neoplasia (CIN) 1, are cleared by the body within 1-2 years of infection (Crow, 2012; Hopfl et al., 2000; Jenson et al., 1991). However, persistent infection causes spontaneous progression to neoplasia in 1 in 200-300 women if left untreated (Kawana et al., 2009).

Several key challenges to the efficient prevention of HPV transmission and treatment of infections have been identified, which if solved, would pave the way to a significant reduction of HPV- induced cervical carcinomas. These include, but are not limited to:

a) the development of a cheap and effective method to produce biologically sound and infectious HPV virions for testing and development of vaccines
b) identification of a reliable immune correlate of protection
c) identification of broadly cross-neutralising epitopes for vaccination against multiple HPV types
d) an overall decrease in cost of testing and vaccine production, to better confront the burden of cervical cancer in developing countries.

While several of these challenges have been solved, at least in part, much remains to be done to decrease the occurrence of HPV-induced cervical cancer, as well as other HPV-related diseases. This review will examine the current state of progress in addressing these challenges, with a particular focus on the production of synthetic virions for testing and development, and a reduction in overall vaccine development costs.

1.2. HPV replication and assembly

1.2.1. HPV infection

HPVs are extremely effective infectious agents, in that they induce only chronic infections, and rely almost wholly on host cell replication and differentiation to spread (Stanley, 2008). Invasive HPV enters the human body through microwounds in the epithelia, which expose the basal epithelium to invasion (see Figure 1.2A) (Longworth and Laimins, 2004). The exact mode of cell entry is unknown, although it is known that cell surface heparan sulphate is required (Giroglou et al., 2001; Joyce et al., 1999). It is has been shown that both the C- and N-terminus of the L2 minor capsid protein possess a nuclear localisation signal (NLS); further evidence shows that L2 is implicated in nuclear localisation of the virus once inside the cell (Fay et al., 2004; Holmgren et al., 2005; Pereira et al., 2009). Once inside the nuclei of the basal epithelia, the viral early ORFs (which encode proteins E1, E2, E4, E5, E6 and E7) are transcribed in a viral gene expression cascade(Fig. 1.2 B)(Conway and Meyers, 2009). E1 and E2 are the first proteins transcribed, and are responsible for the recruitment of cellular replicative machinery (Conger et al., 1999; Mohr et al., 1990). These early events result in between 20 and 100 episomal copies of the HPV minichromosome per cell (Stanley, 2008). Following this step, infected keratinocyte stem cells follow their normal lifecycle. Cells migrate through the epithelial strata while undergoing a process of terminal differentiation (Pang et al., 1993). In contrast with non-infected keratinocytes, HPV-infected keratinocytes remain mitotically active after

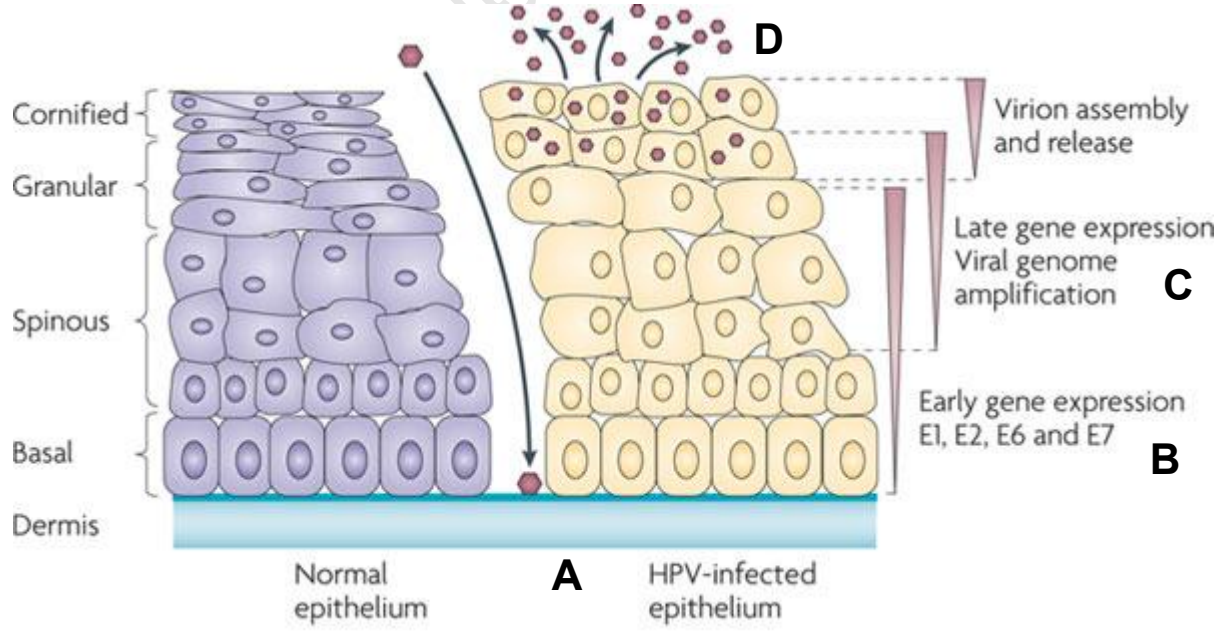


Figure 1.2 | Life cycle of HPV infection (A) HPV entry into the basal stem cells. (B) Episomal replication via early gene expression. (C) Viral genome amplification and late gene expression. (D) Virion assembly and release. Adapted from Moody and Laimins (2010).

detachment from the basal layer, due to the activity of the E7 oncoprotein (Morris et al., 1993). Once the cells are terminally differentiated, this signals the transcription of the L1 and L2 ORFs under control of the late viral promoter (Fig. 1.2C) (Barksdale and Baker, 1993). L1 and L2 proteins then assemble and encapsidate the HPV genome to form infectious virions (Fig. 1.2D).

1.2.2. Virion structure

Fully assembled HPV virions are composed of 360 L1 molecules, and a less clear number of L2 molecules. Current best estimates are that the ratio of L1 to L2 is 30:1, although a estimates of as much as 5:1 have been suggested, based on stochiometric analysis and in vitro production methods (Conway and Meyers, 2009; Finnen et al., 2003; Pereira et al., 2009). The L1 protein forms capsomeres – radially symmetric pentamers of the major capsid protein with a central "hole" or indentation (see Fig 1.1B). There are 72 capsomeres in a mature capsid, with a T=7 icosahedral symmetry (Doorbar, 2005). Cryoelectron microscopy analysis of HPV-16 pseudovirions by Buck et al. (2008) and Trus et al. (1997) has shown individual L2 molecules to be associated with the "holes" in the assembled capsomeres, and that the L1:L2 ratio varies based on concentration of L2 when produced *in vitro*. Their data suggests that an L2 molecule may associate with every capsomere in the virion, resulting in a ratio of 5:1, although evidence from in vivo assembly studies suggests that this is unlikely to be the case in natural virion assembly (Pereira et al., 2009). Virions are formed via intercapsomeric hydrophobic interactions of helices 2, 3 and 4 of the C-terminal region of L1 (Modis et al., 2002). This binding is stabilised by several disulphide bonds, both intra- and intercapsomeric (Buck et al., 2005b; Fligge et al., 2001; Sapp et al., 1998).

1.2.3. Virion assembly

Capsid assembly of HPV virions *in vivo* has not been fully elucidated, in part due to the difficulty of culturing these viruses. However, recent developments in *in vitro* production methods, examined in more depth in Section 1.3., have allowed a new level of understanding of the assembly process and the requirements for production of infectious virions.

The exact molecular interactions required for virion assembly remain unclear (Conway and Meyers, 2009). Replication of the HPV genome has been shown to occur in the vicinity of the nuclear domain ND10 (Swindle et al., 1999). ND10 has been shown to be the localisation site for expressed L2 in natural infections, and it has recently become clear that this localisation event is required for capsid assembly (Day et al., 1998; Florin et al., 2002). Further, Florin et al. (2002) showed that the presence of L2 precedes L1 accumulation by several hours. This was supported by the findings of Zhou et al.

(1993a) that L1 co-localises to L2 *in vitro*, and Becker et al. (2004), who showed that nuclear localisation of L1 was required for L2 incorporation into the capsid. Subsequent to expression of L2, L1 is expressed in the cytoplasm, where it pentamerises to form capsomeres through disulphide bonding (Bird et al., 2008; McCarthy et al., 1998). Capsomeres bind to several karyopherin nuclear transport proteins, via an NLS found in the C-terminal DNA-binding domain of L1, and are transported to the nucleus (Bird et al., 2008; Li et al., 1997). Further, it has been suggested that binding of karyopherins prevents capsid assembly in the cytoplasm (Bird et al., 2008).

The first step in viral capsid assembly is the establishment of a nucleation point, usually a dimer around which the capsid can assemble (Zandi et al., 2006). Several models of nucleation have been suggested for HPV. Early models were based on the closely related polyomaviruses – assembly of these viruses starts as five pentamers of the major capsid protein VP1 around one pentamer (Casini et al., 2004; Stehle et al., 1994). The work of Casini et al.(2004), wherein the assembly kinetics of L1 were investigated using light scattering, suggested an alternative model for *in vitro* capsid formation – that of nucleation around dimerised capsomeres. However, this analysis was performed in the absence of L2, and it is likely that in natural infections, capsid assembly is a far more complex phenomenon. There are several lines of evidence for a role for L2 in nucleation, capsid assembly and encapsidation of DNA, although there is little agreement on the exact role played by L2 in these processes.

L2 possesses a strongly hydrophobic C-terminal domain which has been shown to bind L1. There is evidence that in bovine papillomavirus, L2 binds L1 prior to assembly of the capsid, and it has been suggested that this binding triggers the assembly process (Finnen et al., 2003; Okun et al., 2001). In contrast to this, it has been shown that L2 is involved in intercapsomeric interactions with other L2 molecules via its N- and C-terminals, and these interactions may be responsible for initial capsid formation (Buck et al., 2008; Ishii et al., 2005).

Most studies of the association of DNA with the HPV capsid proteins have utilised synthetic virions such as virus-like particles (VLPs) and pseudovirions (PsVs) *in vitro*. There is some disagreement over the exact requirements for, and respective roles of L1 and L2 in DNA binding. Several investigators have shown that both L1 and L2 are required to package DNA effectively (Ma et al., 2011; Okun et al., 2001; Stauffer et al., 1998), while others have suggested that L2 is not required at all (Touze and Coursaget, 1998; Unckell et al., 1997), and still others that L1 is not required (Zhou et al., 1993a). More recently, Holmgren et al. (2005) showed that L2 incorporation into particles increased

encapsidation of DNA 10-fold. There is no clear evidence for L1 or L2 binding to specific sequences of DNA. Rather, evidence points to non-specific associations of certain protein domains with DNA based on basic interior domains and overall charge – a C-terminal region of L1, and highly charged regions in both the C- and N-terminus of L2 (Fay et al., 2004; Garcea and Gissmann, 2004; Li et al., 1997; Pereira et al., 2009). Notably, incorporation of a putative L1 DNA binding region into an unrelated viral capsid protein conferred the ability to encapsidate DNA (El Mehdaoui et al., 2000).

Interestingly, the L1 NLS overlaps with the C-terminal DNA binding region, and it has been suggested that the karyopherins which bind L1 for nuclear transport play a role as chaperones for virion assembly. This is supported by evidence that L1 binds karyopherin α2 and DNA simultaneously (Bird et al., 2008). The karyopherin α2β1 complex has been shown to facilitate nuclear transport of both L1 and L2, and karyopherin α2 binds L2 in a similar fashion to L1 – via an NLS in the C-terminal DNA binding domain (Bird et al., 2008; Fay et al., 2004). Previous studies have suggested a role for chaperone hsp70 in mediating both assembly and disassembly of papilloma- and polyomavirus capsids (Chromy et al., 2006; Chromy et al., 2003).

DNA incorporated into HPV virions has been shown to affect the structure of the mature virion. Fligge et al. (2001) showed that incorporation of DNA increased disulphide crosslinking between L1 in the assembled capsid, and provided partial protection against trypsin cleavage. DNA in HPV virions is associated with cellular histones in chromatin-like structures (Conway and Meyers, 2009). The presence of histones in mature virions has been shown to increase infectivity 4-5 fold when compared to encapsidated naked plasmid DNA (Fligge et al., 2001).

In summary, HPV virion assembly occurs in several distinct steps. It is initiated by the late viral promoter, and starts with localisation of L2 protein, and the HPV genome, to ND10 sites in the nucleus. L1 expression follows soon after; the protein forms capsomeres in the cytoplasm before being transported to the nucleus by karyopherins. It is likely that L2 binding of capsomeres provides the nucleation point for capsid assembly, and that assembly is mediated by inter-L2 bonds. Encapsidation of HPV DNA occurs through DNA-protein interactions at non-specific DNA binding domains on the interior surfaces of both L1 and L2, and this encapsidation of DNA plays a role in stabilising the assembled virion.

While our current understanding of HPV assembly and DNA encapsidation is not complete, significant steps have been taken toward full understanding of the lifecycle and behaviour of this

virus in recent years. This is due in no small part to the development of recombinant capsid production capabilities, which have allowed a level of study not previously achievable.

1.3. Synthetic HPV particles

Until recently, an inability to produce papillomavirus virions *in vitro* was a major stumbling block to the study of this virus. Two methods have recently been described, namely xenografting and organotypic raft culture, for the production of infectious virus. The xenograft model uses HPV-infected human epithelial tissue, implanted in athymic mice, to produce cysts in which HPV virions are produced. These cysts can be harvested, and purified for HPV virions (Kreider et al., 1987). Organotypic raft culture utilises 3T3 fibroblasts and human epidermal cells to establish stable organotypic 'rafts' *in vitro* (Rheinwald and Green, 1975). This system provides differentiating keratinocytes, in which papillomaviral DNA has been demonstrated to replicate and produce virions (Meyers et al., 1992; Meyers et al., 1997). While these models are important for the study of infectious papillomavirus, they remain expensive, time-consuming, and not particularly efficient (Conway and Meyers, 2009). Synthetic HPV particles (i.e. those particles produced by transfection of papillomaviral DNA into an expression system; namely VLPs, QVs and PsVs) provide a cheaper and easier production approach for the study of papillomavirus structure and infectious behaviour.

1.3.1 Virus-like particles (VLPs)

In vivo, HPV capsids are produced only in terminally differentiated keratinocytes (Barksdale and Baker, 1993), a strategy which allows the virus to avoid the host inflammatory response (Stanley, 2008). For many years, this feature of HPV made it particularly difficult to study the immunogenic and structural features of the mature HPV capsid, and in particular inhibited development of an effective vaccine (Hagensee et al., 1993; Kirnbauer et al., 1992). The last 20 years, however, has seen the development of several methods and systems for the production of HPV VLPs. Early efforts utilised mammalian epithelial cells in culture, transfected with vaccinia viral vectors, to produce VLPs (Hagensee et al., 1993; Kirnbauer et al., 1992). These were quickly followed by production of VLPs of various HPV types in insect cells (Christensen et al., 1994; Roden et al., 1996; Senger et al., 2009; Volpers et al., 1994), bacteria (Nardelli-Haefliger et al., 1997), yeast (Cook et al., 1999; Hofmann et al., 1995; Mach et al., 2006), and more recently in plants (Biemelt et al., 2003; Maclean et al., 2007; Patel et al., 2009). It has been shown that L1 alone can assemble to form stable, uniform VLPs in all of these systems, while L1 and L2 have been produced in most (with the exception of plants). Additionally, and perhaps more importantly, it has been observed that these particles closely

resemble the native papillomavirus virions, and are highly immunogenic (Hagensee et al., 1993; Kirnbauer et al., 1992; Rose et al., 1993).

1.3.2. Quasivirions (QVs) and pseudovirions (PsVs)

Production of recombinant HPV VLPs was an important step forward, in that it allowed researchers to analyse and describe the virus structure and activity in ways not previously possible, and allowed for the development of two highly successful vaccines (Stanley, 2008). Soon after, several researchers showed that these VLPs packaged papillomavirus DNA *in vitro.* Zhou et al. (1993a) showed that recombinant Bovine Papillomavirus (BPV-1) L1 and L2 packaged the BPV-1 genome in B2 cells to produce infectious particles, and Roden et al. (1996) demonstrated that when cells containing an autonomously replicating bovine papillomavirus genome were transfected with HPV-16 L1 and L2, the genomes were packaged efficiently to produce quasivirions (QVs).

HPV QVs are now produced almost exclusively in HEK293T or HEK293TT cells, via a method established by Pyeon et al. (Pyeon et al., 2005; Smith et al., 2007). They have been shown to be similar in infectious titer and neutralisation characteristics to native virions, and can produce papillomas (Conway and Meyers, 2009; Pyeon et al., 2005). While the behaviour and structure of QVs seems to be very similar to native virions, they have not yet been conclusively shown to be indistinguishable, and some work remains before these can be considered as replacements for native virions in experimental procedures.

Soon after the production of the first VLPs and QVs, Unckell et al. (1997), Touze and Coursaget (1998), and Stauffer et al. (1998) demonstrated that the L1 and L2 capsid proteins packaged non-papillomaviral plasmid DNA with similar efficiency. These particles, called pseudovirions (PsVs), were shown to transfer plasmid DNA into epithelial cells in a manner indistinguishable to that of infectious HPV virions. All three groups used a reporter plasmid encoding the *lacZ* gene, while Touze and Coursaget also tested a GFP reporter gene, and Stauffer et al. showed that a puromycin resistance gene could confer antibiotic resistance to pseudovirion-infected cells. Touze and Coursaget further demonstrated that HPV VLPs preferentially packaged plasmid DNA of 5-8kbp, and established that packaging efficiency was proportional to plasmid size (Buck et al., 2004; Touze and Coursaget, 1998)

Since that time, several other groups have achieved PsV synthesis through a variety of methods. These can be roughly sorted into two major groups in terms of approach to production. The first, as pioneered by Touze and Coursaget, relies on the inherent self-assembly and DNA encapsidation

signals of L1 and L2 proteins. Broadly speaking, this approach utilises traditional methods of VLP production as described in Section 1.3.1. These VLPs are then disassembled *in vitro* by chemical means, incubated with the plasmid DNA to be encapsidated, and reassembled through incremental increases in $CaCl_2$ concentration. Several groups have successfully utilised this approach for the production of infectious PsVs, using VLPs produced in insect or mammalian cells (Kawana et al., 1998; Oh et al., 2004; Shi et al., 2001; Touze and Coursaget, 1998; Zhang et al., 2004). A variation of this approach is demonstrated by Yeager et al. (2000), who chemically coupled a reporter plasmid to yeast-produced VLPs.

While the disassembly-reassembly method is nominally successful in producing PsVs, and continues to be used for studies utilising HPV PsVs, a second, more effective method has been developed. This method, best demonstrated by the Schiller group, exploits the intracellular virion assembly mechanisms used by the virus in natural infections to assemble PsVs in mammalian (Liu et al., 2001; Stauffer et al., 1998; Unckell et al., 1997), insect (Zhao et al., 1998), or yeast (Rossi et al., 2000) cells. In mammalian and insect cell systems, this is achieved through transient expression by L1 and L2 expression plasmids, as well as a reporter plasmid for encapsidation (Buck et al., 2004). In yeast, this was achieved through two-step stable transformation of *Saccharomyces cerivisiae*, first for the production of HPV VLPs, and second with the reporter plasmid to be encapsidated (Rossi et al., 2000). Using a specially developed cell line – HEK293TT, encoding two copies of the SV40 T-antigen for improved viral replication – Buck et al. (2004) demonstrated a 10-million fold improvement in efficiency on earlier PsV production methods. This clear evidence for the success of intracellular assembly of PsVs is supported by experimental evidence that despite a significantly higher level of L1 being present in the *in vitro* system, PsVs produced by this intracellular method had higher gene transfer efficiency into infected cells than PsVs produced by reassembly methods (Fleury et al., 2008b; Peng et al., 2011). It is likely that this improved assembly and infectivity is due to not yet fully defined mechanisms of virion assembly present in the nucleus of cell systems utilised for PsV production, which are not present in the chemical milieu found in disassembly-reassembly systems.

1.3.3. Infectivity and immunogenicity of synthetic HPV particles

While advances in synthetic HPV particle technology have allowed giant leaps forward in the study and treatment of the virus and concomitant disease, this progress has rightly been tempered with caution: an important aspect in the production of synthetic HPV virions is whether their morphological, infective and behavioural characteristics accurately match those of the natural virion (Deschuyteneer et al., 2010). This is a necessary caveat to the use of these particles for development

and testing of potential HPV vaccines. Accordingly, there has been some investigation into the comparative morphology, infectivity and immunogenicity of HPV VLPs and PsVs.

While the role of L1 in the virion as a structural protein is clear, the role of L2 is less so, particularly with regards to immunogenicity. In natural infections, L2 plays roles in entry into the cell, entry into the nucleus, and assembly of the virion (reviewed in Pereira, 2008). The importance of L2 for the encapsidation of the plasmid of interest into PsVs has been well established, particularly in systems that rely on intracellular virion assembly (Rossi et al., 2000; Unckell et al., 1997). Several other studies have confirmed that while L1 alone is capable of packaging DNA, L2 vastly improves the packaging efficiency of DNA into PsVs (Kawana et al., 1998; Roden et al., 1996; Zhao et al., 1998). In comparison, Holmgren et al. (2004) showed that in true virus particles (produced by organotypic raft culture) L2 deficiency resulted in a 10-fold reduction in DNA encapsidation, as well as a 100-fold reduction in infectivity. There has been less agreement on whether L2 is required for virion entry into the cell, but it has recently been established that while L2 is not strictly required for infection, it greatly enhances the capacity of PsVs to transfer DNA (Peng et al., 2010). It has been shown that mutation of a single L2 cysteine residue involved intermolecular L2 disulphide bonds negatively affects infection of keratinocytes by inhibiting pseudovirion entry into the cell, while having no effect on assembly (Campos and Ozbun, 2009; Gambhira et al., 2009; Peng et al., 2011). This correlates with findings of Holmgren et al. (2004).

Development of pseudovirions encapsidating a reporter plasmid has allowed for investigators to study the pseudoinfection of the mouse vaginal epithelia *in vivo*. This was achieved using HPV-16 pseudovirions with a gene encoding a fluorescent protein, and has demonstrated that infection by PsVs closely mirrors infections by the natural virion. Vaginal infection occurs only after epithelial disruption, by either chemical or mechanical means. Following wounding of the epithelia, virions are deposited onto the basal membrane, and gene transfer into infected keratinocytes occurs as they migrate to begin wound repair (Graham et al., 2010; Roberts et al., 2007; Shi et al., 2001). Roberts et al. (2007) further demonstrated that L2 was required for successful infection of keratinocytes, and that no differentiated columnar epithelia were infected at any stage of the infection process. Notably, prior vaccination to HPV conferred complete protection against pseudovirion entry into cells in this study.

Graham et al. (2010) demonstrated that intravaginal administration of PsVs resulted in transient, localised expression of the encapsidated gene, followed by rapid clearance (approximately 5 days).

This is a clear difference to natural HPV infections, but is to be expected as HPVs have evolved complex mechanisms to avoid intracellular defence mechanisms. It has been suggested that vaginal administration of PsVs would be useful for delivery of short-term immunomodulatory genes, such as cytokines (Shi et al., 2001).

It is clear that VLPs and PsVs very closely mimic the infectious properties of HPV due to their structural and behavioural similarities, particularly when both capsid proteins are incorporated. This results in comprehensive induction of the immune system. Several groups have confirmed that PsVs elicit a strong humoral and cellular immune response via both intravaginal and intramuscular administration (Oh et al., 2004; Shi et al., 2001; Zhang et al., 2004). This is likely mediated by binding and uptake by dendritic cells (DCs) - an important factor to the use of these particles as vaccines. DCs serve as the sentinels of the immune system, and delivery of antigens to these cells is a critical component of an effective vaccine (Lenz et al., 2001; Peng et al., 2010). In 2001, Lenz et al. showed that both L1 and L1+L2 VLPs are bound and internalised by DCs *in vitro.* This resulted in rapid phenotypic maturation of DCs and upregulation of major histocompatibility complex (MHC) class I and II proteins, cytokines such as Interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α), and induction of a strong Th1 response. This is a clear demonstration of the mechanisms behind the observed immune response to L1 VLP vaccination (Lenz et al., 2001; Yang et al., 2004). More recently, it was demonstrated that HPV PsVs are similarly bound and internalised by DCs, with a comparable immune response, and that the interaction with DCs is mediated by a toll-like receptor (TLR) pathway (Peng et al., 2010; Yang et al., 2004).

It was established relatively early on that L1 VLPs elicited a strong neutralising antibody response, and this has been the basis for the development of current commercially available vaccines (Christensen et al., 1996b; Kirnbauer et al., 1996). L2 also elicits a neutralising antibody response - these antibodies are cross-neutralising for multiple HPV types (Campo et al., 1997; Chandrachud et al., 1995; Christensen et al., 1991; Embers et al., 2002; Gaukroger et al., 1996; Roden et al., 2000). However, Roden et al. (2000) demonstrated that when incorporated into L1+L2 VLPs, there was no cross-neutralising effect, and speculated that the L1 antibodies were immunodominant. It is likely that the lack of strong neutralising antibodies to L2 is due to the structure and composition of the virion: there are 5-30 times less L2 than L1 in a typical virion, and L2 is not naturally exposed until the virion undergoes conformational changes brought about by L1 binding to cell surface receptors (Buck et al., 2008).

In conclusion, it is clear that we are moving towards a comprehensive model of infection and immune stimulation for these synthetic particles. This advanced level of understanding of HPV particle production and biology allows for several sophisticated applications of this technology, such as vaccine development.

1.4. Applications of synthetic HPV particle technology

Viral capsid assembly is efficient, genetically economical, energetically favourable, and reliable (Casini et al., 2004). These features make viral particles an extremely attractive option for the molecular packaging and delivery of various macromolecules, particularly DNA. Coupled with advances in protein fusion technology, recombinant viral particles represent almost unparalleled potential for preventative and therapeutic medicines. In recent times, HPV VLP vaccines have been successfully developed and commercialised, demonstrating the use of these particles in a medical context.

1.4.1. Current HPV VLP vaccines

HPV VLPs are an attractive vaccine candidate: they are stable, relatively straightforward to produce, and elicit strong immune responses. They are natural adjuvants, and stimulate both a B and T cell immune response (Lenz et al., 2001; Rudolf et al., 2001; Shi et al., 2001). Two prophylactic HPV VLP vaccines are commercially available, Gardasil® (Merck) and Cervarix™ (GlaxoSmithKline). Both vaccines are based on the HPV L1 capsid protein: Cervarix is a bivalent HPV-16/18 vaccine, while Gardasil is a quadrivalent vaccine that offers additional protection against the low-risk types HPV-6 and -11 which cause genital warts (Lin et al., 2010). Both these vaccines are delivered by intramuscular injection, and elicit strong, type-specific neutralising antibody responses. These vaccines have both been shown to be safe, highly immunogenic and efficacious over the period that they have been available (reviewed in Stanley et al. (2008) and Mandic (2012)), with no evidence to suggest a loss of efficacy. While these vaccines are already showing early promise in easing the burden of cervical cancer in developed countries (Brotherton et al., 2011), the vaccines leave much to be desired with respect to breadth of type-specificity, prophylactic effect, cost, and ease of production (Kawana et al., 2009). Further, intramuscular administration of VLPs results in low mucosal immunogenicity (Oh et al., 2004). There are accordingly a number of efforts underway to develop second-generation HPV vaccines that improve upon these shortcomings.

1.4.2. Use of HPV particles as gene delivery vehicles

An emerging use for HPV PsVs is as biological gene delivery vehicles (GDVs). Delivery of medically relevant genes *in vivo* is a field with huge potential for the treatment of a large range of diseases and disorders. These include genetic deficiencies, cancers, and acute bacterial and viral infections. Further, they have the potential to revolutionise vaccine technology by providing effective delivery of DNA vaccines to cells. There are many aspects to consider when evaluating the effectiveness of a gene delivery vehicle. These include manufacturing feasibility, cost, stability, ease of storage and administration, replication competence, vector-specific toxicity, pre-existing vector immunity, tissue tropism, host range, and routes of administration (Graham et al., 2010). Currently, the most promising GDVs are viral vectors, such as adenoviral and lentiviral vectors, and synthetic liposomes. However, both of these systems have limitations, such as immune recognition, mutagenic integration, inflammatory toxicity and rapid clearance (Seow and Wood, 2009).

Pseudovirions represent an exciting new type of GDV. They are ideal for this purpose, as they represent the best of both viral and non-viral delivery systems. Gene delivery is efficient, as viruses have well-defined delivery and uptake mechanisms, and due to recent technological advances, PsVs are now easy to modify and produce (Lund et al., 2010; Seow and Wood, 2009). Further, there is no risk of reversion to live virus as no viral DNA is present. Apart from viral and non-viral DNA, pseudovirions can be utilised to encapsidate other non-viral cargos, such as small oligonucleotides, proteins, siRNA, organic polymers and fluorophores (Lu et al., 2012; Lund et al., 2010).

1.4.3. HPV-based PsV candidate vaccines

In the context of gene delivery, pseudovirions have particular promise as vaccines. These may be HPV vaccines, where the DNA could encode an additional antigen or adjuvant, or vaccine delivery vehicles for DNA vaccines of other viruses, where the HPV capsid serves merely as a GDV. There have been several promising early investigations into both of these possibilities.

Pseudovirion vaccines represent aspects of several different types of vaccine, and early evidence suggests that this gives them significant advantages in terms of production and immunogenicity. Like VLPs and killed virus vaccines, they closely resemble the structural and behavioural characteristics of the native virion. Further, incorporation of plasmid DNA allows for stimulation of both humoral and cellular immunity, as seen in DNA vaccines (Gurunathan et al., 2000). Perhaps most importantly, they are relatively easy to produce, and there is no possibility of reversion to live virus.

Various approaches to a pseudovirion vaccine have been tested in the last 10 years. These can be broadly divided into two groups – vaccines to HPV, which utilise the DNA-packaging capabilities to encode an adjuvant or additional epitopes (Combelas et al., 2010; Oh et al., 2004); and vaccines to other viruses, which utilise the immunostimulatory and gene transfer capabilities of PsVs to produce a more effective immune response to a DNA antigen (Gordon et al., 2012; Peng et al., 2010; Renoux et al., 2008; Shi et al., 2001; Zhang et al., 2004).

Despite the success of the recently developed VLP vaccines (Cervarix and Gardasil), there remains some scope for improvement. The major downfall of these vaccines is the cost of production, low mucosal immunogenicity, and the limited cross-reactivity to other HPV types. To address the lack of mucosal immunogenicity, Oh et al. (2004) used L1 VLPs to encapsidate a gene encoding IL-2, to act as an adjuvant. They reported that IL-2 PsVs, delivered intramuscularly, induced the highest vaginal and salivary IgA response when compared to L1 VLPs, or L1 PsVs encapsidating an irrelevant plasmid, up to 6 weeks post-administration. They also demonstrated that encapsidation of an adjuvant plasmid was far more effective in eliciting an immune response when compared to co-delivery of VLP and plasmid. In a different approach, Combelas et al. (2010) encapsidated the HPV31 L2 gene into HPV58 L1+L2 VLPs, and used these PsVs to immunise mice. They demonstrated that PsVs elicited neutralising antibodies to HPV16, 18, 31, 58, demonstrating that including an L2 gene could improve on the range of cross-neutralising antibodies produced by HPV L1 or L1+L2 VLPs. One other interesting HPV vaccine candidate has been demonstrated for therapeutic cervical cancer treatment. This group packaged short hairpin RNAs (shRNAs) to E6 and E7 oncoproteins into HPV-31 PsVs. They showed that these PsVs effectively silenced E6 and E7 expression in cervical carcinoma cells, and dramatically inhibited cancer growth (Bousarghin et al., 2009).

It is well known that the major limitation of DNA vaccines is inefficient delivery into cells (Graham et al., 2010). Recently, it has been extensively demonstrated that PsVs are significantly better at delivering DNA to cells than any other delivery method tested (Graham et al., 2010; Peng et al., 2010; Renoux et al., 2008; Shi et al., 2001). This makes pseudovirions an attractive delivery system for DNA vaccine-expressed antigens. HPV PsVs are particularly attractive due to being mucosatropic viruses, which allows the possibility of inducing mucosal immunity, demonstrated as being key to controlling infection of viruses such as human immunodeficiency virus (HIV) and respiratory syncytial virus (RSV) (Graham et al., 2010). Accordingly, several groups have utilised the mucosatropic properties of HPV pseudovirions to create novel PsV vaccines to non-papillomaviral diseases. These

include simian immunodeficiency virus (SIV), HIV-1, RSV, and lymphocytic choriomeningitis (Gordon et al., 2012; Renoux et al., 2008; Shi et al., 2001; Zhang et al., 2004). Studies have also been performed using an HPV PsV carrying the model antigen gene, ovalbumin (OVA) (Peng et al., 2011; Peng et al., 2010). These studies used both intracellular assembly and *in vitro* disassembly-reassembly methods to create the various HPV pseudovirions used, and most used L1+L2 PsVs. Subjects were immunised via oral, intramuscular or intravaginal delivery of pseudovirions.

The most notable finding from these studies was the potent induction of a mucosal and systemic cytotoxic T-lymphocyte (CTL) response, which was seen in all cases. This is an important finding, particularly in the development of mucosal vaccines. A mucosal CTL response is critical in combatting mucosal infections, as many pathogens initiate infection in epithelial cells, before spreading to the rest of the body (Shi et al., 2001). The presence of CTLs and antibodies in the mucosa are required to regulate viral replications of diseases such as HIV (Zhang et al., 2004). Further, Zhang et al. (2004) and Graham et al. (2010) both showed a significant IgA induction, and demonstrated that oral or vaginal immunisation protected unrelated mucosae in the body from viral challenge. All studies showed induction of both a humoral and cellular immune response.

The success of pseudovirions as vaccine delivery vehicles can be attributed to their ability to mimic a natural viral infection. This allows unprecedented induction of the immune system, and results in a holistic and highly immunogenic response to vaccination, regardless of the antigen in question (Peng et al., 2010). Another potentially valuable facet of PsVs is their stability. Shi et al. (2001) showed that PsVs were resistant to the low pH and proteolysis prevalent in the alimentary canal. This makes pseudovirions attractive for use in oral immunisation, and presents practically limitless scope for vaccines against orally transmitted pathogens. Further, the large number of HPV types and relatively low cross-reactivity between types makes HPV particles particularly attractive for DNA vaccine delivery as they allow repeated boosting without concern for prior immunity (Ma et al., 2011)

1.4.4. Pseudovirion-based neutralisation assay (PBNA)

It has long been recognised that neutralising antibodies confer lasting protection against papillomaviruses; this was first demonstrated for cottontail rabbit papillomavirus (Shope, 1937). VLPs, including the vaccines Gardasil and Cervarix, produce a strong neutralising antibody response, and the current assumption is that this is the mechanism of protection elicited (Schwarz and Leo, 2008; Stanley, 2008). In 2000, Yeager et al. demonstrated a novel approach for the detection of neutralising antibodies to HPV, using what has come to be known as the pseudovirion-based

neutralisation assay (PBNA). This group used an amine-to-sulfhydryl crosslinker (Sulfo-SMCC, Pierce) to attach a β-lactamase reporter gene to the exterior surface of HPV L1 + L2 VLPs produced in yeast cells to produce pseudovirions. They then treated mammalian cells with the pseudovirions, as well as sera from humans immunised with HPV-11 VLPs, and used fluorescence to identify those cells that were expressing β-lactamase. This technique has been further developed by Pastrana et al. (2004) and Steele et al. (2008) to utilise the ability of VLPs to encapsidate plasmid DNA. PBNA using pseudovirions carrying a Secretory Alkaline Phosphatase (SEAP) plasmid is now considered the gold standard for neutralisation assays, and is currently the best assay for efficacy of candidate prophylactic HPV vaccines (Buck et al., 2005a; Fleury et al., 2008b). Another group has also developed a neutralisation assay using a luciferase gene, although production efficacy is much lower (Fleury et al., 2008b). However, despite suggestions that the PBNA method espoused by these groups provides a high-throughput and practical method to evaluate antibody responses in both natural history and prophylactic vaccine studies (Pastrana et al., 2004), it remains expensive and time consuming, particularly for production of pseudovirions (Steele et al., 2008). Thus, alternative systems for pseudovirion production are attractive for both commercial and diagnostic applications.

1.5. Plant production systems

Over the last 20 years, transient and transgenic protein expression in plants has come of age, and now presents an attractive platform for the production of medically and industrially relevant proteins. At this stage, more than 100 different recombinant proteins have been expressed in plants; the list includes human serum proteins, growth regulators, antibodies, subunit vaccines, VLPs, industrial and commercial enzymes, and biopolymers (Schillberg et al., 2005; Tiwari et al., 2009). Further, the regulatory framework has now been put in place to allow for commercial applications of this technology (Rybicki, 2010).

1.5.1. Transient expression as an alternative to transgenic plant production

Historically, proteins produced *in planta* have suffered from low yield, poor and inconsistent quality, and a lack of framework for good manufacturing practice (GMP) (Schillberg et al., 2005; Streatfield and Howard, 2003). Reliance on transgenic protein expression resulted in poor or uncertain yields, and long timeframes for development of a product. Furthermore, several biosafety incidents (such as transgenic crop escape) in the last decade resulted in delayed acceptance of the use of transgenic

plant production systems for commercial and medical applications (Huang et al., 2009; Rybicki, 2010). More recently, however, transient expression of proteins has become an increasingly feasible alternative to stable transformation of plants, and now appears to be a superior approach to protein production.

Transient expression of proteins in plants can be executed in two ways: by utilising viral vectors such as those derived from tobacco mosaic virus (TMV) or potato virus X (PVX), or by agroinfiltration of a plant expression construct into the plant. Viral vectors showed great promise as their action is both rapid and systemic (Schillberg et al., 2005). However, administration can be difficult, particularly in the case of RNA viral vectors, and there has been demonstrated loss of the transgene due to impaired fitness (Rybicki, 2010). Agroinfiltration of viral and non-viral vectors is considered a viable alternative to the use of viral vectors alone, as it allows easier administration, with similar rapid and systemic transgene induction, and without the gene size restraints of traditional viral vectors (Kapila et al., 1997). Originally developed for protein expression in plants recalcitrant for stable transformation, agroinfiltration utilises the DNA transfer behaviour of the infectious bacterium *Agrobacterium tumefaciens* to induce systemic production of a protein of interest (Kapila et al., 1997). Transient expression is favourable to the establishment of transgenic lines, as it is much quicker (3-9 months for transgenic vs. days for transient expression), shows much higher protein yields, is more amenable to scale-up and GMP, and is much easier to contain (Rybicki, 2010; Schillberg et al., 2005; Tiwari et al., 2009). Agroinfiltration was previously thought to be useful only for testing of gene expression before using more traditional approaches to transform plants (Fischer et al., 2004). However, co-infiltration with viral silencing suppressors and the development of industrial-scale vacuum infiltration operations has shown transient expression to be a highly effective tool for large scale production of recombinant protein (D'Aoust et al., 2008).

Of particular interest in our laboratory is the use of replicating DNA viral vectors for transient expression. This technology utilises small ssDNA viruses of the family *Geminiviridae* to create deconstructed viral vectors which replicate to high copy numbers when introduced into plants by agroinfiltration. Geminiviruses in the genus *Mastrevirus* commonly encode only four proteins, namely coat protein (CP), movement protein (MP), and the replication-associated protein Rep and RepA (Liu et al., 1997). Rep and RepA are the only viral components required for replication, which occurs by a rolling circle mechanism and amplifies the circular genome to high copy number (Huang et al., 2009). While early applications simply replaced the CP with a gene of interest (Kammann et al., 1991; Ward et al., 1988), vectors developed more recently use only the essential viral elements,

namely the genes encoding Rep/RepA, and two intergenic regions which contain regulatory elements (Mor et al., 2003). These genetic elements maybe supplied on two or more co-infiltrated vectors (Hefferon and Fan, 2004; Mor et al., 2003; Zhang and Mason, 2006), or on a single vector (Huang et al., 2009; Regnard et al., 2010; Tamilselvi et al., 2004). These recent advances have shown that the incorporation of geminiviral genetic elements into viral vectors is a successful strategy for replication to high copy number and subsequently high protein production (Hefferon, 2012).

In addition to the improvements brought about by transient expression technology, several inherent properties of plants make this system appealing. Recombinant proteins are faithfully expressed in plants: they have also been shown to retain the correct size, as well as tertiary and quaternary structure, and are post-translationally processed similarly to mammalian cells. Plants as a production system represent the possibility of rapid and affordable scale-up to industrial scales, as well as a reduced raw material cost, when compared to other eukaryotic or bacterial production systems. Further, they pose no risk of contamination with human or animal pathogens (Streatfield and Howard, 2003; Thanavala et al., 2006). Many crop species have been used for transgenic and transient production. While protein expression levels have shown to be broadly similar across the many species tested, *Nicotiana* spp. have risen to the forefront of recombinant protein production, due to their high yield, strong biosafety profile, non-feed/non-food crop status, and rapid scalability (Fischer et al., 2004; Schillberg et al., 2005).

In conclusion, transient protein expression in plants is clearly an effective tool for small- and large-scale production of protein. With the current improvements in technology and regulatory framework, plant production offers a scalable and affordable way to increase production capacity to meet rising global demand for recombinant proteins (Fischer 2004). In particular, this is appealing for medical applications.

1.5.2. Antigen production in plants

In 1989, Hiatt et al. produced fully assembled and functional monoclonal mouse antibodies in transgenic tobacco (Hiatt et al., 1989). Following this first successful demonstration of medically relevant protein expression *in planta*, another group produced the first antigen in plants – Hepatitis B surface antigen (HBsAg) – and observed that the protein produced was structurally and antigenically similar to serum-derived HBsAg. Further, they showed that these protein subunits assembled to form particles (Mason et al., 1992). Soon after, Haq et al. (1995) showed that plant-produced *Escherichia coli* heat-labile toxin induced a strong, antigen-specific immune response in

mice, and generated neutralising antibodies. Since that time, many different antigens have been produced by both transgenic and transient expression, from a broad range of human and animal diseases. Importantly, it has been conclusively demonstrated that antigens produced in plants assemble efficiently and are capable of eliciting a strong, antigen-specific B- and T-cell response (Streatfield and Howard, 2003). There are several plant-produced antigens currently in clinical trials (Reviewed in Tiwari et al. (2009). Further, several plant-produced products are licensed. These include an antibody used for recombinant Hepatitis B virus HBV vaccine production (Pujol et al., 2005), and a Newcastle disease virus (NDV) vaccine for chickens (Rybicki, 2009).

1.5.3. HPV vaccine development in plants

Several groups have expressed HPV proteins in plants, by both transient and transgenic expression methods. The oncogenic E7 protein was expressed using a PVX-derived viral vector, and induced cellular and humoral immunity, as well protecting mice against tumour challenge (Franconi et al., 2002). Several groups produced L1 in transgenic plants, and observed that VLPs assembled *in planta* with varying degrees of efficiency (Biemelt et al., 2003; Liu et al., 2005; Varsani et al., 2003; Warzecha et al., 2003). These groups also showed that VLPs produced were morphologically similar to VLPs produced in non-plant systems, and that they elicited a similar immunological response. While this was a promising step for the production of a vaccine candidate in plants, yields were extremely low (<0.5% of total soluble protein (TSP)).

The first demonstration of protection elicited by plant-produced papillomavirus capsid protein followed shortly after. Kohl et al. (2006) showed that transgenic-plant-produced cottontail rabbit papillomavirus L1 protein conferred protective immunity against challenge by live virus. While they demonstrated low neutralising antibody responses, and no VLP formation, this was an important step toward HPV vaccine production in plants. Subsequently, Varsani et al. (2006) used a TMV-derived vector to transiently express L1 in tobacco. This resulted in an approximate 10-fold increase in protein expression compared to transgenic production, but low immunogenicity, which the authors ascribed to an overabundance of L1 subunit proteins relative to capsomeres and assembled VLPs. Soon after, the same group performed a thorough comparison of various codon-optimisation and cellular localisation strategies for increased expression of HPV16 L1, using agroinfiltration of a cauliflower mosaic virus-derived plasmid. The authors reported L1 expression at approximately 17% of TSP, using a human codon-optimised gene and a chloroplast localisation signal (Maclean et al., 2007). Other investigators have shown similar high expression of L1 in the chloroplast, although this was achieved using transplastomic expression (Fernandez-San Millan et al., 2008; Lenzi et al., 2008).

Maclean et al. (2007) further showed that L1 VLPs produced this way were highly immunogenic, and induced a strong neutralising antibody response.

1.6. Objectives of the work

In this project, I set out to to manufacture plant-produced HPV-16 pseudovirions for use in the pseudovirion-based neutralisation assay, with a view to developing a cheaper alternative to the current assay approach. To achieve this, I aimed to modify the previously developed Bean yellow dwarf mastrevirus (BeYDV)-derived pRIC3 vector plasmid to provide the reporter gene plasmid required for the PBNA, and use HPV-16 L1 and L2 proteins to form the pseudovirion protein shell, or capsid. Specifically, the objectives of the project were to:

1) Design and construct three novel replicating vectors to be encapsidated in L1+L2 PsVs
2) Express and purify HPV-16 pseudovirions in *N. benthamiana*
3) Use these plant-produced pseudovirions in a successful neutralisation assay in mammalian cells

To achieve the first objective, I needed to create three novel replicating vectors. These were to be modified from pRIC3, an autonomously replicating shuttle vector developed in our laboratory (Regnard et al., 2010), to include two different mammalian expression cassettes. The entire expression cassette was taken unchanged from the pYSEAP vector developed by Pastrana et al. (2004). The second was taken from the pTH plasmid originally developed by Hanke et al. (1998), and further modified by Tanzer et al. (2011), and modified to carry the luc reporter gene.

The second objective was to use these plasmids, along with non-replicating plant vectors expressing human codon-optimised L1 and L2, to produce PsVs in plants. Autonomous replication of the pRIC3 vectors would be expected to result in approximately 5-7.5Kbp replicons, a size able to be packaged by assembling HPV virions, made up of the co-expressed L1 and L2 proteins. These PsVs would be purified and tested for DNA encapsidation.

The third objective was to demonstrate the infectivity of plant-produced PsVs in a mammalian cell culture system. To this end, particles would be purified and used to infect mammalian cells. Successful infection would result in expression of SEAP or luciferase. Lastly, these PsVs would be pre-incubated with HPV-16 antisera and tested for neutralisation by using the PBNA.

Chapter 2: Construction and testing of replicating vectors for pseudogenome production

2.1. Introduction

This study aimed to produce PsVs *in planta*, to be used for pseudoinfection of mammalian cells with a pseudogenome carrying a mammalian reporter gene. To achieve this, autonomously replicating viral vectors were identified as promising candidates for pseudogenome production. In particular, geminivirus-derived vectors were selected for their small plasmid size and rapid and consistent replication to high copy number in plants. Other genetic elements utilised for production of the pseudogenome include two mammalian reporter gene cassettes for the expression of luc or SEAP, as well as a novel mammalian enhancer element. The newly created replicating vectors were tested for reporter gene expression by transfection of mammalian cells.

2.1.1. Modification of geminiviruses for use as vectors

Single-stranded DNA (ssDNA) plant viruses have recently been utilised as vectors for transient expression of proteins at high volume (Hefferon and Fan, 2004; Mor et al., 2003; Regnard et al., 2010). ssDNA viruses are some of the most widely distributed of all the viruses, infecting prokaryote, plant and animal hosts. The smallest of these are in the *Geminiviridae*, *Nanoviridae*, and *Circoviridae* families. The first two consist of plant viruses, the third of avian and mammalian viruses. These viruses show an organisation characterised by divergent ORFs and highly compact, ambisense genomes - overlapping promoter and *ori* sequences, and promoter sequences within ORFs, are common (Liu et al., 1998; Mankertz and Hillenbrand, 2002). The two elements that remain consistent in the genomes of these viruses are ORFs that code for 1) a replication-associated protein complex (Rep/RepA) that recruits cellular machinery and initiates replication via the rolling circle replication (RCR) model, and 2) a coat protein (CP) which pentamerises to form the units of the viral capsid.

Geminiviruses, which infect plant host cells, are the simplest of the *Geminiviridae* (Stanley, 1993). They are unique amongst the viruses in that they have a geminate capsid structure, made up of two partially assembled capsids (Zhang et al., 2001). One geminivirus in particular, the Bean yellow dwarf virus (BeYDV), has been well characterised, and is an attractive target for vector development (Halley-Stott et al., 2007). This virus is made up of four transcribed ORFs - V1 and V2 (on the virion sense strand) code for movement protein (MP) and CP, respectively, and C1 and C2 on the

complementary-sense strand code for Rep and RepA – as well as short and long intergenic regions (SIR and LIR) (See Fig. 2.1). The movement protein ORF, V1, shows sequence homology with maize streak virus V1, the product of which is responsible for virus movement. The coat protein, once expressed, pentamerises to form the viral capsid. The proteins Rep and RepA are responsible for initiating replication in the host cell, as described earlier. The LIR contains promoter elements and the viral double-stranded *ori*, while the SIR contains the transcription termination signal and primer binding sites for C-strand DNA synthesis. Like all geminiviruses, it replicates via the RCR cruciform model, a version of the RCR model which includes the formation of a cruciform, or stem-loop structure at the double-stranded origin of replication, in the LIR.

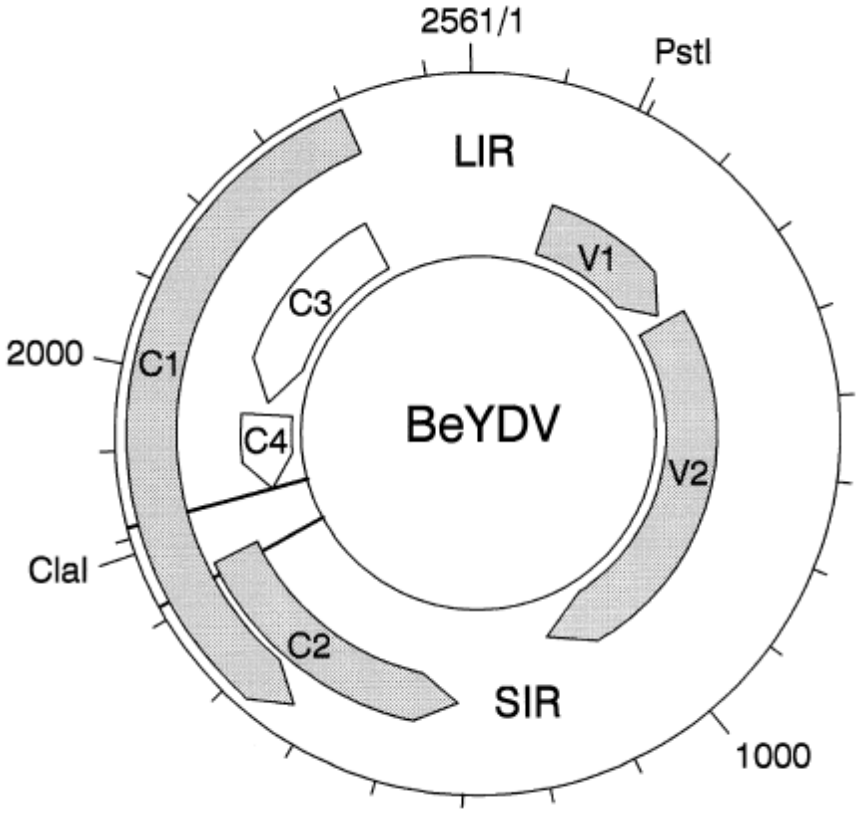


Figure 2.1 | Organization of the BeYDV genome The arrangement of virion-sense (V) and complementary-sense (C) ORFs with the potential to encode proteins in excess of 10 kDa molecular mass are shown relative to the large and small intergenic regions (LIR and SIR, respectively). Shaded ORFs are those that are conserved between BeYDV subgroup I members - V1, movement protein; V2, coat protein; C1, Rep; C2, RepA. Removal of the intron (nucleotides 1722–1807, indicated by lines) serves to fuse ORFs C1 and C2 (ORF C1C2, from which Rep is produced), and ORFs C4 and C1 (ORF C4C1, found only in BeYDV and Tomato Yellow Dwarf Virus (TYDV)). *Pst*I and *Cla*I are restriction enzyme sites. Reproduced from Liu et al. (1997).

BeYDV has been modified for use as an *Agrobacterium*-mediated plant expression vector by several groups (Hefferon and Fan, 2004; Mor et al., 2003; Regnard et al., 2010; Zhang and Mason, 2006). While Zhang and Mason (2006) use the BeYDV replicon to achieve a stable transfection of *Solanum tuberosum*, Regnard et al. (2010) has modified the BeYDV for transient, high level expression, using

Nicotiana benthamiana. To achieve this high level expression, Regnard et al. (2010) replaced the ORFs for the CP and MP with a cauliflower mosaic virus (CaMV) 35S promoter (with duplicated transcriptional enhancer sequence), multiple cloning site (MCS), and polyadenylation signal, as well as a duplicate copy of the LIR. Notably, the introduction of an additional LIR into the vector allows for the formation of a circular replicon within the plant host cell. This duplication is a common feature of all replicating plant expression vectors (Regnard et al., 2010). The two LIR sequences are located within the left and right border sequences (LB and RB, Figure 2.2), which enclose the transfer DNA (T-DNA), that part of the vector that is transferred into plants during agroinfiltration (Zupan et al., 2000). Two LIR copies allows for the formation of the active, circularised dsDNA replicon in plants after T-DNA delivery to the plant cell. This circular form is integral to the cruciform RCR model – the cruciform structure formed by the LIR is the recognition and binding site for the Rep/RepA protein complex (Khan, 2005). Once this complex is formed, the recircularised plasmid, or replicon, forms a double-stranded DNA intermediate, which is replicated to high copy number in the cell.

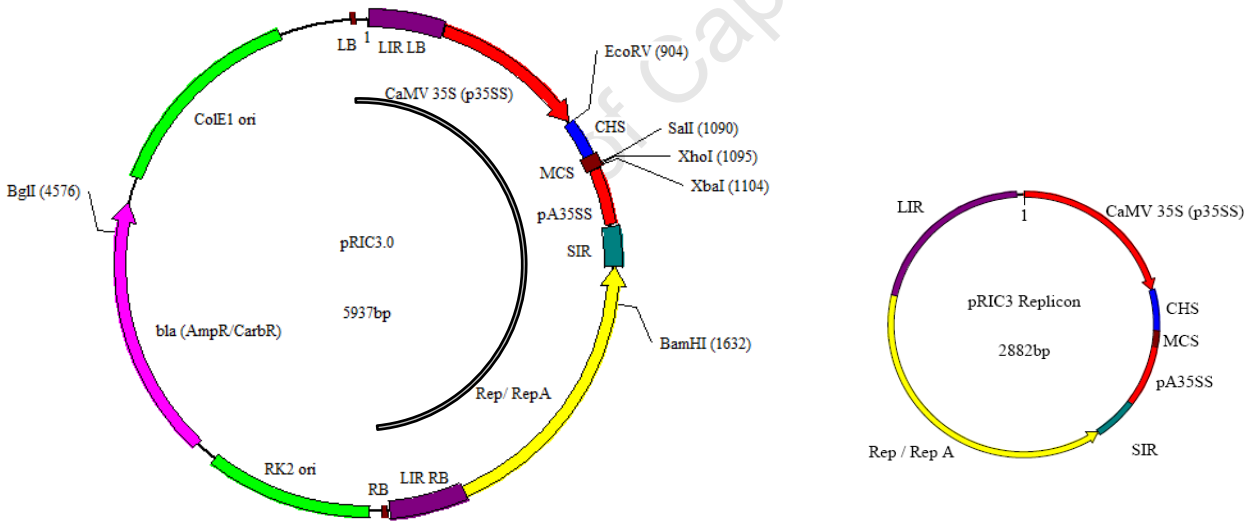


Figure 2.2 | 2nd generation vector pRIC3 and replicon ColE1 ori, origin of replication for Escherichia coli; RK2 ori, origin of replication for Agrobacterium tumefaciens; bla, ampicillin/carbenicillin resistance bla gene; LB and RB, left and right borders for T-DNA integration; P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; CHS, chalcone synthase 5`-untranslated region; MCS, multiple cloning site, pA35S, CaMV 35S polyadenylation signal; LIR, BeYDV long intergenic region; SIR, BeYDV short intergenic region; rep, BeYDV rep gene. The grey bar inside the plasmid maps indicates the T-DNA transferred into the plant cell during transfection.

This replicating vector, designated pRIC, has demonstrated high replication *in planta* (up to two orders of magnitude greater than a highly expressed, non-replicating vector, pTRAc) and thus high protein expression – 3-7 fold higher than pTRAc over a seven-day time trial using transgenes encoding enhanced green fluorescent protein (EGFP), or subunit antigens (HPV-16 L1 and HIV C p24), inserted at the MCS (Regnard et al., 2010). While these constructs showed high protein expression

compared to a similar, non-replicating vector, concerns remain that duplicated structures may lead to recombination (Regnard et al., 2010). This instigated the development of 2nd-generation vectors, by removal of the two scaffolding regions, the polyadenylation signal pA35S, and one of the two CaMV promoter sequences, resulting in the vector shown in Fig. 2.2. This vector, designated pRIC3, was demonstrated to have similar levels of replication to pRIC, as determined by qPCR, and showed higher protein expression at 1 and 3 days post infiltration (dpi) (Ogle, 2008).

Both of these vectors (pRIC and pRIC3) have demonstrated replicational release (formation of the circularised replicon) once they are within the plant cell host, which allows high-copy number replication. This autonomous replication makes pRIC3 an ideal vector for the production of pseudogenome DNA in plants. Insertion of a mammalian promoter, reporter gene, and polyadenylation signal elements (collectively referred to as a cassette) into pRIC3 within the T-DNA will result in replication to high copy number of a replicon incorporating a mammalian reporter cassette. This replicon will be used as a mammalian reporter pseudogenome, which should then be packaged by HPV capsid proteins *in planta* to form PsVs. To further optimise mammalian reporter gene expression we included an enhancer element from porcine circovirus (PCV), which has recently been shown to increase expression of a mammalian gene (Tanzer et al., 2011).

2.1.2. Porcine circovirus

Porcine Circovirus (PCV) is an ssDNA virus of the family *Circoviridae*, which is comprised of viruses such as PCV, beak and feather disease virus and goose circovirus (Finsterbusch and Mankertz, 2009). Circoviruses are single-stranded, circular and non-enveloped viruses, with a size of approximately 1760bp (Tischer et al., 1982), making them the smallest known autonomously replicating mammalian viruses (Mankertz et al., 1998). There are two PCV strains identified, PCV-1 and PCV-2. PCV-1 is non-pathogenic (Tischer et al., 1995), while PCV-2 is the etiological agent in the porcine disease post-weaning multisystemic wasting syndrome (PMWS). Like geminiviruses, PCV-1 is made up of two major ORFs, *rep* and *cap*, which code for the Rep protein and the capsid protein, respectively. These are arranged in an ambisense fashion, with the intergenic region between the 5' ends encoding the viral Ori, as well as the *rep* promoter element, Prep. The promoter for *cap* (Pcap) is found within the *rep* ORF (see Fig. 2.3) (Mankertz et al., 1998).

This simple and extremely compact genome makes PCV-1 a good potential source of mammalian plasmid vector elements. Fiona Tanzer, an investigator in this lab, developed a vector for use as a potential HIV DNA vaccine. To achieve this, she inserted elements from the PCV-1 genome into the DNA vaccine plasmid vector pTHgrttnC. Developed by Hanke (Hanke et al., 1998; Mankertz et al., 1998), this vector was created by removing the neomycin resistance gene and the f1-M13 ori from pRc/CMV (Invitrogen™), then substituting the CMV enhancer/promoter region for a cytomegalovirus enhancer/promoter/intron region. This was further modified into a DNA vaccine, pTHgrttnC by this group and another, the Williamson group at IIDMM (Burgers et al., 2006), by the insertion of the recombinant HIV gene, grttnC. The vector-based vaccine used by Tanzer was created from this. The core and conserved late element (CLE) of the PCV-1 Cap promoter region (Pcap) was sub-cloned immediately adjacent to the CMV i/e promoter region of pTHgrttnC, to form pTHpCapRgrttnC. In the current work, the mammalian promoter, enhancer and polyadenylation signal elements from the pTHpCapRgrttnC construct were used for one of the mammalian cassettes introduced into pRIC3.

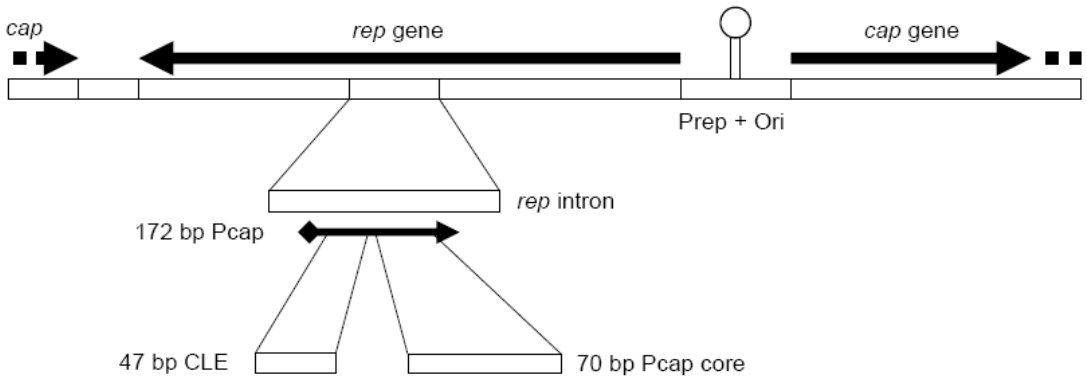


Figure 2.3 | Consensus PCV genome showing ORFs and promoter regions The 172bp Pcap region shown has been used as an enhancer region. Reproduced from Tanzer et al 2010.

2.1.3. Reporter genes and cost of assay

Selection of mammalian reporter cassettes is an important aspect of vector construction. Genes for several reporter proteins have been packaged into PsVs produced in mammalian cells – these include luciferase (*luc*), β-galactosidase, Secreted Alkaline Phosphatase (SEAP) and puromycin resistance gene (*pac*) (Buck et al., 2005a; Stauffer et al., 1998; Touze and Coursaget, 1998; Unckell et al., 1997). In recent years, SEAP has become the standard reporter gene used for PBNA, as originally demonstrated by Buck et al. (2005a). While this assay has been shown to be effective for PBNA, it is one of the most expensive reporter assays available (R36.63/reaction). Luciferase assays, on the other hand, are much cheaper (R16.33/reaction), and have been used for PBNA, though with lower efficiency (Fleury et al., 2008a). Accordingly, both SEAP and luciferase cassettes were selected for

testing. In the reporter plasmid pYSEAP, the SEAP reporter gene is under control of the elongation factor 1 alpha (EF-1α) promoter and the simian virus 40 (SV40) polyadenylation signal, while luciferase is under control of the mammalian expression cassette from the pTH expression plasmid. This is made up of the cytomegalovirus (CMV) intron/enhancer/promoter element with the addition of pCapR enhancer region, and the bovine growth hormone (BGH) polyadenylation signal. Both these cassettes have been extensively utilised for constitutive, high level expression in mammalian cells (Buck et al., 2005a; Burgers et al., 2006; Tanzer et al., 2011).

The aim of this study was to adapt the previously developed geminivirus-derived pRIC3 vector to produce three replicating vectors, which would replicate *in planta* to form pseudogenomes for packaging by HPV L1 and L2 capsid proteins into PsVs. Two different mammalian expression cassettes were chosen, encoding genes for the reporter gene products SEAP (mSEAP cassette) and luc (mluc cassette). Both mammalian cassettes were incorporated into pRIC3 with the extant plant cassette (+) serving to increase the overall replicon size (pRIC3-mSEAP+ and pRIC3-mluc+), while the SEAP cassette was also incorporated in place of the plant cassette to create a smaller replicon (pRIC3-mSEAP). HPV VLPs have been reported to package pseudogenomes of approximately 5-8Kbp in size, whereas larger or smaller pseudogenomes are not packaged at all (Buck et al., 2004; Touze and Coursaget, 1998). To accommodate these size constraints, three vectors were created with different reporter genes and resulting in replicons of different sizes:

a) **pRIC3-mSEAP** – pRIC3 with a mammalian cassette encoding the SEAP reporter gene in place of the current plant cassette (4.8Kbp replicon/pseudogenome)
b) **pRIC3-mSEAP+** – pRIC3 with the addition of a mammalian SEAP cassette, inserted upstream of the plant cassette (6.6Kbp replicon/pseudogenome)
c) **pRIC3-mluc+ -** pRIC3 with the addition of an alternative mammalian cassette encoding the luc reporter gene, inserted upstream of the plant cassette (7.6Kbp replicon/pseudogenome)

2.2. Materials and Methods

2.2.1. Cloning strategy

The previously created pRIC3 (Figure 2.2) will be further developed into three distinct autonomously replicating vectors (See Figure 2.4), for infiltration into plants. To achieve the aims of this project, several modifications of the previously described plasmid pRIC3 were required. These modifications resulted in three final plasmids, shown in Fig. 2.4. These were named pRIC3-mSEAP, pRIC3-mSEAP+, and pRIC3-mluc+. The final plasmids retained most of the original features of pRIC3, differing only in the expression cassettes found on the replicon. A simplified workflow is shown in Fig. 2.5, detailing the major steps in construction of these plasmids.

Vector	Insert	Vector Size (Kbp)	Replicon Size (Kbp)
pRIC3	Plant cassette	6.6	3.5
pRIC3-mSEAP	SEAP cassette	7.9	4.8
pRIC3-mSEAP+	SEAP and plant cassettes	9.7	6.4
pRIC3-mluc+	luc and plant cassettes	10.7	7.4

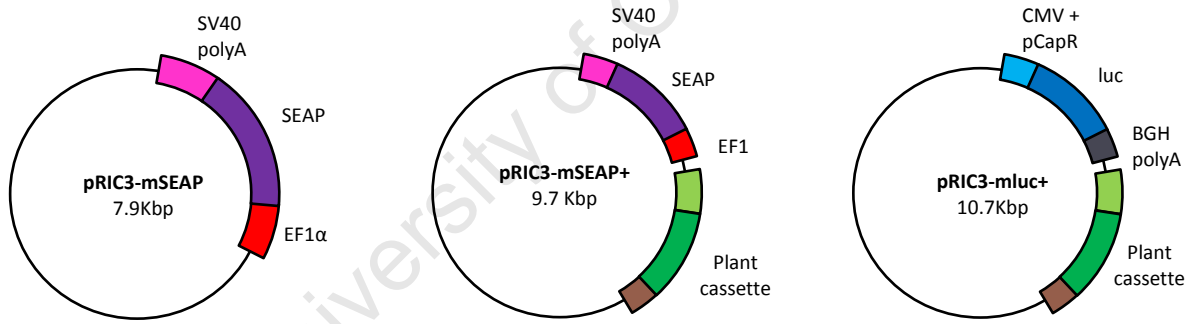


Figure 2.4 | Table and summary diagram of the autonomously replicating vectors created in this project.

2.2.1.1. Modification of pRIC3 to generate pRIC3.2

The backbone used for the constructed plasmids, pRIC3, is a plasmid that has been in development for some time as an autonomously replicating plant vector in this laboratory (Regnard et al., 2010). This plasmid was modified by the re-introduction of *SalI* and *XhoI* sites, at the 5' and 3' ends of the plant expression cassette of pRIC3, respectively (Figure 2.5B). This allowed for ligation of the entire mammalian cassette upstream of the plant cassette. These sites were used in the original construction of the pRIC vector, but were destroyed during cloning. As such, at each site a single nucleotide mutation was required to restore the recognition sequences – G>C for *SalI*, and C>G for *XhoI*.

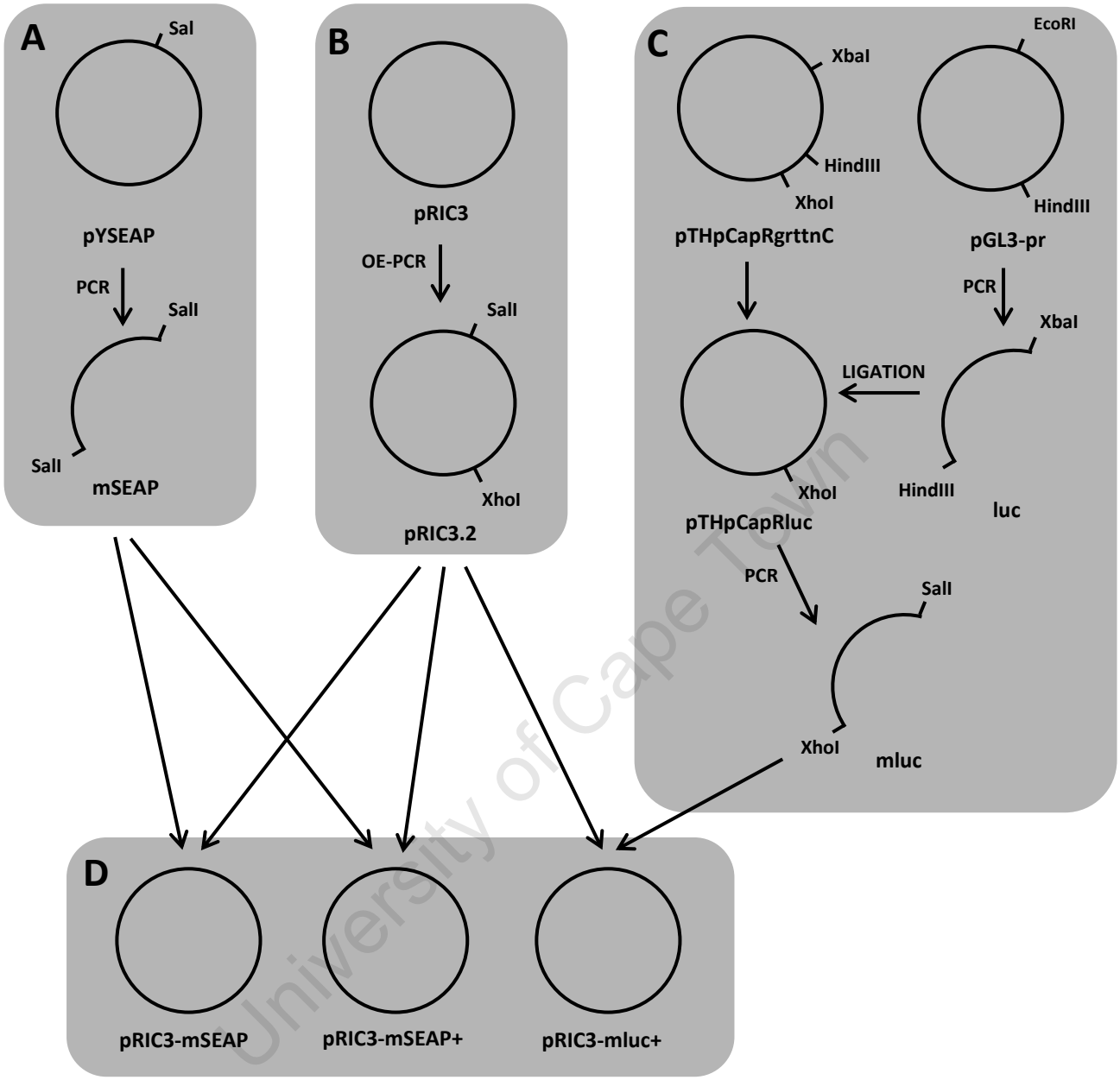


Figure 2.5 | Simplified Cloning Strategy A simplified workflow for cloning of three autonomously replicating vectors used in this study. (A) Creation of SEAP mammalian cassette (mSEAP). (B) Restoration of *SalI* and *XhoI* restriction enzyme sites in pRIC3. (C) Creation of luc mammalian cassette. (D) Final replicating vector construction. Relevant restriction enzyme sites are shown (mluc).

This was achieved via an application of PCR referred to as overlap extension PCR (OE-PCR), first described by Higuchi et al. (1988) (See Figure 2.6). Briefly, forward and reverse primers were designed incorporating the region of the plasmid which contained the destroyed restriction sites, with the appropriate single nucleotide mutation (B and C, Table 2.1, mutation shown in red). A corresponding primer was designed for each (A and D), such that two PCR amplification products, fragment I and fragment II, were generated (See Figure 2.6 and Table 2.2). Each of these fragments included a unique restriction site to allow for re-insertion of the mutated fragment into pRIC3 –

fragment I included a *BglI* site at the 5' end of the fragment, while fragment II included an *EcoRV* site at the 3' end of the fragment. After the amplification of fragments I and II, a second PCR, using primers A and D, and with both fragments I and II as template, was performed, resulting in the amplification product pR3fr-M (reaction 5, table 2.2). This fragment was bounded by a 5' *BglI* site and a 3' *EcoRV* site, and identical to the original sequence, except for the introduced single nucleotide mutation which restored the *SalI* restriction site. Sequence analysis was used to confirm that the correct sequences were generated. An identical process was used for the introduction of *XhoI* into pRIC3 (Primers E, F, G, and H, Table 2.1; reactions 3, 4, and 6, Table 2.2; amplification products III, IV and pR3fr2-M, Figure 2.6 and Table 2.2). The restriction enzyme sites utilised to ligate pR3fr2-M into pRIC3 to yield pRIC3.1 were *BamHI* at the 5' end of fragment III, and *XbaI* at the 3' end of fragment IV. After restriction enzyme digestion, products were separated on a 1% TBE agarose gel, visualised using a 365nm UV lightbox, excised, and purified using the Biospin Gel Extraction Kit (Bioer Technology Co. Ltd.). Purified products were then ligated using the T4 DNA ligase kit (Roche), and ligated at 4° C overnight. Electrocompetent *E.cloni*® cells (Lucigen) were transformed with 5ul of the overnight ligation mix, incubated on ice for 15 minutes, heat shocked for 1min, and incubated on

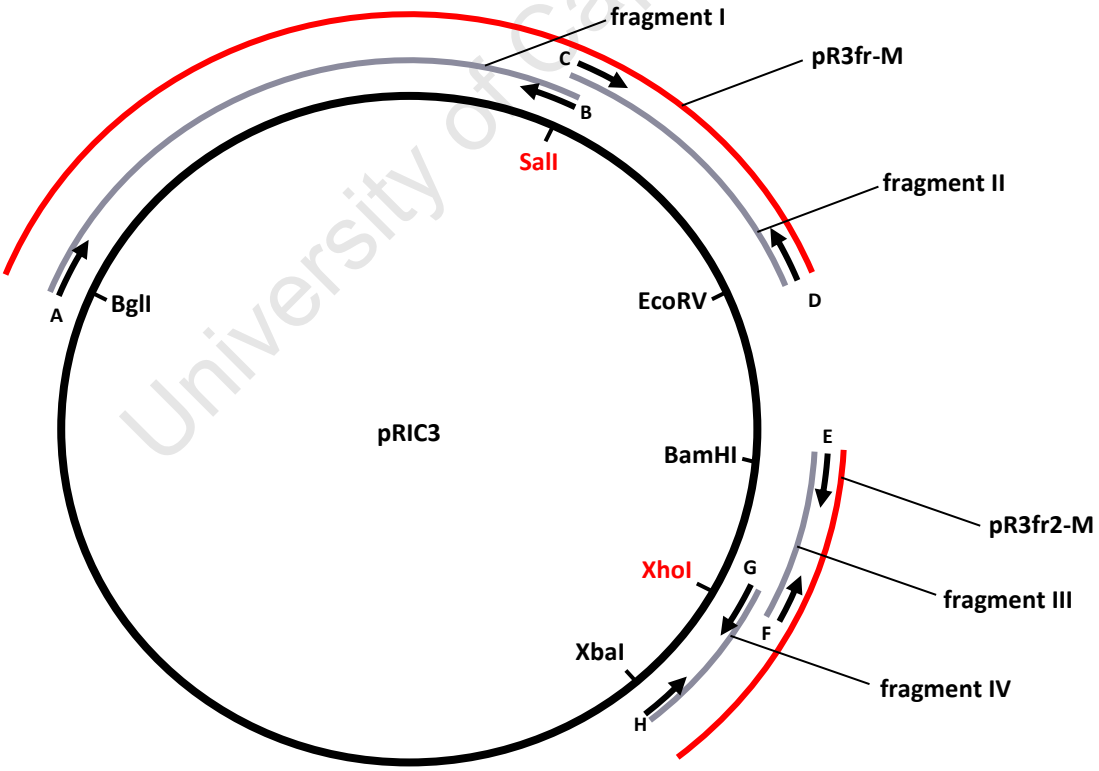


Figure 2.6 |OE-PCR scheme for restriction site rescue Diagram showing all relevant elements involved in restoration of *SalI* and *XhoI* sites in pRIC3. Blue lines indicatePCR fragments with introduced mutations. Red lines indicate full-length fragments cloned into pRIC3. All relevant fragments are indicated. Introduced restriction enzyme sites shown in red.

ice for 2 minutes. 500µl of antibiotic-free media (luria broth) was then added to the cells, and they were incubated at 37° C for 1 hour and grown under ampicillin selection (50µg/ml). Colony PCR (reaction 5, Table 2.2) was used to screen for positive pRIC3.1 colonies. To form pRIC3.2, pRIC3.1 and pR3fr-M were both digested with *BglI* and *EcoRV* and ligated as previously described. Colonies were screened for recombinant pRIC3.2 clones by colony PCR (Table 2.2, reaction 11), and plasmid DNA was extracted for confirmation of successful ligation by restriction digest and sequencing analysis, to yield pRIC3.2.

2.2.1.2 Cloning of mammalian cassettes for ligation into pRIC3.2

The mammalian expression cassettes utilised in vector construction were obtained from a variety of sources. All genes are commercially available as part of reporter plasmids or well-developed assays. The luciferase (luc) gene came from the commercial pGL3-promoter plasmid, from Promega. The secreted alkaline phosphatase (SEAP) gene was taken from the pYSEAP plasmid, used for neutralisation assays in mammalian cells, and originally developed by Pastrana et al. (2004). The pTHpCapRgrttnC plasmid was taken from a study by Tanzer et al. (2011), who showed that the enhancer region pCap, inserted into the promoter region in the reverse orientation, enhanced expression of the gene of interest. This expression vector was originally developed by Hanke et al. (1998).

The entire SEAP expression cassette was cloned directly from the vector pYSEAP (see Figure 2.5A). This vector, developed for use in PBNA, carries the SEAP gene under the control of the EF1alpha constitutive promoter element for high-level expression in mammalian cells. PCR was performed to amplify the 3069bp cassette, and introduce a *Sal*I site at the 3' end for cloning into pRIC3.2 (the cassette also incorporated another existent *SalI* site at the 5' end, which was used for later cloning) using primers SEAPSal-F and SEAP-R (3075bp; reaction 7, Table 2.2).

To create the mluc cassette, luciferase was cloned into the pTHpCapR vector, replacing the grttnC polyprotein, to incorporate the pCapR enhancer element (see Figure 2.5C). The luciferase gene *luc* was excised from the commercial reporter plasmid pGL3-promoter by *HindIII* and *EcoRI* restriction enzyme digestion. The products were separated by gel electrophoresis on a 1% agarose TBE gel and visualised on a longwave (365nm) UV lightbox. DNA was excised and purified using as previously. The pTHpCapRgrttnC vector was similarly digested and purified. The *luc* gene was ligated into pTHpCapRgrttnC in place of the grttnC polyprotein. PCR was performed to amplify the newly created mluc cassette, incorporating a 5' *SalI* site and 3' *XhoI* site flanking the mammalian cassette in the

expression vector pTHpCapRluc (reaction 8, Table 2.2). The PCR product was purified as previously and cloned into the commercial blunt-end ligation vector pJET1/blunt (Promega), to form the intermediary vector pJET-mluc.

2.2.1.3. Cloning of mammalian cassettes into pRIC3.2 to generate pRIC3-mSEAP, pRIC3-mSEAP+, and pRIC3-mluc+

The regeneration of *SalI* and *XhoI* sites created the intermediate pRIC3.2, and allowed for serial cloning of the mammalian expression cassettes mluc and mSEAP into the pRIC3.2 plasmid. To create pRIC3-mSEAP (Figure 2.7A), the mammalian cassette was digested with and *SalI*, and pRIC3.2 was

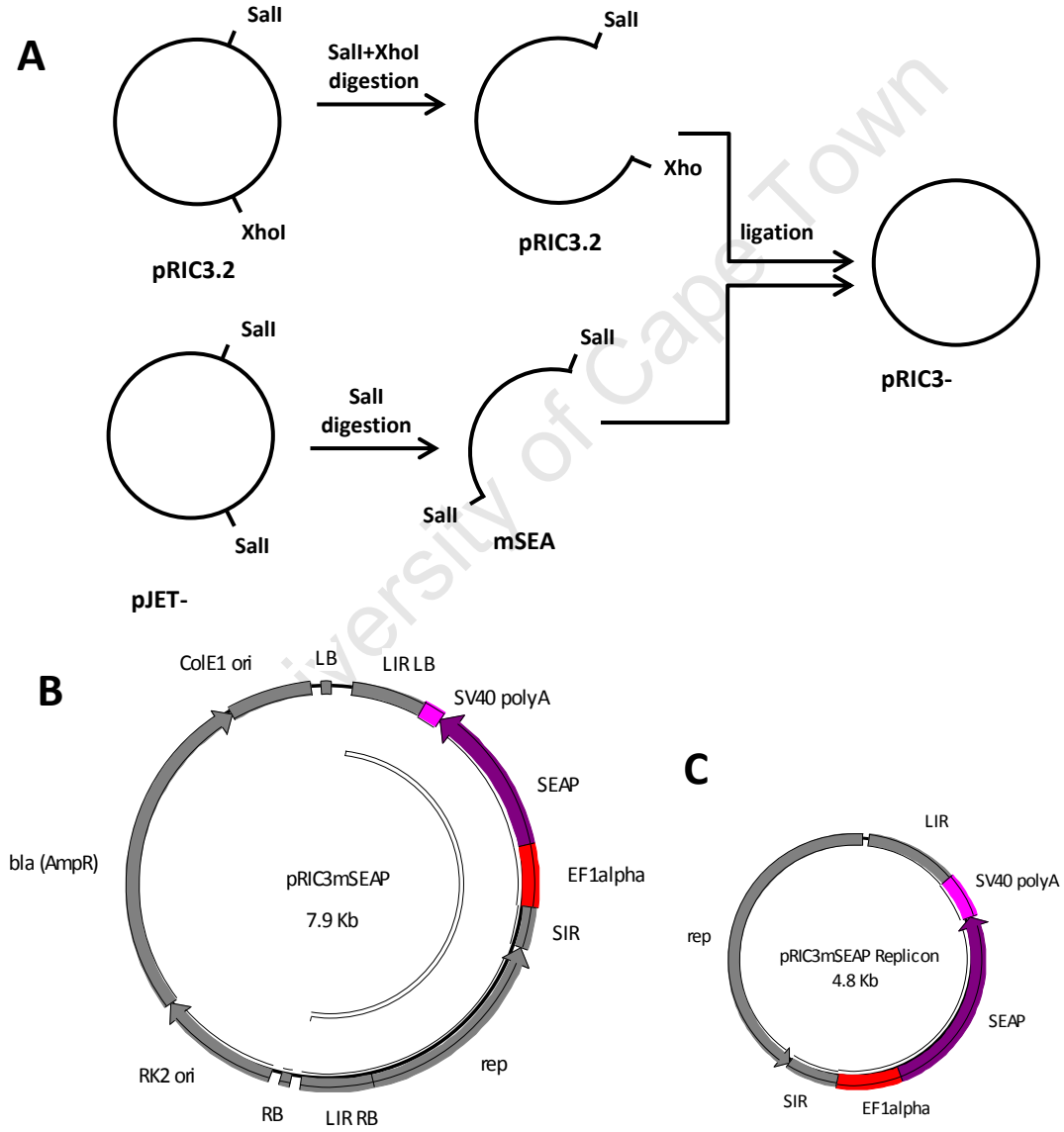


Figure 2.7 | Construction of pRIC3-mSEAP and replicon Autonomously replicating plasmid pRIC3-mSEAP. (A) Final cloning steps to create pRIC3-mSEAP. (B) EF-1α, elongation factor 1 alpha promoter; SEAP, Secreted Alkaline Phosphatase gene; SV40 PolyA, simian virus 40 polyadenylation signal. Grey elements remain unchanged from the original pRIC3 vector; mammalian cassette highlighted in coloured regions. White bar inside plasmid map indicates T-DNA transfected into plant cells. (C) Circularised replicon after release from T-DNA.

digested at the newly regenerated *SalI* and *XhoI* sites. The two digested fragments (i.e. the mSEAP cassette and the pRIC3.2 vector missing the plant cassette) were gel purified as previously, and ligated. Colony PCR was used to screen for positive clones, and positives were chosen for confirmation by plasmid mapping and sequence analysis (See Figure 2.7B and C).To generate pRIC3-mSEAP+ (See Figure 2.8A), pRIC3.2 was digested with *SalI* only, resulting in linearization of pRIC3.2. The mSEAP cassette was digested with *SalI* as previously, and the resulting products were purified and ligated. Colony PCR was performed to screen for positive clones, which contained both a

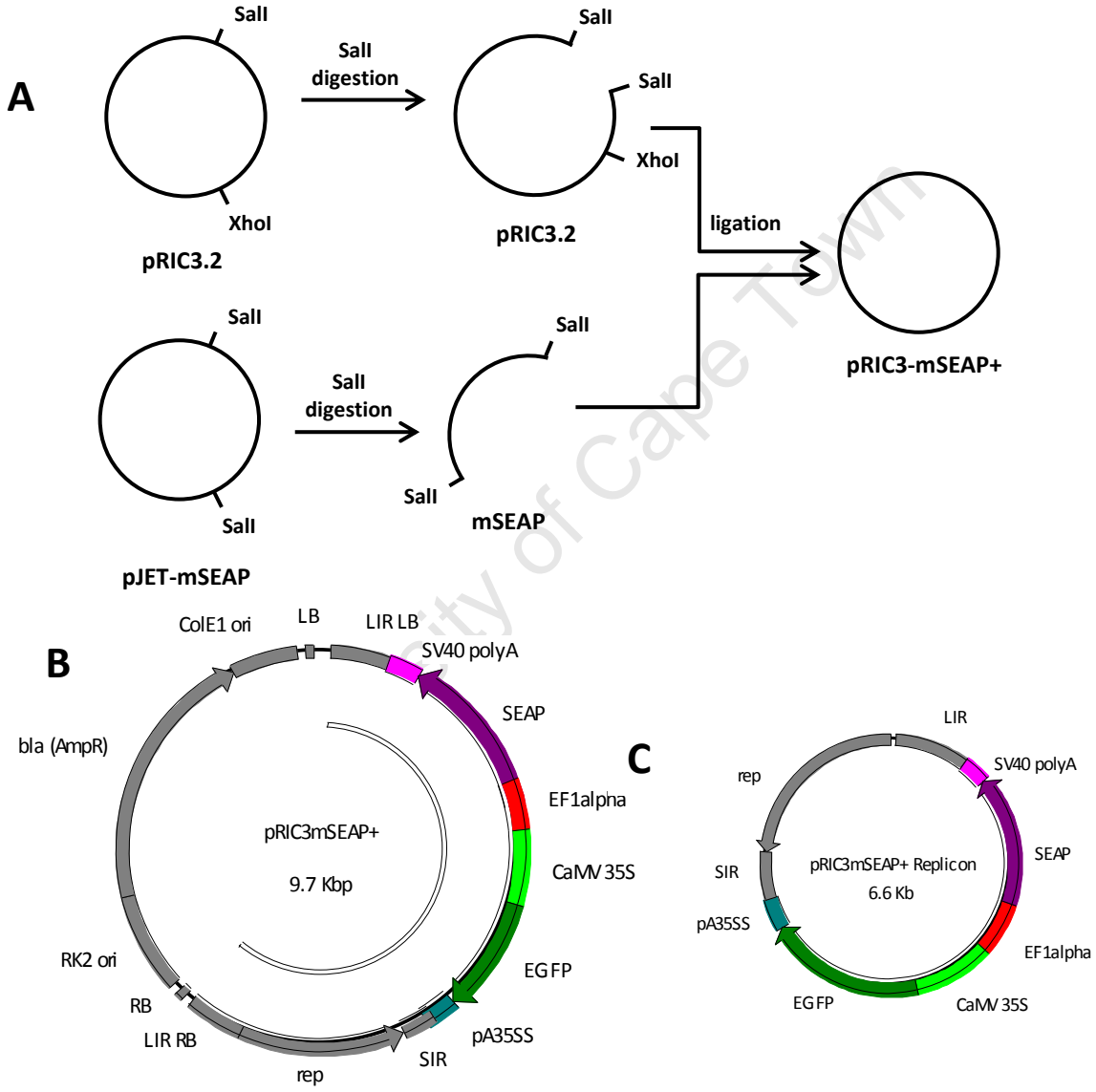


Figure 2.8 | Construction of pRIC3-mSEAP+ and replicon pRIC3-mSEAP+ autonomously replicating plasmid . (A) Final cloning steps in consgtruction of pRIC3-mSEAP+. (B) EF-1α, elongation factor 1 alpha promoter; SEAP, Secreted Alkaline Phosphatase gene; SV40 PolyA, simian virus 40 polyadenylation signal; CaMV 35S, cauliflower mosaic virus promoter region, EGFP, enhanced green fluorescent protein gene; pA35SS, CaMV 35S polyadenylation signal. Grey elements remain unchanged from the original pRIC3 vector; mammalian and plant cassettes highlighted in coloured regions. White bar inside plasmid map indicates T-DNA transfected into plant cells. (C) Circularised replicon after release from T-DNA.

mammalian and plant cassette (See Figure 2.8B and C).Generation of pRIC3-mluc+ was performed in a similar manner to pRIC3-mSEAP+ (Figure 2.9A). The intermediate pJET-mluc was digested with *SalI* and *XhoI* to excise the mammalian cassette from the pJET1/blunt vector backbone. pRIC3.2 was linearised by digestion with *SalI* only. Products were purified, ligated and screened as previously, resulting in the final pRIC3-mluc+ vector (Figure 2.9B and C).

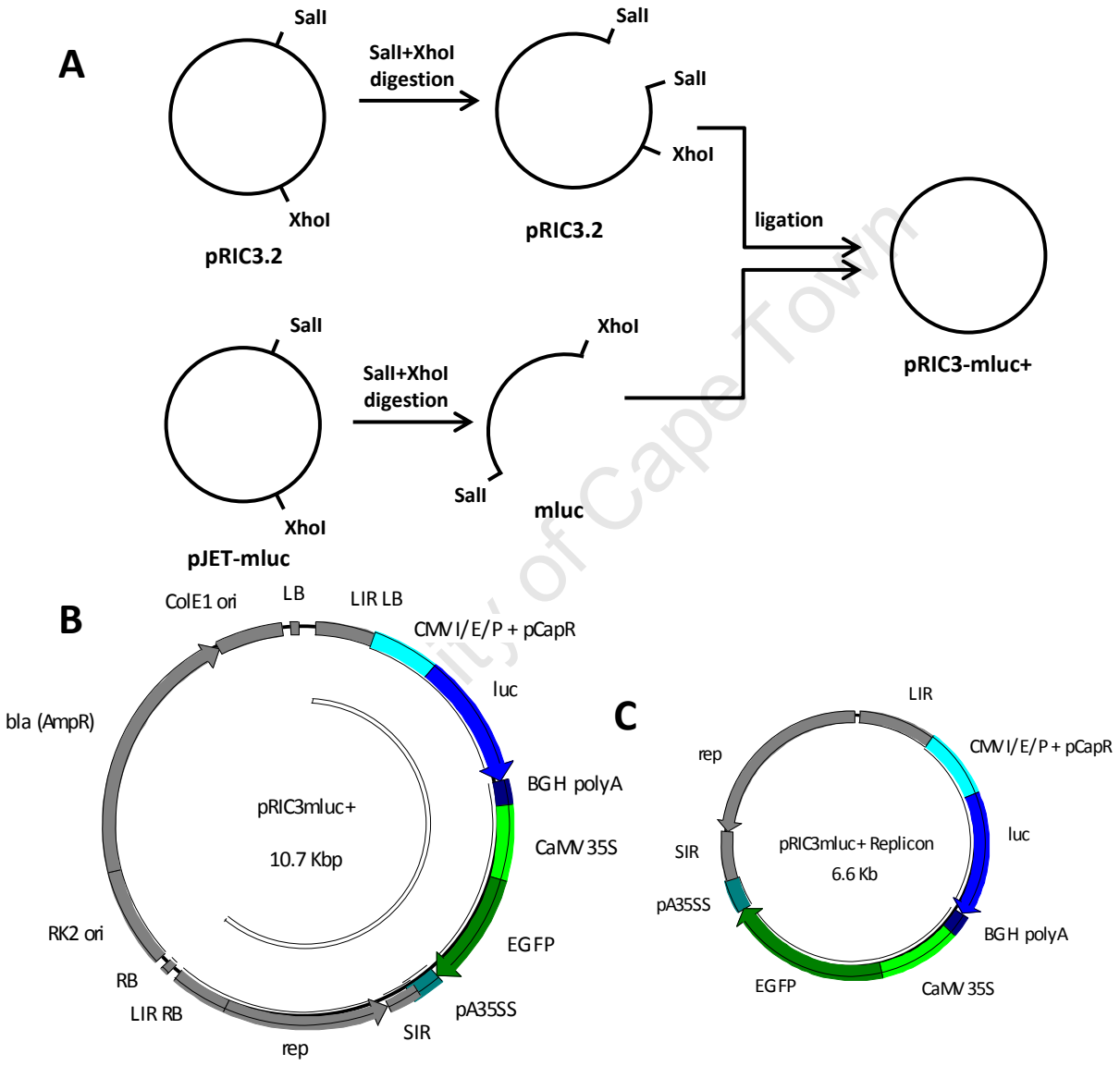


Figure 2.9 | Construction of pRIC3-mluc+ and replicon Autonomously replicating plasmid pRIC3-mluc+. (A) Final cloning steps to create pRIC3-mluc+. (B)CMV I/E/P + pCapR, cytomegalovirus intron/enhancer/promoter region with pCapR enhancer; luc, firefly luciferase reporter gene; BGH polyA, bovine growth hormone polyadenylation signal. CaMV 35S, cauliflower mosaic virus promoter region, EGFP, enhanced green fluorescent protein; pA35SS, CaMV 35S polyadenylation signal. Grey elements remain unchanged from the original pRIC3 vector; mammalian and plant cassettes highlighted in coloured regions. White bar inside plasmid map indicates T-DNA transfected into plant cells. (C) Circularised replicon after release from T-DNA.

2.2.2. PCR and Primer Design

Several applications of PCR were used in this study. All primers used are shown in Table 2.1, and the various PCR reactions are shown in Table 2.2. Polymerase chain reactions 1-7 were performed using ACCUZYME™ Polymerase (Bioline), a high fidelity proofreading polymerase, under the following conditions: 95°C for 3 minutes; 25 cycles of 95°C for 30", variable annealing temperature for 30", 68°C for 1-3 minutes; and 68°C for 5 minutes. PCR reaction 8 was performed using the high fidelity Pfu DNA Polymerase (Fermentas), under the conditions outlined below. All other PCRs were performed using the GoTaq Kit (Promega), under the following conditions: 95°C for 3 minutes; 25 cycles of 95°C for 30", variable annealing temperature for 30", 72°C for 1-3 minutes; and 72°C for 5 minutes. Annealing temperature and Mg^{2+} concentration are indicated on Table 2.2 for each reaction.

Table 2.1 | Primers designed and utilised in this project Nucleotide changes are shown in red.

Name	Primer	Orientation
A	5'-CTCGGCCCTTCCGGCTGGCTGG-3'	F
B	5'-CAGGTCGACCAAATACCATCACATCG-3'	R
C	5'-GATGTGATGGTATTTGGTCGACCTGG-3'	F
D	5'-CCCTTACGTCAGTGGAGATATCACATC-3'	R
E	5'-ACGCTCTAGAGTCCGCAAAAATCACC-3'	F
F	5'-TCGCTCGAGCCGCCGCCCGGTC-3'	R
G	5'-ACCGGGCGGCGGCTCGAGAATG-3'	F
H	5'-GGTGGGATCCCTTCTATAATTCTTTGC-3'	R
pJET-F	5'-GCCTGAACACCATATCCATCC-3'	F
pJET-R	5'-GCAGCTGAGAATATTGTAGCAGATC-3'	R
SEAPSal-F	5'-ATGTCGACTGGGAATTGGCTCCGGTG-3'	F
SEAP-R	5'-GCATCGGTCGACGGATCCTTATCGA-3'	R
pTH-F	5'-GCGTCGACGATATCGCCATT-3'	F
pTH-R	5'-CCATGATTACGCCAAGCTC-3'	R
SEAPi-F	5'-GCCAAGAAAGCAGGGAAGTC-3'	F
SEAPi-R	5'-CACATAGCCTGGACCGTTTC-3'	R
lucQ-F	5'-CAACTGCATAAGGCTATGAAGAGA-3'	F
lucQ-R	5'-ATTTGTATTCAGCCCATATCGTTT-3'	R
SEAPQ-F	5'-CCTTGACCCCGCACAGGTA-3'	F
SEAPQ-R	5'-GGCTCTGTCCAAGACATACAATGTA-3'	R
Rep-F	5'-TCCATCGTGCGTCAGATTTGCG-3'	F
polyA35SS-F	5'-AGGGTTCTTATAGGGTTTCGCTC-3'	F
CMV-R	5'-CCCTGTAACGTATGTGAGA-3'	R
Rep-R	5'-GGAGTCACAACAACTAGTCTTCTGC-3'	R

Table 2.2 | PCR reactions used in this project

Application	Reaction	Primers	Template	Product	Length (bp)	Annealing (°C)	[Mg2+] (mM)
OE-PCR	1	A+B	pRIC3	I	1676	68	3
	2	C+D	pRIC3	II	644	68	3
	3	E+F	pRIC3	III	234	61	1.5
	4	G+H	pRIC3	IV	323	61	1.5
	5*	A+D	I+II	pR3fr-M	2295	68	3
	6*	E+H	III+IV	pR3fr2-M	539	61	1.5
cloning	7*	SEAPSal-F and SEAP-R	SEAP cassette		3075	60	1.5
	8	pTH-F and -R	pTHpCapRluc		4120	51	3
colony PCR	9	pJET-F and -R	pJET1/blunt MCS		2295	60	1.5
	10	SEAPi-F and -R	SEAP gene fragment		795	53	2.5
	11	G and Rep-R	pRIC3 fragment		1275	61	3
	12	pJET-F and CMV-R	luc cassette fragment in pJET		554	51	4.5
inverse PCR	13	polyA35SS-F and CMV-R	mluc+ replicon		2235	60	2.5
	14	Rep-F and SEAPQ-R	mSEAP/mSEAP+ replicon		2192	54	2
qPCR	15*	lucQ-F and lucQ-R	mluc+ replicon		153	57	1.5
	16	SEAPQ-F and SEAPQ-R	mSEAP/mSEAP+ replicon		83	54	1.5

**also used for colony PCR.*

2.2.3. Sequencing

Sequence analysis was used to confirm correct sequence identity of each stage of the cloning process, as well as the final vectors. Sequencing was performed by Macrogen Inc., and multiple sequence alignment was done using CLC Main Workbench 6 bioinformatics analysis software (CLCbio).

2.2.4. Growth and maintenance of HEK293TT cells

HEK293TT cells were used for all mammalian expression experiments. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM), with 10% Fetal Calf Serum (FCS, v/v), 1% nonessential amino acids (NEAA, v/v), and 1% penicillin/streptomycin (v/v). Cells were maintained in T75 cell culture flasks (Corning), and incubated at 37° C, with 5% CO_2, and 95% humidity. Cells were passaged when they reached 90% density, with a seeding density of 10% (approximately $1x10^5$ cells/ml).

2.2.5. Reporter gene expression testing in HEK293TT cells

In order to verify activity of the mammalian reporter cassettes, unencapsidated, endotoxin-free plasmid DNA was prepared from 100ml *E.coli* shake cultures using the EndoFree Plasmid Maxi Kit (QIAGEN) following kit instructions, and used to transfect HEK293TT cells, using the FuGENE 6 Transfection reagent (Promega). The FuGENE transfection protocol was followed; briefly, cells were seeded in 6-well plates at a density of $0.2x10^6$cells/ml, and allowed to grow for 24h (50-60% confluency). FuGENE:DNA ratios of 3:1, 3:2 or 6:1 were tested. 3 or 6µl of FuGENE transfection reagent was incubated in 97µl of serum- and antibiotic-free DMEM for 5 minutes at room temperature. 1-2µg of plasmid DNA was added to this mixture, mixed gently and incubated for a further 30 minutes, before the mixture was added dropwise to cells. Cells were incubated for 72h. For the plasmids containing the SEAP expression cassette, cell culture medium was harvested. For those containing the luciferase expression cassette, media was removed, the cells were washed once with PBS, and an appropriate volume of Cell Culture Lysis Buffer (Promega, 20µl for 96-well plates, 400µl for 6-well plates) was added to the cells. Cells were rocked on an orbital shaker for 15 minutes, and stored at -20° C overnight.

To measure luciferase production in mammalian cells, the Luciferase Assay System kit (Promega) was used, as per kit instructions. 100µl luciferase substrate luciferin was added to 20ul of cell lysate. Luminescence was read on a Modulus Microplate Reader (Turner BioSystems).

Western Blotting was used to confirm SEAP expression after transfection. 32µl of cell culture media from cells transfected with the SEAP cassette was used for SDS-PAGE, as described in Section 3.2.6. Blots were probed with a sheep-produced polyclonal anti-calf intestinal alkaline phosphatase (anti-ciAP) primary antibody (Abcam, ab7330), and mouse anti-sheep alkaline phosphatase-conjugated secondary antibody (Sigma, A8062).

2.3. Results

2.3.1. Introduction of restriction enzymes sites into pRIC3 to create pRIC3.2

In order to ligate new mammalian cassettes into the pre-existing structure of the pRIC3 plasmid, restriction enzyme sites needed to be introduced at the 3' and 5' ends of the extant plant expression cassette in pRIC3. To achieve this, overlap extension PCR was used (OE-PCR, see Fig. 2.6). Proof-reading polymerase system ACCUZYME™ (Bioline) was used for all OE-PCR steps to avoid unwanted mutations. Figure 2.10 shows the PCR reaction to amplify mutated fragments I-IV from pRIC3 (reactions 1-4, Table 2.2). Fragments of the expected size (fragment I, 1676bp; fragment II, 644bp; fragment III, 234bp; and fragment IV, 323bp), as well as positive controls, are indicated by red boxes. Secondary PCR products can be seen in the + and fragment I lanes – fragment I encompasses one of two LIR regions found in pRIC3, and this may lead to non-stringent annealing, and amplification of secondary products. Mutated fragments of the correct size were excised and purified, before being used as a template for the next stage of the overlap extension PCR.

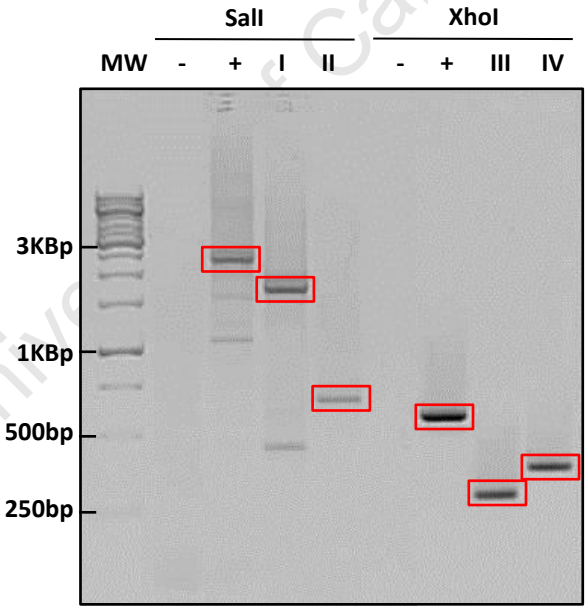


Figure 2.10 | PCR amplification of fragments for OE-PCR PCR was used to introduce *SalI* and *XhoI* restriction enzyme sites. Fragment I was amplified using primers A and B, fragment II using primers C and D, fragment III using primers E and F, fragment IV using primers G and H. Red boxes indicate bands of the expected size. MW, molecular weight marker (sizes are indicated); SalI, reactions for the introduction of *SalI* site; XhoI, reactions for the introduction of *XhoI* site; -, negative control (no primers); +, positive control (primers A and D, or E and H).

OE-PCR product pR3fr-M (2295bp) was amplified using primer pair A and D, from fragments I and II. Colony PCR using pR3fr-M-specific primers (reaction 5, Table 2.2) which amplified a 2295bp DNA

fragment confirmed the successful ligation of pR3fr-M into cloning vector pJET1/blunt (Fig. 2.11A). Lanes 1, 2, 4, 5, 7, 8, and 11 are positive, while smaller products indicate incorrect ligation (lanes 3, 6, 9 and 10). *SalI* restriction enzyme digestion of pJET-pR3fr-M recombinant clones was used to confirm the successful introduction of the 5' *SalI* restriction enzyme site by OE-PCR (Fig 2.11B) by comparison to theoretical digest fragment sizes. The pJET backbone contains a *SalI* site at position 168, approximately 370bp upstream of the MCS, resulting in the two digestion products seen here. Thus, fragments of approximately 3.4Kbp and 2Kbp, or 4.5Kbp and 1Kbp, were expected for presence of *SalI*, in forward or reverse orientation, while no mutation would result in a linearised vector of approximately 5.5Kbp. Colonies 1, 2 and 5 (Figure 2.11B) likely represent pR3fr-m, with the *SalI* mutation, in the forward orientation, while colony 4 represents the fragment in the reverse orientation, resulting in fragment sizes of approximately 4.5Kbp and 1Kbp.

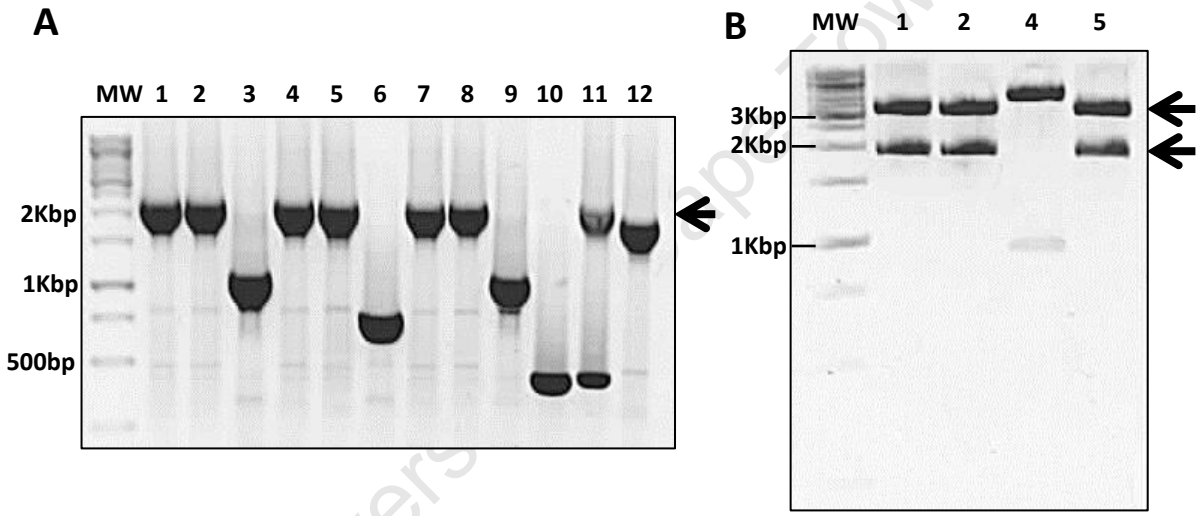


Figure 2.11 | Cloning of fragment pR3fr-M Colony PCR and Restriction enzyme digest of recombinant pJET-pR3fr-M. (A) Colony PCR amplification of a 2295bp fragment (←) confirms successful ligation of pR3fr-M into pJET1/blunt vector. MW, molecular weight; 1-12, recombinant clones. (B) *SalI* digest of positive clones confirms the presence of pR3fr-M insert, and successful introduction of a new *SalI* site. Expected fragments are indicated (←). MW, molecular weight, sizes are indicated; 1, 2, 4, 5, recombinant clones.

Similarly, OE-PCR was used to amplify pR3fr2-M (539bp) from fragments III and IV, using primer pair E and H (Figure 2.6), then ligated into cloning vector pGEM-T Easy (Promega) to form pGEM-pR3fr2-m. Colony PCR with pR3fr2-M-specific primers E and H (reaction 6, Table 2.2), which amplified a 539bp fragment, confirmed the presence of recombinant clones (Fig 2.12A). Positive clones were further screened for successful restoration of the *XhoI* restriction enzyme site by *XhoI* restriction enzyme digestion (Fig 2.12B); linearised vector of approximately 3.5Kbp indicated presence of an

XhoI site, while no digest occurred in those clones lacking the mutation. Several positive pJET-pR3fr-M and pGEM-pR3fr2-M clones were selected for sequencing analysis.

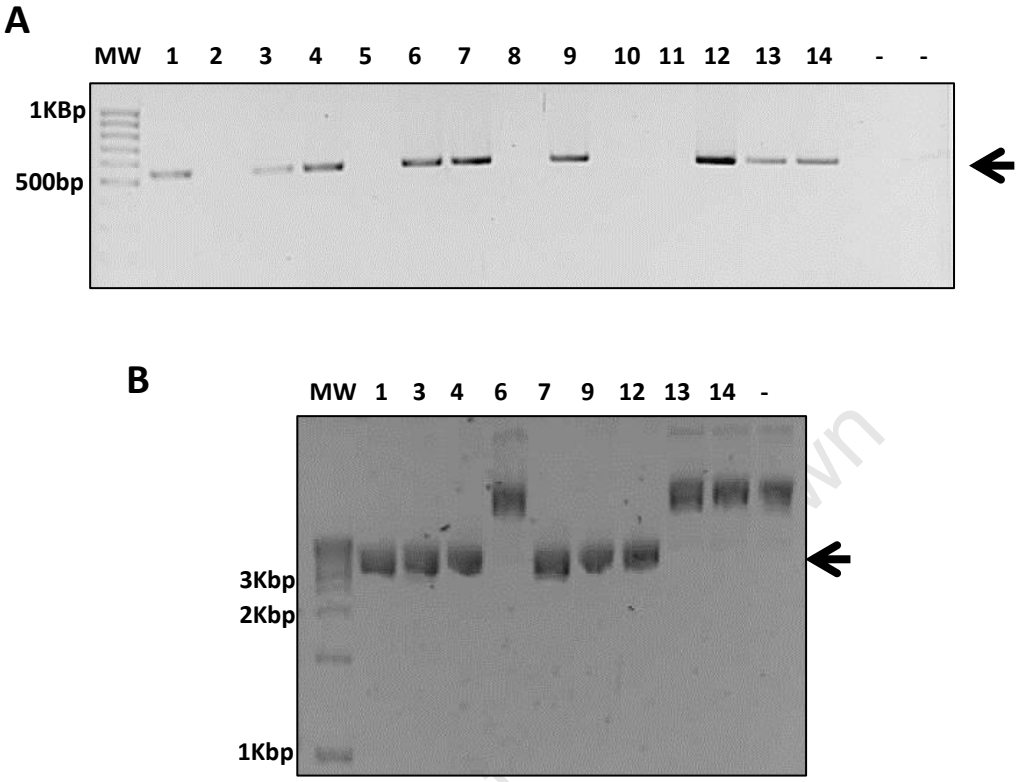


Figure 2.12 | Cloning of fragment pR3fr2-M Colony PCR and restriction enzyme digest of recombinant pGEM-pR3fr2-M. (A) Colony PCR amplification of 539bp fragment (←) confirms successful ligation of pR3fr2-M into pGEM-T Easy vector. MW, molecular weight; 1-14, recombinant clones; -, negative colonies. (B) *XhoI* digest of positive clones confirms the presence of pR3fr2-M insert, and successful introduction of a new *XhoI* site. An indicated fragment (←) indicates the presence of *XhoI* site at the correct location. MW, molecular weight; 1, 3, 4, 6, 7, 9, 12-14, recombinant clones; -, undigested DNA.

pR3fr-M and pR3fr2-M OE-PCR fragments were sequentially ligated into the original pRIC3 vector, using restriction sites located at the 5' and 3' ends of each fragment (*BglII* and *EcoRV* for pR3fr-M; *XbaI* and *BamHI* for pR3fr2-M). Figure 2.13 shows colony PCRs to screen for recombinant clones, as well as restriction enzyme digests to confirm that ligations were performed correctly. PCR primers G and Rep-R (reaction 11, Table 2.2) were designed to amplify a 1275bp fragment containing the newly introduced pR3fr2-M to confirm recombinant pRIC3.1 clones (pRIC3 with an introduced *XhoI* site at the 3' end of the plant cassette, Fig. 2.13A). Lanes 1, 2, 4, 7, 8, 9, and 12, Fig. 2.13A, show positive clones, while religated 'empty' vector resulted in smaller PCR products of approximately 500bp, as seen in lanes 3, 5, 6, 11, 13, and 14, Fig. 2.13A. Similarly, primers A and D (reaction 5, Table 2.2) were used to amplify the pR3fr-M fragment ligated into pRIC3.1, to confirm the presence

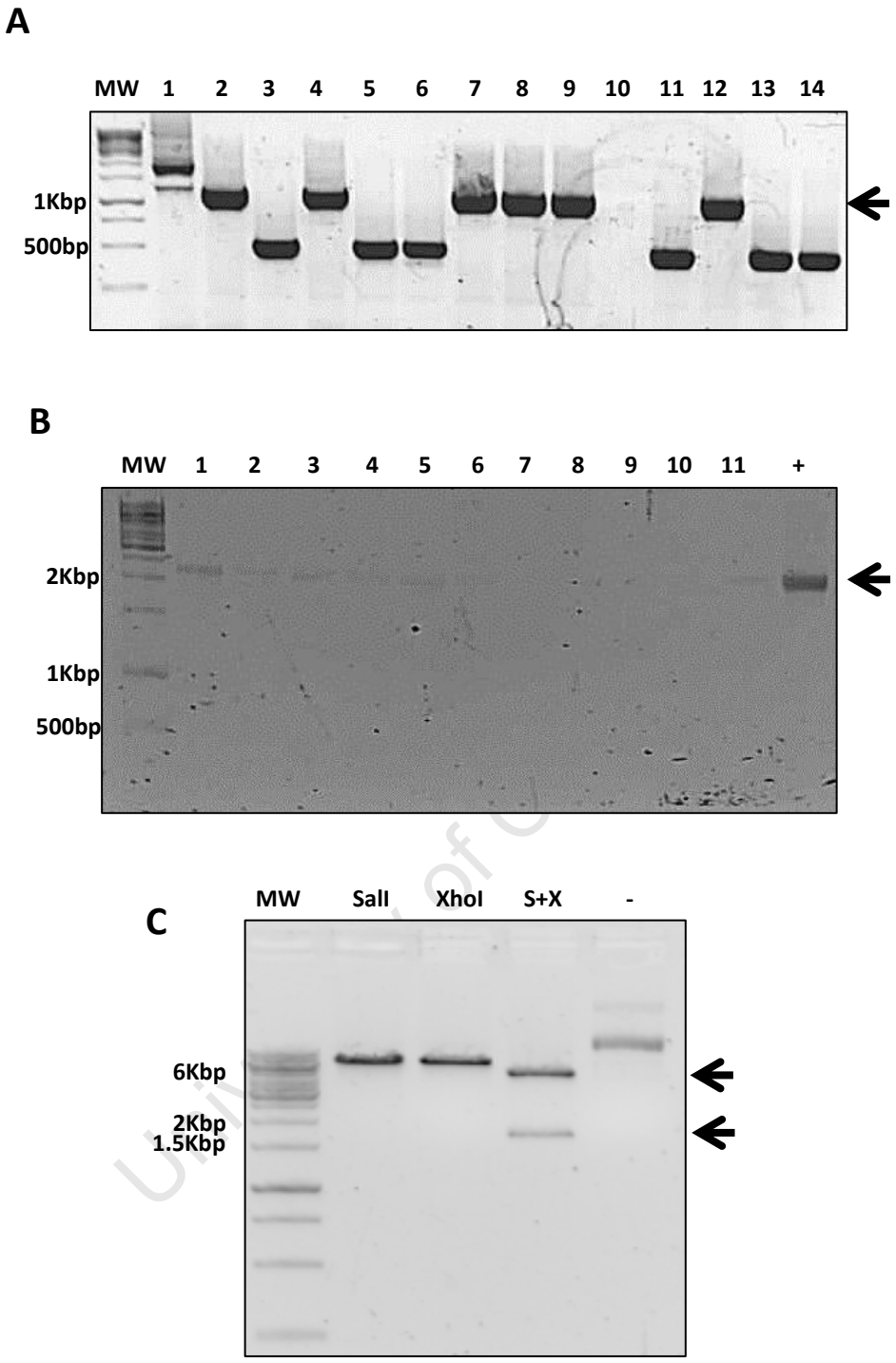


Figure 2.13 | Cloning of intermediates pRIC3.1 and pRIC3.2 Colony PCR of recombinant pRIC3.1 and pRIC3.2, and restriction digest of pRIC3.2. (A) Colony PCR amplification of a 1275bp fragment (←) confirms successful ligation of pR3fr-M into original pRIC3 vector. MW, molecular weight; 1-14, recombinant clones; (B) Colony PCR amplification of a 2295bp fragment (←) confirms successful ligation of pR3fr2-M into pRIC3.1 vector. MW, molecular weight; 1-11, recombinant clones; +, positive control pRIC3. (C) *XhoI* and *SalI* digest of pRIC3.2 confirms the presence of *SalI* and *XhoI* sites. Digested fragments are indicated (←). MW, molecular weight marker; S, *SalI* digest; X, *XhoI* digest; S+X, *SalI* and *XhoI* digest; -, no digest (negative control).

of recombinant pRIC3.2 clones (pRIC3.1 with *SalI* site introduced at the 5' end of the plant cassette, Fig. 2.13B). Although not clear in this image, lanes 1-5, and 11, Fig. 2.12B, were positive, indicating the presence of pR3fr2-M in pRIC3 to form pRIC3.2. Recircularised 'empty' vector would result in no product being amplified, as is the case for lanes 6-10. Restriction enzyme digest of pRIC3.2 with *SalI* and *XhoI* was used to confirm the presence of the introduced restriction sites in recombinant pRIC3.2 clones (Figure 2.13C), with fragments corresponding to expected sizes of 1776 and 4706bp (lane S+X). Digestion of pRIC3.2 with SalI and XhoI alone (lanes SalI and XhoI) indicates linearization of the vector by a single digestion event, while no digestion (lane -) showed coiled and supercoiled plasmid DNA, as expected. Several recombinant clones were picked for DNA sequencing, and were confirmed to possess the correct sequence and nucleotide mutations using CLC Main Workbench 6 bioinformatics analysis software (CLCbio).

2.3.2. Preparation of mammalian expression cassettes for cloning into pRIC3.2

Two mammalian expression cassettes were utilised in this study. The cassette containing the SEAP gene (mSEAP) came from expression vector pYSEAP, developed by Pastrana et al. (2004). The SEAP cassette was successfully amplified by PCR from pYSEAP, with the introduction of a 3' *SalI* site, using primer pair SEAPSal-F and SEAP-R (reaction 7, Table 2.2) to generate a 3075bp fragment with *SalI* sites at both 3' and 5' ends. This PCR product was purified and ligated into the vector pJET1/blunt, to form pJET-mSEAP. Colony PCR using the same primer pair amplified a 3075bp fragment to identify successful recombinant pJET-mSEAP clones – lanes 2, 3, 4, 6, 7, 9, and 1 (Figure 2.14). Recircularised 'empty' pJET1/blunt showed no amplification products – lanes 1, 5, and 8. Lane + shows a PCR

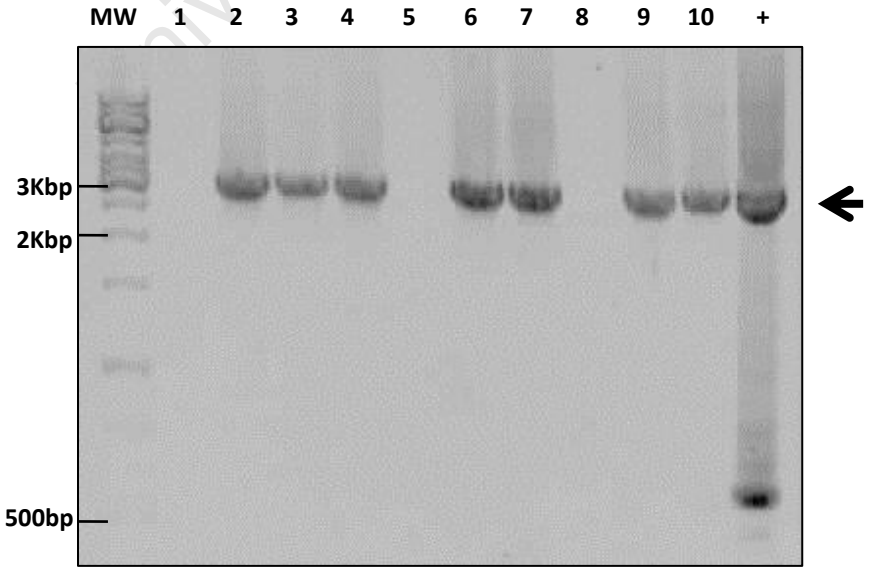


Figure 2.14 | Cloning of SEAP mammalian expression cassette Colony PCR of recombinant pJET-mSEAP clones. Colony PCR (A) amplification of 3075bp fragment (←) confirms successful ligation of mSEAP cassette into pJET1/blunt vector. MW, molecular weight; 1-10, recombinant clones; +, positive control (ligation mix).

performed using the ligation mix as a template – a secondary band in the region of 600bp is likely amplified from incorrectly ligated product.

To form the luciferase cassette (mluc) and incorporate the pCapR enhancer element, luciferase was cloned from commercial vector pGL3-promoter into a modified pTH vector to form pTHpCapRluc (see Figure 2.5C). The mluc cassette was then amplified with incorporated 5' *SalI* and 3' *XhoI* sites from intermediate pTHpCapRluc, and ligated into pJET1/blunt. pJET1/blunt MCS primer pJET-F, and CMV I/E/P-specific primer CMV-R (reaction 12, Table 2.2) were used to identify recombinant pJET-mluc clones (pJET with the full mluc cassette, Figure 2.15). These primers amplified a 554bp product in lane 7, which confirmed the presence of recombinant pJET-mluc clones, while all other lanes showed no product, indicating that the mluc cassette was not present.

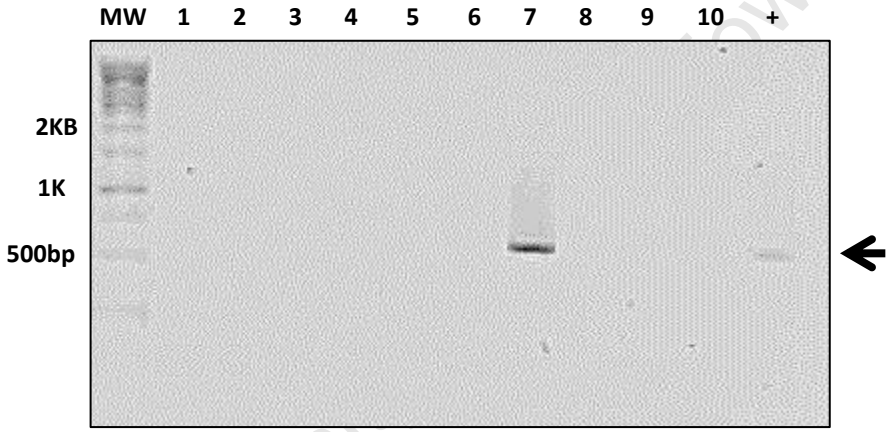


Figure 2.15 | Cloning of luciferase mammalian expression cassette Colony PCR of recombinant pJET-mluc clones. Colony PCR amplification of 554bp fragment (←) confirms successful ligation of mluc cassette into pJET1/blunt vector. MW, molecular weight; 1-10, recombinant clones; +, positive control pTHpCapRluc.

2.3.3. Construction of replicating vectors pRIC3-mSEAP, pRIC3-mSEAP+, and pRIC3-mluc+

The final vectors were created by ligating the mammalian cassettes into the pRIC3.2 intermediate via *SalI* and/or *XhoI* digestion (see Figures 2.7, 2.8, and 2.9). PCR primers SEAPi-F and SEAPi-R (reaction 10, Table 2.2) were designed to amplify a 795bp fragment of the SEAP gene were used to screen for recombinant pRIC3-mSEAP (Fig. 2.16A) and pRIC3-mSEAP+ (Fig. 2.16B) clones by colony PCR. A 795bp band indicated recombinant clones (lanes 1, 3-9 and 11-15, Fig 2.16A; lanes 14 and 17, Fig. 2.16B), while religated 'empty' pRIC3.2 clones showed no amplification product. Primers lucQ-F and lucQ-R (reaction 15, Table 2.2) were designed to amplify a 153bp fragment of the *luc* gene. Colony PCR using these primers resulted in recombinant pRIC3-mluc+ clones, as indicated by a 153bp band

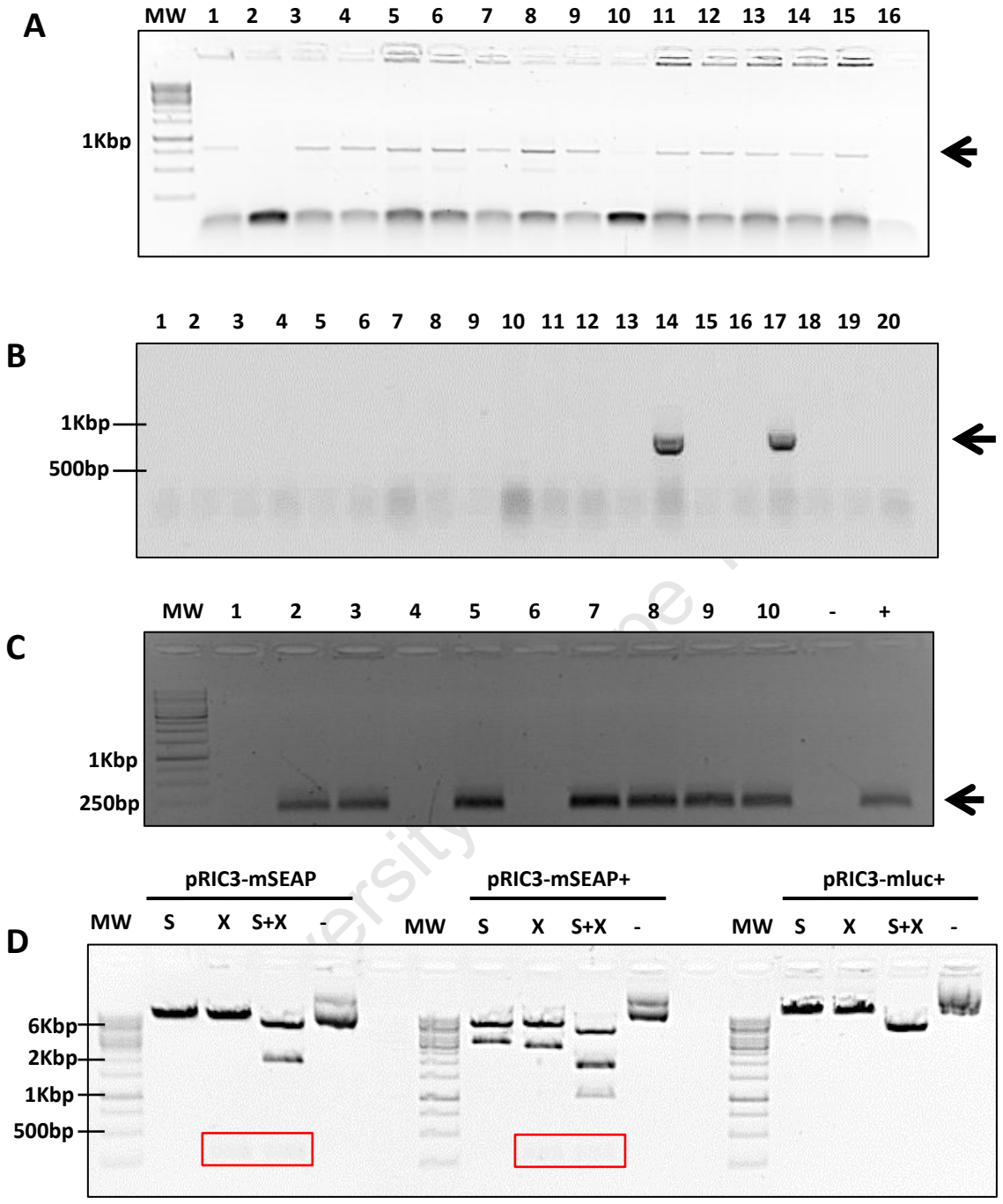


Figure 2.16 | Construction of replicating vectors Colony PCR and restriction enzyme digest of pRIC3-mSEAP, pRIC3-mSEAP+ and pRIC3-mluc+. Colony PCR (A) amplification of a 795bp fragment (←) confirms recombinant pRIC3-mSEAP clones.MW, molecular weight marker; 1-16, recombinant clones. Colony PCR (B) amplification of a 795bp fragment (←) confirms recombinant pRIC3-mSEAP+ clones. 1-16, recombinant clones. Colony PCR (C) amplification of 153bp fragment (←) confirms recombinant pRIC3-mluc+ clones. MW, molecular weight marker; 1-10, recombinant clones; -, negative control (no colony); +, positive control (pTHpCapRluc). *SalI* and *XhoI* restriction enzyme digest (D) of final vectors confirms correct plasmid size and construction. MW, molecular weight marker; S, *SalI* digest; X, *XhoI* digest; S+X, *SalI* and *XhoI* digest; -, no digest (negative control).

In lanes 2, 3, 5, and 7-10 (Fig 2.16C). Religated 'empty' pRIC3.2 vector showed no amplification products. Restriction digest of the final vectors using *SalI* and *XhoI* (Fig 2.16D) was used to confirm that the mammalian cassettes had been ligated as expected. pRIC3-mSEAP digestion showed that *SalI* cut the vector once (the *SalI* site at the 3' end of the mSEAP cassette was destroyed by ligation with *XhoI* on the pRIC3.2 vector), while *XhoI* cut the vector twice – there are two *XhoI* sites in close proximity within the SEAP cassette. Digestion with both *SalI* and *XhoI* resulted in fragments of 241bp (Fig. 2.16, indicated by a red box; could not be visualised), 1855bp and 5885bp. pRIC3-mSEAP+ digestion showed that *SalI* cut the vector twice, at the 3' and 5' ends of the mSEAP cassette, as expected. *XhoI* cut the vector in three places – twice in the interior of the SEAP gene, as for pRIC3-mSEAP, and once at the re-introduced site 5' of the plant cassette, which was not used for pRIC3-mSEAP+ cloning. *SalI* and *XhoI* digestion revealed five bands of 241bp (indicated by a red box), 983bp, 1776bp, 1855bp (not visible as separate fragments), and 4906bp, as expected. pRIC3mluc+ was cut once by each enzyme, *SalI* at the 5' end of the mluc cassette, and *XhoI* at the 3' end. Double digestion resulted in fragments of 4906bp (mluc cassette) and 5864bp (pRIC3 backbone), again visible only as a single band.

2.3.4. Sequencing of vectors

Sequencing of key intermediates and all three final vectors (not shown) was performed, and analysed using CLC Main Workbench 6 bioinformatics analysis software (CLCbio). Sequence analysis confirmed the presence and orientation of all expected elements of the final vectors – see Figures 2.7, 2.8, and 2.9 for complete plasmid maps. The mluc cassette was introduced in the same orientation as the plant cassette, while the mSEAP cassette was introduced in the reverse orientation in both pRIC3-mSEAP and pRIC3-mSEAP+.

2.3.5. Testing of mammalian pseudogenome reporter activity in HEK293TT cells

In order to confirm that the mammalian cassettes introduced into the pRIC3 plasmid would be functional as reporter pseudogenomes, the plasmids were transfected into mammalian cells to test for reporter protein expression. Endotoxin-free plasmid DNA was prepared, and cells were transfected using FuGENE 6 Transfection Reagent. Cells were transfected with various FuGENE:DNA ratios to determine the best ratio for future transfection work. For pRIC3-mSEAP and pRIC3-mSEAP+, cell culture media was harvested 72 hours post-transfection, while for pRIC3-mluc+, cells were lysed and harvested 72h post-transfection. A western blot probed with a rabbit-produced anti-SEAP antibody showed SEAP expression at the expected 65kDa size, at various FuGENE:DNA ratios (Figure 2.17A). SEAP expression in all samples was visible, but low, due to dilution of SEAP protein in cell

culture media. There was no notable difference in expression between constructs or at different ratios, except for 3:2 for pRIC3-mSEAP+, which showed no SEAP expression. Luciferase expression was assayed by adding luciferin substrate to cell lysate, and recording relative luminescence on a luminometer (Figure 2.17B). Transfection at all three ratios resulted in luciferase expression at $2.42x10^3$-$2.55x10^4$ RLU, compared to a baseline value (negative control) of 4.39 RLU. A FuGENE:DNA ratio of 6:1 was marginally better than the other two ratios. These experiments were independently performed at least three times, and images in Fig. 2.17 are representative. These results suggest that these vectors will be useful for the production of reporter pseudogenomes in plants.

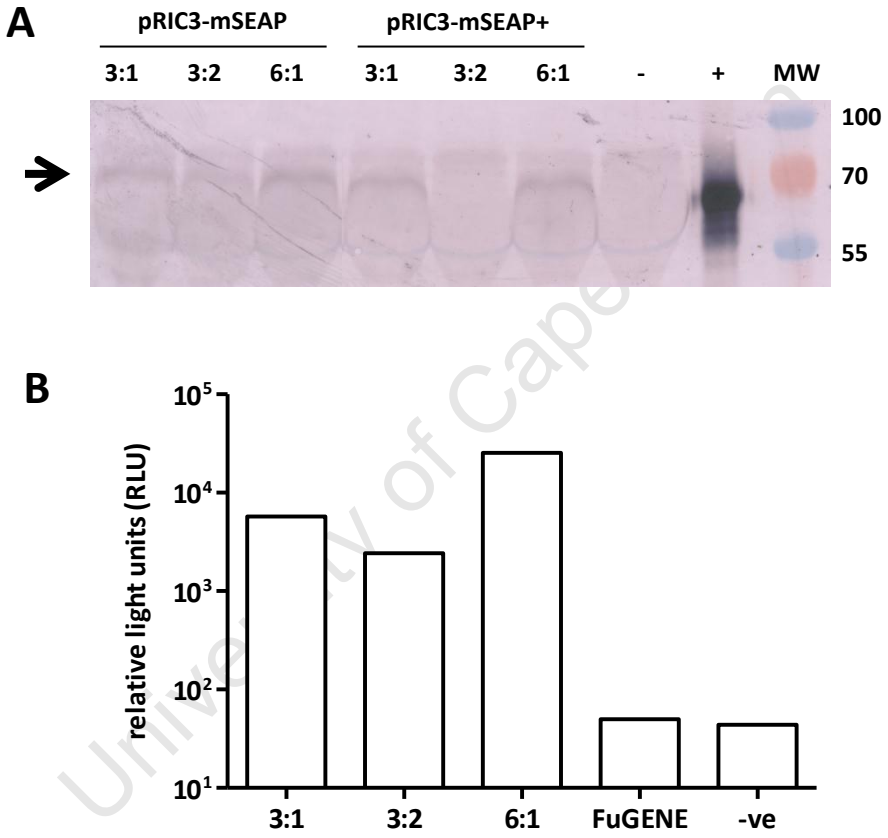


Figure 2.17 | Reporter gene expression in HEK293TT cells (A) Western blots of cell culture media shows SEAP expression (→) 72h post-transfection with pRIC3-mSEAP or pRIC3-mSEAP+ plasmid DNA at various FuGENE:DNA ratios. MW, molecular weight marker, sizes are indicated; -, negative control (FuGENE only); +, positive (commercially available calf intestinal alkaline phosphatase, Sigma). (B) Comparison of luc expression in various FuGENE:DNA transfection ratios 72h post-transfection with pRIC3-mluc+ in relative light units (RLU). FuGENE , negative control (FuGENE only), -ve, negative control (media only)

2.4. Discussion

In order to produce mammalian cassettes for PsV production in plants, autonomously replicating vector pRIC3 was modified to incorporate two different mammalian reporter gene cassettes, luciferase and SEAP. The luciferase vector contained both plant and mammalian expression cassettes, while SEAP-expressing vectors were produced both with and without a plant expression cassette, in order to vary the size of pseudogenomes to be made in plant host cells. Three resulting vectors were created, namely pRIC3-mSEAP, pRIC3-mSEAP+ and pRIC3-mluc+ (replicon sizes of 4.8Kbp, 6.4Kbp, and 7.4Kbp, respectively). The sequence identity of these vectors was confirmed, and they were used to transfect mammalian cells in order to test whether the reporter genes under the influence of mammalian promoters were expressed. All three vectors showed reporter gene expression 72h post-transfection in HEK293TTcells, demonstrating that the mammalian reporter cassettes encoded by these vectors are functional in mammalian cells, and will thus be useful for the production of effective reporter pseudogenomes in plants.

2.4.1. Development of novel replicating constructs

Replicating plant expression vectors have been developed by several researchers, usually using viruses of the family *Geminiviridae*. They have traditionally been developed for use as plant expression vectors, for the transient production of recombinant proteins at large scale (Hefferon, 2012). These replicon systems promise high recombinant protein expression levels, rapid transient expression, and improved versatility over traditional plant expression vectors and transgenic systems (Gleba et al., 2007). Several approaches to replicating plant expression vectors have been attempted – these include constitutive 2- or 3-vector systems (a notable example would be magnifection, as espoused by ICON Genetics) (Gleba et al., 2007; Hefferon and Fan, 2004; Mor et al., 2003), an alcohol-inducible 2-vector system (Zhang and Mason, 2006), and single vector systems (Huang et al., 2009; Palmer et al., 1999; Regnard et al., 2010). Additionally, several replicons have recently been incorporated into a single vector, with no apparent deleterious effect on protein expression and yield (Huang et al., 2010). These replicating vectors all rely on the rolling circle replication (RCR) mechanism to amplify the gene copy number of a transgene by several orders of magnitude, with the aim of improving on transgene expression by non-replicating vectors.

Interestingly, there are also several recent reports on the use of virus-derived replicating mammalian expression vectors. These have been used for a range of purposes, including gene therapy (Kim et al., 2006), protein expression for vaccine development (Faurez et al., 2010) and as a

tool to study replication processes in mammalian cells (Takeda et al., 2005). Of particular interest is the work by Faurez et al. (2010), who constructed PCV-2-derived vectors which replicate via the RCR model, and investigated the rate of rolling circle replication. PCV-2 is an ssDNA circovirus with many similarities to geminiviruses, including a compact, ambisense genome, *rep* gene, and rolling circle replication mechanism. (Mankertz et al., 2004). Further, *rep* sequence analysis for PCV identified nucleotide and amino acid sequence motifs similar to those found in geminivirus *rep*, which in both virus families are required for rolling circle replication (Mankertz et al., 1997).

Genetic elements from a wide variety of sources were used to create the sophisticated replicating vectors used in this project (See Figures 2.7, 2.8, and 2.9). The 'backbone' of each of these vectors is comprised of an origin of replication (ori) for replication in both *E. coli* (ColE1 ori) and *A. tumefaciens* (RK2 ori), as well as an ampicillin resistance gene (*bla*), features common to many other plant expression vectors, such as pTRAc (Maclean et al., 2007) and pEAQ (Sainsbury et al., 2009). Another aspect common to all agroinfiltration is the RB and LB, which are short (25bp) near-identical nucleotide sequences that flank the region of DNA transferred into plant cells during agroinfiltration (T-DNA). In pRIC and pRIC3, two copies of the BeYDV LIR are located immediately inside the borders of the T-DNA. Along with the products of the BeYDV *rep* gene, Rep and RepA, these allow the formation of the initiator complex for the rolling circle replication mechanism (Khan, 2005). The remaining elements found in pRIC3 make up the plant expression cassette – cauliflower mosaic virus 35S promoter and polyA signal were chosen as they have been shown to drive strong constitutive transgene expression in plants (Maclean et al., 2007). An MCS was incorporated to allow for easy cloning of various transgenes.

While replicating vectors, including pRIC3, have proven useful for transient, high-level recombinant protein expression, the replicating ability of pRIC3 was not used for that purpose in this study. Rather, the extremely efficient rolling circle replication mechanism was exploited for the production of PsV pseudogenome DNA at high copy number, and subsequent encapsidation by coexpressed structural proteins for the production of particles. This is a more efficient and elegant solution than delivery of a non-replicating pseudogenome vector for intracellular assembly, or *in vitro* disassembly-reassembly of VLPs in the presence of pseudogenome DNA. To our knowledge, this is the first demonstration of this approach for PsV production.

To facilitate the insertion of mammalian cassettes into the pRIC3 vector, restriction enzyme digest sites needed to be introduced at the 5' and 3' ends of the plant cassette. Original pRIC and pRIC3

vector construction by previous investigators in our lab (Ogle, 2008; Regnard et al., 2010) utilised a *SalI* site located at the 5' end of the CamV35Spromoter element, and an *XhoI* site at the 3' end of pA35SS polyadenylation signal (Figure 2.2). While the sites were destroyed by ligation with compatible restriction enzyme sites, they were able to be restored by the introduction of a single nucleotide mutation at each site. Site-directed mutagenesis thus provided a relatively straightforward way to introduce the mammalian cassettes into pRIC3.

Overlap extension PCR was used for the site-directed mutagenesis required in both cases. This involved several stages of PCR amplification of different fragments in order to incorporate the required nucleotide changes. Following OE-PCR, mutated fragments pR3fr-M and pR3fr2-M were each cloned into a vector for sequencing (Figure 2.6). pR3fr-M was cloned into pJET1/blunt, while pR3fr2-M was cloned into pGEM-T Easy. Different cloning vectors were used due to difficulties in cloning pR3fr2-M into pGEM-T Easy. Cloning PCR products into pJET1/blunt proved an easier method due to the vector being designed for blunt-end cloning. Subsequently, fragments pR3fr-M and pR3fr2-M were ligated into pRIC3 via unique restriction sites located at the 3' and 5' ends of these fragments, namely *BglI* and *EcoRV* for pR3fr-M, and *XbaI* and *BamHI* for pR3fr2-M. This new construct, designated pRIC3.2, was now ready for the introduction of the two new mammalian cassettes.

The choice of mammalian cassette elements was based on past availability and success. Both SEAP and *luc* are widely used as robust and reliable reporter genes in mammalian systems (Jiang et al., 2008). The mSEAP cassette was taken entirely from pYSEAP, a plasmid developed by Pastrana et al. (2004), and chosen due to its wide-spread use for PBNA. The use of SEAP for reporter assays is a relatively recent development, in comparison to other reporter gene assays developed, such as luciferase assays (Berger et al., 1988). It was identified as a sensitive and robust assay in comparison to other common reporter assays, and is considered the superior reporter gene for pseudovirion-based neutralisation assays. It offers a significant advantage over other reporter genes for this purpose, in that it is a secreted protein, and as such there is no need for cell lysis. Luciferase, on the other hand, has been in use for many years as a reporter gene. *Luc* expression is read as luminescence, and it has become widely used due to its rapidity, sensitivity and ease of use (Contag and Bachmann, 2002). Further, it provides an advantage over SEAP, in that it is less than half the cost per reaction when comparing the two kits used in this project (Luciferase Assay System, Promega; and Great EscAPe Chemoluminescence Kit, Clontech Laboratories, Inc.). Two new luciferase assays have recently become available – these are the luciferase from the marine shrimp Gaussia princeps

(Shao and Bock, 2008), and a newly developed Nanoluc, from Promega (Hall et al., 2012). Both these have demonstrated to be far more sensitive than traditional luciferase assays, and may be better choices for this reporter system. The non-gene elements of the mluc cassette, namely a cytomegalovirus intron/enhancer/promoter element, PCV pCapR enhancer element, and a SV40 polyadenylation signal, have been demonstrated to induce high expression of an HIV polyprotein in HEK293 cells (Tanzer et al., 2011). The pCapR enhancer element incorporated into the mluc cassette is comprised of two regions, namely the Pcap core, and the conserved late element (CLE, shown in Fig. 2.3). These regions, identified using the Transcompel database, comprised a slightly larger portion of the genome than that described as the minimal Pcap region (Mankertz and Hillenbrand, 2002), and incorporated previously unidentified putative transcription factor binding sites. This 172bp fragment contains consensus DNA binding sites for c/EBPb, a transcription factor involved in control of cell cycle progression; GATA-1, a constitutive Zn finger transcription factor; and CREB, a cAMP response element binding protein, in the Pcap core, which form a putative composite transcription factor binding site. The 172 bp fragment also contains the smaller CLE, with putative binding sites for transcription factors SP1 and AP3 in. The addition of this region to pTHgrttnC in reverse orientation (pTHPcapRgrttnC) resulted in a stable plasmid and significantly improved expression of GrttnC protein in mammalian cells when compared to pTHgrttnC (Tanzer et al., 2011). As such, its inclusion in the mluc cassette is likely to aid in the expression of luciferase in mammalian cells.

Construction of vectors pRIC3-mSEAP, pRIC3-mSEAP+, and pRIC3-mluc+ was successful, as confirmed by restriction enzyme mapping and sequence analysis. The SEAP cassette was introduced into pRIC3.2 which either had been linearised by digestion at the introduced *SalI* site 5' of the plant expression cassette, or had the plant cassette removed via digestion at the introduced 5' *SalI* and 3' *XhoI* sites. This resulted in the vectors pRIC3mSEAP+ and pRIC3mSEAP, respectively. Notably, orientation of the SEAP cassette was not controlled for, as the SEAP cassette was flanked by *SalI* sites on the 3' and 5' ends. As such, the final vectors contain the SEAP cassette in the reverse orientation to the original plant cassette. This did not have any effect on the functionality of the SEAP reporter cassette, as demonstrated by successful SEAP expression in mammalian cells. The mluc cassette was introduced at the 3' end of the plant cassette to form pRIC3mluc+. The motivation for creation of this vector was two-fold. The first reason was to evaluate more than one reporter gene, due to the relative expense of PBNA using SEAP. The second reason was to create a larger replicon than those produced by pRIC3mSEAP and pRIC3mSEAP+ (4.8 and 6.6 Kbp, respectively). The pRIC3mluc+ replicon is 7.6Kbp in size. This is close to the approximately 7.9Kbp HPV genome, and it

has been suggested that DNA packaging efficiency of HPV PsVs produced in mammalian or insect cells is related to the size of the pseudogenome packaged – several researchers have demonstrated that DNA is packaged more efficiently when the plasmid is close to 8Kbp, and that plasmids smaller than 5Kbp or larger than 8Kbp are not packaged (Buck et al., 2005b; Holmgren et al., 2005; Touze and Coursaget, 1998).

2.4.2. Testing of reporter gene activity in mammalian cells

The results obtained here demonstrate that both of these reporter genes, in all three created vectors, are expressed at appreciable levels in mammalian cells. There is also no evidence to suggest that the presence of a plant expression cassette had any effect whatsoever on mammalian reporter gene expression, at least in the case of SEAP. We can conclude that both SEAP and luc assays are sensitive enough for the purposes of the PBNA. Further, there is little difference in terms of speed and ease of use. Luciferase appears to be a more suitable reporter gene for our purposes, due to its lower cost per assay reaction.

The plant gene present in the larger plasmids pRIC3mSEAP+ and pRIC3mluc+ is not relevant to the project, other than to increase the size of the final replicon. However, it may be useful for further investigations to incorporate a plant reporter gene such as EGFP in this cassette, to allow for easy confirmation of replication in plants (as demonstrated by Regnard et al. 2010 with the pRIC predecessor plasmid). There may be some value in incorporating a gene to improve replication of the replicon; however, it has been suggested that the replication efficiency is close to saturation point for the plant replicative machinery, and as such this approach may have little effect (Zhang and Mason, 2006). Another interesting approach would be to test the inclusion of a chaperone gene. Several studies have shown that HPV virion assembly is mediated by intracellular chaperones, and this inclusion of a chaperone gene in these vectors may assist in PsV assembly *in planta* (Bird et al., 2008; Chromy et al., 2003).

2.5. Conclusion

In this study, three novel replicating vectors were successfully created from the previously developed pRIC3. Two of these carry a SEAP mammalian reporter cassette, while the third carries a luciferase mammalian reporter cassette. Correct construction and orientation of these plasmids was confirmed by restriction enzyme and sequencing analysis. The inclusion of these mammalian cassettes changes the size of the replicon produced by each plasmid to 4.8, 6.6 and 7.6Kbp. These

vectors will be used to agroinfiltrate plants, to allow for autonomous replication to take place, and to determine whether they will be incorporated into PsVs *in planta*.

Chapter 3: Expression of HPV L1/L2 VLPs and PsVs in plants and PsV testing in mammalian cells

3.1. Introduction

Cervical cancer caused by high-risk HPV is the second most prevalent cancer in women in developing countries; Africa in particular has been identified as a high risk region for the disease (Ferlay et al., 2010). Recently developed L1 VLP vaccines, Cervarix® and Gardasil™, protect against HPV-16 and -18, or HPV-6, -11, -16 and -18 infection, respectively. Both currently available vaccines elicit a strong and protracted neutralising antibody response, and have been shown to have sustained efficacy up to 5 years post-administration (Harper et al., 2006; Villa et al., 2006). While these vaccines are showing great promise in reducing the burden of disease, development and production of VLP vaccines remains prohibitively expensive, particularly in developing countries.

A key element of any HPV vaccine development initiative is the pseudovirion-based neutralisation assay (PBNA). Induction of neutralising antibodies is currently the best estimate of vaccine candidate efficacy for second generation HPV vaccine testing. Until recently, the identification of serum neutralising antibodies relied on the use of enzyme-linked immunosorbent assay (ELISA) or neutralisation assays using whole virus (Dessy et al., 2008). However, improvements in HPV pseudovirion production efficiency in the last decade have allowed the development of the pseudovirion-based neutralisation assay (PBNA). Developed by John Schiller's group at the Center for Cancer Research at the NIH, this assay uses mammalian cells for intracellular production of PsVs expressing a SEAP reporter gene (Buck et al., 2005a), and has since become the gold standard for testing neutralisation of candidate HPV vaccines, allowing rapid and un-biased screening of neutralising antibodies and epitopes (Stanley et al., 2008). While this production method has been shown to be extremely effective for production of PsVs, SEAP assay kits are particularly expensive in comparison to other commonly used reporter assays such as luciferase or GFP. There is a need, therefore, to develop alternative PsV production methods to allow for affordable candidate vaccine development and testing.

3.1.1. Development of HPV neutralisation assays

The production of neutralising IgG antibodies in response to vaccination has long been understood to be a key aspect of protective immunity (Robbins et al., 1995). It has been suggested that it may be possible to accurately estimate the required level of neutralising antibody required for protection,

provided that the concentration, isotype and secondary biological activity of these antibodies could be accurately measured (Robbins et al., 1995). Neutralisation assays were developed as a method of accurately quantifying the neutralising capabilities of immune sera, usually in response to a live viral or vaccine candidate challenge, as well as identify neutralising epitopes (Ochsenbauer and Kappes, 2009; Yeager et al., 2000).

The first demonstration of *in vitro* neutralisation of papillomavirus was by Dvoretzky et al. (1980), who demonstrated neutralisation with rabbit-produced BPV-1 antisera to confirm the role of BPV-1 in focus formation in mouse cell lines. Early efforts to establish a robust, sensitive *in vitro* neutralisation assay were hampered by difficulties in production of infectious virus. Production of infectious virions *in vitro* was first achieved by grafting HPV-11-infected material into athymic mice: grafts were left to develop into condylomatous cysts over a period of 3-5 months, before being harvested and purified for HPV virions (Kreider et al., 1987). This method was utilised to produce virions for use in the first *de facto* neutralisation assay. Neutralising monoclonal antibodies were identified and isolated from HPV-11 or BPV-1 antisera. These antibodies were then used to demonstrate neutralisation of intact virions by ELISA, as well as identifying several neutralising conformational epitopes (Christensen et al., 1990). The same group used this method to successfully identify neutralising HPV antibodies in human sera for the first time, and further demonstrated that ELISA was a good indicator of the presence of neutralising antibodies in human sera (Christensen et al., 1992). Another approach coupled the neutralisation of HPV-11 infection with RT-PCR detection of HPV mRNA transcripts to create a semi-quantitative neutralisation assay (Smith et al., 1995). While these approaches were nominally successful in identification of neutralising antibodies, detection remained limited at best, and the procedures used were time-consuming and expensive.

A major step forward in neutralisation assay technology came with the advent of PsV production, which abrogated the need for the expensive and time-consuming xenograft production method. Roden et al. (1996) used hamster BPHE-1 cells to generate BPV-1 or HPV-16 PsVs. These were used to demonstrate focus formation in C127 cells, using the technique demonstrated by Dvoretzky et al. (1980). These researchers further showed that neutralising antibodies in HPV-16 antisera prevented focus formation, demonstrating a quantitative neutralisation assay of a high-risk HPV type using PsVs for the first time (Roden et al., 1996). In this report, the authors noted that the focus transformation assay required 2-3 weeks, and that inclusion of a marker or reporter gene would greatly improve the speed of the assay. This was first attempted by chemically linking a β-lactamase (BLAM) reporter plasmid to VLPs or infectious virions, and incubating these with PV antisera before infecting various

mammalian cell lines. Early attempts demonstrated neutralisation, but resulted in <1% infection of cells with these PsVs (Muller et al., 1995). Yeager et al. (2000) and Bousarghin et al. (2002) demonstrated this approach more successfully, using a BLAM or luc reporter plasmids and an alternative method of attaching the plasmid to VLPs. More importantly, several groups generated PsVs with encapsidated reporter genes, and demonstrated their use for neutralisation assays (Buck et al., 2005a; Fleury et al., 2008a; Kawana et al., 1998; Rossi et al., 2000; Stauffer et al., 1998; Touze and Coursaget, 1998; Unckell et al., 1997). While early attempts were inefficient due to poor PsV production levels, this was improved upon by intracellular generation of high yields of L1/L2 PsVs and incorporation of a SEAP reporter plasmid (Buck et al., 2004). These PsVs were used with a commercially available SEAP detection kit to demonstrate a pseudovirion-based neutralisation assay that was at least as sensitive as, and potentially more type-specific than, the standard ELISA-based neutralisation assay (Pastrana et al., 2004).

While the system developed by Pastrana et al. (2004) is considered the current 'best practice' neutralisation assay, there remains room for improvement. In particular, the costs of PsV production could be greatly decreased by the use of a less expensive production system (Brondyk, 2009). Recombinant protein expression in plants has been demonstrated to have a significantly lower cost of production when compared to production in mammalian cells (Tiwari et al., 2009). Thus, plant expression may provide an attractive alternative for the production of PsVs for use in the PBNA.

3.1.2. Protein expression systems in plants

Expression of recombinant proteins in plants has developed over the last twenty years from a curiosity in the late 1980's to a medically and industrially relevant production system today. Early efforts relied on transformation of plants to produce stable transgenic lines. This was achieved through biolistic delivery or, more recently, agroinfiltration (Daniell et al., 2009). While transgenic protein production remains a useful and viable system, advances in transient expression methods and technology have positioned transient expression as the preferred method for industrial-scale production in plants (Rybicki, 2010). Two key factors that have played a central role in this transition are viral, or virus-derived, expression vectors, and the development of agroinfiltration technology.

Agroinfiltration was originally developed to as an alternative to biolistic bombardment for the stable transformation of plants (Kapila et al., 1997). This process relies on the DNA transfer capability of *A. tumefaciens* to introduce foreign DNA to plant cells. *A. tumefaciens* can be used to transfer a transgene located in the transfer DNA (T-DNA) segment of the Ti plasmid into plants infiltrated with

a bacterial suspension of the transformed bacterium. The T-DNA is transported to the plant nucleus, and this allows for transformation of the plant through integration of the T-DNA into the plant genome (Zupan et al., 2000). Importantly, however, a transgene incorporated into the T-DNA is also transiently expressed from episomal DNA, resulting in systemic expression of a recombinant protein without the need for stable transformation (Kapila et al., 1997).

Viral vectors were the first transient expression method developed for plants. Early efforts simply inserted a recombinant gene or epitope into the genome of viruses such as Tobacco mosaic virus (TMV), Cowpea mosaic virus (CPMV) or (Potato virus X (PVX), either fused to the viral coat protein or separately, under control of a duplicated constitutive viral promoter (Durrani et al., 1998; Gleba et al., 2007; Turpen et al., 1995). While this application produced immunogenic protein, expression levels were lower than those found in transgenic plants. Other problems with these 'first-generation' viral vectors included a tendency to revert to the natural virus, constraints on insert size, difficulty of administration, and an inability to form VLPs (Kohl et al., 2006; Rybicki, 2010; Varsani et al., 2006). These limitations prompted further work to develop 'second generation', or deconstructed, viral vectors. This approach used only the desirable viral elements, in particular the replicative machinery, to manufacture synthetic vectors capable of inducing transgene expression in plants (see Figure 3.1). While these vectors are usually not infectious on their own, when coupled with agroinfiltration

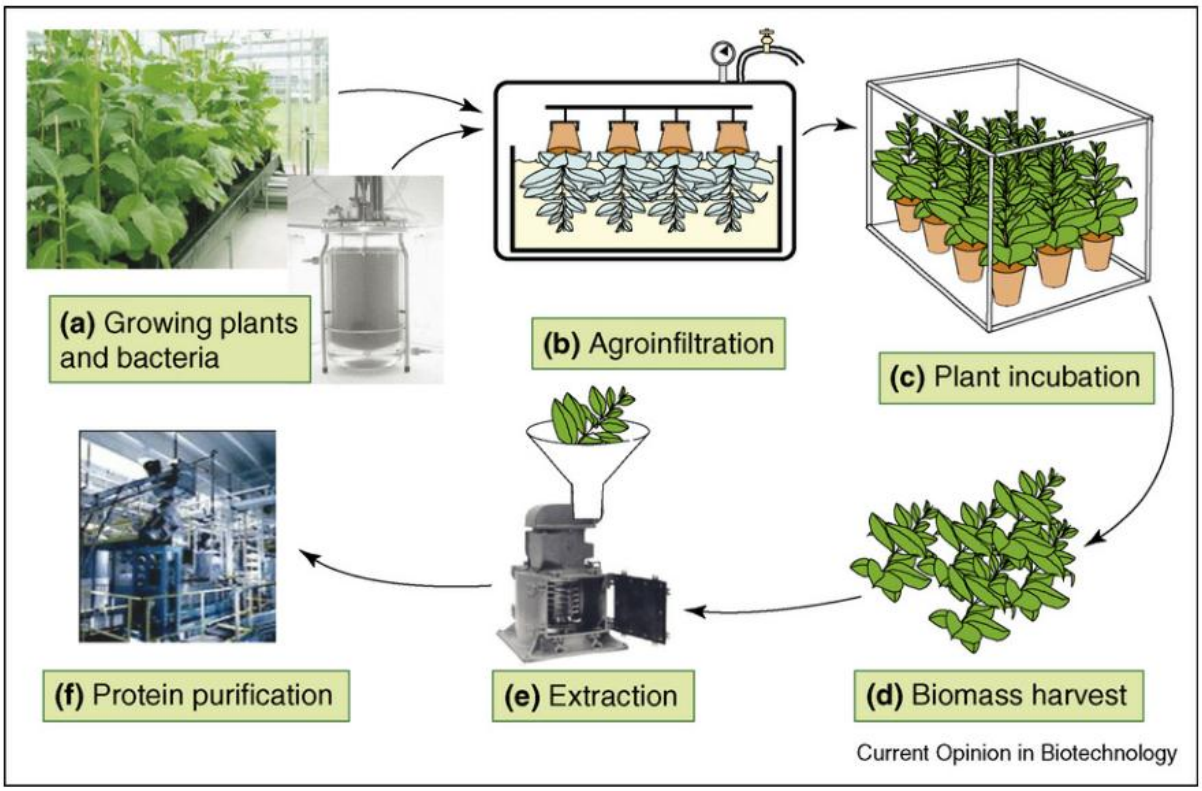


Figure 3.1 | Generalised modern transient expression model Figure reproduced from Gleba et al. (2008).

technology they can result in systemic transient expression of protein at levels comparable to or greater than that of transgenic plants (Tiwari et al., 2009). This approach has the advantages of short time frames (3-7 days) when compared to stable transformation (6-9 months), significant expression levels, and rapid and easy scale-up and purification. This makes agroinfiltration-mediated transient expression via viral vectors an ideal approach for the production of medically relevant proteins and particles in plants. Of particular interest is the use of transient expression for the production of VLPs and PsVs in plants, as there is potential for a reduction in cost when compared to traditional systems (Santi et al., 2006).

3.1.2. HPV VLP production in plants

Papillomavirus L1 VLPs have been produced by several groups in plants. Most have used transgenic plants (Biemelt et al., 2003; Varsani et al., 2003; Warzecha et al., 2003) with resulting low yields. Early attempts at transient expression of L1 also yielded low levels of expression, as well as an apparent inability to form VLPs (Varsani et al., 2006). However, agroinfiltration of a vector encoding a human codon-optimised L1 protein provided a much higher protein yield, and demonstrated that transient expression of HPV-16 VLPs at high levels is a feasible approach for the production of immunogenic HPV candidate vaccines (Maclean et al., 2007).

The non-replicating vector used to produce L1 at such high expression levels – pTRAc – was developed at the Fraunhofer Institute for Molecular Biology and Applied Biology. This vector utilises

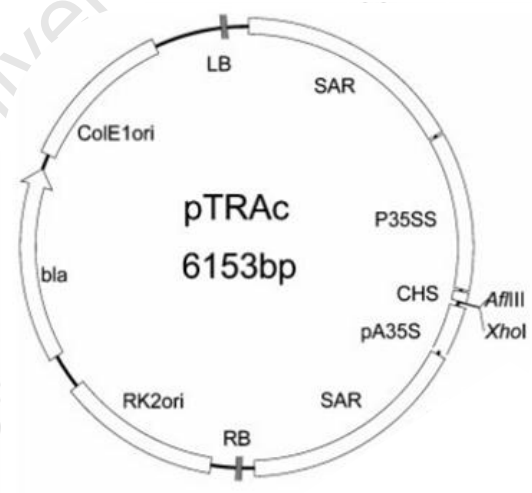


Figure 3.2 | Agrobacterium expression vector pTRAc P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; CHS, chalcone synthase 5' untranslated region; pA35S, CaMV 35S polyadenylation signal; SAR, scaffold attachment region of the tobacco Rb7 gene; LB and RB, the left and right borders for T-DNA integration; ColE1ori, origin of replication for E. coli; RK2ori, origin of replication for Agrobacterium; bla, ampicillin/carbenicillin-resistance gene. Reproduced from Maclean et al. (2007).

a CaMV 35S promoter with duplicated transcriptional enhancer, chalcone synthase 5'-untranslated region, and CaMV 35S polyadenylation signal for foreign gene expression (see Figure 3.2).This vector has also been used to express the minor capsid protein L2 in plants (Pereira, 2008). However, coexpression of L1 and L2 has not previously been conclusively demonstrated to form VLPs *in planta*.

3.1.3. The potential for PsV production in plants

Encapsidation (or covalent attachment) of DNA by HPV VLPs to form PsVs has been demonstrated in yeast, insect, bacterial and mammalian cell systems (Buck et al., 2005a; Roden et al., 1996; Rossi et al., 2000; Unckell et al., 1997). Buck et al. (2005) demonstrated that intracellular encapsidation of the pseudogenome is more efficient than *in vitro* disassembly-reassembly methods for the production of HPV PsVs, probably due to cellular factors that assist in correct assembly of the virions (Buck et al., 2008; Fleury et al., 2008a; Peng et al., 2011) . Currently, HPV pseudovirions have not been successfully expressed in plant expression systems. As discussed above, transient expression in plants offers several significant advantages for this application: protein expression in plants has been shown to be safe, cheaper than other expression systems, and potentially extremely rapid (Ma et al., 2005; Schillberg et al., 2005). A further significant advantage is that there is no need for downstream processing of proteins (e.g. glycosylation), as for bacterial recombinant protein expression systems (Giorgi *et al*, 2010). While it has been noted that *N*-glycosylation may differ in plants (specifically, plants cannot synthesise β-1,4-galactose and sialic acid), this problem can be overcome by recent advances in transgenic tobacco to provide 'humanised' glycosylation machinery (Bakker et al., 2006; Gleba et al., 2007). Further, it has been suggested that glycosylated L1 is not an important part of the assembled virion (Zhou et al., 1993b).

In this study, we aimed to evaluate the feasibility of expressing HPV L1/L2 pseudovirions with an encapsidated mammalian reporter cassette *in planta*. To achieve this, pTRAc plasmids expressing L1 and L2 proteins were co-infiltrated into plants with novel autonomously replicating plasmids, developed in this study, to potentially create HPV L1/L2 PsVs. Further, we aimed to purify these particles by density-based centrifugation, for subsequent testing in a mammalian system.

3.2. Materials and Methods

3.2.1. Plant expression vectors

To express PsVs in *N. benthamiana* plants, several plant expression vectors were utilised. The construction of the replicating vectors used for replicon formation, namely pRIC3-mSEAP, pRIC3-mSEAP+, and pRIC3-mluc+, was covered in Chapter 2. Further to these, the plant expression vector pTRAc (gifted by Prof. Dr. Rainer Fischer; Fraunhofer Institute for Molecular Biology and Applied Ecology, Germany) expressing HPV-16 L1 or L2 human codon-optimised genes (pTRAc-hL1 and pTRAc-hL2, respectively) were used for production of L1 and L2 capsid proteins. This vector, shown in Fig. 3.2, targets L1 and L2 expression to the cytoplasm, and pTRAc-hL1 has demonstrated high transient expression levels for L1 *in planta* (Maclean et al., 2007).

3.2.2. Transformation of *Agrobacterium tumefaciens*

Plasmids were isolated from *E. coli* using a QIAGEN Plasmid Miniprep Kit. These were then introduced into *Agrobacterium tumefaciens* strain GV3101::pMP90RK via electroporation, as described by Maclean et al. (2007). *A. tumefaciens* cells were made electrocompetent by the method described by Shen and Forde (1989). 200ng of plasmid DNA was added to a chilled electroporation cuvette (Molecular BioProducts, Inc.), along with 100µl of electrocompetent cells. After 5 minutes of incubation on ice, cells were electroporated using a Bio-Rad GenePulser™ under the following conditions: 1.8kV, 25µF, 200Ω. 900µl of antibiotic-free Luria broth was added to the electroporated cells, which were incubated for 2 hours at 27°C. Recombinant clones were screened by antibiotic selection with rifampicin (50µg/ml), carbenicillin (50µg/ml), and kanamycin (30 µg/ml). Plates were incubated at 27°C for 48 hours to allow for colony formation, and screened for positive clones by colony PCR.

3.2.3. Agroinfiltration of *N. benthamiana*

Agroinfiltration of *N. benthamiana* plants was performed as described by Maclean et al. (2007). *Nicotiana benthamiana* plants were grown from seed in a controlled plant growth room. The plants were grown at 22°C, with 16 hours of light per day for 6 weeks. Plants were agroinfiltrated by syringe or by vacuum with a bacterial suspension of recombinant *A. tumefaciens* at an optical density (OD) of 0.25, 0.5, 0.75 or 1. Briefly, a syringe was used to force *A.tumefaciens* bacterial suspension into the abaxial air spaces in several leaves per plant. The plants were allowed to grow as normal, and leaf samples were harvested at 1-7 days post infiltration (dpi). For vacuum infiltration,

whole plants were submerged in 500ml of bacterial suspension, and placed in a vacuum chamber. A vacuum of -90 kilopascal (kPa) was maintained for 5 seconds, then rapidly released (10-15kPa.sec^{-1}). Plants were grown as normal, and harvested at 4 dpi.

3.2.4. Quantitative PCR

qPCR analysis was performed to determine whether replication of the replicon was occurring in plants. A single 0.5cm leaf disc was incubated at 95°C for 10 minutes with 100µl Extraction Buffer from the Extract'n'Amp Plant PCR Kit (Sigma Aldrich). This was diluted with 100µl Dilution buffer, and stored at -20°C until needed. qPCR was performed using the 2x SybrGREEN ReadyMix from the same kit. Primers lucQ-F and lucQ-R (reaction 16, Table 2.2; see also Table 2.1) were used to amplify a 153bp fragment of the luciferase gene, and primers SEAPQ-F and SEAPQ-R (reaction 17, Table 2.2) were used to amplify an 83bp fragment of the SEAP gene. All primers were used at a final concentration of 0.4µM. qPCR cycling was performed on a Corbett RotorGene 6000 (Corbett), using cycling parameters as follows: 95°C for 2 minutes; 40 cycles of 95°C for 5 seconds, variable annealing temperature for 5 seconds, and 72°C for 5 seconds; and melt curve analysis from 72-95°C for 5 seconds per degree. qPCR was performed with three technical repeats per sample, with a sample population size of three (N=3). Data was analysed using RotorGene Q Series 2.0.2 software (Corbett). C_t values were converted to replicon copy number in Microsoft Excel 2010 (Microsoft) and values were normalised to total DNA concentration for each sample.

3.2.5. Inverse PCR

A variation of Inverse PCR, as described by Regnard et al. (2010), was utilised to confirm recircularisation of the replicon (Figure 3.3). Primers were designed to amplify a DNA fragment (approximately 2.1Kbp) encompassing the site of recircularisation for each replicon (See table 2.1). Table 2.2, reactions 13 and 14, show the PCR reactions used. Reaction conditions are described in Section 2.2.2.

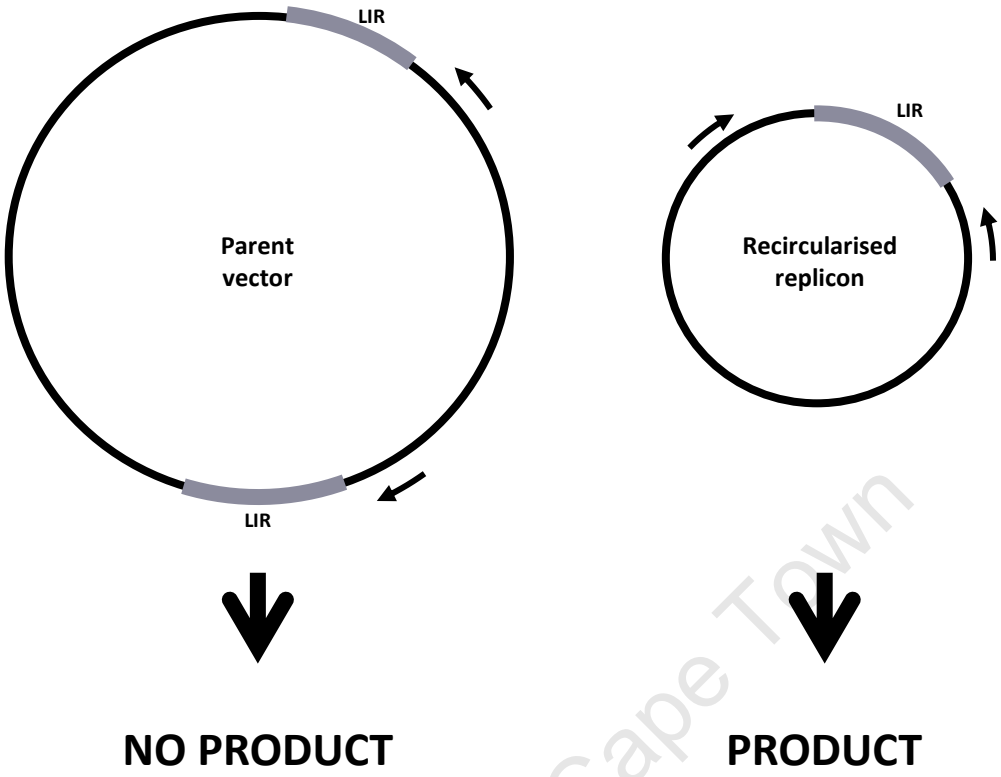


Figure 3.3 |Principle of Inverse PCR Diagram illustrating the principle of inverse PCR. Primers (→) designed to amplify only recircularised replicon DNA, but not the parent vector. Vector recircularised at the duplicated LIR.

3.2.6. SDS-PAGE and western blotting

SDS-PAGE was performed to analyse HPV-16 hL1 and hL2 protein production in plants. Protein was extracted from plants agroinfiltrated with *A. tumefaciens* GV3101::pMP90RK pTRAc-hL1 and/or pTRAc-hL2. Briefly, three 0.5cm leaf discs were harvested at 1, 3, 5, and 7dpi, frozen in liquid N_2, and ground in a microcentrifuge tube using a plastic pestle. 100µl of 0.5M NaCl PBS with 1x Complete EDTA-free protease inhibitor cocktail (Roche) (hL1) or 8M urea in H_2O (hL2) was added to the ground leaf material and mixed thoroughly. Samples were centrifuged at 13000rpm for 5 minutes, and the supernatant was reserved. This centrifugation step was repeated, and the supernatant was stored at -20°C. For SDS-PAGE analysis, 8µl of 5X loading dye containing β-mercaptoethanol was added to 32µl of soluble protein, and samples were incubated at 95°C for 7 minutes. These were then loaded on 10% SDS-polyacrylamide gels using the Mini-PROTEAN® Tetra SDS-PAGE system (Bio-Rad), and electrophoresed at 130V for approximately 120 minutes. These gels, and nitrocellulose membranes, were equilibrated for 10 minutes in transfer buffer before being transferred to a nitrocellulose membrane at 15V for 90 minutes using a Bio-Rad Trans-Blot® SD Semi-Dry Electrophoretic Transfer

Cell. Membranes were incubated with blocking buffer for 60 minutes, then probed for L1 overnight, using commercially available CamVir-1 primary monoclonal antibody (Abcam, ab69) diluted 1 in 10000 in blocking buffer. Membranes were washed for 4x 10 minutes in blocking buffer, probed with goat anti-mouse AP-conjugated secondary antibody (Sigma, A3562) diluted 1 in 5000 in blocking buffer for two hours, washed 4x 10 minutes in blocking buffer without skim milk powder, and visualised using BCIP/NBT Phosphatase substrate (KPL). For L2, a similar protocol was employed. Primary antibody was rabbit-produced anti-L2 primary polyclonal serum produced in our laboratory and used at 1 in 5000 dilution, and secondary antibody was goat anti-rabbit AP-conjugated antibody (Sigma, A3687).

3.2.7. Extraction and purification of particles

To produce particles, plants were vacuum-infiltrated with *A. tumefaciens* GMV3101::pMP90RK containing pTRAc-hL1, pTRAc-hL2 and either pRIC3luc, pRIC3mSEAP or pRIC3iSEAP. Protein and DNA were harvested at 4 dpi, as described in Section 3.2.6. Western blotting, as described in Section 3.2.6., was used to confirm the presence of L1 and L2 protein, and inverse PCR, as described in Section 3.2.5., was used to confirm that replicational release had taken place. Whole plants were harvested 4 dpi. Particles were purified following a variation of the protocol described by Varsani et al. (2003), with some modifications. Whole leaves were weighed, and ground with liquid nitrogen in a pestle and mortar, or macerated thoroughly at room temperature. Cold 0.5M NaCl-PBS was added to the leaf material at a ratio of 1:2 (w:v), and samples were homogenised in an T25 Ultra-Turrax high shear mixer (IICA®) at 13000rpm for 10’ on ice. Homogenate was kept on ice for a further 2 hours before being centrifuged at 8000xg for 20’ at 4° C in a Beckman Coulter Avanti J25i centrifuge with a Beckman JA-14 rotor. Supernatant was filtered through 4 layers of Miracloth (Calbiochem), and layered onto a 7ml, 40% sucrose cushion (w/v). The samples were centrifuged at 100000g for 3h at 4° C in a Optima™ L-100 XP centrifuge (Beckman Coulter) with a Beckman Coulter SW32Ti rotor. The supernatant and sucrose cushion were removed, the pellet was resuspended in 1ml 0.4g/ml CsCl in PBS, and clarified on an Eppendorf 5424 tabletop centrifuge at 13000rpm for 10’. The supernatant was diluted in 5ml of 0.4g/ml CsCl in PBS, and subjected to centrifugation at 100000xg for 24h in an L-100 XP ultracentrifuge with a Beckman SW55Ti rotor at 10° C.

3.2.8. Identification of VLPs and PsVs in CsCl gradient

After centrifugation, the CsCl gradient was fractionated manually or using a Foxy Jr. fractionator (ISCO). The density of each fraction was determined using a hand refractometer (ATAGO) to read the

refractive index at 25° C, and International Critical Tables (Kellogg, 1927) were used to convert refractive index to buoyant density.

A dot blot was performed to confirm the presence of L1 in the CsCl fractions. Briefly, 1 µl of each fraction was dropped onto a nitrocellulose membrane. The membrane was blocked for 30' in blocking buffer, then probed for L1 as described in Section 3.2.6. Membranes were scanned and analysed using GeneTools densitometry software (SynGene), and relative spot intensity was normalised to L1 presence in crude plant extract. L1-positive fractions were pooled and dialysed overnight against 0.5M NaCl in PBS to remove CsCl.

In order to confirm the presence of the DNA replicon in the PsVs, inverse PCR was performed as previously (Section 3.2.5). Proteinase K was added to the fractions, which were incubated at 55° C for 3h to allow full digestion of the PsV protein shell, before undergoing inactivation at 95° C for 10'. PCR reactions 13 and 14 (Table 2.2) were used to amplify an approximately 2.1Kbp DNA fragment in these samples.

3.2.9. Electron microscopy

To confirm the presence of VLPs and PsVs, transmission electron microscopy (TEM) was used. Copper grids were rendered hydrophilic by glow-discharge using a Model 900 SmartSet Cold Stage Controller (Electron Microscopy Sciences) at 25mA for 30 seconds. Grids were incubated for 1-30' with VLP or PsV samples, washed three times with dH_2O, and particles were stained with 2% uranyl acetate (w/v). Grids were viewed on a Tecnai F20 transmission electron microscope (FEI) or a LEO912 transmission electron microscope (Zeiss) at 14500X, 19000X or 50000X magnification. 10 fields of view were captured at 50000X magnification for all samples, and three fields of view were captured at 19000X magnification for L1/L2 VLP samples, and 14500X for PsV samples. PsV proportions were estimated by counting 3 fields of view for each PsV type, and calculating that as a percentage of the total number of PsVs per field.

3.2.10. Pseudovirion neutralisation assay

To determine whether plant-produced PsVs were useful for PBNA, mammalian cells were pseudoinfected with plant-produced PsVs. HEK293TT cells were trypsinised and resuspended in neutralisation media (standard growth media, using DMEM lacking in phenol red) at a density of $0.3x10^6$cells/ml, and plated at 100µl/well in a 96-well plate. Cells were grown at 37°C for 3-4h. 60µl of each PsV was added per well, in triplicate, and grown for 72 hours. Samples were harvested as

described in Section 2.2.5. Luc activity was assayed as described previously (Section 2.2.5.). SEAP activity was assayed using the Great EscAPe SEAP Chemiluminescence Kit (Clontech Laboratories, Inc.), at 0.6 volumes of those described in kit instructions. Briefly, 50µl of cell culture media was harvested at 72h post-transfection. 15µl were added to 45µl dilution buffer, and incubated at 65°C for 30 minutes. Samples were placed on ice for 5 minutes, before 60µl of SEAP Substrate Solution was added, and samples were incubated at room temperature for 30-60 minutes. Luminescence was detected on a Modulus Microplate Reader (Turner BioSystems) for 10 seconds. All samples were assayed in triplicate, and standard deviation was calculated for all samples

Neutralisation of PsVs was assayed using a known HPV-16 neutralising antibody, following the protocol described by Buck et al. (2005a). HEK293TT cells were prepared as described in Section 4.2.3. 60µl of PsVs were incubated with 15µl neutralising antibody HPV-16.V5 (developed by Christensen et al. (1996a)) at a dilution of 1 in 4000 (for a final dilution 1 in 20000) on ice for 60 minutes. 75µl of PsVs were added dropwise to cells in triplicate, and cells were incubated for 72 hours. Luciferase and SEAP activity were assayed as previously described (luciferase, see section 2.2.5.; SEAP, see section 4.2.3.). Standard deviation was calculated for all samples.

3.3. Results

3.3.1. Replicating vectors undergo replicational release in plants

Three novel vectors, pRIC3-mSEAP, pRIC3-mSEAP+ and pRIC3-mluc+ were designed and tested in *N. benthamiana*. All three vectors were cloned into *A. tumefaciens* GV3101::pMP90RK, and plants were infiltrated at an OD_{600} of 0.5. DNA was harvested from plants at 3dpi, and tested for replicational release by PCR. Primers were designed to amplify a 2.1Kbp fragment of the replicon, incorporating the LIR (see Figures 2.7C, 2.8C and 2.9C) – reaction 13 for pRIC3mluc+, and reaction 14 for pRIC3-mSEAP and pRIC3-mSEAP+ (Table 2.2). A PCR amplification product would only be produced in the

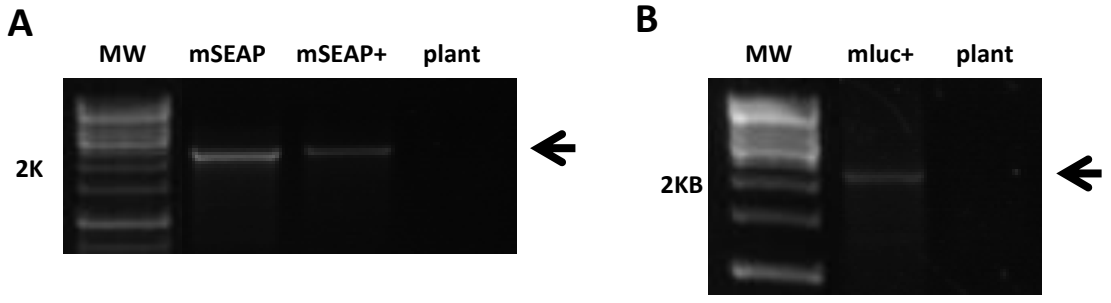


Figure 3.4 | Novel constructs demonstrate replication in plants PCR amplification of (A) pRIC3-mSEAP and pRIC3-mSEAP+ and (B) pRIC3-mluc+ replicons shows replicational release of T-DNA. MW, molecular weight marker; mSEAP, pRIC3-mSEAP; mSEAP+, pRIC3-mSEAP+, mluc+, pRIC3-mluc+, plant, uninfiltrated plant DNA (negative control).

presence of recircularised replicon. PCR amplification (Figure 3.4) of an approximately 2.1Kbp product confirmed that the replicon was formed in plants individually infiltrated with pRIC3-mSEAP (Fig. 3.4A), pRIC3-mSEAP+ (Figure 3.4A), and pRIC3-mluc+ (Fig. 3.4B). This confirms that these vectors form a recircularised replicon in plant cells, and are suitable vectors for pseudogenome production.

3.3.2. Optimisation of protein and DNA expression in plants

Expression of hL1 and hL2 was optimised by a 1-7dpi time trial. Plants were agroinfiltrated with a range of bacterial suspension OD_{600} values (0.25-1.0). Protein was harvested at 1, 3, 5, and 7dpi, and separated by SDS-PAGE. hL1 and hL2 expression was analysed by western blotting using anti-L1 CamVir-1, and an anti-L2 polyclonal antibody raised in rabbits (Figure 3.5), respectively. Expression of recombinant protein was detected at all OD_{600} values tested, from 3dpi, for both hL1 and hL2, at

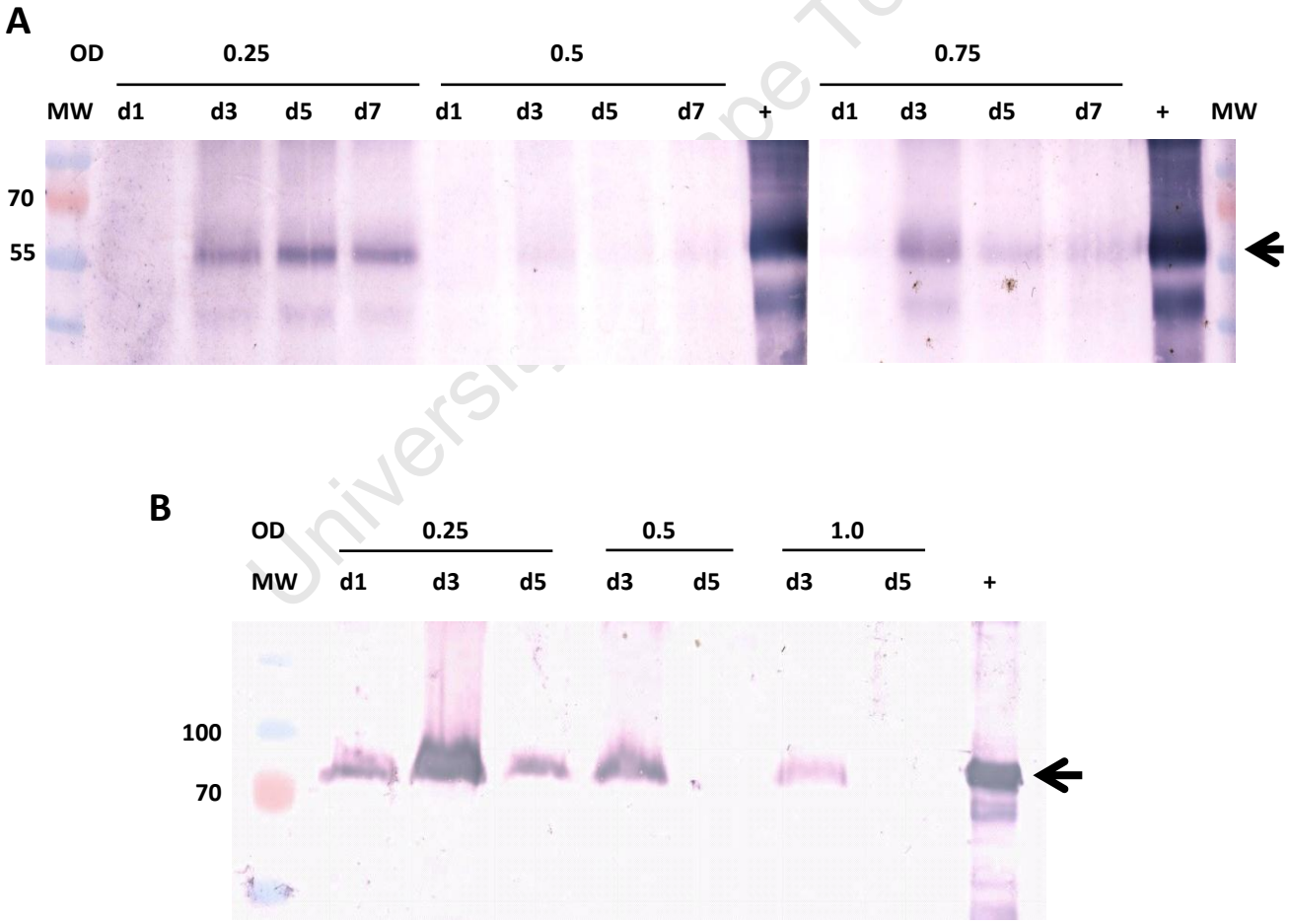


Figure 3.5 | Optimisation of L1 and L2 expression Timetrial of (A) hL1 and (B) hL2expression levels at various infiltration ODs, at 1-7 and 1-5 dpi, respectively. Protein is indicated (→) at 55kDa (hL1) and approximately 65kDa (hL2). MW, molecular weight marker, with sizes indicated in kDa; +, crude plant-produced hL1 (A) and bacterially-produced hL2 (B)

the expected size. While L2 is an approximately 50kDa protein, it has been widely observed to migrate at approximately 80kDa (Muller et al., 1995). The highest expression detected for hL1 was at OD_{600} 0.25, from 3-7dpi (Fig. 3.5A). The highest expression of hL2 was also seen in those plants infiltrated at OD_{600} 0.25, at 3dpi (Fig. 3.5B). Agroinfiltration parameters of OD_{600} 0.25 at 4dpi were chosen for optimal hL1 and hL2 expression in further experiments.

The pRIC3 vector backbone has been previously demonstrated to form replicons that replicate to high copy number within the plant cell, relative to non-replicating vector pTRAc (Regnard et al., 2010). Plants were infiltrated individually with each replicating vector at OD 0.5, and DNA was harvested at 1, 3, 5 and 7dpi. qPCR was used to determine the increase in replicon copy number from 1 to 7dpi with each of the replicating vectors. Reactions 15 and 16 (Table 2.2) were used for pRIC3mluc+, and pRIC3-mSEAP and pRIC3-mSEAP+, respectively. Analysis showed a 100-1000-fold increase in gene copy number for all three vectors at from 1 to 3dpi, with maintenance at similar copy number up to 7dpi (Figure 3.6). pRIC3-mSEAP (Fig. 3.6A), pRIC3-mSEAP+ (Fig. 3.6B) and pRIC3-

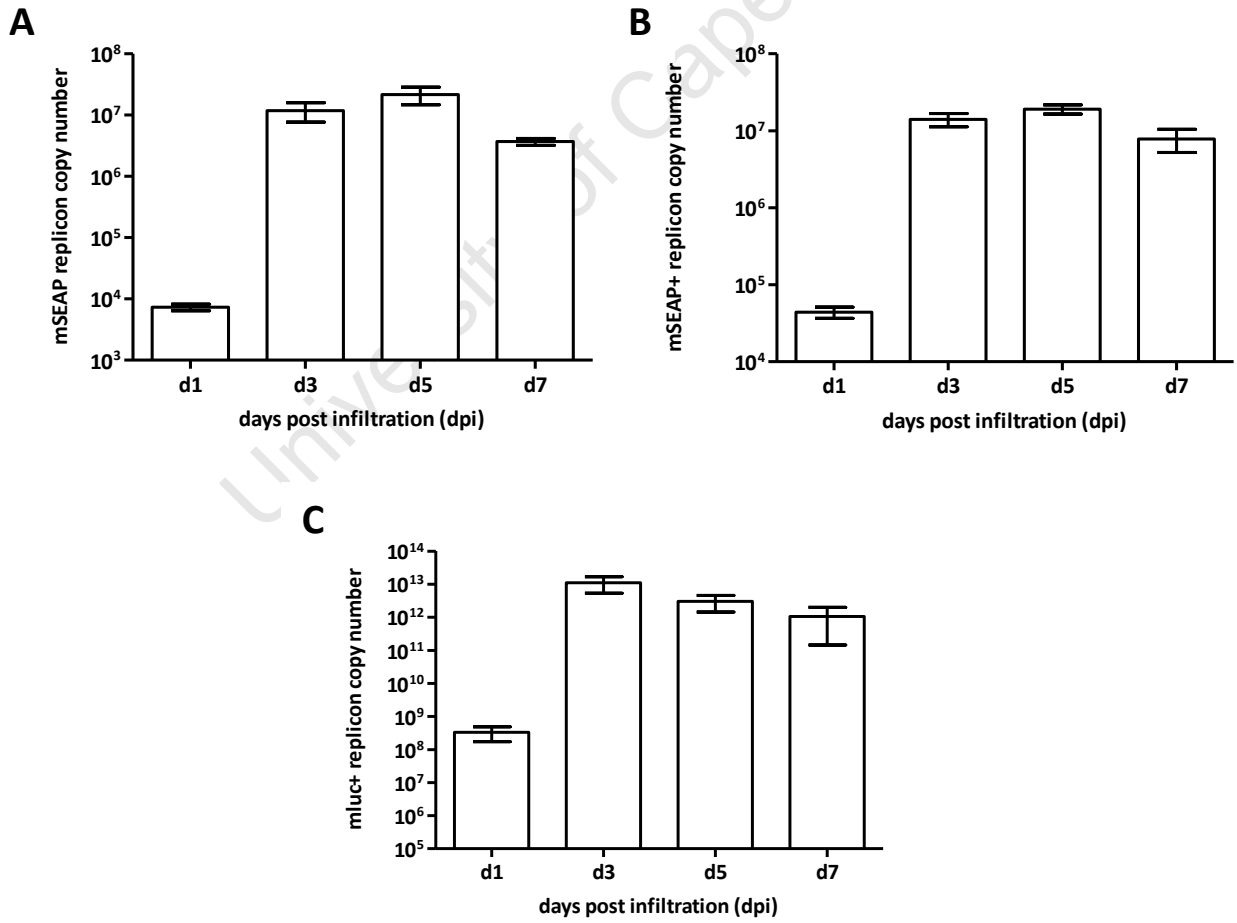


Figure 3.6 | qPCR timetrial of replicating vectors qPCR analysis of DNA extracted from plants infiltrated with (A) pRIC3-mSEAP, (B) pRIC3-mSEAP+, and (C) pRIC3-mluc+, 1-7dpi. Copy number is calculated from total DNA concentration and shown as log10 scale. Error bars indicate standard error of the mean (N=3)

mluc+ (Fig3.6C) all show very similar increases in copy number. This is consistent with previous observations for pRIC and pRIC3 (Ogle, 2008; Regnard et al., 2010).

3.3.2. Production of PsVs in plants

To produce HPV PsVs, *N. benthamiana* plants were co-infiltrated with pTRAc-hL1 and pTRAc-hL2, along with one of pRIC3-mSEAP, pRIC3-mSEAP+, or pRIC3-mluc+. Based on data shown in Section 3.3.2, agroinfiltration with pTRAc vectors was at an OD_{600} of 0.25, while replicating vectors were agroinfiltrated at OD_{600} of 0.5, and particles were harvested at 4dpi. pTRAc-hL1 and pTRAc-hL2 were also co-infiltrated without a replicating vector, with the intention of producing HPV L1/L2 VLPs. This was performed by vacuum infiltration for the production of large volumes of biomass. DNA and crude protein were extracted at 4dpi, in order to confirm the presence of all components necessary for PsV formation by PCR and western blotting (Figure 3.7). PCR amplification of a 2.1Kbp fragment confirmed that replicon formation was occurring for all three replicating constructs at 4dpi (Figure 3.7, panels A and B). Lanes demarcated 'PsV' indicate replicon formation in plants co-infiltrated with pTRAc-hL1 and -hL2, while those marked '+' are from plants infiltrated with replicating vector alone, and serve as a positive control. Western blotting analysis with CamVir-1 (hL1) and a rabbit polyclonal antibody (hL2) confirmed expression of both L1 (Fig. 3.7C) and L2 (Fig. 3.7D) at 4dpi, in plants infiltrated with L1 and L2 alone ('VLP'), or L1 and L2 coinfiltrated with a replicating vector ('mSEAP', 'mSEAP+' and 'mluc+'). This was independently confirmed in at least three separate co-infiltration experiments. Notably, the intensity of the band corresponding to L2 (Fig. 3.7D) showed marked variability between repeats in all constructs.

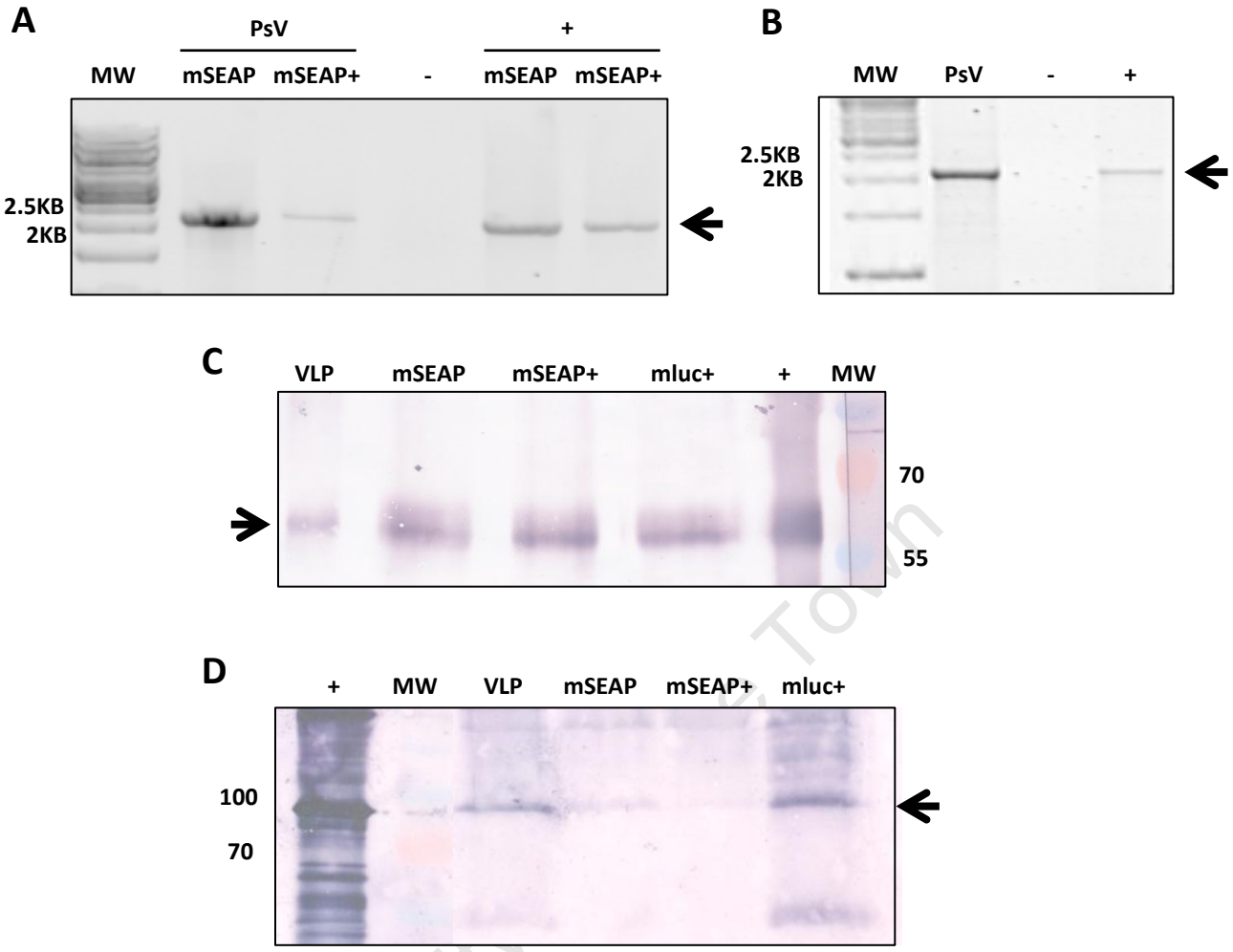


Figure 3.7 | Expression of PsV structural elements PCR was used to confirm presence of (A) pRIC3-mSEAP, pRIC3-mSEAP+, and (B) pRIC3-mluc+ replicons 3dpi, in plants co-infiltrated with pTRAc-hL1, pTRAc-hL2 and pRIC3-mSEAP, -mSEAP+ or -mluc+, respectively. A band at approximately 2.1Kbp (→) indicates replicational release. MW, molecular weight marker, sizes shown on left; PsV, crude plant extract; +, DNA only (positive control); -, plant DNA (negative control). Western blotting for L1 (C) and L2 (D) confirm presence of both structural HPV proteins 4dpi in crude extract from plants co-infiltrated with pTRAc-hL1, pTRAc-hL2 and pRIC3-mSEAP, -mSEAP+ or -mluc+. VLP, pTRAc-hL1 and –hL2 only; mSEAP, pRIC3-mSEAP PsV, mSEAP+, pRIC3-mSEAP+ PsV; mluc+. pRIC3-mluc+ PsV; +, crude plant-produced hL1 (C) or bacterially produced L2 (D); MW, molecular weight marker, sizes indicated in kDa.

3.3.3. Purification and identification of plant-produced PsVs

Having confirmed the presence of all necessary elements comprising PsVs in infiltrated plants (L1 protein, L2 protein, as well as mSEAP, mSEAP+ and mluc+ replicons), VLPs and PsVs were isolated from crude plant extract using variations of the method described by Varsani et al. (2003). Briefly, homogenised PsV-containing plant material was subjected to ultracentrifugation on a 40% sucrose cushion. The resulting pellet was resuspended in 0.4g/ml CsCl in PBS, and subjected to isopycnic ultracentrifugation to separate particles on the basis of buoyant density.

After centrifugation, samples were fractionated and analysed for the presence of L1 by dot blotting, using anti-L1 CamVir-1, as it was thought that this would indicate the presence of VLPs and PsVs as a result of its association with L1, a vital component of these particles. Densitometry analysis of L1 signal on the dot blots indicated the presence of putative HPV VLPs or PsVs, and compared to the buoyant density of each fraction of the gradient, calculated as a function of refractive index (Figure 3.8). Previous work has reported that HPV L1/L2 VLPs with encapsidated DNA (PsVs) have a buoyant density of 1.32-1.34 g/ml, while VLPs (without DNA) have a buoyant density of 1.26-1.28g/ml (Rossi et al., 2000; Touze and Coursaget, 1998). L1 was seen to be present in all fractions, with a distinct peak in signal corresponding to a buoyant density of 1.33g/ml, suggesting that these particles contain encapsidated DNA (Figure 3.8). Interestingly, L1/L2 VLPs demonstrated an L1 peak at a buoyant density of 1.30-1.33g/ml, which corresponds to a 'heavy' particle (Figure 3.8A). This suggests that these particles had possibly encapsidated nucleic acid with similar efficiency to those co-infiltrated with replicating vectors. A secondary peak was seen at a density of 1.25g/ml in L1/L2 VLPs (Fig. 3.8A) and at a density of 1.27g/ml in particles purified from plants infiltrated with pRIC3-mSEAP (Figure 3.8B). This suggests that in these two samples, small quantities of particles were formed without encapsidated DNA. These results are each representative of at least three separate purification procedures. Fractions 8-11 were pooled and dialysed against high-salt PBS to obtain purified PsVs, and fractions 17-18 were pooled and dialysed as a non-PsV control. These were used for further analysis by electron microscopy, western blotting, and PCR.

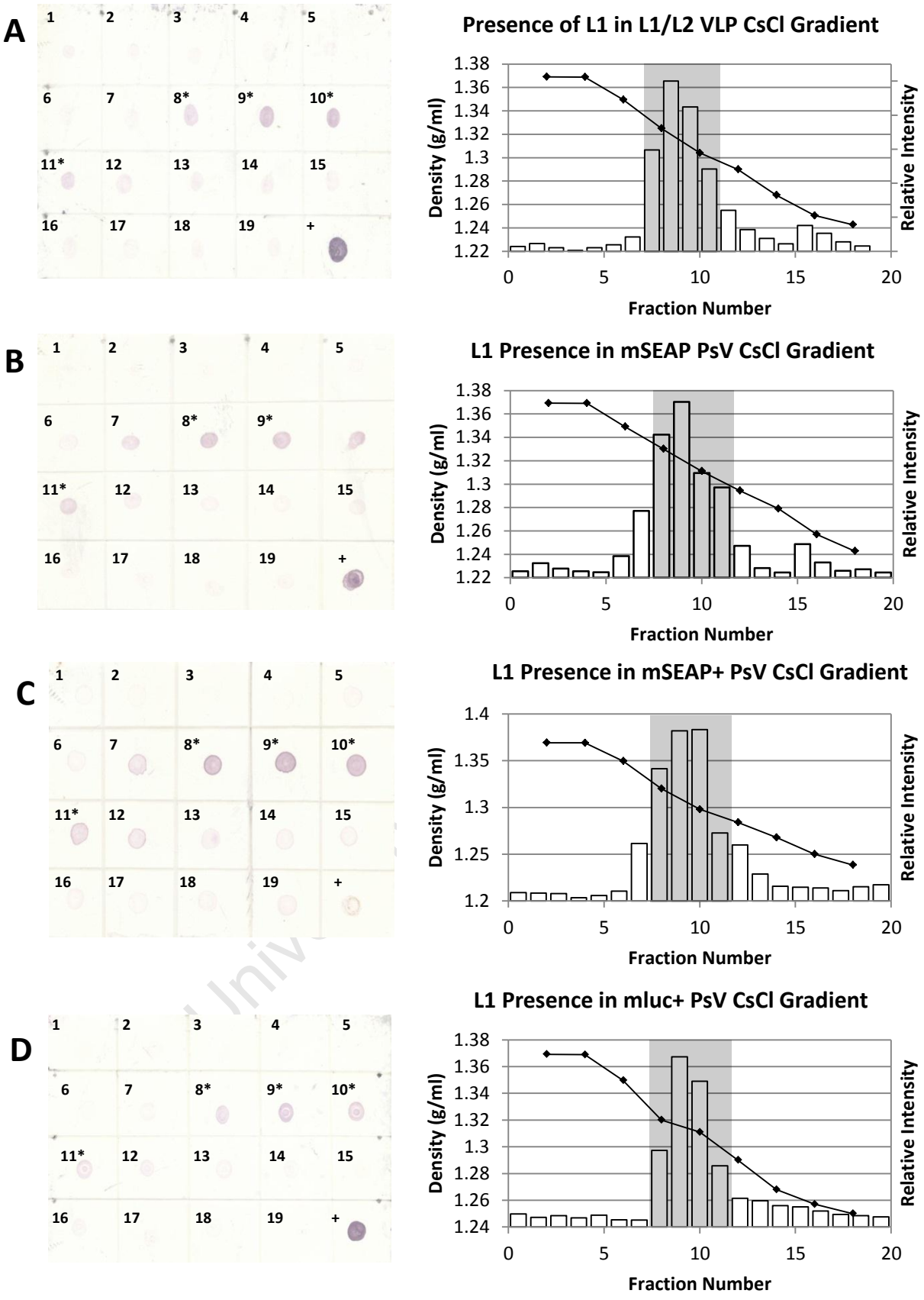


Figure 3.8 | Caesium chloride gradient ultracentrifugation of VLPs and PsVs Densitometry analysis of L1 dot blots shows distribution of L1 after Caesium chloride ultracentrifugation and fractionation. Dot Blots (left panels) of CsCl fractions 1-19 were probed with CamVir-1 anti-L1 antibody. * on dot blots, and grey panel on graphs, indicate fractions pooled for dialysis; +, crude plant extract (positive control). Density of CsCl fractions (♦) was compared to relative intensity (□, arbitrary units) of L1 (right panels) to estimate density of purified particles. (A) L1/L2 VLPs, (B) pRIC3-mSEAP, (C) pRIC3-mSEAP+, (D) pRIC3-mluc+.

Figure 3.9 shows several key stages in the purification process, separated on and SDS-PAGE gel. Coomassie staining reveals the removal of the majority of protein contaminants from the purified samples (Fig. 3.9A and B). A protein band is present at approximately 55kDa in purified samples in both Figure 3.9A and 3.9B, which is probably purified L1. L2, which migrates at approximately 70kDa, is not visible in the Coomassie-stained gels. This is expected, as L2 is present in HPV VLPs and PsVs in much smaller quantities than L1 (a maximum ratio of L1:L2 is estimated at 5:1). Western blotting analysis with CamVir-1 shows a clear increase in concentration and purity of L1 in all samples (3.9C and D).

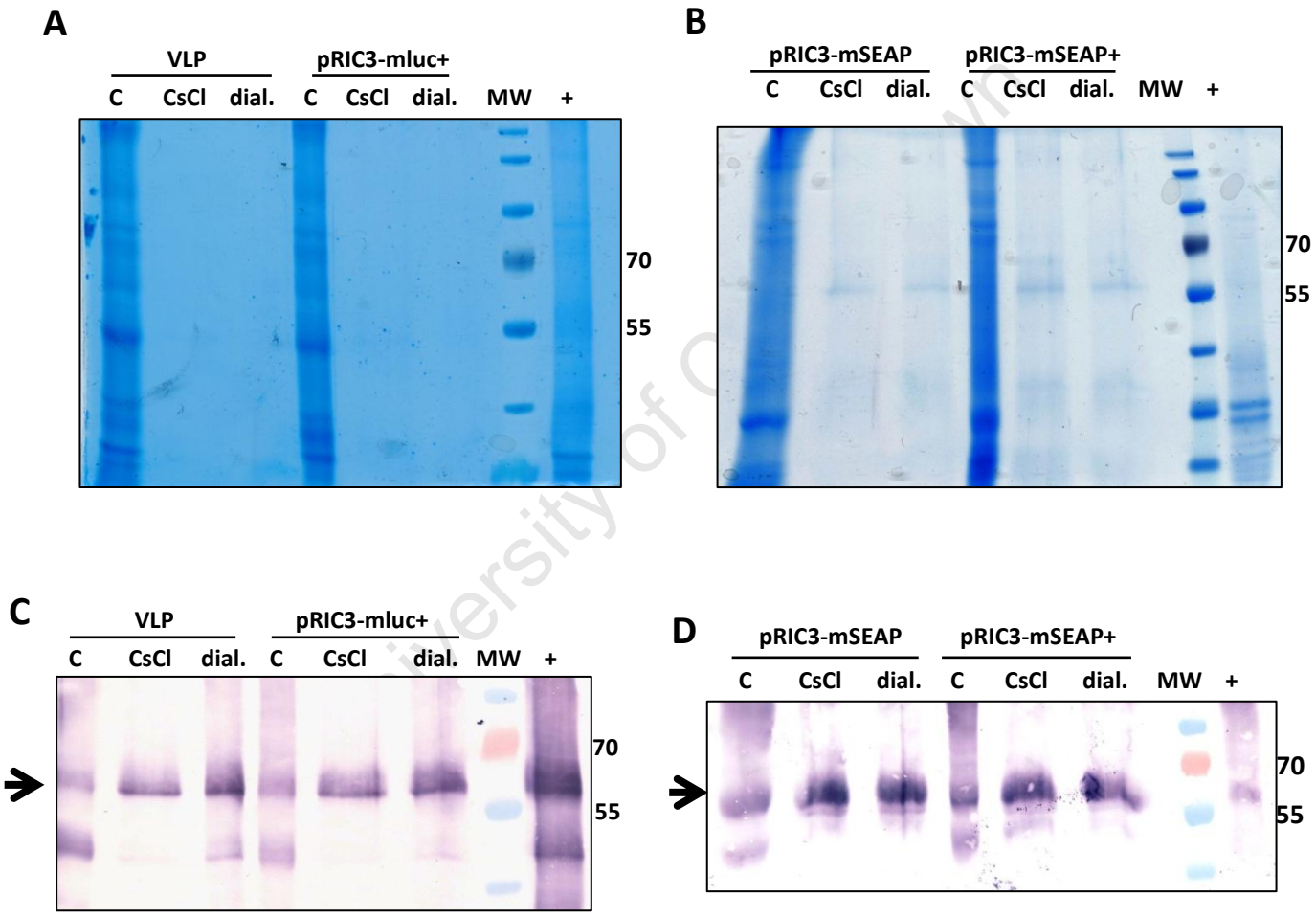
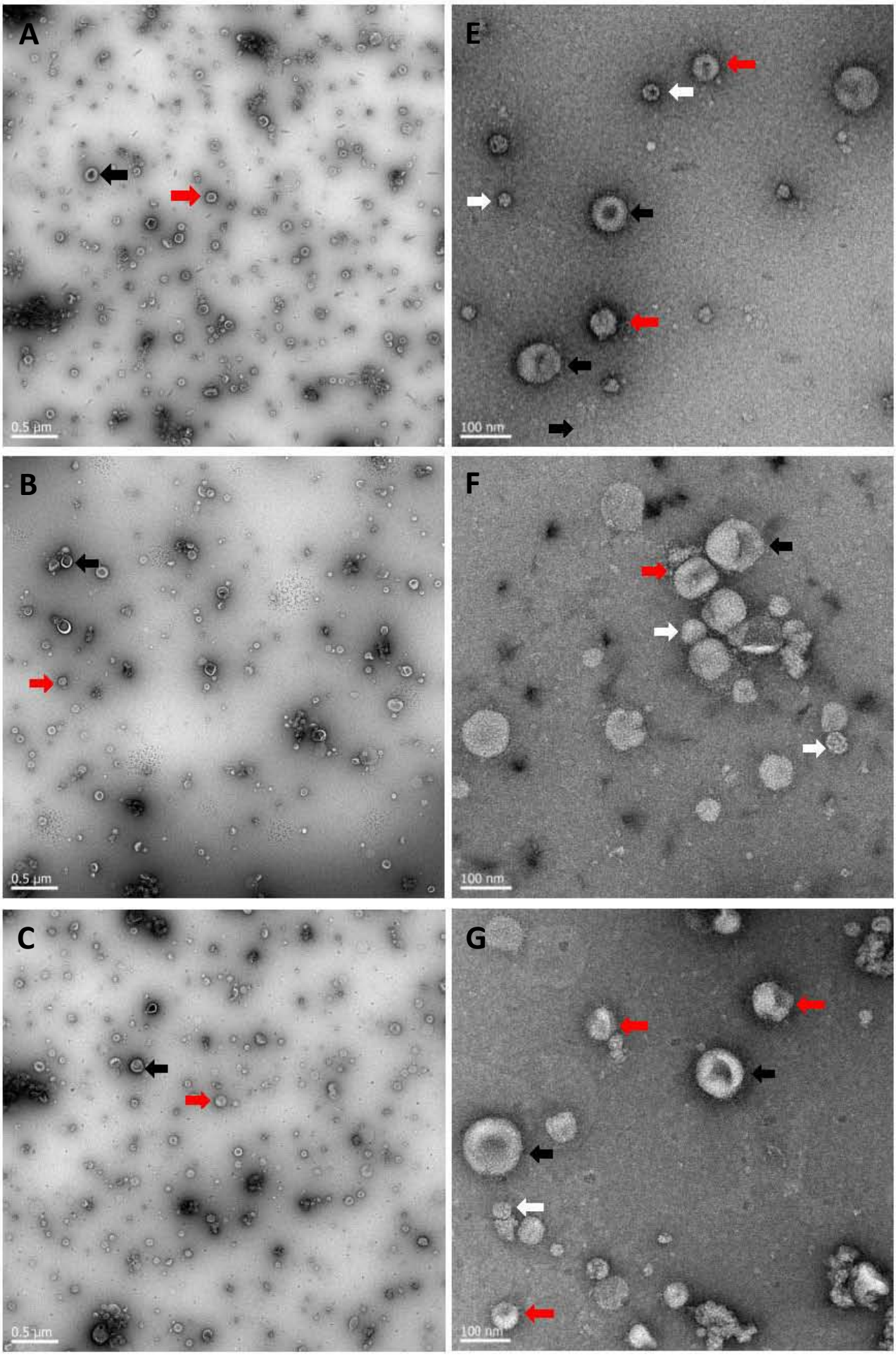


Figure 3.9 Purification of PsVs Coomassie staining (A, B) and western blot for L1 (C, D) of various stages of purification of PsVs, separated by SDS-PAGE. Western blots were probed for L1 using the commercial anti-L1 antibody CamVir-1. MW, molecular weight marker, sizes shown on right; C, crude plant extract; CsCl, pooled caesium chloride gradient fractions; dial., dialysed pooled fractions, +, crude hL1 extract (positive control).


A
0.5 µm
E
100 nm
B
0.5 µm
F
100 nm
C
0.5 µm
G
100 nm

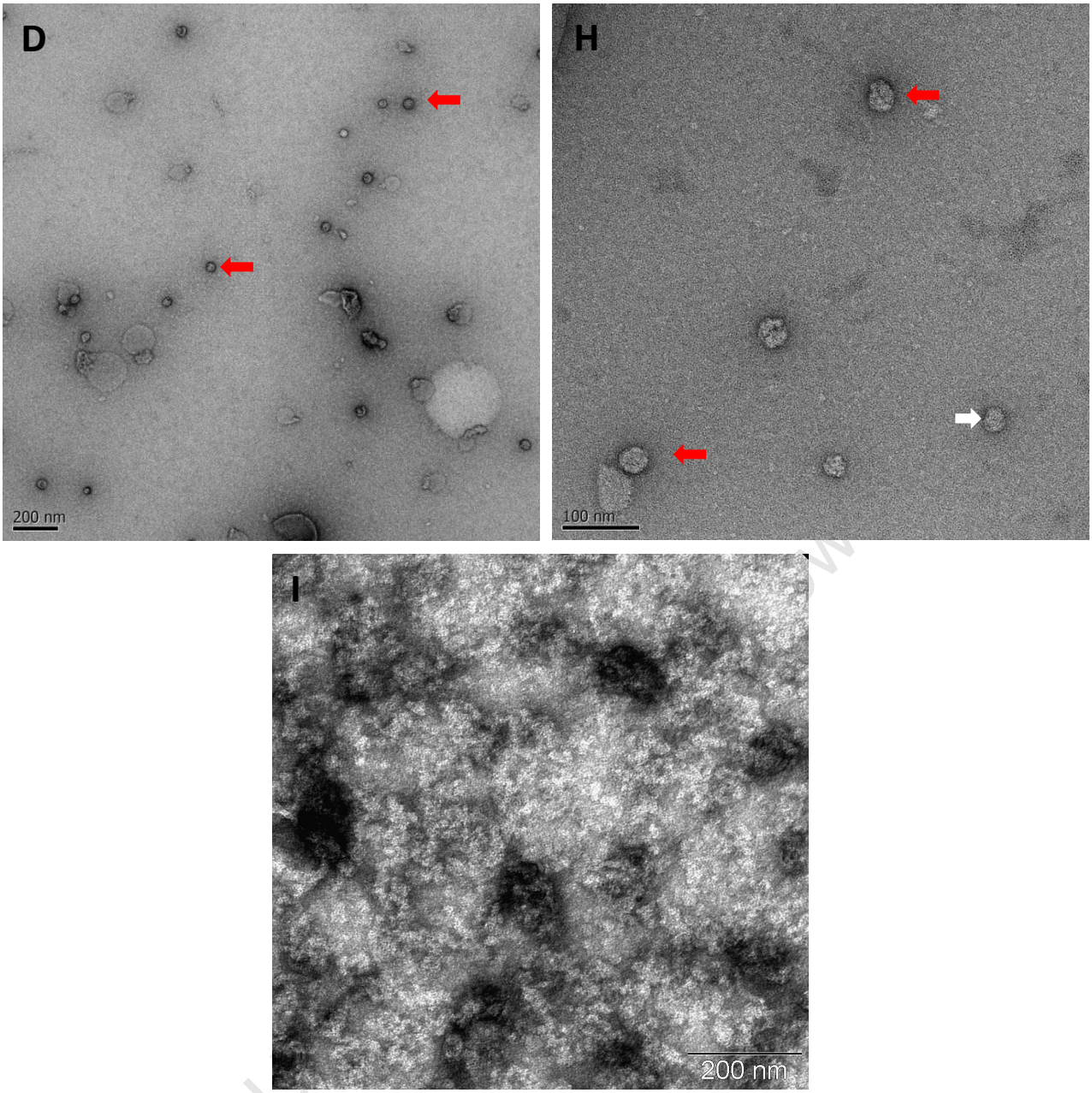


Figure 3.10 | Electron micrographs of purified PsVs (A) and (E) pRIC3-mSEAP , (B) and (F) pRIC3-mSEAP+, (C) and (G) pRIC3-mluc+ PsVs (previous page) and (D) and (H) L1/L2 VLPs were purified by CsCl gradient ultracentrifugation. Purified PsVs size varied from 30-120nm in diameter. White arrows indicate small (30-40nm) particles, Red arrows indicate standard-sized HPV PsV particles (50-60nm), and black arrows indicate large (100-120nm) particles. (I) Crude plant extract serves as negative control. Scale bars are indicated (left panel, 0.5µm; right panel and (D), 200nm, (I), 100nm).

In order to further confirm the presence of L1/L2 VLPs and PsVs, dialysed samples were examined by transmission electron microscopy (Figure 3.10). All samples showed abundant particles, of sizes ranging from 30-120nm. 54% of mSEAP-PsVs (Fig. 3.10A and E) were 40-70nm in diameter, while 47% of mSEAP+-PsVs (Fig. 3.10B and F) and 50% of mluc+-PsVs (Fig. 3.9C and G) 73% of L1/L2 VLPs (Fig. 3.10D and H) were of a similar size. Infectious HPV virions are usually between 50 and 60nm in

diameter. These particles showed a similar morphology to other examples of plant-produced HPV L1-only particles (Maclean et al., 2007; Warzecha et al., 2003).

To confirm that replicon DNA was encapsidated to form PsVs, pooled L1-containing fractions were digested with proteinase K to release the encapsidated pseudogenome, followed by PCR with replicon-specific primers (as described in Section 3.2.5.) to confirm the presence of replicon DNA (Figure 3.11). PCR amplification confirmed that mSEAP, mSEAP+ (Fig. 3.11A) and mluc+ (Fig. 3.11B) PsVs contained the expected DNA replicon. No replicon DNA was amplified in samples not treated with proteinase K, indicating that the DNA was encapsidated, and not found outside of the virion shell. Fractions 17 and 18 from each sample were pooled and dialysed. PCR amplification of these fractions, before or after proteinase K digestion, as well as amplification of proteinase K-treated L1/L2 VLPs, yielded no amplification products.

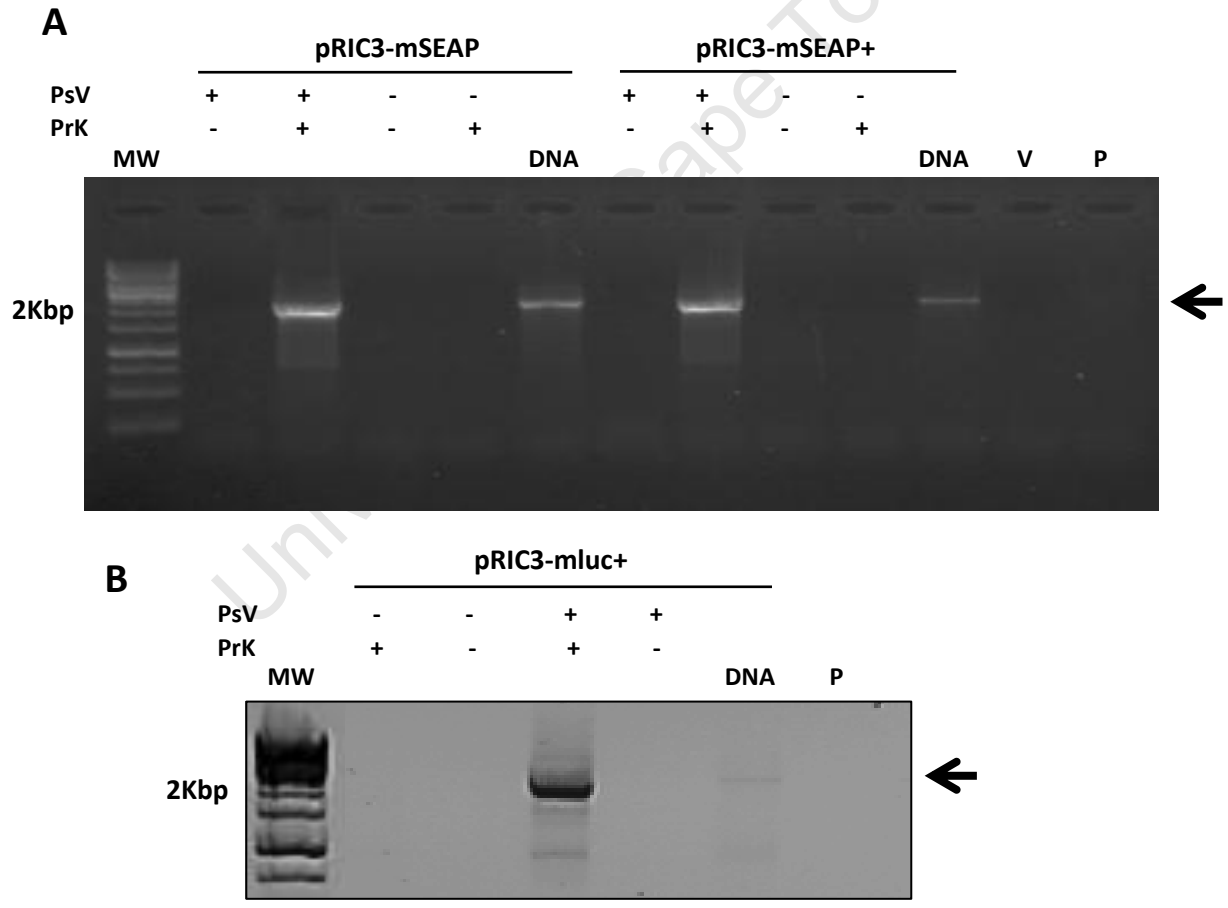


Figure 3.11 | Presence of DNA in purified PsVs PCR amplification of (A) pRIC3-mSEAP, pRIC3-mSEAP+, and (B) pRIC3-mluc+ replicon (2.1Kbp, →) indicates presence of DNA in purified PsV particles after digestion with proteinase K (PrK). MW, molecular weight marker; PrK +/-, PrK digestion; PsV +, pooled L1-containing fractions, PsV -, non-L1 containing fraction (fraction 18); DNA, replicon DNA extract (positive control), V, PrK-digested purified L1/L2 VLPs (negative control); P, plant DNA (negative control).

As a preliminary measure of quantity of PsVs in each sample, DNA concentration of proteinase K-treated samples was read using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Table 3.1. shows NanoDrop readings for all three PsV types. As a broad first estimate at particle concentration for each type, DNA concentration was used to calculate the maximum number of pseudogenomes present per microliter, using the formula

$$no.of\ molecules = \frac{total\ DNA\ (ng)}{660.bp.N_A\ /_{10^{-9}}}$$

where ng is nanograms of DNA in 1µl, bp is pseudogenome size in base pairs, and N_A is Avogadro's constant. Results can be seen in Table 3.1. Concentration of molecules for all three PsV types was in the billions of particles per milliliter. This data assumes that all DNA present was pseudogenome DNA, and that each PsV packaged exactly one copy of the pseudogenome. Taken together, these results indicate the successful production *in planta* of PsVs containing a reporter gene for the first time.

Table 3.1 | Estimated DNA and particle concentration of purified PsVs

	mSEAP	mSEAP+	mluc+
DNA (ng/µl)	8.56	14.33	14.95
PsVs (pseudogenomes/ µl)	$1.63x10^9$	$2.04x10^9$	$1.84x10^9$

3.3.4. Pseudovirion-based neutralisation assay using plant-produced PsVs

In order to demonstrate that plant produced HPV-16 PsVs are an effective biological tool for use in the PBNA, PsVs were tested for reporter expression in mammalian cells, as well as for neutralisation with a commonly used HPV-16 neutralising antibody, HPV16.V5. HEK293TT cells were grown in 96 well plates, and pseudoinfected with 60µl of purified, undiluted PsVs in 0.5M NaCl-PBS, with or without prior incubation with HPV16.V5 monoclonal antibodies diluted 1:20000 in neutralisation media. Successful infection of mammalian cells with PsVs, as well as neutralisation of PsVs, was demonstrated by *luc* or SEAP reporter gene expression in these cells. Figure 3.12 shows reporter gene expression for cells 72h post-infection with mSEAP (3.12A), mSEAP+ (3.12B) and mluc+ (3.12C) PsVs, with or without the presence of neutralising antibodies (PsV, or PsV+NAb, respectively). The negative control (-ve) for each experiment – mammalian cells with 60µl of neutralisation media added - provides a baseline reading in RLU, while transfection with the corresponding endotoxin free

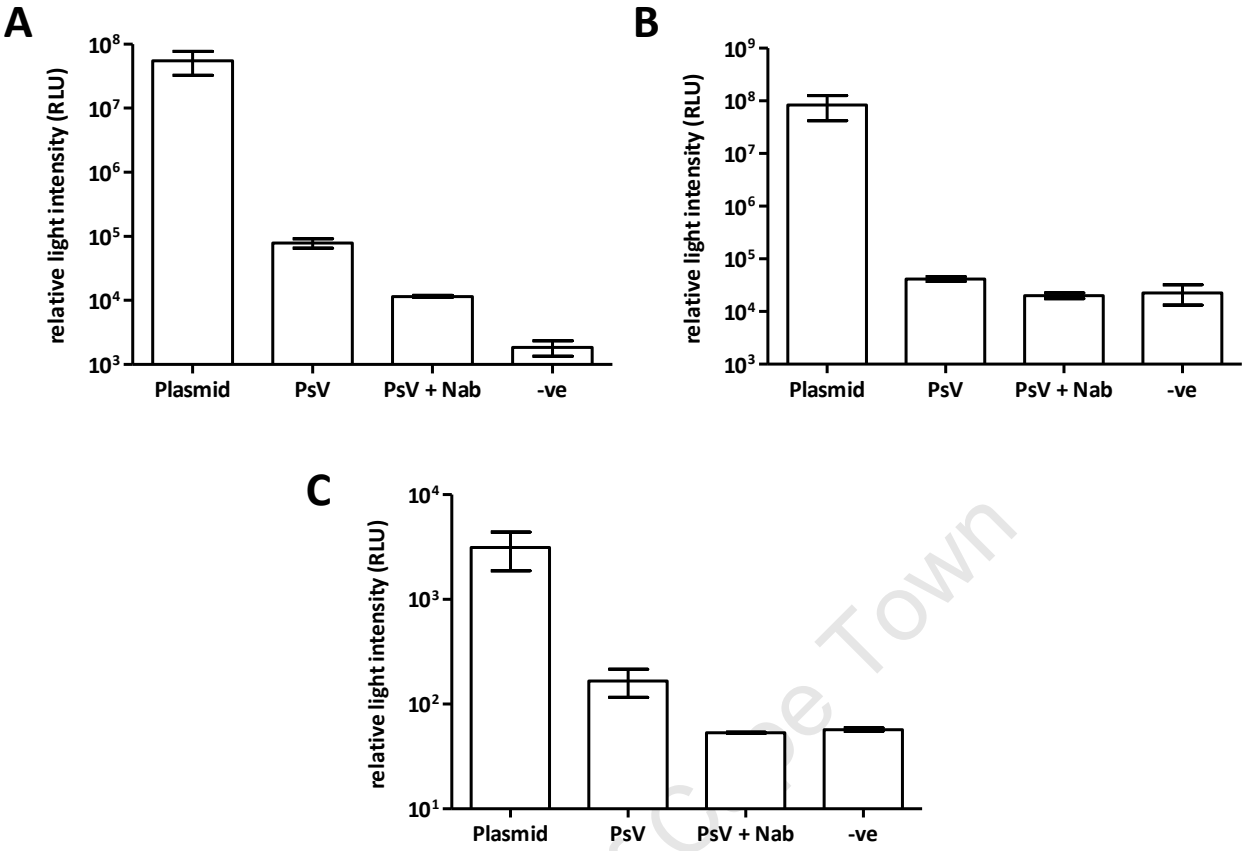


Figure 3.12 | Reporter gene expression in PsV-pseudoinfected mammalian cells (A) SEAP expression 72 hours post pRIC3-mSEAP transfection (Plasmid) or mSEAP-PsV pseudoinfection (PsV) in relative light units (RLU), expressed in logarithmic scale (log10). (B) SEAP expression for pRIC3-mSEAP+, as previously. (C) luc expression for pRIC3-mluc+, as previously. –ve, negative control (media only). All experiments performed in triplicate. Error bars show standard deviation between triplicates.

plasmid DNA is used for the positive control. Pseudoinfection with mSEAP PsVs elicited a clear positive SEAP response (Fig. 3.12A), although not as strong as that in cells transfected with plasmid DNA by lipofection. Incubation with neutralising antibody partially neutralised infection, as demonstrated by a decrease in SEAP signal. Pseudoinfection with mSEAP+ PsVs did not show a strong SEAP signal above the baseline level provided by the negative control (Fig. 3.12B). As such, neutralisation of mSEAP+ PsVs was not observed. Pseudoinfection with mluc+ PsVs elicited a weak luciferase signal, although clearly above that of the negative control. Incubation with HPV16.V5 NAb completely neutralised luciferase expression, resulting in expression identical to the negative control.

3.4. Discussion

In this study, novel autonomously replicating vectors were used in conjunction with previously developed, non-replicating vectors to produce HPV-16 PsVs *in planta*. Preliminary expression trials established optimal conditions and timeframes for production of each individual element required for assembly of HPV PsVs. The structural elements required for PsV production are hL1 and hL2, produced by non-replicating plant expression vectors pTRAc-hL1 and pTRAc-hL2, respectively; and one of three replicons derived from pRIC3-mSEAP, pRIC3-mSEAP+ or pRIC3-mluc+. Putative PsV particles, as well as L1/L2 VLPs, were harvested from plants and purified by successive gradient ultracentrifugation steps. Gradient fractions containing L1 were pooled and dialysed against high-salt (0.5M) NaCl PBS, to obtain purified PsVs. These were confirmed by electron microscopy to be conformationally similar to VLPs and PsVs produced in other systems, and by PCR to contain the corresponding encapsidated replicon DNA. Purified PsVs were used to demonstrate their use in a neutralisation assay. Two of the three PsVs created, namely mSEAP and mluc+ PsVs demonstrated successful pseudoinfection and neutralisation with a common HPV16 neutralising antibody, while mSEAP+ PsVs showed no reporter gene expression after pseudoinfection of mammalian cells. This is the first known report of the production and purification of HPV PsVs, as well as L1/L2 VLPs *in planta*, as well as the first demonstration of the PBNA using plant-produced PsVs.

3.4.1. Replicational release in plants

The three novel vectors discussed in Chapter 2 were tested for their ability to replicate autonomously in plants. PCR analysis confirmed that the appropriate replicons were formed as expected. To elucidate whether the autonomously replicating vectors were, in fact, producing high copy numbers of the replicons *in planta*, qPCR analysis was employed. qPCR analysis of DNA samples harvested up to 7dpi showed that replicon copy number for all plasmids was amplified between 100- and 1000-fold between 1 and 3dpi, and that this high copy number was maintained up to 7dpi. This is similar to the results obtained by Regnard et al. (2010), who showed a near-identical increase in the pRIC vectors used to generate replicons encoding the HIV p24 gene or EGFP. Replicating vectors developed by other groups have demonstrated similar increases in replicon copy number (Huang et al., 2009; Zhang and Mason, 2006). Our result demonstrates that the use of these replicating vectors for the generation of high quantities of double-stranded replicon DNA in plants is a feasible strategy for producing sufficient pseudogenome DNA in plant host cells for PsV production.

3.4.2. Production of PsVs *in planta*

The production of HPV-16 PsVs in plants was successful for each of the three replicating vectors constructed. This work relied on the findings of several earlier papers, in particular that of Maclean et al. (2007). That study demonstrated that humanised L1 was expressed at high levels using the pTRAc vector, and spontaneously assembled into VLPs *in planta*. This, along with unpublished results for pTRAc-hL2 from the same group, demonstrated the feasibility of these vectors for HPV particle production in plants.

It has been widely demonstrated that both HPV L1 and L2 are required for efficient packaging of DNA into the HPV virion, in both natural virions and PsVs (Ma et al., 2011; Okun et al., 2001; Stauffer et al., 1998). Further, it was recently established that the presence of L2 in the PsV capsid increases DNA packaging efficiency 10-fold (Holmgren et al., 2005). In this study, both L1 and L2 were co-expressed to allow for maximum potential DNA encapsidation. No investigations into differential packaging in the presence and absence of L2 were performed; however, little or no L1 signal was visible in fractions 14-16 (buoyant density 1.26-1.28g/ml - corresponding to the density of VLPs with no encapsidated DNA). This lack of 'light' particles suggests that packaging of DNA by HPV particles in plants is very efficient indeed, resulting in few or no particles without encapsidated DNA. This is in contrast to other PsV production methods, all of which show a peak corresponding to 'light' particles, or VLPs. This is in particular true for the VLP disassembly-reassembly method (as demonstrated by Touze and Coursaget (1998)), which usually has a packaging efficiency well below 50% (Touze and Coursaget, 1998; Unckell et al., 1997).

This efficient packaging is a distinct advantage for the plant production approach, although this must be tempered with the observation that not all DNA packaged is necessarily pseudogenome DNA. This is clearly demonstrated by the L1/L2 VLPs produced here, which were seen to be mostly 'heavy' particles, indicating encapsidated DNA. As these were produced in the absence of a replicating vector, DNA packaged was either the pTRAc plasmids used to produce VLPs, or miscellaneous plant DNA. Both pTRAc-hL1 and pTRAc-hL2 (7.7Kbp and 7.5Kbp, respectively) fall below the cut-off mark of 7.9-8Kbp for pseudogenomes that can be packaged effectively into HPV L1/L2 PsVs (Buck et al., 2005b; Touze and Coursaget, 1998). It is possible that these plasmids, or miscellaneous DNA, were packaged into assembling HPV particles instead of the intended replicon DNA – it has been suggested that VLPs produced in mammalian cells encapsidate miscellaneous cellular DNA (Roden et al., 1996). In any event, the extremely efficient replication observed in the three replicating vectors used here, as well as that observed when pRIC was compared to pTRAc in a previous study (Regnard

et al., 2010), suggest that the vast majority of plasmids present in the plant during PsV assembly would be the reporter pseudogenomes. The potential for pTRAc plasmids to be packaged may not hold any relevance to the outcome of this study. Future work to elucidate all DNA species encapsidated into plant-produced PsVs is important for a full understanding of the PsV assembly process *in planta*, as well as for their use in neutralising assays.

Structural analyses of L1 and L2 suggest that DNA associates in a non-specific manner, based on the overall pH and charge of internal structural motifs (Fay et al., 2004; Garcea and Gissmann, 2004; Li et al., 1997; Pereira et al., 2009). Presumably, this allows for *in vitro* PsV assembly, demonstrated by several investigators, in the absence of mammalian cellular factors (Oh et al., 2004; Shi et al., 2001; Touze and Coursaget, 1998). In mammalian cells, there is evidence to suggest that chaperones (particularly karyopherins) play a role in the assembly and DNA packaging of natural HPV virions, and it seems likely that these are responsible for efficient intracellular PsV production (Bird et al., 2008; Chromy et al., 2006). Chaperones, in particular Heat shock protein 70 (Hsp70) and karyopherins, have been shown to play a role in the assembly of diverse viruses, including plant viruses (Kunik et al., 1999; Sullivan and Pipas, 2001). Interestingly, the ER-associated chaperone Binding Protein (BiP) has been demonstrated to take part in folding and assembly of recombinant antibodies in transgenic plants (Nuttall et al., 2002). These data suggest that the molecular machinery required for papillomavirus assembly and DNA encapsidation is conserved across all eukaryote systems, and is responsible for the efficient PsV assembly observed here.

3.4.3. Purification of plant-produced PsVs

Purification of PsVs was performed using a protocol developed for extracting HPV VLPs from harvested plant material (Varsani et al., 2003). This protocol has been used successfully to extract VLPs of several different HPV types from plants (Maclean et al., 2007; Matic et al., 2012). This method proved to be successful, too, for the purification of PsVs. One concern was the use of liquid N_2 for preliminary grinding of plant material. While this step was not, in itself, a problem, cycles of freezing and thawing, as well as freezing plant material for long-term storage at -70°C, resulted in degradation of PsV particles (data not shown). As such, the protocol was modified slightly to replace grinding of frozen leaf material with finely chopping the leaf material in high-salt PBS before proceeding directly to the homogenisation step. This alteration noticeably decreased degradation of PsVs – PCR amplification of the replicon before purification of PsVs showed much more amplification product in fresh plant material when compared to frozen (data not shown).

Electron micrographs clearly demonstrate the successful assembly and isolation of HPV L1/L2 VLPs and PsVs in plants. The PsVs produced demonstrated an unusual variability in size, when compared to other VLP and PsV production methods (Buck et al., 2004; Maclean et al., 2007; Touze and Coursaget, 1998). The broad size range, from 30nm to 120nm in diameter, may be due to pooling of fractions corresponding to CsCl density of 1.30-1.33g/ml. HPV L1/L2 PsVs should be found at a density of 1.32-1.34g/ml, and as such, some PsVs smaller and larger than the expected 50-60nm size range may have been pooled, resulting in the variability shown. Other researchers have seen similar results in transgenic plants, and suggest that the smaller-sized particles may be assembly intermediates (Biemelt et al., 2003). It is also possible that the differential sizes seen here are due to an assembly process that differs substantially to that of HPV virions in mammalian cells.

Previous work has included a maturation step, to allow PsV capsid formation in vitro (Buck et al., 2004). This approach was tested by the inclusion of a 2-hour incubation at 4C after plant tissue homogenisation. EM data revealed no differences in quantity or quality of PsVs produced with or without this step, and as such it was omitted from future purifications.

Previous reports on the production of HPV PsVs has used benzonase treatment coupled with PCR to demonstrate that DNA is encapsidated within the virion shell, and not merely associated with the virion (Rossi et al., 2000; Unckell et al., 1997). Benzonase also decreases the viscosity of the lysate, and frees capsid proteins associated with cellular DNA. PsVs produced in this study were not degraded by the 95°C PCR denaturation step, as demonstrated by no amplification of pseudogenome DNA in PsV samples not digested with proteinase K. As such the protein shell needed to be digested prior to PCR pseudogenome amplification in order to demonstrate the presence of pseudogenome DNA. The significance of this is twofold. Firstly, benzonase treatment was not required to demonstrate encapsidation of DNA, and was subsequently not used. Second, and possibly more importantly, this demonstrates that these PsVs are remarkably stable, even under mildly denaturing conditions. This is an important observation. VLPs are generally relatively unstable, and need to be treated with some care to avoid collapsing the particle (Mach et al., 2006). While PsVs are generally more stable, most that have been produced in other systems are not as stable as these plant-produced PsVs have been demonstrated to be. Accordingly, this suggests an important advantage over traditional PsV production systems.

The data presented here is the first clear evidence for successful production and purification of plant-produced L1/L2 VLPs. While this was not the primary aim of the project, production of L1/L2

VLPs was useful, in that it allowed a comparison of VLPs and PsVs produced in plants. Electron micrographs clearly show regular particles of 40-70nm in diameter. The low number of VLPs shown relative to the numbers of PsVs is a result of less starting material – VLPs were purified from approximately 25% (by fresh leaf weight) of the crude plant material used for PsV production. Western blots seen in Figure 3.6 (panels C and D) clearly show the presence of both L1 and L2 in plants co-infiltrated with pTRAc-L1 and pTRAc-L2. Levels of L2 varied noticeably between different co-infiltration experiments, including co-infiltrations for the production of PsVs. This is not unsurprising, in that the ratio of L1:L2 has been shown to vary between 5:1 and 30:1 in HPV virions and L1/L2 VLPs. This first evidence of L1/L2 VLP production in plants is an encouraging new landmark in plant-based production of HPV VLP vaccines.

A first estimate of final concentration of PsVs yielded figures in the billions per microliter. This estimate is an inherently rough one: several assumptions are made, and the starting data – DNA concentration obtained by spectrophotometry, is far from accurate. These assumptions are 1) that all DNA present was encapsidated pseudogenome DNA, and 2) that each PsV packaged exactly one copy of the pseudogenome. However, it is safe to assume these estimates would not be out by more than two orders of magnitude. Several other researchers have tried to quantitate PsV concentration from various systems, usually using L1 quantitation by ELISA (Fleury et al., 2008a), L1 ELISA in combination with PCR (Unckell et al., 1997) or estimating transducing units from reporter assay data (Buck et al., 2004). Future improvements on this system will require an accurate estimate of PsV concentration, such as that provided by ELISA quantitation.

Total yield and concentration factor were not determined. It is clear from electron micrographs and western blots that there was a marked concentration of particles. Western blotting of various stages of purification (Figure 3.9) clearly shows an increase in L1 signal, suggesting an approximate doubling in concentration from the crude plant sample to the dialysed PsVs. However, this is by no means a quantitative assay, and as such no firm conclusions can be reached on that basis. Further work to determine PsV concentration is an important next step in evaluating the efficiency of plant production of PsVs.

This study successfully demonstrated the feasibility of producing PsVs in plants. However, much work remains to fully elucidate the production method and efficiency of production for plant-produced PsVs to be a feasible alternative to current methods. Most importantly, quantitation of PsVs produced is a necessary next step. This could be achieved with relative simplicity, by L1 ELISA,

as demonstrated by Touze and Coursaget (1998). Another important step is the investigation of all DNA species incorporated into virions, to avoid issues of contamination when using these PsVs. Lastly, an exciting possibility is a simplified purification protocol. The complexity of the current protocol was necessitated by the instability of VLPs. However, the demonstrated stability of these PsVs suggest that a much 'harsher' virus extraction protocol, such as those used for plant virus extraction (E.P. Rybicki, personal communication), could be equally successful in purifying PsVs at a fraction of the time and cost.

3.4.4. PsV testing and PBNA in mammalian cells

For plant-produced PsVs to be a useful tool for vaccine testing, it is vital to demonstrate their use in the pseudovirion-based neutralisation assay. PsVs were tested for pseudogenome reporter gene expression and PBNA activity using the Great EscAPe SEAP Chemoluminescence Kit (Clontech Laboratories, Inc.) or the Luciferase Assay Sytem (Sigma). The Great EscAPe kit is used for the widely accepted PBNA protocol developed by the Schiller laboratories, for its sensitivity and ease of use (Buck et al., 2005a). Luciferase has seen broad utility as an easy and sensitive reporter assay, and was chosen due to its low cost Great EscAPe system, as well as to test an alternative pseudogenome size and reporter system.

Of the three PsV types produced, two (mSEAP PsVs and mluc+ PsVs) showed low-level reporter activity after pseudoinfection of mammalian cells, while one (mSEAP+) showed little or no reporter activity. A preliminary neutralisation assay using a well-established mouse monoclonal HPV-16 neutralising antibody HPV16.V5 (Christensen et al., 1996a), demonstrated partial neutralisation of mSEAP PsV infection, and complete neutralisation of mluc+ PsV infection. It is unclear why mSEAP+ PsVs failed to induce reporter gene expression in mammalian cells. The SEAP cassette is clearly functional, as demonstrated by successful reporter gene expression by mSEAP PsVs. The plant cassette incorporated into the pseudogenome is unlikely to be the cause – mluc+ PsVs also incorporated an identical plant expression cassette without affecting expression. It is possible that it was due to low concentration of particles in comparison to the other two PsV types – while estimates of concentration based on presence of DNA revealed no major differences, electron micrographs showed fewer particles in the mSEAP+ PsV samples compared to the other two PsV types. While preliminary, these data provide an initial proof-of-concept for the production of PsVs *in planta* for use in the PBNA.

Reporter gene expression after pseudoinfection was considerably lower than expected. Most previous PBNA studies have needed to dilute PsVs up to 1000000-fold in order to be within the linear range of the SEAP assay. Preliminary calculations determined PsV particle concentration to be similar to that obtained by Buck et al. (2005a). Accordingly, it would be expected that infectivity would be similar. However, this was not the case: the PsVs tested here showed limited reporter gene expression, even though they were added undiluted to cells. Expression by pseudoinfection was lower than DNA transfected by lipofection. Total DNA added by FuGene transfection to each well of a 96-well plate was approximately 200ng per well, while total DNA in a 60µl PsV sample, as determined by NanoDrop spectrophotometry, was 500-900ng, depending on the sample. Previous work has shown that infectivity of PsVs can be quite low - Roden et al. (1996) estimated an infectivity of 1 in 10000 cells, while Unckell et al. (1997) and Touze and Coursaget (1998) estimated ratios of 1:2000, 1:1000, respectively. However, this does not fully explain the poor expression following pseudoinfection. One consideration is that the PBNA demonstrated by Pastrana et al. (2004) used a plasmid (pYSEAP) which included an SV-40 ori. The high level of SEAP expression in HEK293TT has been attributed to this feature, and the lack of an SV-40 ori in the SEAP plasmid may have resulted in lower-than-expected expression levels. However, it is likely that the major cause of this discrepancy is the buffer that the PsVs were dialysed into after purification, namely 0.5M NaCl-PBS. Changes in cell culture media osmolality (a measure of the concentration of particles in solution) have a marked effect on mammalian cells. Physiological osmolality is estimated at 290-320mOsm/kg for mammalian tissues (Waymouth, 1970). Any major deviation from this, such as the introduction of large quantities of NaCl into cell culture media, is likely to seriously affect the growth of the cells, as well as their ability to produce recombinant protein. This is likely to be the case here, and a priority for future work is to repeat these experiments with PsVs in a buffer with less salt. This buffer was chosen because it has been shown to aid in stability of VLPs produced in plants (Varsani et al., 2003). However, these PsVs have demonstrated marked stability to denaturing conditions, and it is likely that they will be stable in PBS.

3.5. Conclusion

In conclusion, this study has demonstrated, for the first time, the successful production and testing of HPV PsVs in plants. HPV L1/L2 VLPs, as well as PsVs containing a mammalian reporter cassette pseudogenome, were produced in large quantities *in planta*. The particles readily encapsidated the pseudogenome DNA provided by the replicating vectors. Further, they were easily purified, stable at high temperature, and were conformationally indeterminable from PsVs produced in other systems. Most importantly, they were successfully used to perform a PBNA in mammalian cells. While this is a

preliminary proof-of-concept study only, it demonstrates that transient plant-based production of HPV PsVs is a feasible strategy, and should be further investigated as low cost alternative to mammalian cell culture for PsV production.

Chapter 4: Conclusions

Production of neutralising antibodies to HPV is currently regarded as the means of protection elicited by HPV vaccines. The best estimate of protective efficacy relies on using the widely accepted pseudovirion neutralisation assay (PBNA) for evaluation of the neutralising potential of anti-HPV sera. While the currently accepted PBNA, as described by Buck et al. (2005a), is a sensitive and efficient assay, production of PsV stocks used in this assay relies on expensive mammalian cell culture. As such, there is a perceived need to reduce the costs of current PsV production technology. Transient expression in plants offers an attractive alternative to mammalian cell culture for this purpose, with the potential for a more cost effective PsV production method. The aims of this project were to 1) develop replicating plant vectors for the production of PsV pseudogenomes in plants, 2) co-infiltrate plants with these vectors, along with HPV-16 L1 and L2 expression vectors, to produce PsVs *in planta* by transient expression, and purify those particles, and 3) test the efficacy of these plant-produced PsVs for use in a pseudovirion-based neutralisation assay.

4.1. Design and construction of replicating vectors for pseudogenome production in *N. benthamiana* and testing in mammalian cells

Three novel replicating vectors were successfully created from the replicating plant expression vector pRIC3; namely pRIC3-mSEAP, pRIC3-mSEAP+, and pRIC3-mluc+. The autonomously replicating vector pRIC, and its successor pRIC3, were previously developed from Bean yellow dwarf mastrevirus (BeYDV) for transient expression of recombinant protein at high levels in plants. pRIC and pRIC3 have both been demonstrated to form an autonomously replicating replicon in plant cells which replicates to high copy number as a dsDNA episome (Regnard et al., 2010). This replication to high copy number makes pRIC3 an ideal vector for the production of large quantities of reporter pseudogenomes in plant cells. pRIC3-mSEAP was created by insertion of mammalian EF-1α promoter element, SEAP gene, and mammalian SV40 polyadenylation signal, in place of the extant plant cassette. pRIC3-mSEAP+ was created by insertion of the same mammalian cassette, at the 5'-end of the plant cassette. pRIC3-mluc+ was created by insertion of a mammalian CMV intron/enhancer/promoter element, pCapR enhancer element, luciferase reporter gene, and BGH polyadenylation signal, at the 5' end of the extant plant cassette. These three vectors were designed to test a range of replicon sizes (4.8, 6.6 and 7.6Kbp, respectively) when agroifiltrated into plants, for efficient packaging as pseudogenome DNA into assembling HPV-16 PsVs.

All three vectors were confirmed to have the correct sequences, and were tested for reporter gene functionality in mammalian cells. Transfection of mammalian cells with plasmid DNA resulted in reporter gene expression for all three vectors, as determined by western blotting for SEAP, and commercial luciferase assay for luc. This confirmed that these vectors were fully functional under the control of a mammalian promoter, and that they would provide a suitable mechanism of pseudogenome production for PsVs produced *in planta*. Further, it validated the choice of SEAP and luc as robust and reliable reporter genes for this application.

4.2. Production and purification of HPV-16 VLPs and PsVs in *N. benthamiana* via agroinfiltration of transient expression vectors

In order to use the replicating vectors for transient production of pseudogenome DNA, these vectors needed to demonstrate replicon formation and replication to high copy number *in planta*. Vector-specific replicon formation was demonstrated for each replicating vector by inverse PCR. qPCR was used to evaluate the gene copy number increase after agroinfiltration of plants. All three replicating vectors showed a 100-1000-fold increase in gene copy number from 1 to 3dpi. This increase closely mimics the copy number increase seen in pRIC and pRIC3 (Regnard et al., 2010)

For transient expression of the HPV-16 major and minor capsid proteins in plants, non-replicating plant expression constructs pTRAc-hL1 and pTRAc-hL2 were chosen. pTRAc is a transient expression vector, which targets protein expression to the cytoplasm of plants. pTRAc constructs encoding human codon-optimised HPV16 L1 and HPV16 L2 proteins (hL1 and hL2) have demonstrated high levels of protein expression in plants, compared to wildtype and plant codon-optimised genes, as well as to other vectors (Maclean et al., 2007; Pereira, 2008). The highest expression of all elements (L1, L2 and DNA replicons) was seen at 3-5dpi. Accordingly, 4dpi was chosen as the time at which to harvest plants co-infiltrated with pTRAc-hL1 and pTRAc-hL2 alone, as well as with each of pRIC3-mSEAP, pRIC3-mSEAP+, and pRIC3-mluc+ individually. Purification of particles was successful, using a density gradient ultracentrifugation method for purification developed by Varsani et al. (2003) for the purification of HPV-16 VLPs from plants. Dot blot testing for presence of L1 in density gradient fractions identified putative PsVs in fractions corresponding to the expected buoyant density of PsVs, for all three PsV types (i.e. mSEAP, mSEAP+ and mluc+ PsVs), with little or no L1 signal at the expected buoyant density for empty VLPs. These fractions were pooled and electron microscopy confirmed the presence of abundant PsV particles, of a size range from 30-120nm. PsVs of the expected size (40-70nm) constituted approximately 54% (mSEAP PsVs), 47% (mSEAP+ PsVs) and 50% (mluc+ PsVs) of the total PsVs visible in electron micrographs. Western blotting confirmed that both

L1 and L2 were present in all PsVs, as well as VLPs, produced. L1 presence was fairly consistent, while levels of L2 expression (as seen by western blot) varied between different PsV types and biological repeats. This is unsurprising, considering that the ratio of L1:L2 is variously reported at 5:1, 12:1 and 30:1, and there is some evidence for variation of this ratio based on amount of L2 present during VLP assembly (Buck et al., 2008). Inverse PCR testing of DNA from PsVs revealed that each PsV type successfully encapsidated pseudogenome DNA, and that this DNA was protected by the PsV capsid shell. Proteinase K digestion of PsVs abrogated this protection, to confirm that DNA was encapsidated by PsVs, rather than merely associated with them.

4.3. Pseudoinfection of mammalian cells with purified plant-produced PsVs, and their use for the PBNA

In order for plant-produced HPV-16 PsVs to be useful for vaccine testing, there is a need to demonstrate their effectiveness for pseudoinfection of mammalian cells, as well as neutralisation of PsVs with HPV-16 neutralising antibodies. To achieve this, plant produced HPV PsVs were used to pseudoinfect mammalian cells. Reporter gene activity was assayed 72 hours post-infection, using luciferase or SEAP assays. pRIC3-mSEAP and pRIC3-mluc+ demonstrated successful reporter activity following pseudoinfection of mammalian cells, while pRIC3-mSEAP+ showed no activity at 72 hours post-infection. Further, incubation of PsVs with a well-known neutralising HPV16 antibody HPV16.V5 resulted in partial neutralisation of mSEAP PsVs, and complete neutralisation of mluc+ PsVs, as shown by abrogation of reporter gene signal. Overall, reporter gene expression following pseudoinfection of mammalian cells was lower than expected, given the high concentration of PsVs produced. However, this is likely as a result of the high-salt PBS buffer that purified PsVs were stored in. Further work is needed to test whether this hypothesis is accurate. As a result, this work has not yet produced usable PsVs, but has demonstrated a feasible production and purification strategy.

4.4. Conclusions and further work

Replicating vectors were successfully designed and constructed for the production of HPV-16 PsV pseudogenomes, incorporating luc and SEAP mammalian expression cassettes. Pseudogenomes of 4.8, 6.4 and 7.4Kbp were all produced and packaged with similar efficacy, and L1/L2 VLPs assembled readily in the absence of replicon DNA. Two out of the three PsVs produced (MSEAP and mluc+) were successfully used for a PBNA, while mSEAP+ PsVs did not result in reporter gene expression in mammalian cells. This preliminary proof-of-concept study was successful using two of the three PsV types produced, but there are several facets of the work that require further investigation. An

accurate estimate of PsV concentration would be of great value to determine whether plant-based production is favourable to the current mammalian cell culture production protocol. An evaluation of all DNA species present in the PsVs produced would be of great value if these particles are ever to be used for vaccine formulations. The PBNA work performed was preliminary, and requires optimisation. Repeating this work using PsVs in a low-salt buffer is a vital step to properly evaluate the potential of these particles for PBNA applications. In conclusion, transient plant-based expression of HPV capsid proteins, used in conjunction with replicating vectors for pseudogenome production, is a viable strategy to generate HPV PsVs for use in the PBNA.

Appendix A: Recipes

Complete DMEM (cDMEM)
500ml Dulbecco's Modified Eagles Medium (DMEM)
50ml Foetal Calf Serum (FCS)
5ml penicillin/streptomycin (10mg/ml)
5ml Non-essential amino acids (NEAA)

Neutralisation media
500ml Dulbecco's Modified Eagles Medium (DMEM) without phenol red
50ml Foetal Calf Serum (FCS)
5ml penicillin/streptomycin (10mg/ml)
5ml Non-essential amino acids (NEAA)

Blocking Buffer
100ml 10X PBS
1ml 10% Tween-20
5g Skim-milk powder
ddH_2O to 100ml

Transfer Buffer (1X)
28.8g Glycine
6.04g Tris Base
200ml Methanol
ddH_2O to 1L

Running Buffer (1X)
28.8g Glycine
6.04g Tris base
2g Sodium dodecyl sulphate (SDS)
ddH2O to 2L

Coommassie Stain
1g Coomassie R250
100ml glacial acetic acid
400ml methanol
ddH2O to 1L

Coomassie Destain
200ml Methanol
100ml Glacial acetic acid
700ml ddH_2O

LB Medium
10g Tryptone

10g NaCl
5g Yeast Extract
ddH_2O to 1L
Autoclave

LB Agar
10g Tryptone
10g NaCl
5g Yeast Extract
ddH2O to 1L
15g Bacterial agar
Autoclave

Induction Medium
10mM MES
20µM acetosyringone
LB Medium to 1L
pH 5.6
Autoclave

Infiltration medium
10mM $MgCl_2$
10mM MES
30g sucrose
200µM Acetosyringone
ddH2O to 1L
pH to 5.6
Autoclave

5X Protein loading dye
940µl 10% SDS
470µl 1M TrisCl
95µl 100mM Ethylenediaminetetraacetic acid (EDTA)
2.45ml glycerol
545µl ddH_2O
205µl β-mercaptoethanol
1mg bromophenol blue

10X Tris-Borate-EDTA (TBE) Electrophoresis Buffer
108g Tris base
55g Boric acid
7.5g EDTA
ddH2O to 1L

10X Phosphate Buffered Saline (PBS)
8g NaCl
2g KCl
14.4g Na_2HPO_4
2.4g KH_2PO_4
Adjust to pH 7.4
ddH_2O to 1L
Autoclave

High-Salt PBS
100ml 1X PBS
29.22g NaCl
ddH_2O to 1L

CsCl-PBS
100ml 1X PBS
40g CsCl

References

Bakker, H., Rouwendal, G.J., Karnoup, A.S., Florack, D.E., Stoopen, G.M., Helsper, J.P., van Ree, R., van Die, I. and Bosch, D. (2006) An antibody produced in tobacco expressing a hybrid beta-1,4-galactosyltransferase is essentially devoid of plant carbohydrate epitopes. *Proc Natl Acad Sci U S A* **103**, 7577-7582.

Barksdale, S.K. and Baker, C.C. (1993) Differentiation-specific expression from the bovine papillomavirus type 1 P2443 and late promoters. *Journal of virology* **67**, 5605-5616.

Becker, K.A., Florin, L., Sapp, C., Maul, G.G. and Sapp, M. (2004) Nuclear localization but not PML protein is required for incorporation of the papillomavirus minor capsid protein L2 into virus-like particles. *Journal of virology* **78**, 1121-1128.

Berger, J., Hauber, J., Hauber, R., Geiger, R. and Cullen, B.R. (1988) Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene* **66**, 1-10.

Biemelt, S., Sonnewald, U., Galmbacher, P., Willmitzer, L. and Muller, M. (2003) Production of human papillomavirus type 16 virus-like particles in transgenic plants. *Journal of virology* **77**, 9211-9220.

Bird, G., O'Donnell, M., Moroianu, J. and Garcea, R.L. (2008) Possible role for cellular karyopherins in regulating polyomavirus and papillomavirus capsid assembly. *Journal of virology* **82**, 9848-9857.

Bosch, F.X., Burchell, A.N., Schiffman, M., Giuliano, A.R., de Sanjose, S., Bruni, L., Tortolero-Luna, G., Kjaer, S.K. and Munoz, N. (2008) Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. *Vaccine* **26 Suppl 10**, K1-16.

Bousarghin, L., Combita-Rojas, A.L., Touze, A., El Mehdaoui, S., Sizaret, P.Y., Bravo, M.M. and Coursaget, P. (2002) Detection of neutralizing antibodies against human papillomaviruses (HPV) by inhibition of gene transfer mediated by HPV pseudovirions. *Journal of clinical microbiology* **40**, 926-932.

Bousarghin, L., Touze, A., Gaud, G., Iochmann, S., Alvarez, E., Reverdiau, P., Gaitan, J., Jourdan, M.L., Sizaret, P.Y. and Coursaget, P.L. (2009) Inhibition of cervical cancer cell growth by human papillomavirus virus-like particles packaged with human papillomavirus oncoprotein short hairpin RNAs. *Molecular cancer therapeutics* **8**, 357-365.

Brondyk, W.H. (2009) Selecting an appropriate method for expressing a recombinant protein. *Methods in enzymology* **463**, 131-147.

Brotherton, J.M., Fridman, M., May, C.L., Chappell, G., Saville, A.M. and Gertig, D.M. (2011) Early effect of the HPV vaccination programme on cervical abnormalities in Victoria, Australia: an ecological study. *Lancet* **377**, 2085-2092.

Buck, C.B., Cheng, N., Thompson, C.D., Lowy, D.R., Steven, A.C., Schiller, J.T. and Trus, B.L. (2008) Arrangement of L2 within the papillomavirus capsid. *Journal of virology* **82**, 5190-5197.

Buck, C.B., Pastrana, D.V., Lowy, D.R. and Schiller, J.T. (2004) Efficient Intracellular Assembly of Papillomaviral Vectors. *Journal of virology* **78**, 751-757.

Buck, C.B., Pastrana, D.V., Lowy, D.R. and Schiller, J.T. (2005a) Generation of HPV pseudovirions using transfection and their use in neutralization assays. *Methods in molecular medicine* **119**, 445-462.

Buck, C.B., Thompson, C.D., Pang, Y.-Y.S., Lowy, D.R. and Schiller, J.T. (2005b) Maturation of Papillomavirus Capsids. *Journal of virology* **79**, 2839-2846.

Burchell, A.N., Winer, R.L., de Sanjose, S. and Franco, E.L. (2006) Chapter 6: Epidemiology and transmission dynamics of genital HPV infection. *Vaccine* **24 Suppl 3**, S3/52-61.

Burgers, W.A., van Harmelen, J.H., Shephard, E., Adams, C., Mgwebi, T., Bourn, W., Hanke, T., Williamson, A.L. and Williamson, C. (2006) Design and preclinical evaluation of a multigene human immunodeficiency virus type 1 subtype C DNA vaccine for clinical trial. *J.Gen.Virol.* **87**, 399-410.

Campo, M.S., O'Neil, B.W., Grindlay, G.J., Curtis, F., Knowles, G. and Chandrachud, L. (1997) A peptide encoding a B-cell epitope from the N-terminus of the capsid protein L2 of bovine papillomavirus-4 prevents disease. *Virology* **234**, 261-266.

Campos, S.K. and Ozbun, M.A. (2009) Two highly conserved cysteine residues in HPV16 L2 form an intramolecular disulfide bond and are critical for infectivity in human keratinocytes. *PloS one* **4**, e4463.

Casini, G.L., Graham, D., Heine, D., Garcea, R.L. and Wu, D.T. (2004) In vitro papillomavirus capsid assembly analyzed by light scattering. *Virology* **325**, 320-327.

Chandrachud, L.M., Grindlay, G.J., McGarvie, G.M., O'Neil, B.W., Wagner, E.R., Jarrett, W.F. and Campo, M.S. (1995) Vaccination of cattle with the N-terminus of L2 is necessary and sufficient for preventing infection by bovine papillomavirus-4. *Virology* **211**, 204-208.

Christensen, N.D., Dillner, J., Eklund, C., Carter, J.J., Wipf, G.C., Reed, C.A., Cladel, N.M. and Galloway, D.A. (1996a) Surface conformational and linear epitopes on HPV-16 and HPV-18 L1 virus-like particles as defined by monoclonal antibodies. *Virology* **223**, 174-184.

Christensen, N.D., Höpfl, R., DiAngelo, S.L., Cladel, N.M., Patrick, S.D., Welsh, P.A., Budgeon, L.R., Reed, C.A. and Kreider, J.W. (1994) Assembled baculovirus-expressed human papillomavirus type 11 L1 capsid protein virus-like particles are recognized by neutralizing monoclonal antibodies and induce high titres of neutralizing antibodies. *Journal of General Virology* **75**, 2271-2276.

Christensen, N.D., Kreider, J.W., Cladel, N.M., Patrick, S.D. and Welsh, P.A. (1990) Monoclonal antibody-mediated neutralization of infectious human papillomavirus type 11. *Journal of virology* **64**, 5678-5681.

Christensen, N.D., Kreider, J.W., Kan, N.C. and DiAngelo, S.L. (1991) The open reading frame L2 of cottontail rabbit papillomavirus contains antibody-inducing neutralizing epitopes. *Virology* **181**, 572-579.

Christensen, N.D., Kreider, J.W., Shah, K.V. and Rando, R.F. (1992) Detection of human serum antibodies that neutralize infectious human papillomavirus type 11 virions. *The Journal of general virology* **73 (Pt 5)**, 1261-1267.

Christensen, N.D., Reed, C.A., Cladel, N.M., Han, R. and Kreider, J.W. (1996b) Immunization with viruslike particles induces long-term protection of rabbits against challenge with cottontail rabbit papillomavirus. *Journal of virology* **70**, 960-965.

Chromy, L.R., Oltman, A., Estes, P.A. and Garcea, R.L. (2006) Chaperone-mediated in vitro disassembly of polyoma- and papillomaviruses. *Journal of virology* **80**, 5086-5091.

Chromy, L.R., Pipas, J.M. and Garcea, R.L. (2003) Chaperone-mediated in vitro assembly of Polyomavirus capsids. *Proc Natl Acad Sci U S A* **100**, 10477-10482.

Combelas, N., Saussereau, E., Fleury, M.J., Ribeiro, T., Gaitan, J., Duarte-Forero, D.F., Coursaget, P. and Touze, A. (2010) Papillomavirus pseudovirions packaged with the L2 gene induce cross-neutralizing antibodies. *Journal of translational medicine* **8**, 28.

Conger, K.L., Liu, J.S., Kuo, S.R., Chow, L.T. and Wang, T.S. (1999) Human papillomavirus DNA replication. Interactions between the viral E1 protein and two subunits of human dna polymerase alpha/primase. *The Journal of biological chemistry* **274**, 2696-2705.

Contag, C.H. and Bachmann, M.H. (2002) Advances in in vivo bioluminescence imaging of gene expression. *Annual review of biomedical engineering* **4**, 235-260.

Conway, M.J. and Meyers, C. (2009) Replication and Assembly of Human Papillomaviruses. *Journal of Dental Research* **88**, 307-317.

Cook, J.C., Joyce, J.G., George, H.A., Schultz, L.D., Hurni, W.M., Jansen, K.U., Hepler, R.W., Ip, C., Lowe, R.S., Keller, P.M. and Lehman, E.D. (1999) Purification of virus-like particles of recombinant human papillomavirus type 11 major capsid protein L1 from Saccharomyces cerevisiae. *Protein expression and purification* **17**, 477-484.

Crow, J.M. (2012) HPV: The global burden. *Nature* **488**, S2-3.

D'Aoust, M.A., Lavoie, P.O., Couture, M.M., Trepanier, S., Guay, J.M., Dargis, M., Mongrand, S., Landry, N., Ward, B.J. and Vezina, L.P. (2008) Influenza virus-like particles produced by transient expression in Nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice. *Plant biotechnology journal* **6**, 930-940.

Daniell, H., Singh, N.D., Mason, H. and Streatfield, S.J. (2009) Plant-made vaccine antigens and biopharmaceuticals. *Trends in plant science* **14**, 669-679.

Day, P.M., Roden, R.B., Lowy, D.R. and Schiller, J.T. (1998) The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. *Journal of virology* **72**, 142-150.

de Villiers, E.M., Fauquet, C., Broker, T.R., Bernard, H.U. and zur Hausen, H. (2004) Classification of papillomaviruses. *Virology* **324**, 17-27.

Deschuyteneer, M., Elouahabi, A., Plainchamp, D., Plisnier, M., Soete, D., Corazza, Y., Lockman, L., Giannini, S. and Deschamps, M. (2010) Molecular and structural characterization of the L1 virus-like particles that are used as vaccine antigens in Cervarix, the AS04-adjuvanted HPV-16 and -18 cervical cancer vaccine. *Human vaccines* **6**, 407-419.

Dessy, F.J., Giannini, S.L., Bougelet, C.A., Kemp, T.J., David, M.P., Poncelet, S.M., Pinto, L.A. and Wettendorff, M.A. (2008) Correlation between direct ELISA, single epitope-based inhibition ELISA and pseudovirion-based neutralization assay for measuring anti-HPV-16 and anti-HPV-18 antibody response after vaccination with the AS04-adjuvanted HPV-16/18 cervical cancer vaccine. *Human vaccines* **4**, 425-434.

Doorbar, J. (2005) The papillomavirus life cycle. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* **32 Suppl 1**, S7-15.

Durrani, Z., McInerney, T.L., McLain, L., Jones, T., Bellaby, T., Brennan, F.R. and Dimmock, N.J. (1998) Intranasal immunization with a plant virus expressing a peptide from HIV-1 gp41 stimulates better mucosal and systemic HIV-1-specific IgA and IgG than oral immunization. *Journal of immunological methods* **220**, 93-103.

Dvoretzky, I., Shober, R., Chattopadhyay, S.K. and Lowy, D.R. (1980) A quantitative in vitro focus assay for bovine papilloma virus. *Virology* **103**, 369-375.

El Mehdaoui, S., Touze, A., Laurent, S., Sizaret, P.Y., Rasschaert, D. and Coursaget, P. (2000) Gene transfer using recombinant rabbit hemorrhagic disease virus capsids with genetically modified DNA encapsidation capacity by addition of packaging sequences from the L1 or L2 protein of human papillomavirus type 16. *Journal of virology* **74**, 10332-10340.

Embers, M.E., Budgeon, L.R., Pickel, M. and Christensen, N.D. (2002) Protective immunity to rabbit oral and cutaneous papillomaviruses by immunization with short peptides of L2, the minor capsid protein. *Journal of virology* **76**, 9798-9805.

Faurez, F., Dory, D., Henry, A., Bougeard, S. and Jestin, A. (2010) Replication efficiency of rolling-circle replicon-based plasmids derived from porcine circovirus 2 in eukaryotic cells. *Journal of virological methods* **165**, 27-35.

Fay, A., Yutzy, W.H.t., Roden, R.B. and Moroianu, J. (2004) The positively charged termini of L2 minor capsid protein required for bovine papillomavirus infection function separately in nuclear import and DNA binding. *Journal of virology* **78**, 13447-13454.

Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C. and Parkin, D.M. (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International journal of cancer. Journal international du cancer* **127**, 2893-2917.

Fernandez-San Millan, A., Ortigosa, S.M., Hervas-Stubbs, S., Corral-Martinez, P., Segui-Simarro, J.M., Gaetan, J., Coursaget, P. and Veramendi, J. (2008) Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like particles that are highly immunogenic. *Plant biotechnology journal* **6**, 427-441.

Finnen, R.L., Erickson, K.D., Chen, X.S. and Garcea, R.L. (2003) Interactions between papillomavirus L1 and L2 capsid proteins. *Journal of virology* **77**, 4818-4826.

Finsterbusch, T. and Mankertz, A. (2009) Porcine circoviruses--small but powerful. *Virus Res.* **143**, 177-183.

Fischer, R., Stoger, E., Schillberg, S., Christou, P. and Twyman, R.M. (2004) Plant-based production of biopharmaceuticals. *Current opinion in plant biology* **7**, 152-158.

Fleury, M.J., Touze, A., de Sanjose, S., Bosch, F.X., Klaustermeiyer, J. and Coursaget, P. (2008a) Detection of human papillomavirus type 31-neutralizing antibodies from naturally infected patients by an assay based on intracellular assembly of luciferase-expressing pseudovirions. *Clinical and vaccine immunology : CVI* **15**, 172-175.

Fleury, M.J.J., Touzé, A., de Sanjosé, S., Bosch, F.X., Klaustermeiyer, J. and Coursaget, P. (2008b) Detection of Human Papillomavirus Type 31-Neutralizing Antibodies from Naturally Infected Patients by an Assay Based on Intracellular Assembly of Luciferase-Expressing Pseudovirions. *Clinical and Vaccine Immunology* **15**, 172-175.

Fligge, C., Schafer, F., Selinka, H.C., Sapp, C. and Sapp, M. (2001) DNA-induced structural changes in the papillomavirus capsid. *Journal of virology* **75**, 7727-7731.

Florin, L., Sapp, C., Streeck, R.E. and Sapp, M. (2002) Assembly and translocation of papillomavirus capsid proteins. *Journal of virology* **76**, 10009-10014.

Franconi, R., Di Bonito, P., Dibello, F., Accardi, L., Muller, A., Cirilli, A., Simeone, P., Dona, M.G., Venuti, A. and Giorgi, C. (2002) Plant-derived human papillomavirus 16 E7 oncoprotein induces immune response and specific tumor protection. *Cancer research* **62**, 3654-3658.

Gambhira, R., Jagu, S., Karanam, B., Day, P.M. and Roden, R. (2009) Role of L2 cysteines in papillomavirus infection and neutralization. *Virology journal* **6**, 176.

Garcea, R.L. and Gissmann, L. (2004) Virus-like particles as vaccines and vessels for the delivery of small molecules. *Current opinion in biotechnology* **15**, 513-517.

Gaukroger, J.M., Chandrachud, L.M., O'Neil, B.W., Grindlay, G.J., Knowles, G. and Campo, M.S. (1996) Vaccination of cattle with bovine papillomavirus type 4 L2 elicits the production of virus-neutralizing antibodies. *The Journal of general virology* **77 (Pt 7)**, 1577-1583.

Gérard, O. (2006) Genetics of epidermodysplasia verruciformis: Insights into host defense against papillomaviruses. *Seminars in Immunology* **18**, 362-374.

Giroglou, T., Florin, L., Schafer, F., Streeck, R.E. and Sapp, M. (2001) Human papillomavirus infection requires cell surface heparan sulfate. *Journal of virology* **75**, 1565-1570.

Gleba, Y., Klimyuk, V. and Marillonnet, S. (2007) Viral vectors for the expression of proteins in plants. *Current opinion in biotechnology* **18**, 134-141.

Gordon, S.N., Kines, R.C., Kutsyna, G., Ma, Z.M., Hryniewicz, A., Roberts, J.N., Fenizia, C., Hidajat, R., Brocca-Cofano, E., Cuburu, N., Buck, C.B., Bernardo, M.L., Robert-Guroff, M., Miller, C.J., Graham, B.S., Lowy, D.R., Schiller, J.T. and Franchini, G. (2012) Targeting the vaginal mucosa with human papillomavirus pseudovirion vaccines delivering simian immunodeficiency virus DNA. *J Immunol* **188**, 714-723.

Graham, B.S., Kines, R.C., Corbett, K.S., Nicewonger, J., Johnson, T.R., Chen, M., LaVigne, D., Roberts, J.N., Cuburu, N., Schiller, J.T. and Buck, C.B. (2010) Mucosal delivery of human papillomavirus pseudovirus-encapsidated plasmids improves the potency of DNA vaccination. *Mucosal immunology* **3**, 475-486.

Gurunathan, S., Wu, C.Y., Freidag, B.L. and Seder, R.A. (2000) DNA vaccines: a key for inducing long-term cellular immunity. *Current opinion in immunology* **12**, 442-447.

Hagensee, M.E., Yaegashi, N. and Galloway, D.A. (1993) Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. *Journal of virology* **67**, 315-322.

Hall, M.P., Unch, J., Binkowski, B.F., Valley, M.P., Butler, B.L., Wood, M.G., Otto, P., Zimmerman, K., Vidugiris, G., Machleidt, T., Robers, M.B., Benink, H.A., Eggers, C.T., Slater, M.R., Meisenheimer, P.L., Klaubert, D.H., Fan, F., Encell, L.P. and Wood, K.V. (2012) Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS chemical biology* **7**, 1848-1857.

Halley-Stott, R.P., Tanzer, F., Martin, D.P. and Rybicki, E.P. (2007) The complete nucleotide sequence of a mild strain of <i>Bean yellow dwarf virus</i&gt. *Archives of Virology* **152**, 1237-1240.

Hanke, T., Schneider, J., Gilbert, S.C., Hill, A.V. and McMichael, A. (1998) DNA multi-CTL epitope vaccines for HIV and Plasmodium falciparum: immunogenicity in mice. *Vaccine* **16**, 426-435.

Haq, T.A., Mason, H.S., Clements, J.D. and Arntzen, C.J. (1995) Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* **268**, 714-716.

Harper, D.M., Franco, E.L., Wheeler, C.M., Moscicki, A.B., Romanowski, B., Roteli-Martins, C.M., Jenkins, D., Schuind, A., Costa Clemens, S.A. and Dubin, G. (2006) Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. *Lancet* **367**, 1247-1255.

Hefferon, K.L. (2012) Plant virus expression vectors set the stage as production platforms for biopharmaceutical proteins. *Virology* **433**, 1-6.

Hefferon, K.L. and Fan, Y. (2004) Expression of a vaccine protein in a plant cell line using a geminivirus-based replicon system. *Vaccine* **23**, 404-410.

Hiatt, A., Cafferkey, R. and Bowdish, K. (1989) Production of Antibodies in Transgenic Plants. *Nature* **342**, 76-78.

Higuchi, R., Krummel, B. and Saiki, R.K. (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic acids research* **16**, 7351-7367.

Hofmann, K.J., Cook, J.C., Joyce, J.G., Brown, D.R., Schultz, L.D., George, H.A., Rosolowsky, M., Fife, K.H. and Jansen, K.U. (1995) Sequence determination of human papillomavirus type 6a and assembly of virus-like particles in Saccharomyces cerevisiae. *Virology* **209**, 506-518.

Holmgren, S.C., Patterson, N.A., Ozbun, M.A. and Lambert, P.F. (2005) The Minor Capsid Protein L2 Contributes to Two Steps in the Human Papillomavirus Type 31 Life Cycle. *Journal of virology* **79**, 3938-3948.

Hopfl, R., Heim, K., Christensen, N., Zumbach, K., Wieland, U., Volgger, B., Widschwendter, A., Haimbuchner, S., Muller-Holzner, E., Pawlita, M., Pfister, H. and Fritsch, P. (2000) Spontaneous regression of CIN and delayed-type hypersensitivity to HPV-16 oncoprotein E7. *Lancet* **356**, 1985-1986.

Huang, Z., Chen, Q., Hjelm, B., Arntzen, C. and Mason, H. (2009) A DNA replicon system for rapid high-level production of virus-like particles in plants. *Biotechnology and bioengineering* **103**, 706-714.

Huang, Z., Phoolcharoen, W., Lai, H., Piensook, K., Cardineau, G., Zeitlin, L., Whaley, K.J., Arntzen, C.J., Mason, H.S. and Chen, Q. (2010) High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. *Biotechnology and bioengineering* **106**, 9-17.

Ishii, Y., Ozaki, S., Tanaka, K. and Kanda, T. (2005) Human papillomavirus 16 minor capsid protein L2 helps capsomeres assemble independently of intercapsomeric disulfide bonding. *Virus Genes* **31**, 321-328.

Jenson, A.B., Kurman, R.J. and Lancaster, W.D. (1991) Tissue effects of and host response to human papillomavirus infection. *Dermatologic clinics* **9**, 203-209.

Jiang, T., Xing, B. and Rao, J. (2008) Recent developments of biological reporter technology for detecting gene expression. *Biotechnology & genetic engineering reviews* **25**, 41-75.

Joyce, J.G., Tung, J.S., Przysiecki, C.T., Cook, J.C., Lehman, E.D., Sands, J.A., Jansen, K.U. and Keller, P.M. (1999) The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. *The Journal of biological chemistry* **274**, 5810-5822.

Kammann, M., Matzeit, V., Schmidt, B., Schell, J., Walden, R. and Gronenborn, B. (1991) Geminivirus-based shuttle vectors capable of replication in Escherichia coli and monocotyledonous plant cells. *Gene* **104**, 247-252.

Kapila, J., De Rycke, R., Van Montagu, M. and Angenon, G. (1997) An Agrobacterium-mediated transient gene expression system for intact leaves. *Plant Science* **122**, 101-108.

Kawana, K., Yasugi, T. and Taketani, Y. (2009) Human papillomavirus vaccines: current issues & future. *The Indian journal of medical research* **130**, 341-347.

Kawana, K., Yoshikawa, H., Taketani, Y., Yoshiike, K. and Kanda, T. (1998) In vitro construction of pseudovirions of human papillomavirus type 16: incorporation of plasmid DNA into reassembled L1/L2 capsids. *Journal of virology* **72**, 10298-10300.

Kellogg, V. (1927) International Critical Tables. *Science* **65**, 273.

Khan, S.A. (2005) Plasmid rolling-circle replication: highlights of two decades of research. *Plasmid* **53**, 126-136.

Kim, Y.D., Park, K.G., Morishita, R., Kaneda, Y., Kim, S.Y., Song, D.K., Kim, H.S., Nam, C.W., Lee, H.C., Lee, K.U., Park, J.Y., Kim, B.W., Kim, J.G. and Lee, I.K. (2006) Liver-directed gene therapy of diabetic rats using an HVJ-E vector containing EBV plasmids expressing insulin and GLUT 2 transporter. *Gene therapy* **13**, 216-224.

Kirnbauer, R., Booy, F., Cheng, N., Lowy, D.R. and Schiller, J.T. (1992) Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc Natl Acad Sci U S A* **89**, 12180-12184.

Kirnbauer, R., Chandrachud, L.M., O'Neil, B.W., Wagner, E.R., Grindlay, G.J., Armstrong, A., McGarvie, G.M., Schiller, J.T., Lowy, D.R. and Campo, M.S. (1996) Virus-like particles of bovine papillomavirus type 4 in prophylactic and therapeutic immunization. *Virology* **219**, 37-44.

Kohl, T., Hitzeroth, II, Stewart, D., Varsani, A., Govan, V.A., Christensen, N.D., Williamson, A.L. and Rybicki, E.P. (2006) Plant-produced cottontail rabbit papillomavirus L1 protein protects against tumor challenge: a proof-of-concept study. *Clinical and vaccine immunology : CVI* **13**, 845-853.

Kreider, J.W., Howett, M.K., Leure-Dupree, A.E., Zaino, R.J. and Weber, J.A. (1987) Laboratory production in vivo of infectious human papillomavirus type 11. *Journal of virology* **61**, 590-593.

Kunik, T., Mizrachy, L., Citovsky, V. and Gafni, Y. (1999) Characterization of a tomato karyopherin α that interacts with the Tomato Yellow Leaf Curl Virus (TYLCV) capsid protein. *Journal of Experimental Botany* **50**, 731-732.

Lenz, P., Day, P.M., Pang, Y.Y., Frye, S.A., Jensen, P.N., Lowy, D.R. and Schiller, J.T. (2001) Papillomavirus-like particles induce acute activation of dendritic cells. *J Immunol* **166**, 5346-5355.

Lenzi, P., Scotti, N., Alagna, F., Tornesello, M.L., Pompa, A., Vitale, A., De Stradis, A., Monti, L., Grillo, S., Buonaguro, F.M., Maliga, P. and Cardi, T. (2008) Translational fusion of chloroplast-expressed human papillomavirus type 16 L1 capsid protein enhances antigen accumulation in transplastomic tobacco. *Transgenic research* **17**, 1091-1102.

Li, M., Cripe, T.P., Estes, P.A., Lyon, M.K., Rose, R.C. and Garcea, R.L. (1997) Expression of the human papillomavirus type 11 L1 capsid protein in Escherichia coli: characterization of protein domains involved in DNA binding and capsid assembly. *Journal of virology* **71**, 2988-2995.

Lin, K., Roosinovich, E., Ma, B., Hung, C.F. and Wu, T.C. (2010) Therapeutic HPV DNA vaccines. *Immunologic research* **47**, 86-112.

Liu, H.L., Li, W.S., Lei, T., Zheng, J., Zhang, Z., Yan, X.F., Wang, Z.Z., Wang, Y.L. and Si, L.S. (2005) Expression of human papillomavirus type 16 L1 protein in transgenic tobacco plants. *Acta biochimica et biophysica Sinica* **37**, 153-158.

Liu, L., Davies, J.W. and Stanley, J. (1998) Mutational analysis of bean yellow dwarf virus, a geminivirus of the genus Mastrevirus that is adapted to dicotyledonous plants. *The Journal of general virology* **79 (Pt 9)**, 2265-2274.

Liu, L., van Tonder, T., Pietersen, G., Davies, J.W. and Stanley, J. (1997) Molecular characterization of a subgroup I geminivirus from a legume in South Africa. *The Journal of general virology* **78 (Pt 8)**, 2113-2117.

Liu, Y., Frazer, I.H., Liu, W.J., Liu, X.S., McMillan, N. and Zhao, K.N. (2001) Efficiency of delivery of DNA to cells by bovine papillomavirus type-1 L1/L2 pseudovirions. *Applied microbiology and biotechnology* **56**, 150-156.

Longworth, M.S. and Laimins, L.A. (2004) Pathogenesis of Human Papillomaviruses in Differentiating Epithelia. *Microbiology and Molecular Biology Reviews* **68**, 362-372.

Lu, X., Thompson, J.R. and Perry, K.L. (2012) Encapsidation of DNA, a protein and a fluorophore into virus-like particles by the capsid protein of cucumber mosaic virus. *The Journal of general virology*.

Lund, P.E., Hunt, R.C., Gottesman, M.M. and Kimchi-Sarfaty, C. (2010) Pseudovirions as vehicles for the delivery of siRNA. *Pharmaceutical research* **27**, 400-420.

Ma, B., Roden, R.B., Hung, C.F. and Wu, T.C. (2011) HPV pseudovirions as DNA delivery vehicles. *Therapeutic delivery* **2**, 427-430.

Ma, J.K., Chikwamba, R., Sparrow, P., Fischer, R., Mahoney, R. and Twyman, R.M. (2005) Plant-derived pharmaceuticals--the road forward. *Trends in plant science* **10**, 580-585.

Mach, H., Volkin, D.B., Troutman, R.D., Wang, B., Luo, Z., Jansen, K.U. and Shi, L. (2006) Disassembly and reassembly of yeast-derived recombinant human papillomavirus virus-like particles (HPV VLPs). *Journal of pharmaceutical sciences* **95**, 2195-2206.

Maclean, J., Koekemoer, M., Olivier, A.J., Stewart, D., Hitzeroth, II, Rademacher, T., Fischer, R., Williamson, A.L. and Rybicki, E.P. (2007) Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. *The Journal of general virology* **88**, 1460-1469.

Mandic, A. (2012) Primary prevention of cervical cancer: prophylactic human papillomavirus vaccines. *Journal of B.U.ON. : official journal of the Balkan Union of Oncology* **17**, 422-427.

Mankertz, A., Caliskan, R., Hattermann, K., Hillenbrand, B., Kurzendoerfer, P., Mueller, B., Schmitt, C., Steinfeldt, T. and Finsterbusch, T. (2004) Molecular biology of Porcine circovirus: analyses of gene expression and viral replication. *Vet.Microbiol.* **98**, 81-88.

Mankertz, A. and Hillenbrand, B. (2002) Analysis of transcription of Porcine circovirus type 1. *J.Gen.Virol.* **83**, 2743-2751.

Mankertz, A., Persson, F., Mankertz, J., Blaess, G. and Buhk, H.J. (1997) Mapping and characterization of the origin of DNA replication of porcine circovirus. *J.Virol.* **71**, 2562-2566.

Mankertz, J., Buhk, H.J., Blaess, G. and Mankertz, A. (1998) Transcription analysis of porcine circovirus (PCV). *Virus Genes* **16**, 267-276.

Mason, H.S., Lam, D.M. and Arntzen, C.J. (1992) Expression of hepatitis B surface antigen in transgenic plants. *Proc Natl Acad Sci U S A* **89**, 11745-11749.

Matic, S., Masenga, V., Poli, A., Rinaldi, R., Milne, R.G., Vecchiati, M. and Noris, E. (2012) Comparative analysis of recombinant Human Papillomavirus 8 L1 production in plants by a variety of expression systems and purification methods. *Plant biotechnology journal*.

McCarthy, M.P., White, W.I., Palmer-Hill, F., Koenig, S. and Suzich, J.A. (1998) Quantitative disassembly and reassembly of human papillomavirus type 11 viruslike particles in vitro. *Journal of virology* **72**, 32-41.

Meyers, C., Frattini, M.G., Hudson, J.B. and Laimins, L.A. (1992) Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. *Science* **257**, 971-973.

Meyers, C., Mayer, T.J. and Ozbun, M.A. (1997) Synthesis of infectious human papillomavirus type 18 in differentiating epithelium transfected with viral DNA. *Journal of virology* **71**, 7381-7386.

Modis, Y., Trus, B.L. and Harrison, S.C. (2002) Atomic model of the papillomavirus capsid. *The EMBO journal* **21**, 4754-4762.

Mohr, I.J., Clark, R., Sun, S., Androphy, E.J., MacPherson, P. and Botchan, M.R. (1990) Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. *Science* **250**, 1694-1699.

Moody, C.A. and Laimins, L.A. (2010) Human papillomavirus oncoproteins: pathways to transformation. *Nature reviews. Cancer* **10**, 550-560.

Mor, T.S., Moon, Y.S., Palmer, K.E. and Mason, H.S. (2003) Geminivirus vectors for high-level expression of foreign proteins in plant cells. *Biotechnol.Bioeng.* **81**, 430-437.

Morris, J.D., Crook, T., Bandara, L.R., Davies, R., LaThangue, N.B. and Vousden, K.H. (1993) Human papillomavirus type 16 E7 regulates E2F and contributes to mitogenic signalling. *Oncogene* **8**, 893-898.

Muller, M., Gissmann, L., Cristiano, R.J., Sun, X.Y., Frazer, I.H., Jenson, A.B., Alonso, A., Zentgraf, H. and Zhou, J. (1995) Papillomavirus capsid binding and uptake by cells from different tissues and species. *Journal of virology* **69**, 948-954.

Nardelli-Haefliger, D., Roden, R.B., Benyacoub, J., Sahli, R., Kraehenbuhl, J.P., Schiller, J.T., Lachat, P., Potts, A. and De Grandi, P. (1997) Human papillomavirus type 16 virus-like particles expressed in attenuated Salmonella typhimurium elicit mucosal and systemic neutralizing antibodies in mice. *Infection and Immunity* **65**, 3328-3336.

Ngoma, T. (2006) World Health Organization cancer priorities in developing countries. *Annals of Oncology* **17**, viii9-viii14.

Nuttall, J., Vine, N., Hadlington, J.L., Drake, P., Frigerio, L. and Ma, J.K. (2002) ER-resident chaperone interactions with recombinant antibodies in transgenic plants. *European journal of biochemistry / FEBS* **269**, 6042-6051.

Ochsenbauer, C. and Kappes, J.C. (2009) New virologic reagents for neutralizing antibody assays. *Current opinion in HIV and AIDS* **4**, 418-425.

Ogle, R. (2008) Expression of HPV16 L1 and L1/L2 chimeras using a novel replicating Bean Yellow Dwarf Virus (BeYDV) vector. In: *Molecular Cell Biology*. CapeTown: University of Cape Town.

Oh, Y.K., Sohn, T., Park, J.S., Kang, M.J., Choi, H.G., Kim, J.A., Kim, W.K., Ko, J.J. and Kim, C.K. (2004) Enhanced mucosal and systemic immunogenicity of human papillomavirus-like particles encapsidating interleukin-2 gene adjuvant. *Virology* **328**, 266-273.

Okun, M.M., Day, P.M., Greenstone, H.L., Booy, F.P., Lowy, D.R., Schiller, J.T. and Roden, R.B. (2001) L1 interaction domains of papillomavirus l2 necessary for viral genome encapsidation. *Journal of virology* **75**, 4332-4342.

Palmer, K.E., Thomson, J.A. and Rybicki, E.P. (1999) Generation of maize cell lines containing autonomously replicating maize streak virus-based gene vectors. *Arch.Virol.* **144**, 1345-1360.

Pang, Y.Y., Schermer, A., Yu, J. and Sun, T.T. (1993) Suprabasal change and subsequent formation of disulfide-stabilized homo- and hetero-dimers of keratins during esophageal epithelial differentiation. *Journal of cell science* **104 (Pt 3)**, 727-740.

Partridge, J.M. and Koutsky, L.A. (2006) Genital human papillomavirus infection in men. *The Lancet infectious diseases* **6**, 21-31.

Pastrana, D.V., Buck, C.B., Pang, Y.Y., Thompson, C.D., Castle, P.E., FitzGerald, P.C., Kruger Kjaer, S., Lowy, D.R. and Schiller, J.T. (2004) Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. *Virology* **321**, 205-216.

Patel, M.C., Patkar, K.K., Basu, A., Mohandas, K.M. and Mukhopadhyaya, R. (2009) Production of immunogenic human papillomavirus-16 major capsid protein derived virus like particles. *Indian Journal of Medical Research* **130**, 213-218.

Peng, S., Ma, B., Chen, S.H., Hung, C.F. and Wu, T. (2011) DNA vaccines delivered by human papillomavirus pseudovirions as a promising approach for generating antigen-specific CD8+ T cell immunity. *Cell & bioscience* **1**, 26.

Peng, S., Monie, A., Kang, T.H., Hung, C.F., Roden, R. and Wu, T.C. (2010) Efficient delivery of DNA vaccines using human papillomavirus pseudovirions. *Gene therapy* **17**, 1453-1464.

Pereira, R. (2008) Expression of HPV-16 L2 in Plants. In: *Molecular Cell Biology*. Cape Town: University of Cape Town.

Pereira, R., Hitzeroth, II and Rybicki, E.P. (2009) Insights into the role and function of L2, the minor capsid protein of papillomaviruses. *Arch Virol* **154**, 187-197.

Pujol, M., Ramirez, N.I., Ayala, M., Gavilondo, J.V., Valdes, R., Rodriguez, M., Brito, J., Padilla, S., Gomez, L., Reyes, B., Peral, R., Perez, M., Marcelo, J.L., Mila, L., Sanchez, R.F., Paez, R., Cremata, J.A., Enriquez, G., Mendoza, O., Ortega, M. and Borroto, C. (2005) An integral approach towards a practical application for a plant-made monoclonal antibody in vaccine purification. *Vaccine* **23**, 1833-1837.

Pyeon, D., Lambert, P.F. and Ahlquist, P. (2005) Production of infectious human papillomavirus independently of viral replication and epithelial cell differentiation. *Proc Natl Acad Sci U S A* **102**, 9311-9316.

Regnard, G.L., Halley-Stott, R.P., Tanzer, F.L., Hitzeroth, II and Rybicki, E.P. (2010) High level protein expression in plants through the use of a novel autonomously replicating geminivirus shuttle vector. *Plant biotechnology journal* **8**, 38-46.

Renoux, V.M., Fleury, M.J., Bousarghin, L., Gaitan, J., Sizaret, P.Y., Touze, A. and Coursaget, P. (2008) Induction of antibody response against hepatitis E virus (HEV) with recombinant human papillomavirus pseudoviruses expressing truncated HEV capsid proteins in mice. *Vaccine* **26**, 6602-6607.

Rheinwald, J.G. and Green, H. (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* **6**, 331-343.

Robbins, J.B., Schneerson, R. and Szu, S.C. (1995) Perspective: hypothesis: serum IgG antibody is sufficient to confer protection against infectious diseases by inactivating the inoculum. *The Journal of infectious diseases* **171**, 1387-1398.

Roberts, J.N., Buck, C.B., Thompson, C.D., Kines, R., Bernardo, M., Choyke, P.L., Lowy, D.R. and Schiller, J.T. (2007) Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. *Nature medicine* **13**, 857-861.

Roden, R.B., Greenstone, H.L., Kirnbauer, R., Booy, F.P., Jessie, J., Lowy, D.R. and Schiller, J.T. (1996) In vitro generation and type-specific neutralization of a human papillomavirus type 16 virion pseudotype. *Journal of virology* **70**, 5875-5883.

Roden, R.B., Yutzy, W.H.t., Fallon, R., Inglis, S., Lowy, D.R. and Schiller, J.T. (2000) Minor capsid protein of human genital papillomaviruses contains subdominant, cross-neutralizing epitopes. *Virology* **270**, 254-257.

Rose, R.C., Bonnez, W., Reichman, R.C. and Garcea, R.L. (1993) Expression of human papillomavirus type 11 L1 protein in insect cells: in vivo and in vitro assembly of viruslike particles. *Journal of virology* **67**, 1936-1944.

Rossi, J.L., Gissmann, L., Jansen, K. and Muller, M. (2000) Assembly of human papillomavirus type 16 pseudovirions in Saccharomyces cerevisiae. *Human gene therapy* **11**, 1165-1176.

Rudolf, M.P., Fausch, S.C., Da Silva, D.M. and Kast, W.M. (2001) Human dendritic cells are activated by chimeric human papillomavirus type-16 virus-like particles and induce epitope-specific human T cell responses in vitro. *J Immunol* **166**, 5917-5924.

Rybicki, E.P. (2009) Plant-produced vaccines: promise and reality. *Drug discovery today* **14**, 16-24.

Rybicki, E.P. (2010) Plant-made vaccines for humans and animals. *Plant biotechnology journal* **8**, 620-637.

Sainsbury, F., Thuenemann, E.C. and Lomonossoff, G.P. (2009) pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant biotechnology journal* **7**, 682-693.

Santi, L., Huang, Z. and Mason, H. (2006) Virus-like particles production in green plants. *Methods* **40**, 66-76.

Sapp, M., Fligge, C., Petzak, I., Harris, J.R. and Streeck, R.E. (1998) Papillomavirus assembly requires trimerization of the major capsid protein by disulfides between two highly conserved cysteines. *Journal of virology* **72**, 6186-6189.

Schillberg, S., Twyman, R.M. and Fischer, R. (2005) Opportunities for recombinant antigen and antibody expression in transgenic plants--technology assessment. *Vaccine* **23**, 1764-1769.

Schwarz, T.F. and Leo, O. (2008) Immune response to human papillomavirus after prophylactic vaccination with AS04-adjuvanted HPV-16/18 vaccine: Improving upon nature. *Gynecologic oncology* **110**, S1-S10.

Senger, T., Schädlich, L., Gissmann, L. and Müller, M. (2009) Enhanced papillomavirus-like particle production in insect cells. *Virology* **388**, 344-353.

Seow, Y. and Wood, M.J. (2009) Biological gene delivery vehicles: beyond viral vectors. *Molecular therapy : the journal of the American Society of Gene Therapy* **17**, 767-777.

Shao, N. and Bock, R. (2008) A codon-optimized luciferase from Gaussia princeps facilitates the in vivo monitoring of gene expression in the model alga Chlamydomonas reinhardtii. *Current genetics* **53**, 381-388.

Shen, W.J. and Forde, B.G. (1989) Efficient transformation of Agrobacterium spp. by high voltage electroporation. *Nucleic acids research* **17**, 8385.

Shi, W., Liu, J., Huang, Y. and Qiao, L. (2001) Papillomavirus pseudovirus: a novel vaccine to induce mucosal and systemic cytotoxic T-lymphocyte responses. *Journal of virology* **75**, 10139-10148.

Shope, R.E. (1937) Immunization of Rabbits to Infectious Papillomatosis. *The Journal of experimental medicine* **65**, 219-231.

Smith, J.L., Campos, S.K. and Ozbun, M.A. (2007) Human papillomavirus type 31 uses a caveolin 1- and dynamin 2-mediated entry pathway for infection of human keratinocytes. *Journal of virology* **81**, 9922-9931.

Smith, L.H., Foster, C., Hitchcock, M.E., Leiserowitz, G.S., Hall, K., Isseroff, R., Christensen, N.D. and Kreider, J.W. (1995) Titration of HPV-11 infectivity and antibody neutralization can be measured in vitro. *The Journal of investigative dermatology* **105**, 438-444.

Stanley, J. (1993) Geminiviruses: plant viral vectors. *Curr.Opin.Genet.Dev.* **3**, 91-96.

Stanley, M. (2008) Immunobiology of HPV and HPV vaccines. *Gynecologic oncology* **109**, S15-21.

Stanley, M., Gissmann, L. and Nardelli-Haefliger, D. (2008) Immunobiology of human papillomavirus infection and vaccination - implications for second generation vaccines. *Vaccine* **26 Suppl 10**, K62-67.

Stauffer, Y., Raj, K., Masternak, K. and Beard, P. (1998) Infectious human papillomavirus type 18 pseudovirions. *Journal of molecular biology* **283**, 529-536.

Steele, J., Collins, S., Wen, K., Ryan, G., Constandinou-Williams, C. and Woodman, C.B. (2008) Measurement of the humoral immune response following an incident human papillomavirus type 16 or 18 infection in young women by a pseudovirion-based neutralizing antibody assay. *Clinical and vaccine immunology : CVI* **15**, 1387-1390.

Stehle, T., Yan, Y., Benjamin, T.L. and Harrison, S.C. (1994) Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature* **369**, 160-163.

Streatfield, S.J. and Howard, J.A. (2003) Plant-based vaccines. *International journal for parasitology* **33**, 479-493.

Sullivan, C.S. and Pipas, J.M. (2001) The virus-chaperone connection. *Virology* **287**, 1-8.

Swindle, C.S., Zou, N., Van Tine, B.A., Shaw, G.M., Engler, J.A. and Chow, L.T. (1999) Human papillomavirus DNA replication compartments in a transient DNA replication system. *Journal of virology* **73**, 1001-1009.

Takeda, D.Y., Shibata, Y., Parvin, J.D. and Dutta, A. (2005) Recruitment of ORC or CDC6 to DNA is sufficient to create an artificial origin of replication in mammalian cells. *Genes & development* **19**, 2827-2836.

Tamilselvi, D., Anand, G. and Swarup, S. (2004) A geminivirus AYVV-derived shuttle vector for tobacco BY2 cells. *Plant cell reports* **23**, 81-90.

Tanzer, F.L., Shephard, E.G., Palmer, K.E., Burger, M., Williamson, A.L. and Rybicki, E.P. (2011) The porcine circovirus type 1 capsid gene promoter improves antigen expression and immunogenicity in a HIV-1 plasmid vaccine. *Virology journal* **8**, 51.

Thanavala, Y., Huang, Z. and Mason, H.S. (2006) Plant-derived vaccines: a look back at the highlights and a view to the challenges on the road ahead. *Expert review of vaccines* **5**, 249-260.

Tischer, I., Bode, L., Peters, D., Pociuli, S. and Germann, B. (1995) Distribution of antibodies to porcine circovirus in swine populations of different breeding farms. *Arch.Virol.* **140**, 737-743.

Tischer, I., Gelderblom, H., Vettermann, W. and Koch, M.A. (1982) A very small porcine virus with circular single-stranded DNA. *Nature* **295**, 64-66.

Tiwari, S., Verma, P.C., Singh, P.K. and Tuli, R. (2009) Plants as bioreactors for the production of vaccine antigens. *Biotechnology advances* **27**, 449-467.

Touze, A. and Coursaget, P. (1998) In vitro gene transfer using human papillomavirus-like particles. *Nucleic acids research* **26**, 1317-1323.

Trus, B.L., Roden, R.B., Greenstone, H.L., Vrhel, M., Schiller, J.T. and Booy, F.P. (1997) Novel structural features of bovine papillomavirus capsid revealed by a three-dimensional reconstruction to 9 A resolution. *Nature structural biology* **4**, 413-420.

Turpen, T.H., Reinl, S.J., Charoenvit, Y., Hoffman, S.L., Fallarme, V. and Grill, L.K. (1995) Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. *Biotechnology (N Y)* **13**, 53-57.

Unckell, F., Streeck, R.E. and Sapp, M. (1997) Generation and neutralization of pseudovirions of human papillomavirus type 33. *Journal of virology* **71**, 2934-2939.

Varsani, A., Williamson, A.L., de, V.D., Becker, I., Christensen, N.D. and Rybicki, E.P. (2003) Chimeric human papillomavirus type 16 (HPV-16) L1 particles presenting the common neutralizing epitope for the L2 minor capsid protein of HPV-6 and HPV-16. *J.Virol.* **77**, 8386-8393.

Varsani, A., Williamson, A.L., Stewart, D. and Rybicki, E.P. (2006) Transient expression of Human papillomavirus type 16 L1 protein in Nicotiana benthamiana using an infectious tobamovirus vector. *Virus research* **120**, 91-96.

Villa, L.L., Costa, R.L., Petta, C.A., Andrade, R.P., Paavonen, J., Iversen, O.E., Olsson, S.E., Hoye, J., Steinwall, M., Riis-Johannessen, G., Andersson-Ellstrom, A., Elfgren, K., Krogh, G., Lehtinen, M., Malm, C., Tamms, G.M., Giacoletti, K., Lupinacci, L., Railkar, R., Taddeo, F.J., Bryan, J., Esser, M.T., Sings, H.L., Saah, A.J. and Barr, E. (2006) High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up. *British journal of cancer* **95**, 1459-1466.

Volpers, C., Schirmacher, P., Streeck, R.E. and Sapp, M. (1994) Assembly of the major and the minor capsid protein of human papillomavirus type 33 into virus-like particles and tubular structures in insect cells. *Virology* **200**, 504-512.

Walboomers, J.M., Jacobs, M.V., Manos, M.M., Bosch, F.X., Kummer, J.A., Shah, K.V., Snijders, P.J., Peto, J., Meijer, C.J. and Munoz, N. (1999) Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *The Journal of pathology* **189**, 12-19.

Ward, A., Etessami, P. and Stanley, J. (1988) Expression of a bacterial gene in plants mediated by infectious geminivirus DNA. *The EMBO journal* **7**, 1583-1587.

Warzecha, H., Mason, H.S., Lane, C., Tryggvesson, A., Rybicki, E., Williamson, A.L., Clements, J.D. and Rose, R.C. (2003) Oral immunogenicity of human papillomavirus-like particles expressed in potato. *Journal of virology* **77**, 8702-8711.

Waymouth, C. (1970) Osmolality of mammalian blood and of media for culture of mammalian cells. *In vitro* **6**, 109-127.

Xu, Y.F., Zhang, Y.Q., Xu, X.M. and Song, G.X. (2006) Papillomavirus virus-like particles as vehicles for the delivery of epitopes or genes. *Archives of Virology* **151**, 2133-2148.

Yang, R., Murillo, F.M., Cui, H., Blosser, R., Uematsu, S., Takeda, K., Akira, S., Viscidi, R.P. and Roden, R.B. (2004) Papillomavirus-like particles stimulate murine bone marrow-derived dendritic cells to produce alpha interferon and Th1 immune responses via MyD88. *Journal of virology* **78**, 11152-11160.

Yeager, M.D., Aste-Amezaga, M., Brown, D.R., Martin, M.M., Shah, M.J., Cook, J.C., Christensen, N.D., Ackerson, C., Lowe, R.S., Smith, J.F., Keller, P. and Jansen, K.U. (2000) Neutralization of human papillomavirus (HPV) pseudovirions: a novel and efficient approach to detect and characterize HPV neutralizing antibodies. *Virology* **278**, 570-577.

Zandi, R., van der Schoot, P., Reguera, D., Kegel, W. and Reiss, H. (2006) Classical nucleation theory of virus capsids. *Biophysical journal* **90**, 1939-1948.

Zhang, H., Fayad, R., Wang, X., Quinn, D. and Qiao, L. (2004) Human immunodeficiency virus type 1 gag-specific mucosal immunity after oral immunization with papillomavirus pseudoviruses encoding gag. *Journal of virology* **78**, 10249-10257.

Zhang, W., Olson, N.H., Baker, T.S., Faulkner, L., Agbandje-McKenna, M., Boulton, M.I., Davies, J.W. and McKenna, R. (2001) Structure of the Maize streak virus geminate particle. *Virology* **279**, 471-477.

Zhang, X. and Mason, H. (2006) Bean Yellow Dwarf Virus replicons for high-level transgene expression in transgenic plants and cell cultures. *Biotechnol.Bioeng.* **93**, 271-279.

Zhao, K.N., Sun, X.Y., Frazer, I.H. and Zhou, J. (1998) DNA packaging by L1 and L2 capsid proteins of bovine papillomavirus type 1. *Virology* **243**, 482-491.

Zhou, J., Stenzel, D.J., Sun, X.Y. and Frazer, I.H. (1993a) Synthesis and assembly of infectious bovine papillomavirus particles in vitro. *The Journal of general virology* **74 (Pt 4)**, 763-768.

Zhou, J., Sun, X.Y. and Frazer, I.H. (1993b) Glycosylation of human papillomavirus type 16 L1 protein. *Virology* **194**, 210-218.

Zupan, J., Muth, T.R., Draper, O. and Zambryski, P. (2000) The transfer of DNA from agrobacterium tumefaciens into plants: a feast of fundamental insights. *The Plant journal : for cell and molecular biology* **23**, 11-28.

zur Hausen, H. (2000) Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *Journal of the National Cancer Institute* **92**, 690-698.