An investigation into the development of plant-derived vaccines against Human papillomavirus type 11 and Cottontail rabbit papillomavirus

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Certification by Supervisors

In terms of paragraph GP9 of the regulations for the degree of Doctor of Philosophy at the University of Cape Town, we certify that we approve of the inclusion in this thesis of material already published, or submitted for publication by candidate Thomas O. Kohl.

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Abstract

The principal object of this thesis was to investigate the feasibility of developing a novel plant-derived vaccine against Human papillomavirus type 11 (HPV-11), the causal agent of genital warts and laryngeal condylomas. A secondary objective was a proof-of-concept study using Cottontail rabbit papillomavirus (CRPV), as this is a viable animal model for human papillomavirus vaccines. Accordingly, I investigated and compared the potential of two different plant expression systems.

The HPV-11 major capsid protein gene (*L1*) was successfully transferred via *Agrobacterium*-mediated transformation into *Arabidopsis thaliana* and two *Nicotiana tabacum* cultivars (Soulouk and Xanthi). The complete *L1* gene appeared to be toxic; successful regeneration of putative transgenic callus was only possibly once the C-terminal *L1* nuclear localization signal (*L1 NLS*) had been removed. Thereafter consecutive generations of transgenic plant systems showed stable integration and constitutive expression of the *L1 NLS*⁻ transgene. The transgenic plant-derived HPV-11 L1 NLS⁻ protein formed virus-like particles (VLPs): these were characterized by ELISA using a panel of monoclonal antibodies (MAbs), and showed similar antigenic properties to baculovirus-produced L1 NLS⁻ VLPs. Transgenic plants were calculated to yield up to 11.5mg (*A. thaliana*) and 2.2mg (*N. tabacum*) of HPV-11 L1 NLS⁻ protein per kilogram of fresh leaf material. Injection of concentrated L1 protein extract into New Zealand white rabbits induced an antigen-specific antibody response; however, evaluation of antisera showed that these sera predominantly recognized HPV-11 L1 NLS⁻ and not NLS⁺ insect cell-derived VLPs. This suggests the possible modification of the plant-derived protein, resulting in antigenic differences between plant-derived and wild-type VLPs, including the possible exposure of a novel immunodominant epitope on the surface of the plant-produced NLS⁻ VLPs.

An alternative plant production system was explored to maximize the expression of the HPV-11 *L1 NLS*⁻ gene. The transient gene delivery system used was a recombinant Tobacco mosaic virus (TMV) vector. Production of antigenically similar HPV-11 L1

NLS$^-$ VLPs in *N. benthamiana* increased yields by a factor of 5 for TMV-mediated expression in a *Nicotiana* species. Evaluation of the TMV-produced HPV-11 L1 NLS$^-$ protein by inoculation of guinea pigs again resulted in a predominantly HPV-11 L1 NLS$^-$ targeted immune response as described above. To validate this observation, guinea pigs were inoculated with HPV-11 L1 NLS$^-$ or NLS$^+$ VLPs made by recombinant baculovirus: results indicated no apparent differences in antiserum reactivity, suggesting possible post-transcriptional modifications of the L1 NLS$^-$ protein in plants.

A study exploring the transgenic and TMV-mediated plant expression of the CRPV *L1* gene was undertaken with the aim of validating the efficacy of a plant-derived PV vaccine in an animal model. Stable gene expression was confirmed in the transgenic *N. tabacum* system; however, gene expression appeared to be short-term in the TMV expression system due to transgene deletion. Expression levels of the CRPV L1 protein were calculated as 1mg and 600µg per kilogram of fresh leaf material harvested from transgenic and recombinant TMV-infected plants respectively. The protein derived from both expression systems was found not to assemble into VLPs, and instead formed only pentameric aggregates that were detected using MAbs. Animal data suggests that inoculation of New Zealand white rabbits with concentrated CRPV L1 protein extract, derived from both expression systems, is capable of inducing an antibody response.

All CRPV L1 vaccinated rabbits were challenged with infectious virus and were protected against papilloma development. However, serum collected before the challenge experiment was not capable of neutralising the virus *in vitro*. Thus the protective effect is believed to originate from a cellular immune response. In addition, serum collected from all HPV-11 L1 NLS$^-$ immunized animals also did not neutralize the HPV-11 L1 pseudovirus *in vitro*, thus calling into question the use of this plant-derived HPV-11 L1 protein as a suitable vaccine candidate for the prevention of HPV-11 infection.

Abbreviations

BCIP	5-bromo-4-chloro-3-indoyl-phosphate
bp	base pair
BPV	Bovine papillomavirus
CaMV35S	Cauliflower mosaic virus 35S promoter
CCE	cornified cell envelopes
cDNA	complementary deoxyribonucleic acid
CIN	cervical intraepithelial neoplasia
cm^2	centimetres squared
CRPV	*Cottontail rabbit papillomavirus*
ColE1	origin of replication for high copy maintenance in *E. coli*
COPV	*Canine oral papillomavirus*
CsCl	caesium chloride
DAS	double-antibody sandwich
DIG	digoxigenin
DNA	deoxyribonucleic acid
dpi	days post inoculation
E. coli	*Escherichia coli*
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FBS	foetal bovine serum
g	gravity
GST	glutathione S-transferase
H_2O	water
HBV	*Hepatitis B virus*
HPV	*Human papillomavirus*
hr(s)	hour(s)
HSIL	high-grade squamous intraepithelial lesion
IgG	immunoglobulin G
kg	kilogram
LA	Luria agar
M	molar
MAb(s)	monoclonal antibody(ies)
mg	milligram
mg/kg	milligram per kilogram
min	minutes
mm	millimeter
mM	milli molar
MS	Murashige and Skoog
MW	molecular weight
NBT	4-nitro blue tetrazolium chloride
NLS^-	nuclear localization signal not included
NLS^+	nuclear localization signal included
nm	nanometer
npt	neomycin phosphotranferase

ocs 3'	octopine synthase gene terminator
ORF	open reading frame
pAB	polyclonal antiserum
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pNPP	p-nitrophenyl phosphate
PNS	plant nutrient sucrose medium
PV	papillomavirus
RK2	minimal replicon for replication and maintenance in *E. coli* and *Agrobacterium*
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
R_0	regenerated transgenic generation
s	seconds
SDS	sodium dodecyl (lauryl) sulfate
*Sf*21	*Spodoptera frugiperda* cell line
T/C	tissue culture
TCA	trichloroacetic acid
TMB	3,3',5,5'-tetra-methyl-bezidine
TMV	*Tobacco mosaic virus*
Tn7	spectinomycin/streptomycin resistance gene for bacterial selection
T_x	transgenic generation
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
µl	microlitre
µg	microgram

Statement for animal experiments

Animal experiments reported in this thesis were approved by the **Animal Ethics Committee** of the Faculty of Health Sciences, **University of Cape Town**.

This work was carried out by qualified animal technicians.

Für meine Eltern

Chapter 1

Literature review

Contents

1.1 INTRODUCTION

The infectious nature of the agents causing human and different animal warts was first established at the beginning of the 20th century. Early events describing the carcinogenic potential of Cottontail rabbit papillomavirus (CRPV), the first visualization of virions, the unravelling of the papillomavirus genome and the discovery of tumour-inducing Bovine papillomavirus (BPV) and the description of papillomavirus-induced *Epidermodysplasia verruciformis* (EV) culminated in renewed interest in papillomavirus biology and the elucidation of its significant role in cancer in the 1970s (zur Hausen H., 1996).

The isolation and characterization of Human papillomavirus (HPV) types 6 and 11 from genital warts in 1983 and 1986 respectively, and types 16 (Seedorf *et al.* 1985) and 18 (Cole & Danos, 1987) from cervical cancer biopsies, drastically changed the field of papillomavirus research in the 1980s. However, another 10 years passed before the association between certain human papillomaviruses and cervical cancer became generally accepted. Today research on HPV spans the globe. Many laboratories do research on understanding the different aspects of the virus life cycle, its interaction with the host cells, and on virus-induced oncogenesis.

HPV infections, including types 16, 18, 33 and 58, are the leading cause of cancer of the cervix, a disease burden that results in the second biggest cause of female cancer mortalities worldwide: approximately half a million new cases of cervical cancer are reported annually, and there are an estimated 288000 mortalities annually. Developing countries report the highest prevalence rate; that is, about 80% of all reported cases. Approximately 68000 cases are annually reported on in Africa, followed by about 77000 new cases in Latin America and 245000 in Asia (taken from http://www.who.int/vaccine_research/diseases/hpv/en/). These shocking statistics have influenced the focus on the development of vaccines against this group of carcinoma-causing viruses.

It was thought until recently that HPV-16 associated cancer of the uterine cervix is a disease which progresses over decades, and is directly attributable to the persistence of the viral DNA on the cervix. However, a recent publication by Winer *et al.*, (2005) described the HPV DNA testing and cytologic evaluation of 602 women over a mean period of 36 months, and findings that included the progression of disease from the incident of infection to development of cervical intraepithelial neoplasia (CIN) grade 2 and 3 lesions, to occur within half the number of incident cases over a time period of 14 months. HPV-16 was primarily implicated in the development of high-grade squamous intraepithelial lesions (HSILs) early after the incident of infection. Within the same study, findings revealed that the development of genital warts, resulting from infection with either HPV-6 and/or HPV-11, took 2.9 months (IQR, 0-5.7 months) to develop from incident infection with either virus. In light of these findings, it now appears that disease progression from incident HPV infection occurs within a relatively short time period and re-affirms that infection by HPV-11 is easily detectable and that genital warts develop over a period of weeks to months. In general, HPV-11 is recognized as one of the most prevalent anogenital papillomaviruses, and is associated with more severe infections resulting in benign genital and laryngeal warts (Gerein *et al.*, 2004). In addition, HPV-11 DNA has also been found to be associated with squamous cell carcinomas of the tongue (Fife *et al.* 1996), has been identified to be the predominant type in oesophageal cancer samples (Matsha *et al.* 2002) and has been detected in rare verrucous carcinomas of the penis, vulva and in Buschke-Löwenstein tumours (zur Hausen H., 1996) and in nasal inverted papillomas (Shen *et al.* 1996).

Given the high global HPV-11 prevalence rate (Chan *et al.* 2002) a more recent concern is the impact of steadily increasing Human immunodeficiency virus (HIV) infection rates and the associated immunosuppression of HIV-positive individuals on HPV type 6 and 11 co-infections and genital warts. The possibilities of becoming re-infected with HPV-11 are high. Koutsky *et al.* (1997) have shown that 60% of HPV-11 infected men and women between the ages of 15 and 49 years showed a history of prior HPV-11 infection. HPV infections and their associated disease have been found to be the most common co-infection and co-morbidity in immunosuppressed individuals (Jay & Moscicki, 2000) and

have become a clinically relevant issue. Not only has it been shown that HPV infections are more readily detected in HIV-seropositive women, but they have been found to be more persistent, more severe, more difficult to treat than HPV infections in HIV-seronegative women (Jay & Moscicki, 2000) and recurrences have been found to be more frequent (de Panfilis *et al.* 2002). Silverberg *et al.* (2002) have found the prevalence of HPV-6 and HPV-11 to be up to 5.6 times higher in HIV-seropositive women, thereby increasing the prevalence of genital warts by a factor of 3.2. It has been postulated that HIV infection and the resulting immunodeficiency synergistically modify the relation between HPV-6 and / or HPV-11 and the prevalence of genital warts.

The use of highly active antiretroviral therapy (HAART) by HIV-seropositive women has been hypothesized to effectively decrease the rate of genital HPV infection and related cancer development (Hanna, 1999), yet the role of HAART in controlling HPV-associated oral lesions in HIV-seropositive individuals remains undefined (Aboulafia, 2002; Hagensee *et al.* 2004). Cusini *et al.* (2004) have explored the effects of applying 5% imiquimod cream to external anogenital warts in HIV-infected individuals undergoing HAART therapy. Their results concluded that 31% of HIV-seropositive patients were able to achieve complete clearance of warts, whereas partial clearance was only obtained in 24% of HIV/HPV co-infected individuals. Overall, they concluded that application of 5% imiquimod cream to external genital warts had an acceptable efficacy and was safe to be applied by HIV-positive patients.

The potential attributes of HAART therapy with or without simultaneous application of 5% imiquimod cream to genital warts, have been hypothesized to possibly decrease and control already existing HPV co-infections; however, this strategy only remains a temporary solution to the ever increasing occurrence of HIV/HPV co-infection.

Worldwide efforts to develop a subunit vaccine against the predominantly cervical cancer causing HPV type 16 have thus far been evaluated in phase III clinical trials and have yielded promising results, as described in section 1.9. Concurrent attention is aimed at the genital wart causing HPV type 11. Although it is not a cancer-causing virus, the

complications of HPV-11 co-infection in HIV-seropositive individuals, as described above, reemphasizes the need for the development of a safe and efficacious vaccine against HPV-11.

1.2 PAPILLOMAVIRUS TAXONOMY, NOMENCLATURE AND PHYLOGENY

Up until the VIIth Report of the International Committee on the Taxonomy of Viruses (ICTV), the taxonomic family *Papovaviridae* included papillomaviruses and polyomaviruses, deriving its name from the first two letters of the first viruses to be grouped together: rabbit *pa*pillomavirus, mouse *po*lyomavirus and simian *va*cuolating virus (SV40) (Howley, 1995). Polyomaviruses and papillomaviruses (PVs) were separated in the VIIth Report of the ICTV and now appear as individual families. There is a single genus *Papillomavirus* in the family *Papillomaviridae*, which is divided into different species. The multitude of different papillomaviruses isolated from *Homo sapiens sapiens* is represented as one species within the genus *Papillomavirus* (van Regenmortel *et al.* 2000). However, de Villiers *et al.* (2004) have proposed a new phylogenetic interpretation that separates different PV types into genera and species respectively.

PVs are apparently ubiquitous among higher vertebrates. Different viruses have been isolated from humans, cattle, rabbits, horses, dogs, sheep, elk, deer, non-human primates, the harvest mouse, the multimammate mouse, parrots and chaffinches (Howley, 1995).

PVs have been found to be highly species-specific. It is unknown for a particular papillomavirus to be able to cause and sustain a productive infection within another host different to its own (Chan *et al.* 1997). This includes the much debated association of HPV-7 with the formation of the "butcher's warts": HPV-7 has been firmly established as a human virus, displays no homology with bovine PV sequences and does not originate from the handling of slaughtered animals (Majewski *et al.* 2001). The predominant association of butcher's warts with slaughterhouse workers has been speculated to result

from the activation of latent HPV-7 infections, possibly due to growth factors present in bovine serum and meat (Majewski *et al.* 2001). However, there is one exception to the general rule of PV species-specificity. Two ungulate fibropapillomaviruses of the genus *Delta papillomavirus* species, *Bovine papillomavirus* types 1 and 2 (BPV-1 & BPV-2) are the only PVs to be linked to a disease common in horses termed equine sarcoid. Chambers *et al.*, (2003) have found these particular neoplasms to contain detectable levels of either BPV-1 or BPV-2 viral DNA and RNA and to express the respective major transforming protein E5. In horses these infections result in local fibroplastic skin tumours, but remain non-productive with no production of infectious virions.

In animals, papillomaviruses have predominantly been found to be associated with the formation of benign warts. Squamous epithelial proliferative lesions or commonly known as warts can be purely cutaneous in nature; or result from an infection of the squamous epithelium found throughout the mucosal surfaces of the oral pharynx, the oesophagus and the genital tract. In humans, the same holds true for infections by human papillomaviruses. Infection by certain HPVs results in the formation of an array of different benign papillomas. Among those are included the common- flat- plantar- and genital warts; myrmecia or pigmented warts; epidermoid cysts; keratoacanthomas and intraepithelial neoplasias as well as laryngeal papillomas (zur Hausen H., 1996). However, not all papillomaviruses known to infect humans are benign, some mucosa-infecting viruses have been identified to be the causative agents of malignant neoplasias; the most prevalent being cancer of the cervix (Bosch *et al.*, 2001; Walboomers *et al.*, 1999; zur Hausen H., 1996).

In order to understand the division of viruses, it is necessary to define their classification. Unlike bacteria which are defined as strains, HPVs are classified into types. Initial typing of HPV isolates relied on the comparisons of the *E6, E7* and *L1* nucleotide sequences to those of established prototypes, where a difference of more than 10% within those open reading frames (ORFs) was used to define a new type (zur Hausen H., 1996). In 1995 it was decided that nucleotide differences of more than 10% within the *L1* gene only would be used for further definition of new types. The term "variant" is used to describe a PV

isolate that differs from the same type by 5% within the nucleotide sequence under investigation (Chan *et al.*, 1995, 1997; Heinzel *et al.*, 1995).

In an effort to classify the multitude of papillomaviruses (PVs), Chan *et al.* (1995) proposed an internal taxonomic organization based on the *L1* phylogeny that divided papillomaviruses of human and non-human origin into five supergroups (A to E). This classification system distinguishing between supergroups and further differentiating into subgroups, has been replaced by the classification system proposed by de Villiers *et al.*, (2004).

Three different criteria have been introduced to differentiate PVs. The term supergroup has been replaced with genus, based on a nucleotide sequence identity within the *L1* gene of less than 60% between genera. "Species", formerly known as subgroups, form the lower-order clusters of human papillomaviruses (HPVs) found within the different genera and are based on a 60% to 70% nucleotide identity. Various different PVs sharing a nucleotide sequence identity between 71% and 89% within the complete *L1* gene are classified as different types within a species.

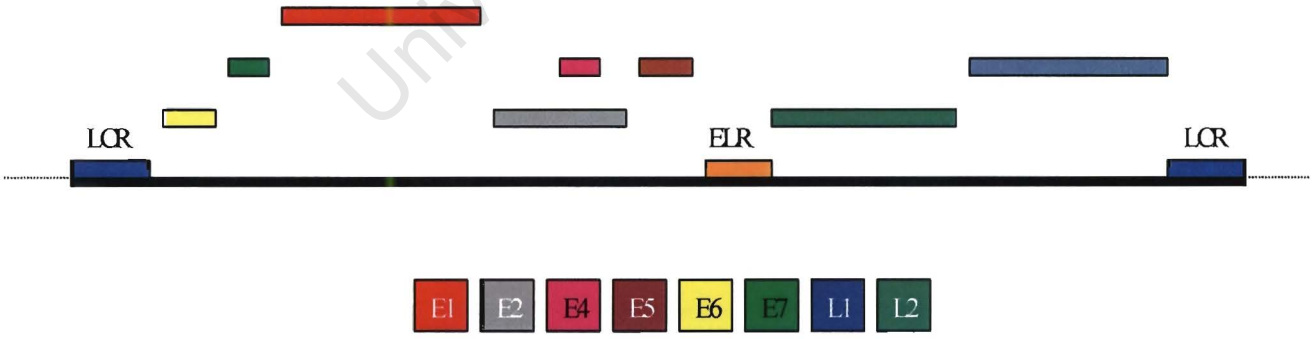


Figure 1.1: Schematic diagram of the PV genomic organization (not drawn to scale) showing the early and late genes in their respective translational frames, the non-coding long control region (LCR) and the region between the early and the late genes (ELR).

Within the new classification of PVs (Figure 1.2), viruses of a particular genus share two characteristics: the organization of their respective genomes together with their biological properties. Figure 1.1 shows a general representation of the PV genome, displaying the early open reading frames (ORFs) *E1* through to *E7*; with the exception of *E3*; and both the structural ORFs *L1* and *L2*. An *E3* ORF has been found to be present in only the following papillomaviruses: *Bovine papillomavirus* types 2 and 4 (BPV-2 & BPV-4) and the *European elk papillomavirus* (EEPV). This *E3* ORF overlaps the *E2* and *E4* genes in BPV-2 and EEPV, whereas in the BPV-4 genome the same has been found to overlap the *E1* gene. It is not known as to whether this particular *E3* ORF is transcribed or not.

There are additional differences in PV genomes between the genera: some viruses lack particular genes, whereas others possess an additional gene not commonly found among PVs. Table 1.1 summarizes these differences in genome genetic make-up and lists the biological properties of the diseases these viruses cause. The proposed genus Alpha-papillomavirus includes high- and low-risk HPVs responsible for mucosal (pre- and malignant) and cutaneous (benign) lesions. HPV-11, the causal agent of genital warts, is included in the genus *Alpha papillomavirus* and is a type representative of species number 10 (Figure 1.2). In addition to the above, Table 1.1 also lists the various aspects of the other genera, their respective hosts, biological properties and genome differences.

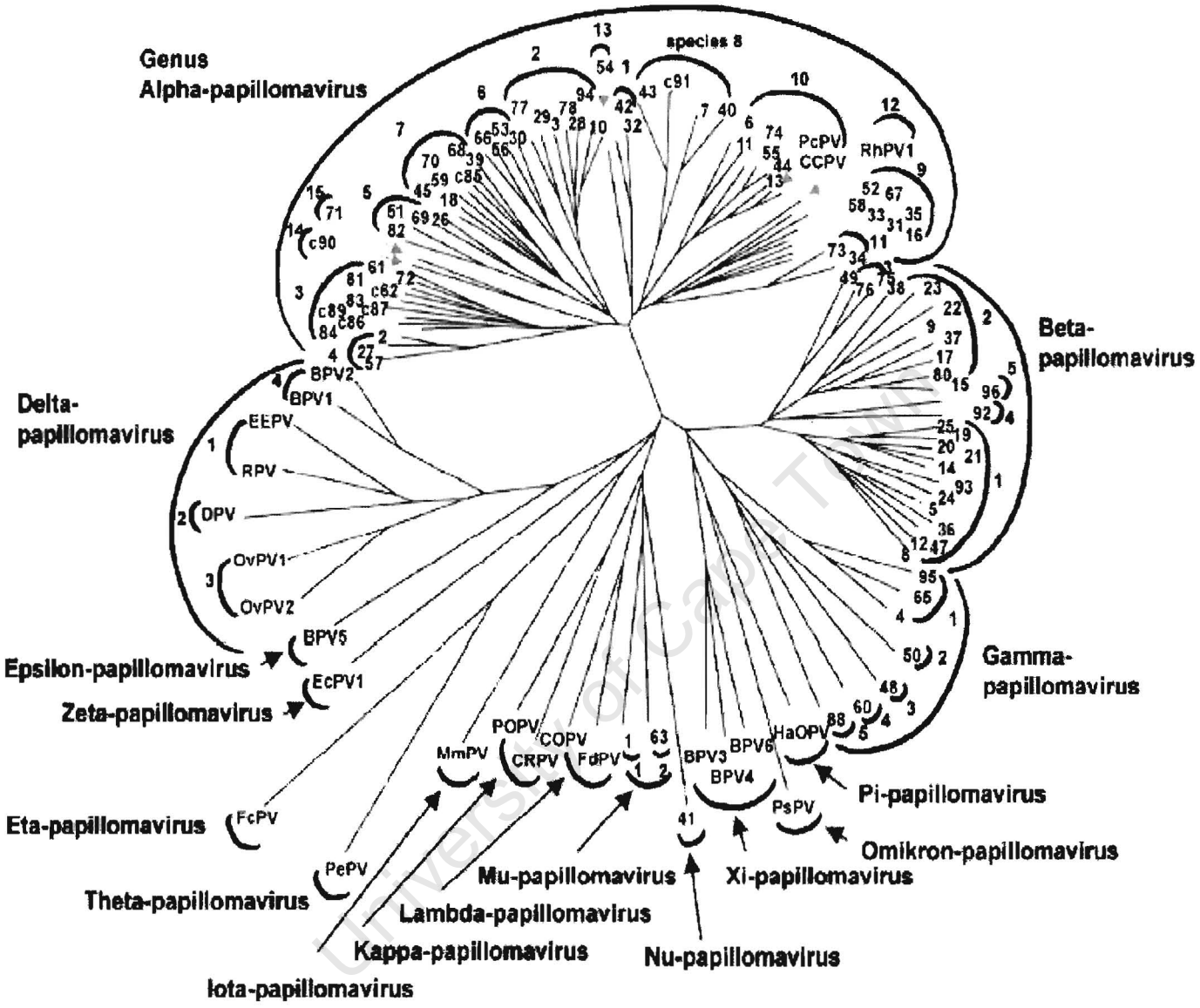


Figure 1.2: "Phylogenetic tree containing the sequences of 118 papillomavirus types" (reproduced with permission from author Hans-Ulrich Bernard, Department of Molecular Biology and Biochemistry, University of California, Irvine, USA). For the comparison of the *L1* ORFs, the authors used a modified version of Phylip version 3.572, based on a weighted version of the neighbour-joining analysis. Different HPV types are identified by numbers at the end of each branch, whereas c-numbers refer to candidate HPV types. All other abbreviations are those of animal PV types. PV genera are identified by the outermost semicircular symbols, e.g. the genus beta-papillomaviruses. The number at the inner semicircular symbol indicates the PV species. HPV-11, when used as an example, would be part of the HPV species 10 in the genus alpha-papillomaviruses. All unabbreviated PV names and sequence accession numbers are presented in Tables A.1 and A.2 (Appendix A).

Table 1.1: Summary of the different PV genera, including their hosts, biological properties and genome differences (adapted from de Villiers *et al.* 2004)

PV genus	Species (human / non-human)	Biological Properties	Genome differences (absent / additional genes)
Alpha	Human and primates	Mucosal (pre- and malignant) and cutaneous (benign) lesions	
Beta	Human	Cutaneous lesions (*Epidermodysplasia verruciformis*)	absent E5
Gamma	Human	Cutaneous lesions (intracytoplasmic inclusion bodies)	absent E5
Delta	Ungulates	Lesions (fibropapillomas)	
Epsilon	Bovine	Cutaneous papillomas in cattle	
Zeta	Horses	Cutaneous lesions	
Eta	Avian (chaffinch)	Cutaneous lesions	absent E4 E5 E6 E7
Theta	Avian (parrot)	Cutaneous lesions	absent E4 E5 E6 E7
Iota	Rodent	Cutaneous lesions	absent E5
Kappa	Rabbits	Cutaneous and mucosal lesions	additional E8
Lambda	Animal (dog and cat)	Benign mucosal and cutaneous lesions	
Mu	Human	Cutaneous lesions (intracytoplasmic inclusion bodies)	
Nu		Benign and malignant cutaneous lesions	
Xi	Bovine	True papillomas	absent E6 additional E8
Omikron	Cetaceans	Genital warts	absent E7
Pi	Hamster	Mucosal lesions	

The avian papillomaviruses *Fringilla coelebs papillomavirus* (FcPV) and *Psittacus erithacus timneh papillomavirus* (PePV) (Figure 1.2, Table 1.1), lack the canonical E6 and E7 ORFs, suggesting that they serve adaptive functions during papillomavirus evolution (Terai *et al.*, 2002).

1.3 PAPILLOMAVIRUS EVOLUTION

In comparison to RNA viruses like Human immunodeficiency virus (HIV) and Hepatitis C virus (HCV), the double-stranded DNA papillomaviruses seem to evolve slowly, as they use the host's enzymes for replication and proofreading of their genome (Halpern, 2000). Some insertions and deletions have been reported for PVs, mostly within the non-coding regions of the genome (Figure 1.1) (Rubben *et al.* 1996). Occasional and rare substitutions and/or rearrangements of coding regions have been reported for HPV-16 (Bernard *et al.* 1994; Pushko *et al.* 1994). The occurrence of distinct recombination patterns among HPVs has been documented by Halpern *et al.*, (1995), but no such signs of recombination have been found in other studies (Chan *et al.* 1992a; Yamada *et al.* 1995, 1997).

If PV diversification of the most variable parts of the genomes is estimated to occur at a rate of 0.25% over a period of 10000 or 20000 years (Chan *et al.* 1997), how can the enormous diversity among papillomaviruses be explained?

A number of animal PVs are found grouped together with HPVs in the phylogenetic tree (Figure 1.2). Pygmy chimpanzee PV (PcPV) and colobus monkey PV (CCPV) are most closely related to HPV-13 in species 10 of the alpha-papillomavirus genus. Additional non-human primate PVs, including the Rhesus monkey PV (RhPV-1), the *Micromys minutus* PV (MmPV) and the *Mastomys natalensis* PV (MnPV), can also be linked most closely to certain HPV types (Myers *et al.*, 1996b). This strongly suggests a zoonotic event in terms of evolutionary terminology. However, there is no evidence for such cross-species transmissions to have occurred amongst primates, unlike the frequently occurring zoonotic events with other viruses such as the outbreak of the Hong Kong chicken flu in 1997 and the Severe acute respiratory syndrome (SARS) virus in 2003 (Cyranoski, 2001; Liu *et al.* 2004; Lun & Qu 2004; Ng *et al.* 2004; Shortridge *et al.* 2000; Wuethrich, 2003).

In contrast to HIV, there is little evidence of dramatic adaptive ability within the PVs. Unlike HIV, which seems to display the mutational potential for the sole purpose of escaping the selectional pressures of the immune system (Moore *et al.* 2002; Price *et al.* 1997; Yusim *et al.* 2002), HPVs apparently avoid fully activating the immune system, rather than having to escape it (Halpern, 2000; Tindle, 2002).

The diversity of PVs is argued to have arisen through coupled evolution between species and host. To explain why some primate PVs are found interspersed among HPVs, Chan *et al.*, (1997) proposed that interspecies transmission did indeed occur, not between modern humans and rhesus monkeys, but instead between their ancestral species. Divergent HPV type variants are calculated to be older than 100 000 years (Halpern, 2000) and probably already existed at the time humans originated as a species (Chan *et al.* 1997). The multitude of additional variants of HPV types has been suggested to have emerged in conjunction with the expansion and migration of modern human populations.

In summary, the response to host speciation, together with mechanisms of drift and selection, not only accounts for the evolution of the different PV branches, but also for the intrahost species diversity we see today (Chan *et al.* 1997).

1.4 THE BIOLOGY OF HUMAN PAPILLOMAVIRUSES

Based on the criterion of greater than 10% nucleotide variation within the L1 major capsid protein, (Laimins, 1993, zur Hausen H. 2000), more than a hundred HPVs have been identified, of which more than eighty genomes have been completely sequenced.

HPVs are a group of heterogeneous, non-enveloped viruses that contain a circular double-stranded DNA genome, varying in size from 7.2 kb up to 8.4 kb. All HPVs display a similar genomic organization, the early region of the genome codes for the E1, E2, E4, E5, E6 and E7 proteins, involved in DNA replication and cellular transformation, whereas the latter part of the genome contains the open reading frames encoding the two structural proteins. The *L1* and *L2* genes, encoding the major and minor capsid proteins

respectively, make up the late region of the genome and are only transcribed in fully differentiated keratinocytes. A non-coding region known as the long control region (LCR) contains control elements for transcription and replication (Figure 1.3).

HPVs associated with genital tract lesions are divided into the "high-risk" or "low-risk" category based on the risk of malignant progression of the hyperprolific lesions they cause (Howley, 1995). Twenty seven of the characterized HPVs have been identified as the causal agents of anogenital infections, the most prevalent being HPV types 6, 11, 16, 18, 33, 35 and 42 (Chow *et al.* 1994; Kirnbauer, 1996). The epidemiological and biochemical association with urogenital carcinomas, including cervical, vulvar and penile cancer has been attributed to HPV types 16, 18, 31, 33, 35, 52, 56 and 58 (Cason *et al.* 1998; Shah *et al.* 1996). Within the time period of the above mentioned publications to this present day, the list of papillomaviruses has been expanded to include additional genotypes known for their association with cancer. Genotypes 39, 45, 51, 59, 66, 68 and 73 have been added to the types listed above, thereby raising the number of the most common oncogenic papillomaviruses to fifteen (Muñoz *et al.* 2004). These types form part of the genus alpha-papillomaviruses (Liu *et al.* 2000a; Mor *et al.* 1998) (Figure 1.2) and are classified as being of high-risk for the progression to cancer. Unlike high-risk HPV-16 which has been found in 95% of cervical cancers, HPV-6 and / or HPV-11 are rarely associated with carcinomas. They have been found to be the causal agents of benign genital warts (condyloma accuminata) and are thus classified as low-risk for cancer. With over two dozen human papillomaviruses able to infect the anogenital epithelia, HPVs have been termed the most prevalent sexually transmitted disease of viral etiology. However transmission does not necessarily occur through sexual intercourse. Studies have indicated that infants can acquire high-risk HPV infections from their mothers at birth (Antonsson *et al.* 2003; Cason *et al.* 1998; Kaye *et al.* 1996; Zhao *et al.* 2001). In addition to non-sexual parent-to-child transmission; investigators have been able to detect HPV DNA in swabs from virginal women, in cervical-virginal specimens from young girls, and in vulval swabs from 15% of women who claimed no history of sexual contact (Cason *et al.* 1998).

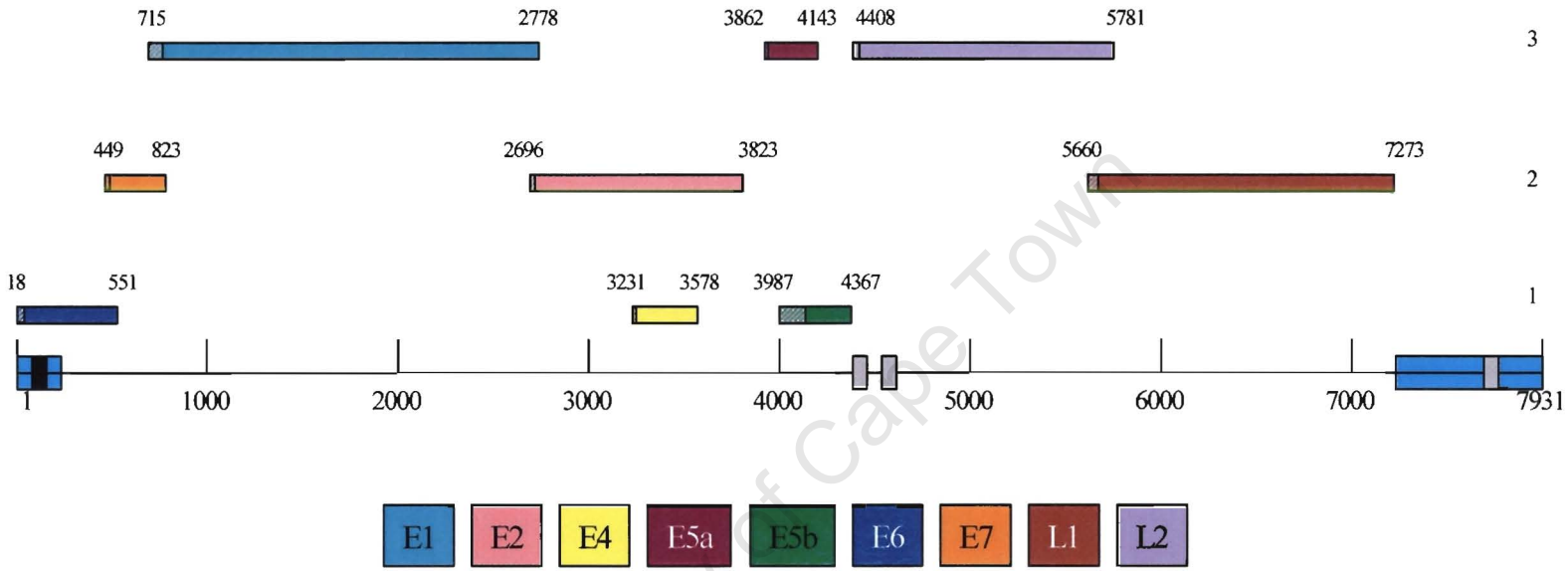


Figure 1.3: Schematic representation of the HPV-11 genome, displaying all the genes in their respective translational frames. Positions of the genes are indicated by nucleotides at the 5' and 3' ends. The TATA box spanning nucleotides 66 – 71 is indicated by ▮, whereas the poly(A) signals at positions 4371 – 4376, 4545 – 4551 and 7457 – 7462 are indicated by ▮. The 5' untranslated region, including the long control region, spanning nucleotides 1 – 101 and 7277 – 7931 is indicated by ▮.

1.4.1 HPV replication and gene expression

HPVs display a strict tropism for undifferentiated cutaneous or mucosal squamous epithelial cells, whose differentiation status is synonymous with their life cycle (Laimins, 1993). Rapid binding and attachment of virions via heparin sulfate on the cell surface (Joyce *et al.* 1999; Roden *et al.* 1994a) of compromised cells located in the basal layer (Giroglou *et al.* 2001a), progresses to the uptake of virions (Müller *et al.* 1995), whereafter the DNA is uncoated in the cytoplasm and transported to the nucleus (Figure 1.4).

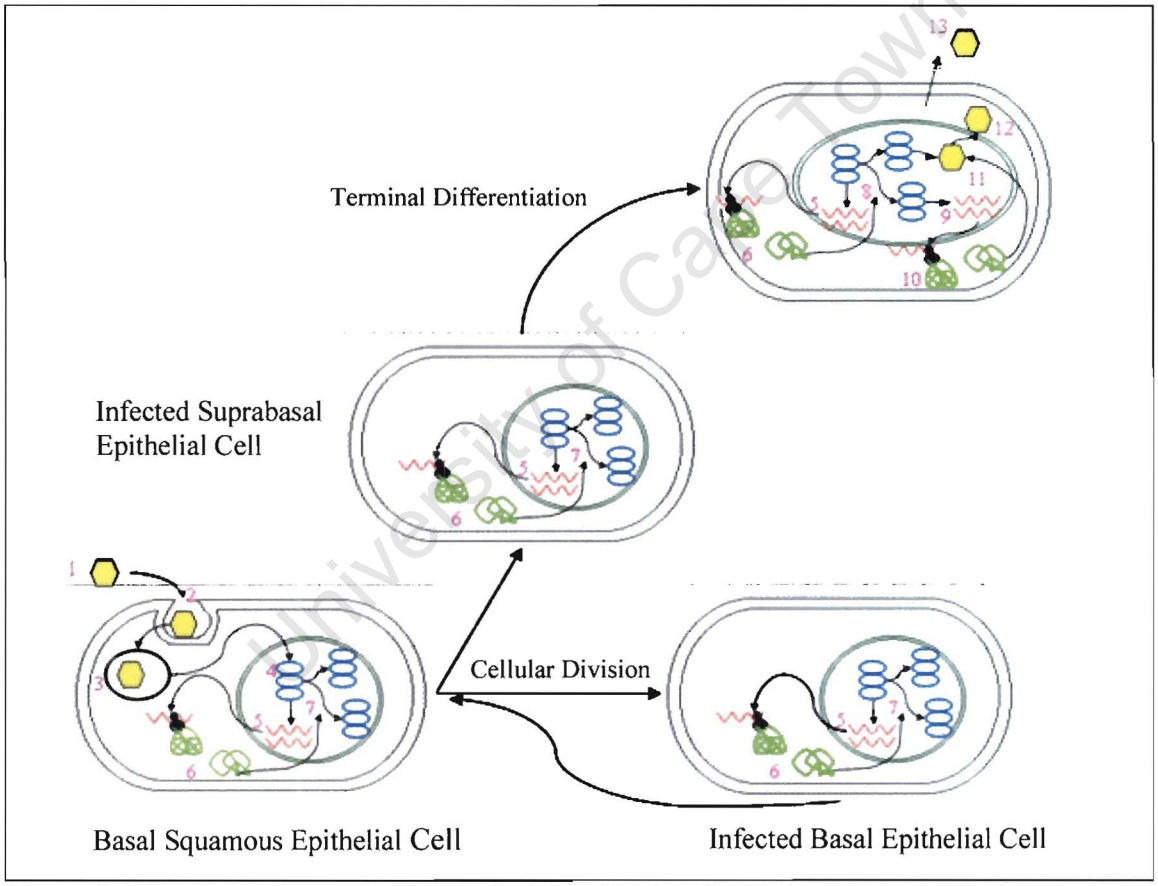


Figure 1.4: Papillomavirus replication cycle (after Howley, 1995). In order to establish a wart or papilloma, the virus must infect a basal epithelial cell. Virus attachment (1) is followed by uptake (2), endocytosis (3), and transport to the nucleus and uncoating of the viral DNA (4). Early region transcription (5), translation of the early proteins (6), and steady state viral DNA replication (7) all occur in the basal cell and in the infected suprabasal epithelial cell. Events in the viral life cycle leading to the production of virion particles occur in the differentiated keratinocyte: vegetative viral DNA replication (8), transcription of the late region (9), production of the capsid proteins L1 and L2 (10), assembly of the virion particles (11), nuclear breakdown (12) and release of the virus (13).

Recent discoveries have shown that the L2 minor capsid protein plays an important part during virus infection. Bossis *et al.*, (2005) have reported that the L2 protein has the ability to mediate the entry of virions into the endoplasmic reticulum (ER). This entry is facilitated by the interaction of L2 with the tSNARE syntaxin 18 protein, a member of the SNAP (soluble *N*-ethylmaleimide-sensitive factor-attachment protein) receptor family whose role is to traffic vesicles within the cell into or out of the ER.

Wound healing promotes the proliferation of infected basal epithelial cells, a process through which the viral genomes are stably maintained as episomes and replicated together with the chromosomal DNA. This process is initiated by the replication factors E1 and E2 (see Table 1.2) and results in production of approximately 20 to 100 extrachromosomal copies of viral DNA per cell (Longworth & Laimins, 2004). As the wound healing process comes to an end, most of the basal cells will return to a state of quiescence. Replenishment of the stratified epithelial layer is accomplished by cellular division of parabasal cells. Mitotic cell division of infected basal epithelial cells helps maintain a steady state of low copy numbers of viral episomes and progressively leads to migration away from the basal epithelial layer. Throughout this migration, cells no longer conform to the mitotic cell cycle and begin to differentiate, a cellular process that in turn activates the expression of the HPV late genes. Expression of the late genes in differentiating epithelia is promoted by the E5 protein (Fehrmann *et al.* 2003). As the differentiation process of the epithelial cells comes to an end, it leaves behind an increased thickness of the spinous cells leading to the formation of warty or condylomatous tissue. The HPV life cycle comes to completion in fully differentiated cells; further viral genome amplification and late HPV gene synthesis from a differentiation-dependent promoter facilitates assembly of virions that will eventually be released from the infected cells (Howley, 1995; Longworth & Laimins, 2004).

HPVs do not induce cell lysis to escape infected cells; instead it has been shown that the cornified cell envelopes (CCE) of HPV-11 infected differentiated keratinocytes are thin and fragile compared to those found in uninfected epithelium (Lehr *et al.* 2002). The major component loricrin is normally incorporated into the CCE of differentiating

keratinocytes, however no loricrin was detected among proteins extracted from human genital epithelium infected with HPV-11. The reduced expression (70% to 26%, uninfected versus infected) of loricrin is believed to be due to alterations in transcription. An increase in HPV replication leads to the reduction of loricrin. Lehr *et al.* (2002) speculated that expression of the HPV E1^E4 protein could potentially play a role in the altercation of the CCE, but cautioned that this needed further investigation and that other viral gene products could also contribute to the CCE abnormalities. The diminished amount of loricrin to be incorporated into the CCE renders the CCEs more fragile and susceptible to mechanical breakage upon which the mature virions would be released from the infected cells (Lehr *et al.* 2002). The number of HPV-11 early genes, *E1* through to *E7*, and their respective functions during the replication of the virus are listed in Table 1.2.

Table 1.2: Summary of the HPV early genes and their respective functions

Proteins	Function	References	
E1	ATPase and helicase activity ATP binding protein essential for viral DNA replication	Chen *et al.* 2001a Chow *et al.* 1994 Conger *et al.* 1999 Deng *et al.* 2003 Hughes *et al.* 1993	Lin *et al.* 2002 Lusky *et al.* 1993 Mohr *et al.* 1990 Sanders & Stenlund, 1998 Zou *et al.* 2000
E2	primary ori-recognition protein, transcriptional transactivator, binds E1 to facilitate initiation of viral DNA replication, important in genome encapsidation	Chiang *et al.* 1992 Chow *et al.* 1994 Deng *et al.* 2003 Dong *et al.* 1994 Hou *et al.* 2002 Hartley *et al.* 2002	Howley, 1995 Kong *et al.* 2001 Liu *et al.*1995 McMichael *et al.* 2002 Mason *et al.* 1996 Surabhi *et al.* 2002
E4	VEGETATIVE VIRAL DNA REPLICATION Interacts with cytoskeletal proteins, allows viral assembly	Brown *et al.* 1995, 1996 Howley, 1995 Doorbar *et al.* 1997 Roberts *et al.* 1993	
E5a E5b	ONSET OF CELLULAR TRANSFORMATION weak transforming activity, upregulates growth factor, receptors	Chen *et al.* 1990 Cohen *et al.* 1993 Fehrmann *et al.* 2003 Howley, 1995 Hwang *et al.* 1995	Leechanachai *et al.* 1992 Oelze *et al.* 1995 Pim *et al.* 1992
E6 E7	EPISOMAL MAINTENANCE Binds p53, directs p53 ubiquitin mediated degradation, with E7 immortalises primary keratinocytes Binds pRb, deregulates the G1/S checkpoint, cooperates with E6 to immortalise primary cells	Chen *et al.* 1995 Degenhardt *et al.* 2001 Farthing & Vousden, 1994 Flores *et al.* 2000 Galloway *et al.* 1996 Gao *et al.* 1999, 2000 Gardiol *et al.* 1999 Haq *et al.* 1995 Horner *et al.* 2004 Hou *et al.* 2002	Huibregtse *et al.* 1993 Huibregtse & Beaudenon, 1996 Kuerbitz *et al.* 1992 Li *et al.* 1999 Liang *et al.* 1993 Longworth & Laimins, 2004 Kiyono *et al.* 1997 Pim *et al.* 2000 Ronco *et al.* 1998 Tong & Howley, 1997

A detailed description of the late genes, the major (*L1*) and minor (*L2*) capsid protein genes, with emphasis on the L1 protein is followed in section 1.4.2.

1.4.2 Papillomavirus capsid proteins

Two structural genes, encoding the major capsid protein (L1) and the minor coat protein (L2) respectively, are expressed late in the papillomavirus life cycle and only when the host cells reach an appropriate stage of differentiation (Howley, 1995). The 1367 bp long HPV-11 *L2* minor capsid protein gene spans nucleotides 4417 through 5784 and is followed by the 1505 bp long *L1* major capsid protein gene spanning nucleotides 5771 through 7276. Translation in different open reading frames results in the synthesis of the 50 kDa L2 and the 55 kDa L1 proteins (Figure 1.3).

The structure of the HPV-16 L1 monomeric protein was determined by X-ray crystallography (Chen *et al.*, 2000) and reveals the highly conserved secondary and tertiary structure found among PV L1 proteins (Figure 1.5).

A number of β-strands, labelled B through J, form the core of the monomeric L1 protein, whereas distinct α-helices (h2 through h5) are located within the carboxyl terminal region. The less conserved and most variable region of PV L1 proteins is the nuclear localization signal (NLS) found at the carboxy terminus. First identified by Zhou *et al.* (1991) for HPV-16 L1, the NLS has been described as a specific sequence of basic amino acids located at the carboxyl terminal end.

The HPV-11 L1 NLS is considered to be bipartite in nature. The first cluster of basic residues constituting the NLS is situated at amino acid positions 493 through 502 (KRKRtKtKK), and is separated from the second NLS (KRpavsK; 481 through 487) by 5 amino acids (Figure 1.10). Although the HPV-11 L2 tertiary structure has not been resolved by crystallography, its NLS spans amino acids 440 through 445 (RRRRKR) (Sun *et al.*, 1995).

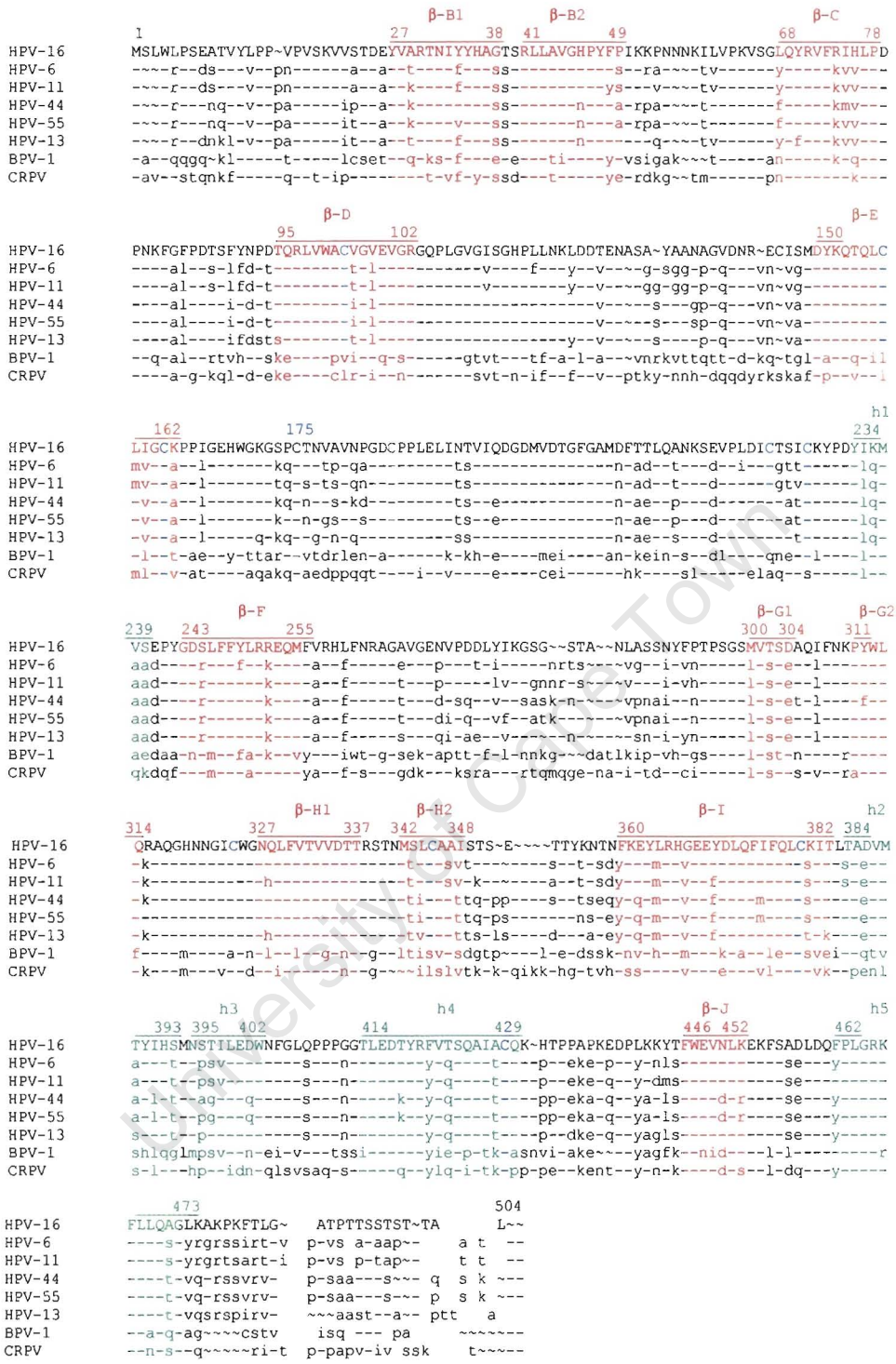


Figure 1.5: L1 amino acid sequence alignment of low-risk HPV types 6, 11, 13, 44 and 55 and the high-risk HPV type 16, BPV-1 and CRPV; illustrating the conserved L1 secondary structure. Sequences were aligned using DNAMAN (Lynnon BioSoft, Quebec, Canada) and the resolved secondary structure of the HPV-16 L1 major capsid protein (Chen *et al.*, 2000) is used as a reference. β-strands are indicated in red, whereas α-helices are represented in green. The specific sequences of basic amino acids constituting the NLS at the carboxy terminus are shaded in purple. The highly conserved cysteine residues are in blue (Ishii *et al.*, 2003). Amino acid positions for all HPV-16 L1 secondary structures are given above the respective elements.

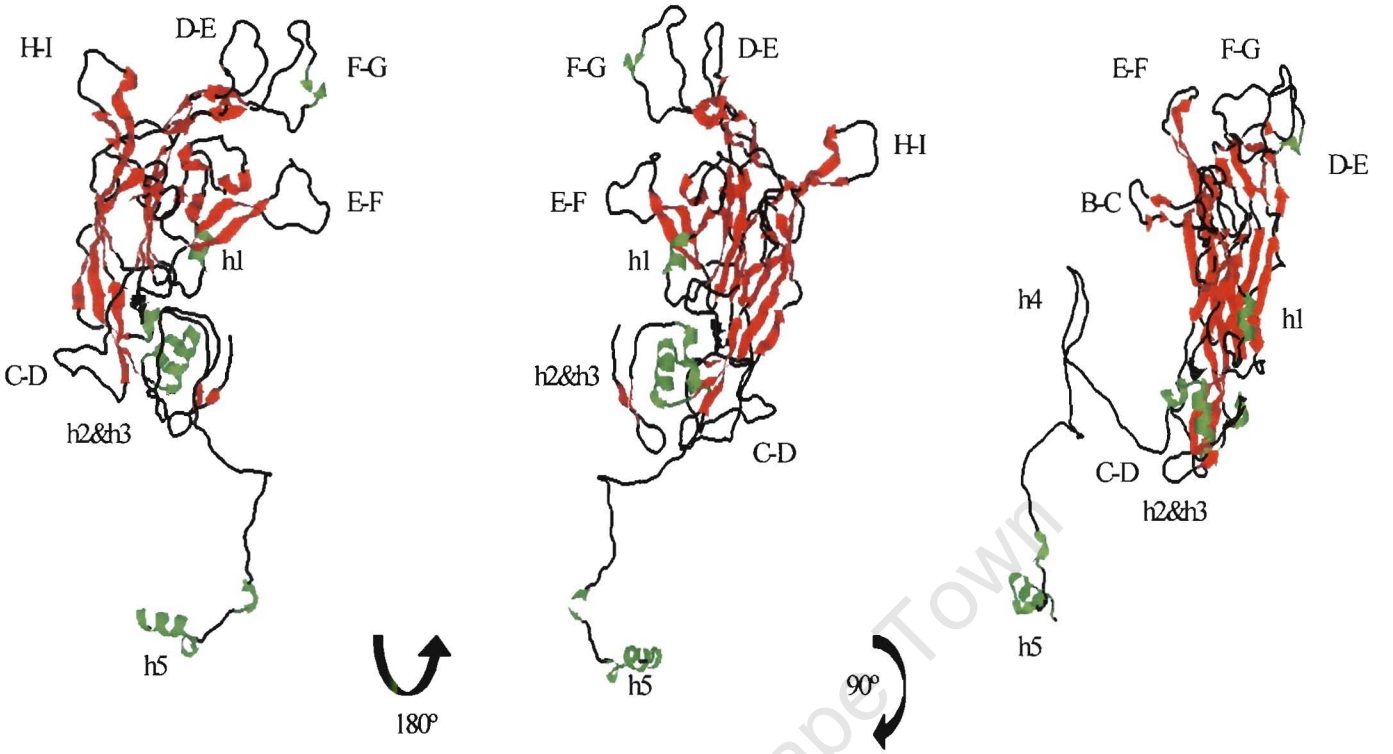
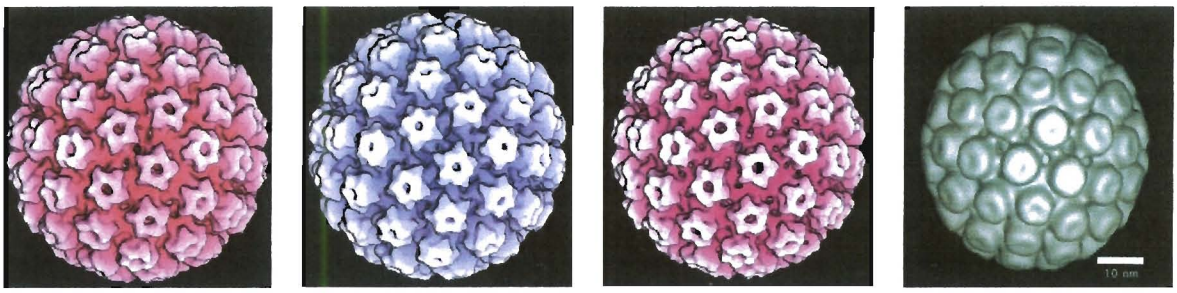


Figure 1.6: 3-D images of the HPV L1 monomer in three different orientations displaying the hypervariable loop regions (B-C, C-D, D-E, E-F, F-G- and H-I). Images were drawn in RASMOL (Sayle & Milner-White, 1995). β strands are displayed in red, and α-helices are represented in green.

The L1 monomer has been described as a classical "jelly roll" β sandwich by Chen *et al.* (2000) (Figure 1.6). The hypervariable loops are responsible for sculpting the outer surface appearance of the pentameric structure, whereas the L1 C-terminal end consists mostly of α-helical structures, providing contact surfaces between monomers (h2-h4), with helix 5 (h5) and β-strand J anchoring the projection back into the jelly roll. Monomer interactions resulting in capsomer formation and the assembly of virions are described in section 1.5 of this chapter.

1.5 VIRION ARCHITECTURE



BPV-1 CRPV HPV-1 SV-40

Figure 1.7: Reconstructed, surface-shaded representations of papilloma and polyomaviruses displaying the differences in capsid morphology. (Belnap *et al.* 1996, Images taken from http://bilbo.bio.purdue.edu/~baker/icons/proj_pics/papova/papilloma).

Polyoma- and papillomaviruses are spherical particles whose capsids are made up of 72 morphological units, termed capsomers and are composed of 5 identical protein subunits. Described to be mushroom-like protrusions, the pentameric "star-shaped" heads of papillomaviruses formed in part by the outward projecting L1 E-F loops on the edge of the pentamers (Chen *et al.* 2000), are 11 to 12 nm in diameter, whereas polyomavirus capsomeric heads are only 8 nm in diameter and display a "barrel-shaped" morphology (Figure 1.7). Despite their difference in appearance, both virus families exhibit conserved capsid features. The 72 capsomers are arranged on an icosahedral lattice with a triangulation number of 7 (Belnap *et al.* 1996).

In total, the 72 PV capsomers contain 360 copies of the major capsid protein L1. Sixty of these capsomers are each surrounded by six neighbours, and are centred on vertices that lie between the 5-fold icosahedral axes. The remaining 12 capsomers are surrounded by only five neighbours, and are centred on the 5-fold axes. In addition to the L1 protein, 12 copies of the minor capsid protein (L2) are estimated to be associated and located at the 12 pentavalent capsomers (Combita *et al.* 2002; Sapp *et al.* 1998).

The T=7 lattice either exists as a left (T=7 *laevo*) or right (T=7 *dextro*) handed enantiomorph, where handedness is defined by the arrangement of lattice points between neighbouring 5-fold vertices. Energetically favourable interactions determine the

arrangement of the protein subunits, ensuring a particular configuration (Belnap *et al.* 1996).

Although all capsids within a particular virus species are assumed to have the same lattice hand, it is not known whether the same applies to different viruses within a genus or family. Indeed, earlier findings reported that the Cottontail rabbit papillomavirus (CRPV) was the only member of the papillomaviruses to display a T = 7 *laevo* capsid (Klug & Finch, 1965; Finch & Klug, 1965), but more recent reports have shown that all papillomavirus capsids display the same T = 7 *dextro* handedness (Belnap *et al.* 1996).

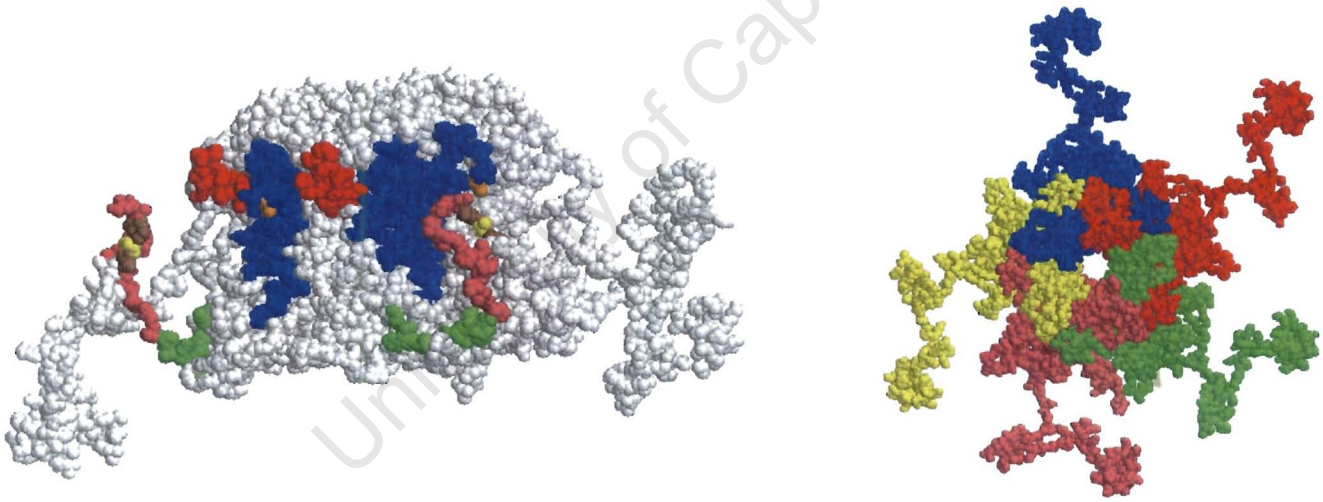
1.5.1 Capsomer formation

The L1 amino-terminal end is highly conserved among PVs. As has been shown with other HPVs, the first 20 amino acids constituting the N-terminus of HPV-11 L1 are essential for the formation of pentamers. Chen *et al.* (2001b) have shown that pentamer formation of trypsin digested N-terminal mutants decreased with an increase of residue deletions from the amino-terminus. Deletion of only 5 (ΔN5) or 8 (ΔN8) amino acids still allowed for the formation of trypsin-resistant pentamers, but removal of a further 6 (ΔN14) or 11 (ΔN19) amino acids, yielded only small amounts or no pentamers respectively. Modis *et al.* (2002) proposed that the first 10 N-terminal amino acids block the invading C-terminal arm from returning to its pentamer of origin.

The C-D loop found within the N-terminal end has been proposed to form important contacts between subunits in pentavalent and hexavalent pentamers, and may thus act as another element allowing for the T = 7 icosahedral assembly of PVs (Modis *et al.* 2002). The L1 C-terminal arm has been found to be involved in the principal interpentamer contacts (Li *et al.* 1997). The "invading arm" model has been proposed by Modis *et al.* (2002), in which the C-terminal arm extends away from its subunit of origin and invades a subunit in an adjacent pentamer. Thus, any one pentamer would receive five invading arms from its surrounding pentamers and at the same time would donate five arms to its neighbours. This pattern of interaction has been observed throughout the polyomaviruses

and has been described in both SV40 and murine polyomavirus (Liddington *et al.* 1991; Stehle *et al.* 1994).

The conserved proline- and glycine-rich region spanning amino acids 400 through 409 (HPV-11) acts as a flexible hinge region, not only bridging the gaps between donor and acceptor pentamers, but also allowing any given pentamer to donate its C-terminal arms in either a pentavalent or hexavalent environment (Modis *et al.* 2002). The C-terminus of HPV-11 L1 helix 4 (amino acids 410 through 425) invades an adjacent pentamer, inserting into the space between the B-C and E-F loops, thereby positioning the helix 4 (h4) cysteine residue 424 (C424) directly opposite the cysteine residue 172 (C172) in the adjacent pentamer (Figure 1.8). This interaction, resulting in the formation of a critical disulphide bond, and thus allowing for HPV particle assembly is completely abolished, if the entire h4 of either HPV-11 or 16 is deleted (Chen *et al.* 2001b).



A **B**

Figure 1.8: Pentameric image displaying the groove between the B-C (red) and E-F (blue) loops into which the invading helix 4 is inserted. The flexible hinge region is in green, the helix 4 C-terminus in pink and the helix 4 N-terminus in brown. Through helix 4 insertion, the cysteine residues (yellow and orange) are brought into contact, allowing for disulphide bond formation (**A**). (**B**) Image of the interactions of 5 L1 monomers, resulting in pentamer formation. Images were drawn using RASMOL (Sayle & Milner-White, 1995)

Once h4 is inserted into the acceptor pentamer, amino acid residues 426 through 443 (HPV-11) extend around the circumference of the target pentamer, thereby linking h4 with the C-terminal segment.

The segment spanning amino acids 444 through 471 (HPV-11), including the β-strand J (444 through 450) and helix 5 (h5, 460 through 471), inserts into the acceptor pentamer at the interface between the two L1 subunits, thereby linking the donor and acceptor pentamers (Modis *et al.* 2002). This interaction is capped by the amino acid residues (400 through 409) of yet another invading C-terminal arm, thereby blocking access of h5 and the C-terminus to the outer viral surface and possibly enhancing overall virion stability (Modis *et al.* 2002).

The L1 major capsid protein is capable of L2-independent self-assembly into virus-like particles (VLPs) (Hagensee *et al.* 1993, 1994; Kirnbauer *et al.* 1992, 1993; Zhou *et al.* 1991, 1993). To characterize the position of the L2 protein within the PV capsid, Finnen *et al.* (2003) have defined the domains on HPV-11 L2 that interact with L1 pentamers. A hydrophobic, non-negatively charged region consisting of amino acid residues PVTPALP (amino acids 413 through 419) contained within 44 amino acid residues (396 through 439) near the C-terminal end of L2 has been identified as the L1 binding domain. Binding of L2 to L1 is predominantly facilitated by hydrophobic interactions to the extend that treatment with 5 M urea could not completely disrupt the L1-L2 complex, indicating that these hydrophobic residues form the principal domain responsible for anchoring L2 within the PV capsid (Finnen *et al.* 2003).

1.6 HOST DEFENCE AND IMMUNITY

Most human pathogens initiate infection at mucosal surfaces, where they encounter the body's first and most effective line of defence: this is the mucosal immune system (MIS), which in many ways differs from the systemic immune system (Mason *et al.* 1995; Mor *et al.* 1998). Its localization at the epithelial border involves the action of several immunomodulatory cytokines and cellular effectors, including macrophages, monocytes, natural killer (NK) and antigen presenting cells (APCs) (Hilleman, 2000; Stern *et al.* 2000). HPVs are capable of evading the induction of cellular distress signals. Once infection has occurred, the mucosotropic HPV virions remain at the original site of infection and do not induce lysis of the infected cells. The expression of the capsid

proteins genes occurs only towards the end of the natural replication cycle and only in fully differentiated epithelia where dendritic cells are not capable of recognizing them (Scheurer *et al.* 2005). HPV infected cells are resistant to NK cells, but as mentioned by Scheurer *et al.* (2005) can be destroyed by cytokine-activated NK cells and macrophages.

Following infection, the presentation of mostly linear viral epitopes on the surface of APCs triggers their recognition as non-self components by cytotoxic T lymphocytes (CTLs) (Berzofsky & Berkower, 1994). The activation of T lymphocytes in turn leads to the secretion of multiple cytokines, including interferon γ (IFNγ), perforin and the tumour necrosis factor (TNF) (Hines *et al.* 1995). Although the natural regression of plane (Iwatsuki *et al.* 1986) and genital warts has been observed in 22% of infected patients (Coleman *et al.* 1994), IFN-α has been licensed for the treatment of genital warts (Barnard & McMillian, 1999). Although IFN treatment shows considerable variations between patients (Arany & Tyring, 1996), it has shown to be effective against infections of low-risk HPVs. However the use of interferon for the treatment of HPV infections remains quite controversial. In the 2005 publication on possible therapy methods for genital HPV-related disease, the author Charles J. N. Lacey states that the application of interferon therapy has never gained a therapeutic niche. Not only is this therapy costly, but the author also warns of potential side effects and does not recommend it for routine clinical use. However, interferon-α-2b has been successfully used for the treatment of epithelial tumours of the ocular surface. HPV-16/18 was detected in one of the CIN patients and the application of interferon-α-2b showed to be safe and effective and was thus preferred to more invasive and destructive treatment methods (Fuchsluger *et al.* 2005).

HPV-6 and/or 11 infected patients have been treated with a combination of IFN-γ and IFN-α 2a and showed regression in lesions. This was attributed to the marked increase in infiltrating T-helper 1 (Th1) inflammatory cells combined with elevated levels of macrophages, natural killer (NK) cells and activated $CD4^+$ lymphocytes (Shepherd *et al.* 1996), indicating a proliferative response to the major capsid protein (L1) of either HPV-6 and -11 (Hong *et al.* 1997; Stern *et al.* 2000). However patients with a depleted number

of Langerhans cells (LC), important HLA class II antigen presenting cells (Breitburd *et al.* 1996), did not respond to the IFN-γ/α 2a treatment and showed less T cell infiltration and reduced levels of interleukin 1 (IL-1), granulocyte-macrophage-colony stimulating factor (GM-CSF) and TNF. In conjunction with the decreased treatment effectiveness in non-responding patients, Arany and Tyring, (1996) have observed high expression of the HPV *E7* gene. It has been reported that the E7 protein of high-risk HPVs is capable of inhibiting IFN-α inducible genes (Barnard & McMillan, 1999) and that HPV-16 and 18 patients who did not respond to IFN-α treatment showed elevated levels of the E7 protein (Schneider *et al.* 1987).

In addition to the observed inhibitory effects of high-risk HPV E7 protein levels on the IFN signalling pathway, the effects of the HPV E6 proteins on IFN signalling have been elucidated by Li *et al.* (1999). HPV-18 E6 has been found to bind Tyk2 with greater affinity than the HPV-11 E6 protein, thereby collapsing the Jak-STAT pathway and blocking IFN synthesis. These findings are consistent with the ability of HPV-18 E6, but not of HPV-11 E6, to inhibit the IFN-α signalling and helps explain the difference in susceptibility between high- and low-risk lesions to IFN treatment, it being one of the strongest antiviral and anti-proliferative cytokines.

Not only are the activities of IFN-α and IFN-β down-regulated in the presence of the high-risk HPV E6 and E7 oncoproteins, but recent discoveries have shown that the E5 and E7 proteins are capable of suppressing the major histocompatibility complex (MHC) class I heavy chain promoter activity and interfere with the transporter associated with antigen presentation (TAP)-1 and the latent membrane protein (LMP)-2 required for the correct presentation of antigens on the cell surface. Not only is the MHC class I, but also the MHC class II affected. It has been suggested that the E5 and E7 proteins also suppress the MHC class II antigen presentation. The E5 protein is said to interrupt the normal trafficking of endosomal proteins, while the overexpression of the E7 protein in Langerhans cells reduces their function as antigen presenting cells (Scheurer *et al.* 2005).

Once lymphocytes present at the site of infection are activated, they possess the capability to enter into the systemic circulation, thereby moving from one mucosal tissue to other places in the body, including different mucosal and glandular tissues. The sensitization of one part of the MIS will in turn stimulate other parts of the MIS and will ultimately elicit a systemic immune response once the innate immune system is breached. Once the adaptive immune response is triggered, antigen specific effector cells and their products are generated, specifically targeting the invading pathogen or the pathogen infected cells (Stern *et al.* 2000). *In vitro* serological examination using HPV virus-like particles (VLPs) has revealed a systemic response to HPV capsid proteins that is undoubtedly a consequence of exposure to the antigen. In naturally occurring virus infections, it is generally found that seroconversion to antibodies against the invading virus might take up to one year after HPV DNA has been detected in infected tissues. This holds true for infections by high-risk HPV types, but not the low-risk types. In this instance, seroconversion is closely followed after initial infection (Scheurer *et al.* 2005). A typical sequence of events takes place during the induction of an antibody-mediated immune response against HPV infection. The initial and rapid antibody response primarily consists of immunoglobulins M (IgM) and A (IgA). While the IgM response tends to disappear as quickly as it was induced, the IgA response was found to decline at a much slower rate. The overall disappearance of this initial immunoglobulin response is linked to the appearance of a more stable and longer lasting IgG antibody response (Scheurer *et al.* 2005). Altogether serum antibodies have been detected in female patients with anogenital warts (HPV-11) (Christensen *et al.* 1992; Rose *et al.* 1994), in 13-year-old-school girls (HPV-11) (Cubie *et al.* 1998), in HIV-infected women (HPV-16) (Marais *et al.* 2000; Williamson *et al.* 2002), in the oral fluid from women with cervical neoplasia (HPV-16) (Marais *et al.* 2001), in homosexual men (HPV-6, 16) (Hagensee *et al.* 1997), and in women (HPV-16) (Carter *et al.* 1996; Hagensee *et al.* 2000); (HPV-6, 16 and 18) (Carter *et al.* 2000), (HPV-16, 18, 31 and 45) (Wideroff *et al.* 1999) and (HPV-16, 18, 31, 33 and 45) (Marais *et al.* 2000).

Animal research has shown that high titres of systemic antibodies are induced when animals are injected with virus-like particles (VLPs) (Breitburd *et al.* 1995; Kirnbauer *et*

al. 1996; Suzich *et al.* 1995). These sera are capable of virus neutralisation, with antibodies recognizing conformation-specific epitopes of assembled VLPs (Zinkernagel, 1993), an aspect that is crucial to the development of potential human counterpart vaccines and is further discussed in detail in section 1.7.

1.7 PROPHYLACTIC IMMUNIZATION: THE ANIMAL MODELS

Naturally occurring PV infections in non-human species have been described throughout the history of papillomavirus research (zur Hausen H., 1996): Cottontail rabbit papillomavirus (CRPV), Rabbit oral papillomavirus (ROPV), Canine oral papillomavirus (COPV) and Bovine papillomavirus (BPV) all induce papillomas in their respective hosts.

HPVs are highly species-specific and the evaluation of potential prophylactic HPV vaccines would not be possible in animals. Thus the means of evaluating the efficacy of prophylactic vaccines *in vivo* is by vaccination of animals with their specific PV VLPs and subsequent challenge with live virus. The effectiveness of prophylactic vaccines in preventing PV infections has been studied using the CRPV (Breitburd *et al.* 1995; Christensen *et al.* 1991; Jansen *et al.* 1995; Lin *et al.* 1992), the COPV (Suzich *et al.* 1995) and the BPV (Kirnbauer *et al.* 1996) animal models. Animals were immunized with their PV-specific VLPs and then challenged with live virus. In addition, a review on animal models of papillomavirus pathogenesis has been compiled by Campo (2002), in which various animal models including the COPV, BPV-1, -2 and -4 and CRPV are described (Campo, 2002). It also touches on aspects not described in this thesis. Those include the first generation vaccines based on inactivated virus and the use of DNA vaccines as alternative therapeutic approaches for the treatment of already established infections. Among these are included the recent publication by Brandsma *et al.* (2004) in which the vaccination of rabbits with an adenovirus vector construct expressing the CRPV E2 protein is described to result in clearance of papillomas and infection. Furthermore, this thesis does not deal with the published research on immunotherapeutic alternatives targeting the HPV E1, E2, E6 and E7 proteins as described in these

publications (Baldwin *et al.* 2003; Stanley, 2003). It primarily focuses on the PV L1 protein for the use as a prophylactic vaccine against PV infection.

Breitburd *et al.* (1995), Christensen *et al.* (1996c) and Jansen *et al.* (1995) have shown that immunization of rabbits with either CRPV L1 or L1/L2 VLPs results in full protection upon challenge with infectious CRPV. Passive transfer of serum and IgG immunoglobulin isolated from serum conferred protection of non-vaccinated animals, meaning that protection is probably largely mediated by neutralising antibodies. Jansen *et al.* (1995) have confirmed that the antiserum from yeast-derived CRPV VLP vaccinated animals is capable of neutralising infectious virus *in vitro*. While these results established that VLP vaccination protects from cutaneous papillomavirus infection, it needs to be noted that critical differences exist between the cutaneous and mucosal immunity, important enough to validate a VLP vaccination approach in a mucosal papillomavirus animal system. BPV type 4 (BPV-4) induces papillomas of the alimentary canal mucosa (Campo *et al.* 1993) which can undergo malignant transformation and best resembles mucosal infections by their human counterparts. Kirnbauer *et al.* (1996) have reported a 100% and 71% protection of calves vaccinated with BPV-4 L1 VLPs and BPV-4 L1/L2 VLPs respectively, indicating that a VLP vaccination approach can protect from PV infections of the mucosa (Campo, 1995). Similar results describing the immunization with BPV-2 L1 and L2 fusion proteins and subsequent protection from challenge have been reported by Jarrett *et al.* (1991). COPV L1 VLP vaccination has shown to protect from COPV viral challenge, a PV that naturally infects the oral mucosa of dogs (Suzich *et al.* 1995).

The success achieved in these studies justifies the extensive clinical trials currently being carried out on human beings, as they are the only real model for testing the efficacy of potential HPV candidate vaccines (Campo, 2002; Hilleman, 2000).

1.8 PROPHYLACTIC VACCINE CHARACTERISTICS: NEUTRALISING ANTIBODIES

To elicit the desired antibody response, vaccination with native HPV L1 VLPs is critical. Generally PVs cannot be propagated in tissue culture, although thus far HPV types 11, 40, 59, 83 and MM7/LVX82 virions have been produced in the athymic mouse xenograft system (Christensen *et al.* 1997; Brown *et al.* 1998; Bryan *et al.* 2000; Lehr *et al.* 2003; McClowry *et al.* 2002). Given these restrictions, the only means of achieving an antibody immune response is by vaccination with antigenically identical HPV VLPs. Only under non-denaturing conditions will various conformation-specific epitopes be displayed on the outer surface of the particles to trigger the induction of high titre virus-neutralising immunoglobulins.

Antibodies that are capable of virus neutralisation can be divided into different sets (Booy *et al.* 1998). Antibodies that neutralise virus by inhibiting viral binding to the cell surface are grouped together in a different set to those that prevent infection without significantly inhibiting virion binding to the cell surface. It has been proposed that neutralising antibodies that do not prevent virus-cell-surface-receptor interactions, act in a way to prevent the capsid from uncoating and releasing the viral DNA into the nucleus. BPV-specific monoclonal antibody 5B6 has been shown to bind between capsomers, thereby cross-linking the capsid between at least two pairs of epitopes (Booy *et al.* 1998).

Christensen and Kreider (1991) have shown that infection by CRPV could be neutralised by monoclonal antibody (MAb) 5A that identifies a conformation-specific epitope on intact virions. Yuan *et al.* (2001) have evaluated the display of conformation-specific epitopes on COPV GST-L1 fusion and purified COPV L1 proteins with a rabbit polyclonal antiserum recognizing type-specific, conformation-dependent epitopes on both COPV virions and VLPs. The antiserum reacted strongly with both the L1 fusion and purified L1 proteins, which are incapable of assembling beyond the pentameric form, indicating that assembly beyond pentamers is not required for the correct display of conformation-dependent, neutralising epitopes.

However, complete denaturation of the L1 protein abolishes all capability to induce a neutralising antibody response (Ghim *et al.* 1991; Breitburd *et al.* 1995; Kirnbauer *et al.* 1992; Lin *et al.* 1992; Suzich *et al.* 1995), reemphasizing the importance of native L1 conformation and its recognition by immunoglobulins.

Various neutralising epitopes are present in the HPV-11 L1 major capsid protein (Christensen *et al.* 1990a, 1990b, 1992, 1994a, 1994b, 1995; Rose *et al.* 1998, 1999; Smith *et al.* 1995). Neutralising epitopes found throughout the L1 major capsid protein of PVs other than HPV-11 have been identified for BPV-1 (Christensen *et al.* 1993), BPV-1 & CRPV (Christensen *et al.* 1990a), HPV-16 & 18 (Christensen *et al.* 1996a), HPV-16, 31, 33, 45, 58 & 59 (Combita *et al.* 2002) and HPV-33 (Fligge *et al.* 2001), but are not restricted to the major capsid protein alone. Epitopes recognized by virus neutralising antibodies are found within the PV L2 minor capsid protein as well [BPV-4 (Gaukroger *et al.* 1996), HPV-16 (Kawana *et al.* 1998a; 1999), BPV (Roden *et al.* 1994b) and HPV-6, 16 & 18 (Roden *et al.* 2000)].

Neutralising antibodies have been found generally to be highly type-specific (Chen *et al.* 2000; Roden *et al.* 1996); however cross-reactivity of particular MAbs has been reported for HPV-6 and 11 (Christensen *et al.* 1994b; 1996b). It has been found that HPV-11 specific MAbs H11:A3.2 and H11:B2 could precipitate both HPV-6 and 11 L1 proteins, indicating that these two closely related HPV types (92.4% L1 protein sequence identity) share one non-neutralising and one neutralising epitope (Hines *et al.* 1994). The HPV-11 neutralising MAb H11:H3 does not cross-react with HPV-6.

HPV-31 and 45 VLPs can induce low levels of neutralising antibodies against HPV-33 and 18, but cross-neutralisation is less efficient than direct neutralisation, supporting the fact that sufficient differences in the L1 protein sequence not only define distinct PV genotypes, but also unique viral serotypes (Giroglou *et al.* 2001b; Heino *et al.* 1995; Rose *et al.* 1996).

The multitude of MAbs against HPV types 6 and 11 produced by the Christensen lab are listed in Table 1.3 below. Conformation-dependent and linear epitope-recognizing MAbs used throughout this study are highlighted in green.

Table 1.3: Summary of monoclonal antibodies against HPV types 6 and 11, including the nature of the epitope and the cross-reactivity potential.

Monoclonal antibody	Isotype	Neutralisation	Nature of Epitope	Cross-reactivity	References
H11:A3.2	IgG2a	No (HPV-11)	conformation-specific	Yes (HPV-11&6)	(Christensen *et al.* 1990b; 1996b) (Ludmerer *et al.* 1996) (Chen *et al.* 2000)
H11:B2	IgG2b	Yes (HPV-11)	conformation-specific	Yes (HPV-11&6)	
H11:F1	IgG2a	Yes (HPV-11)	conformation-specific		
H11:G5	IgG2a	Yes (HPV-11)			
H11:H3	IgG2b	Yes (HPV-11)	conformation-specific	No	
H6.A1	IgM	Unknown	conformation-specific	No	
H6.B10.5	IgG2b	Unknown	conformation-specific	No	
H6.C6	IgG2a	No (HPV-11)	linear (surface)	Yes (HPV-6&11)	
H6.D10	IgG3	No (HPV-11)	linear (buried)	Yes (HPV-6&11)	
H6.E51	IgG1	No (HPV-11)	linear (surface)	Yes (HPV-6&11&18)	
H6.F62	IgM	Yes (HPV-11)	conformation-specific	Yes (HPV-6&11)	
H6.H9	IgG2a	No (HPV-11)	conformation-specific	Yes (HPV-6&11)	
H6.I2	IgM	Yes (HPV-11)	linear (surface)	Yes (HPV-6&11)	
H6.J54	IgG2b	Yes (HPV-11)	conformation-specific	Yes (HPV-6&11)	
H6.K57	IgG2b	No (HPV-11)	conformation-specific	Yes (HPV-6&11)	
H6.L12	IgM	Yes (HPV-11)	conformation-specific	Yes (HPV-6&11)	
H6.M48	IgG1	Unknown	conformation-specific	No	
H6.N8	IgG1	Unknown	conformation-specific	No	

1.9 PROPHYLACTIC HPV VACCINES: EFFICACY TRIALS IN HUMANS

As described by Scheurer *et al.* (2005): 'Naturally occurring HPV infections at mucosal surfaces of the anogenital area are very poor in eliciting an immune response. A successful prophylactic vaccine would be the ultimate public health tool to immunize and reduce the exposure to the virus among any population by means of herd immunity.' The prerequisites for a HPV vaccine trial with respect to target population, behavioural changes, study group size, acceptability and cost-effectiveness have been discussed by Paavonen *et al.* (2000) and Lethinen *et al.* (2000, 2001). Based on these criteria, various HPV vaccine efficacy trials have been conducted and are described below.

Independent clinical trials have taken place to evaluate the efficacy of a potential HPV-11 L1 VLP vaccine (Brown *et al.* 2001; Emeny *et al.* 2002; Fife *et al.* 2004). A HPV-11

VLP vaccine was tested in 18 to 25 year old women, who tested negative for HPV-6/11 DNA sampled from various anogenital sites and showed no detectable serum antibodies (Brown *et al.* 2001; Gibbs *et al.* 2000). Women were to receive placebo or HPV-11 L1 VLP vaccine formulated on aluminium adjuvant on a random basis. Equal doses of vaccine formulations (10, 20, 50 or 100μg) and placebo were administered intramuscularly at enrolment, followed by two booster injections at 2 and 6 months (Brown *et al.*, 2001) and a 4th at 12 months (Emeny *et al.* 2002; Fife *et al.* 2004).

Serum collected before and after vaccination was evaluated for HPV-11 specific antibodies by competitive radioimmunoassay (cRIA), and for HPV-11 neutralisation (Brown *et al.* 2001). Brown *et al.* (2001) and Fife *et al.* (2004) recorded that no subject in the placebo group developed a measurable HPV-11 antibody titre after 7 months, but observed an apparent doubling on the geometric mean titres (GMTs) between the 10 and 20μg groups and the 50 and 100μg groups. The lower band of the 95% confidence interval (CI) of month 7 cRIA titres for the 20, 50 and 100μg groups exceeded 200 milli-Merck units/ml (mMU/ml); a titre that correlated with virus neutralisation.

Overall, the vaccination of college-age women elicited a vigorous HPV-specific immune response, not only indicated by the presence of IgM, IgA, IgG1 through IgG4 and IgE antibodies, but also proliferate T-cell responses (Emeny *et al.* 2002). Williams *et al.* (2002) have used 95 15-mer HPV-11 L1-specific peptides spanning the entire L1 molecule to analyze which particular epitopes were recognized by the prominent $CD4^+$ T-cell response. $CD4^+$ T-cells lines were established and examined for every blood donor. Results indicated that cells from every donor responded to at least 1 HPV-11 L1 peptide pool; recognition of 10 peptide pools by cells from one donor was recorded as well. HPV-11 L1 peptide pool recognition patterns were different among donor cells, with no single peptide pool being immunodominant; however it was found that HPV-11 L1 $CD4^+$ T-cell responses were cross-reactive with skin (HPV-1 through 4) and genital (HPV-6, 16 and 18) HPVs, where the number of amino acids differences between variant peptides and HPV-11 was inversely related to the strength of the cross-reactive proliferative response (Williams *et al.* 2002).

With regards to the "low-risk" papillomaviruses HPV-11 and HPV-6, another clinical trial has been conducted in which the efficacy of an HPV-6b VLP vaccine was evaluated. This trial, as reported on by Zhang *et al.* (2000), involved the recruitment of HPV-6b positive individuals with external genital warts. Subjects (27 females and 6 males, average age = 33) received intramuscular immunizations of either 1μg (n = 8), 5μg (n = 13) or 10μg (n = 12) of HPV-6b VLPs without adjuvant at 0, 4 and 8 weeks. Patients were observed for a 20-week period, during which serum was collected at two week intervals and warts were examined. Wart regression was defined as a loss of at least 50% of the wart area present at enrolment. Patients not showing wart clearance were offered further immunizations at weeks 12, 16 and 20.

Results indicate that 25 out of 36 individuals (76%) showed complete regression of warts over a period of 20 weeks. Of the remaining 8 individuals still showing residual disease, 5 had a substantial partial regression of more than 50%. All subjects were followed up on after a period of 9 months. None of the 25 subjects with initial complete clearance showed recurrence of the disease. Of the 8 individuals that initially showed partial regression, 3 individuals showed complete wart clearance, although such was achieved by destructive treatment in 1 individual. One of the remaining 5 individuals showed persistent disease whereas the other 4 showed substantial partial regression. The overall rate of wart regression was similar in individuals that had received the 1μg VLP dosage compared to those of the higher VLP dosage (5μg and 10μg).

Concluding remarks made by the authors supported the concept that HPV L1 VLPs are good candidates for both prophylactic and therapeutic vaccines against HPV infection, however cautioned that further investigations as to the therapeutic effect of VLPs on the natural history of genital warts had to be evaluated.

A doubled-blinded, randomized HPV-16 vaccine trial was conducted by Koutsky *et al.* (2002) and Fife *et al.* (2004), during which the efficacy of a HPV-16 L1 VLP vaccine was tested for the prevention of HPV-16 infection in women. Approximately 2400 women from 16 different centres throughout the US, aged between 16 and 23 years,

underwent gynaecological examination and serum analysis, before being admitted. 40μg of HPV-16 VLPs formulated on 225μg of aluminium adjuvant, in a total volume of 0.5ml was administered to the individuals within the study group, whereas the placebo group received only 225μg of adjuvant in a total volume of 0.5ml (Koutsky *et al.* 2002). Having undergone the identical screening procedure, a 109 women between the ages of 18 and 25 received three formulations of an HPV-16 L1 VLP vaccine of either 10, 40 or 80μg per injection (Fife *et al.* 2004). Intramuscular injections were administered at day 0, and at 2 and 6 months.

Follow-up results concluded that after the third 40μg dose, the geometric mean titre of HPV-16 antibodies was 1510 mMU/ml (95% CI) (Koutsky *et al.* 2002), whereas Fife *et al.* (2004) reported on a geometric mean antibody titres of 478.7 mMU/ml (10μg), 807.6 mMU/ml (40μg) and 732.3 mMU/ml (80μg) (95% CI) of women who had received the HPV-16 L1 VLP vaccine. Antibody titres of less than 6mMU/ml (95% CI, <6) were found among the group that had received the placebo (Koutsky *et al.* 2002).

An additional independent multi-centre clinical trial evaluating the efficacy of a bivalent HPV16/HPV-18 VLP vaccine has been conducted by Harper *et al.* (2004), who describe the assessment of a cohort of 4939 women from North (USA and Canada) and South America (Brazil). Furthermore, this study was conducted in two phases with the aim to evaluate the efficacy of the administered HPV-16/HPV-18 VLP vaccine against incident and persistent infections with HPV-16 and HPV-18 (phase I); and to determine the vaccine efficacy against cytological abnormalities and CIN (phase II). Out of the 4939 women, a final number of 1113 women were enrolled. Women eligible to enter into the first phase of this trial were healthy women aged between 15 and 25 years of age who displayed no abnormal cytology, high risk HPV DNA positivity and were seronegative for HPV-16 or HPV-18. Approximately half the women were randomly selected to be given the vaccine, whereas the other half were administered a placebo.

Women selected to receive the vaccine were given a 0.5ml dose formulated to contain a mixture of 20μg of HPV-16 and 20μg HPV-18 VLPs together with 500μg aluminium

hydroxide and 50μg 3-deacylated monophosphoryl lipid A. The placebo consisted of 500μg of aluminium hydroxide in a final dosage of 0.5ml. Participants received inoculations at 0 months, 1 month and 6 months. Women were assessed for HPV infection and cervical cytology over a period of 18 (phase I) and 27 (phase II) months. Results concluded that the HPV-16/HPV-18 VLP vaccine proved to be 91.6% efficacious against incident infection and showed to be 100% effective against already persistent HPV-16/HPV-18 infections (Harper *et al.* 2004). Results obtained from the second phase of this trial showed that the vaccine was 95.1% efficacious against persistent cervical infection with HPV-16/HPV-18 and 92.9% effective in reducing the cytological abnormalities resulting from infection with HPV-16 and HPV-18.

Overall Harper *et al.* (2004) concluded that this bivalent vaccine was efficacious in preventing incident and persistent cervical infections with HPV-16 and HPV-18. Not only was this vaccine well tolerated and appeared to be safe without any reports on adverse events, but it was shown to be effective against cytological abnormalities and lesions resulting from infection.

In summary, these clinical trials have proven that HPV-6b and HPV-11 VLPs (Brown *et al.* 2001; Emeny *et al.* 2002; Fife *et al.* 2004; Zhang *et al.* 2000), as well as HPV-16 and HPV-18 VLPs (Fife *et al.* 2004; Harper *et al.* 2004; Koutsky *et al.* 2002) can safely be administered to act as a highly efficacious prophylactic vaccine for the prevention of infection.

A different approach to HPV vaccination has been described by Kawana *et al.* (2003). This approach entailed the vaccination of volunteers with a synthetic peptide consisting of the HPV-6/16 cross-neutralising HPV-16 L2 epitope (amino acids 108 through 120) by means of nasal immunization (Kawana *et al.* 1999). Results indicated the generation of antibodies that reacted to both HPV-16 and HPV-52 (Kawana *et al.*, 2003), was well tolerated and displays the potential to be integrated and used in the development of broad-spectrum prophylactic vaccines.

2.0 THE POTENTIAL OF VACCINATION TO PROTECT FROM AND ERADICATE PAPILLOMAVIRAL DISEASE: AN AFRICAN PERSPECTIVE

The relatively high incidence of HPV infection worldwide emphasizes the importance of vaccine development and practical delivery thereof to large populations.

The costs of a potential HPV vaccination program have been modelled based on school-based HBV vaccination programs. The overall costs including vaccine materials, personnel and administration are estimated to amount to US$ 300, assuming a three-injection protocol with an additional booster shot (US$ 100) required every 10 years (Sanders *et al.* 2003). Costs of this magnitude are unaffordable to populations in developing countries, which evidently display the highest numbers of HPV-related cervical cancers; the leading cause of cancer deaths among women (Schiller *et al.* 1996).

Even if costs of implemented vaccination programs were to be state-subsidized, most of the infrastructure to produce vaccines on a large-scale is primarily found to be non-existent in African developing countries. Storage of vaccines, distribution and the administration by qualified medical professionals form part of a list of limitations that virtually make it impossible for populations of developing countries to be vaccinated.

Different vaccine production systems have been developed: these include prokaryotic- (bacteria), plant viruses- and eukaryotic- (yeast, mammalian cell cultures, transgenic plants) expression systems; alternative production systems that have been extensively used for the synthesis of a number of recombinant PV proteins as discussed in detail in section 2.1.

2.1 PRODUCTION SYSTEMS: A DETAILED DESCRIPTION WITH REGARD TO HPV VACCINES

2.1.1 Prokaryotic expression of PV antigens

Escherichia coli remains one of the most attractive expression systems, because bacteria can rapidly be grown up to great densities on relatively inexpensive substrates. A variety of cloning vectors have been engineered to facilitate the expression of foreign genes in *E. coli*, simultaneously ensuring cell death upon loss of the plasmid (Baneyx, 1999).

A variety of different HPV L1 proteins including HPV-6 and 16 (Banks *et al.* 1987); HPV-1a (Doorbar *et al.* 1987); HPV-6a (Jenison *et al.* 1988, Thompson *et al.* 1987) and HPV-16 (Kelsall *et al.* 1995; Zhang *et al.* 1998) as well as the HPV-16 L2E7E6 fusion protein vaccine (de Jong *et al.* 2002) have been expressed in *E. coli.*

Disadvantages of the system have been described by Baneyx (1999) including the *E. coli* mRNA instability and preferred codon usage. Nucleotides AGA and AGG code for the amino acid arginine, commonly found in eukaryotic organisms including *Saccharomyces cerevisiae*; they are hardly ever found in *E. coli* genes.

Protein misfolding and segregation into insoluble aggregates, known as inclusion bodies, often accompanies overproduction of the protein. This in turn affects the amount of biologically active product that can be harvested. Bacterial expression systems are inducible, but another factor to be considered is the *E. coli* strain of choice, which often affects protein expression (Li *et al.* 1997).

The expression of HPV-11 L1 in *E. coli* has been described by Li *et al.* (1997). Although the expression was good, they found the subsequent purification of the HPV-11 L1 protein somewhat more difficult. Only 50% of the L1 protein was soluble after cell lysis, with the other 50% remaining in the bacterial cell pellet. Partial recovery of the insoluble protein was achieved using a buffer containing 1M NaCl (Li *et al.* 1997).

To increase the quantity of assembly-competent pentamers derived from *E. coli*, HPV-11 L1 has been expressed as a GST fusion protein. Not only did this greatly facilitate the subsequent purification, but also aided in the separation of native L1 protein from the GroEL-bound complex (Chen *et al.* 2001b). GroEL release was accomplished by the addition of ATP-$MgCl_2$ to the cell lysate, followed by treatment with 3.5M urea. Under these conditions the authors obtained approximately 3 to 5mg of near homogeneous assembly-competent L1 protein per litre of bacterial cell culture (Chen *et al.* 2001b).

2.1.2 Live bacterial delivery systems

Bacille Calmette-Guérin, otherwise known as BCG, is a live, highly attenuated tubercle bacillus (*Mycobacterium bovis*), that is currently the most widely used vaccine against tuberculosis in the world (Edelmann *et al.* 1998). The potential to use BCG as a safe and effective multi-vaccine carrier has been explored.

Limited success has been achieved by expressing the HPV-6b L1 protein or the HPV-16 E7 protein by recombinant BCG (rBCG) (Jabbar *et al.* 2000), in comparison to the induction of cytotoxic T lymphocytes by a BCG heat shock HPV-16 E7 fusion protein (Chu *et al.* 2000). Prophylactic immunization with this fusion protein has shown that mice were protected against challenge and re-challenge with the tumour cell line TC-1. In therapeutic use, palpable tumours showed $CD8^+$-dependent regression, indicating the potential of this fusion protein for immunotherapy in humans.

Other live bacterial systems, including *Salmonella typhimurium* and *Listeria monocytogenes* have been engineered to deliver various antigens. Recombinant *S. typhimurium* expressing HPV-16 VLPs or HPV-16 E7 epitope integrated into the Hepatitis B virus core antigen have shown to induce moderate immune responses against HPV-16, a fact that could possibly be explained by a competition for immunodominance between the recombinant proteins with native *Salmonella* antigens in the host (Benyacoub *et al.* 1999; Londoño *et al.* 1996). Recombinant *L. monocytogenes*

expressing the CRPV-E1 protein has shown 77% protective immunity and tumour regression in rabbits after challenge with infectious CRPV (Jensen *et al.* 1997).

2.1.3 Eukaryotic expression of PV antigens

Numerous reports have described the stable expression of HPV major and minor capsid proteins in *Saccharomyces cerevisiae* (Angeletti *et al.* 2002; Buonamassa *et al.* 2002; Cook *et al.* 1999; Fife *et al.* 2004; Hofmann *et al.* 1996; Neeper *et al.* 1996; Rossi *et al.* 2000; Sasagawa *et al.* 1995) as well as the expression of merozoite surface protein 1 from *Plasmodium falciparum*, a malaria vaccine antigen (Brady *et al.* 2001), and the external glycoprotein encoded by the HIV-2 *env* gene (Zhang *et al.* 2001) in the methylotrophic yeast *Pichia pastoris*.

The expression of HPV-11 L1 protein in *S. cerevisiae* had initially been reported to be very low (Neeper *et al.* 1996). A truncation of the HPV-11 mRNA was found to be the reason for the reduced expression levels. Complete synthetic reconstruction of the entire HPV-11 *L1* gene based on the HPV-6 *L1* gene resulted in a 7-fold increase in the production of HPV-11 VLPs. The HPV-6/11 hybrid gene was used for protein expression by Cook *et al.* (1999). Galactose induction of this expression system ensured high cell densities, before expression of the heterologous L1 protein. The advantages over bacterial or mammalian expression systems have been summarized as follows. Not only did it allow growth of large amounts of *S. cerevisiae* in relatively simple culture medium, but unlike protein expression in *E. coli*, large amounts of correctly folded and soluble recombinant protein, free of contamination by toxins or infectious viruses, could be harvested by microfiltration and cation-exchange chromatography. Recombinant L1 protein accounted for approximately 15% of the total soluble protein in yeast cell lysate (Cook *et al.* 1999). VLPs were indistinguishable from native virions and were able to induce a variety of virus-specific neutralising antibodies. Antibodies directed against conformation-specific epitopes were found in sera from Balb/c mice immunized with *S. cerevisiae*-derived HPV-6/16 L1 and L2 hybrid VLPs, concluding that co-expression of

different L1 capsid proteins may provide new tools in the generation of antibody responses against multiple HPV types (Buonamassa *et al.*, 2002).

2.1.4 Insect cell-derived PV antigens

Different baculovirus expression vectors facilitate the reliable and versatile production of recombinant proteins in insect cells. The production of baculovirus particles results in budding from the cell, after which new cells are infected. The ability to directly ligate foreign genes into the baculovirus expression vectors has made it an attractive way to express foreign proteins (Possee, 1997). A variety of PV VLPs have been produced by recombinant baculovirus expression in insect cells. These include, amongst others, VLPs from COPV (Suzich *et al.* 1995), CRPV (Breitburd *et al.* 1995; Christensen *et al.* 1996c), HPV-16 (Ishii *et al.* 2003) and HPV-11 (Christensen *et al.* 1994a; Rose *et al.* 1999).

Although the production of recombinant proteins in tissue culture remains one of the most attractive and time efficient methods, the baculovirus expression system associated limiting factor is the post-translational modification of proteins, showing marked differences between insect and mammalian cells. Fang *et al.* (2000) have compared post-transcriptional differences between recombinant Vaccinia virus and insect cell-derived HPV-6b L1 protein and have noted a higher degree of phosphorylation in proteins derived from baculovirus expression. Although a number of reports have established that insect cell-derived HPV VLPs are morphologically and antigenically indifferent from virions, the varying degree of post-transcriptional modifications should be considered if proteins are destined for human vaccination.

2.1.5 Chimaeric VLPs

The use of virus-like particles to present foreign epitopes to the immune system is relatively new for vaccine development against HPV infection. Chimaeric VLPs have been engineered to deliver a variety of different HPV epitopes. Factors to be considered when engineering chimaeric VLPs include the preservation of the particle integrity and

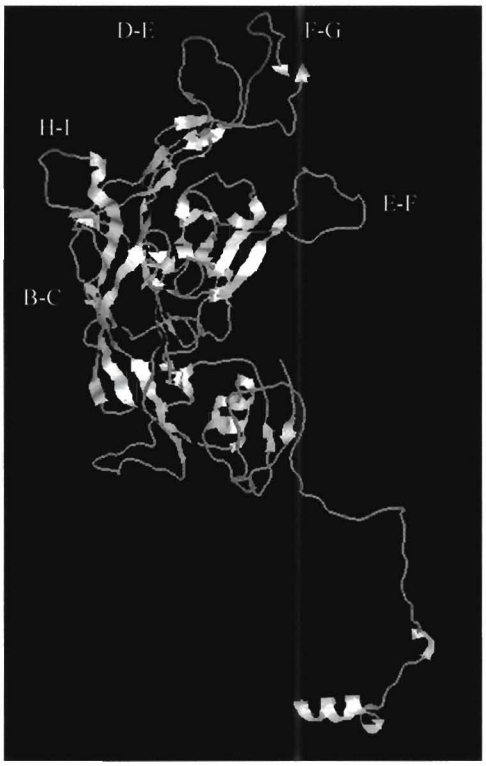


Figure 1.9: Various foreign epitopes have been introduced into the loops of the PV L1 backbone. These are as follows: Insertion of the Hepatitis B core antigen (DPASRE) at HPV-16 L1 positions 56/57, 140/141, 179/180, 266/267, 283/284 and 352/353 indicated in yellow (Sadeyen *et al.* 2003).
Insertion of the HIV-1 gp41 envelope glycoprotein epitope (LELDKWAS) at HPV-16 L1 positions 286/287, replacement of amino acids 281-287 and fused to the C-terminus, indicated in green (Slupetzky *et al.* 2001).
The same HIV epitope was inserted at BPV-1 *L1* positions 133/134, and replaced amino acid 282-286 and 351-355 indicated in orange (Slupetzky *et al.* 2001).
Insertion of the HPV-16 L2 cross-neutralising epitope (LVEETSFIDAGAP) (Kawana *et al.* 1999) into the following regions of the HPV-16 *L1* backbone, thereby replacing the following regions: amino acids 174-186, 131-143, 431-443, 414-426 and 81-93 (Varsani *et al.* 2003).

However, identification of mutation-permissive regions within the PV capsid that do not alter the overall immunogenic and neutralisation capabilities support the potential of virus particles as alternative vaccine carriers (Zinkernagel & Hengartner, 1997).

The potential of engineering the PV L2 protein while not altering the PV L1 protein has been explored (Greenstone *et al.*, 1998). Fusion of the full-length HPV-16 E7 gene to the C-terminus of either the full-length HPV-16 L2 or BPV-1 L2 gene, followed by co-expression of these chimaeric L2 proteins with their respective L1 counterparts in insect cells resulted in the assembly into VLPs. The HPV-16 L1/L2-HPV-16 E7 chimaeric VLPs showed full protection of mice against tumour challenge, indicating that prophylactic VLP-based vaccines might increase in therapeutic potential by presenting non-structural proteins to the immune system.

Christensen *et al.* (2001) have constructed chimaeric VLPs by rearrangement of L1 regions from both HPV-11 and 16 and have found that by replacing the hypervariable F-G and H-I loops from HPV-11 with the those of HPV-16 yielded a hybrid VLP that resulted in the stimulation of neutralising antibodies to both viruses upon immunization

maintenance of the ability to interact with cell surface receptors. Müller *et al.* (1997) have found fusion of 30 amino acids from the influenza matrix protein to the C-terminus of HPV-16 L1 protein only produced a small number of intact VLPs. However, Liu *et al.* (2000) have replaced the 23 amino acid carboxyl-terminal of the BPV-1 L1 major capsid protein with an artificial "polytope" containing known HPV-16 E7 protein, HIV gp120 P18, Nef, reverse transcriptase (RT) cytotoxic T cell epitopes and a HPV-16 E7 linear B epitope. When expressed by recombinant baculovirus, chimaeric L1 protein assembled into particles that were found to be highly immunogenic upon immunization of mice, eliciting a PV L1 antibody response together with cytotoxic T lymphocyte (CTL) precursors against the incorporated epitopes. The stability of chimaeric VLPs not only depends on the length of the insert, but also on the nature of the inserted foreign sequence (Liu *et al.* 2000b). Various other reports have shown that replacement of the HPV-16 L1 C-terminus with approximately 60 amino-terminal HPV-16 E7 residues (Schafer *et al.* 1999) or C-terminal fusions thereof to the L1 protein (Wakabayashi *et al.* 2002), are capable of inducing CTLs and prevent tumour formation. A number of different epitopes have been inserted into the different loop regions of the PV L1 monomer, potentially resulting in their display on the surface of VLPs upon particle assembly (Figure 1.9). Results indicated that insertion of epitopes resulted in either loss or change of function for immunogenic capsid regions, indicating that surface loops act as neutralisation determinants (Sadeyen *et al.* 2003; Slupetzky *et al.* 2001, Varsani *et al.* 2003)

of rabbits. This represents the possibility of designing prophylactic VLP vaccines that could potentially target and neutralise unrelated HPV types.

2.1.6 Live viral vector-encoded PV antigens

Different human viruses have been engineered to act as delivery systems of foreign genes and have displayed wide application in live recombinant vaccine applications. Virus isolates belonging to the species *Vaccinia virus* (VV), the prototypic member of the *Orthopoxvirus* genus of the *Poxviridae* family, have been engineered to be replication-incompetent in humans. Two highly attenuated Vaccinia virus strains, NYVAC and modified vaccinia virus Ankara (MVA), have been approved for use without safety precautions. These virus vectors can stably integrate up to 25 kilobases of foreign DNA without the loss of infectivity. Integrated genes are expressed in the cellular cytoplasm of a variety of vertebrates (Carroll *et al.* 1997).

Recombinant poxviruses induce both innate and systemic immune responses and are able to elicit strong cytotoxic T-cell responses, a potential that has been explored in the development of both prophylactic and therapeutic HPV vaccines. Recombinant Vaccinia virus-derived PV L1 and L2 VLPs have shown to induce an antibody response against BPV-1 (Zhou *et al.* 1993), CRPV (Lin *et al.* 1992), HPV-1 (Hagensee *et al.* 1993; 1995), HPV-16 (Zhou *et al.* 1991, 1992) and HPV-33 (Volpers *et al.* 1994).

Therapeutic use of recombinant vaccinia virus has the advantage that target antigens are directly processed and presented by APCs, resulting in the stimulation of a MHC class I restricted CTL response (Adams *et al.* 2001). Boursnell *et al.* (1996) have developed a recombinant Vaccinia virus, based on the Wyeth strain, which had been used for large scale smallpox vaccinations in the US and had a long safety record. This encoded modified forms of the HPV-16 and 18 E6 proteins, and protected against tumour challenge. Similar results focusing on VV-encoded HPV-16 E2, E6 and E7 have been reported (Davidson *et al.* 2001; Lamikanra *et al.* 2001; Meneguzzi *et al.* 1991; Nindl *et al.* 1996).

Adenoviruses are double-stranded DNA viruses, commonly found in upper respiratory infections in humans. Advantages as vectors include high titre production in culture, the ability to infect a wide variety of postmitotic cell types and the ability to accommodate up to 8 kb of foreign DNA, which have favoured the use of manipulated adenoviruses as vectors for the transfer and/or expression of foreign genes (Kovesdi *et al.* 1997).

Recombinant adenovirus-derived vectors have been used for the expression of Hepatitis B virus (HBV) surface antigens (Morin *et al.* 1987; Tacket *et al.* 1992), Herpes simplex virus 2 (HSV-2) glycoprotein B (Prevec *et al.* 1989) and HIV antigens (Natuk *et al.* 1993, 1994). Their potential use as vectors for therapeutic vaccines against PVs has been demonstrated by Liu *et al.* (2000a) and Brandsma *et al.* (2004), in which adenovirus-delivered HPV-16 E5 and CRPV E2 showed protection form tumour formation and resulted in clearance of papillomas and infection, respectively. Further, Liu *et al.* (2005) have explored the prophylactic use of virus-vector-based vaccines and have shown that a recombinant adeno-associated virus vaccine elicited strong levels of neutralising antibodies against HPV-16. Reuter *et al.* (2002) have shown that a Vesicular stomatitis virus (VSV) live recombinant virus expressing the CRPV L1 protein protects rabbits against challenge with CRPV.

The application of virus-derived vector vaccines for large scale human vaccination programs is still in the infant stages. Although studies in animals have been successful, a number of concerns still need to be addressed. These include the potential pathogenicity in young children and in the ever increasing numbers of immunocompromised patients due to HIV (Schiller *et al.* 1996). Recombinant virus production requires stringent safety procedures, the foremost being the production of clinical-grade vaccines for either application in therapeutic or prophylactic use.

2.1.7 Plant-derived PV antigens

An ever-increasing demand for large amounts of safe, inexpensive, recombinant proteins to protect from human disease has led to a focus on the potential use of transgenic plants

as an alternative source for their safe and rather inexpensive expression (Fischer *et al.* 1999). As in other eukaryotic expression systems, discussed in sections 2.1.3 and 2.1.4, plant expression systems produce fully-folded functional molecules, and mostly complete the appropriate post-translational modifications required for the optimal biological activity of the recombinant proteins.

The local production of transgenic plants is applicable to settings in third world countries, yielding human pathogen free, biomedically required products; and using the existing infrastructure would drastically reduce storage, transportation and distribution costs (Ma *et al.* 2003). Transgenic plants provide a cheap source of recombinant proteins (Giddings *et al.* 2000), estimated to be almost 50 times less than production of the identical protein in *E. coli* (Kusnandi *et al.* 1997).

The genetic manipulation of plants for the production of recombinant proteins has resulted in beneficial applications in both developed and developing countries. Table 1.4 lists various recombinant protein subunit vaccines and MAbs that have thus far been produced in transgenic plants.

Table 1.4: Overview of recombinant antibodies and protein subunit vaccines expressed in plants.

Target	Host	Protein	Expression system	Reference
Indirect thrombin inhibitors	**Ethiopian mustard, Tobacco, oilseed**	Human hirudin variant 2	AMT	(Parmenter *et al.* 1995) (Boothe *et al.* 1997)
Gaucher's disease	**Tobacco**	Glucocerebrosidase	AMT	(Cramer *et al.* 1996)
Autoimmune diabetes	**Potato**	*Vibrio cholerae* toxin B subunit-human insulin fusion	AMT	(Lam *et al.* 2000) (Arakawa *et al.* 1998b)
		Glutamic acid decarboxylase	AMT	(Ma *et al.* 1997)
Cholera and *E. coli* Diarrhea	**Tobacco / potato**	*E. coli* heat labile enterotoxin (LT-B)	AMT	(Mason *et al.* 1998) (Tacket *et al.* 1999) (Richter *et al.* 2000) (Lauterslager *et al.* 2001) (Haq *et al.* 1995)
	Maize	Cholera toxin B subunit/insulin fusion		(Streatfield *et al.* 2001)
	Potato	Cholera toxin B subunit	AMT	(Arakawa *et al.* 1998b)
		Cholera toxin subunit B & A2	AMT	(Arakawa *et al.* 1998a) (Yu *et al.* 2001)

Target	Host	Protein	Expression system	Reference
Dental caries	**Tobacco**	*Streptococcus mutans* surface protein SpaA	AMT	(Tacket *et al.* 1999)
Foot and mouth disease virus	**Blackeyed bean**	Foot and mouth disease virus epitope (VP1)	CPMV	(Tacket *et al.* 1999) (Beachy *et al.* 1999)
	Arabidopsis thaliana		AMT	(Carrillo *et al.* 1998)
	Alfalfa		AMT	(Wigdorovitz *et al.* 1999a) (Wigdorovitz *et al.* 1999b)
	Tobacco		TMV	
Hepatitis B virus	**Tobacco**	Hepatitis B surface antigen	AMT	(Mason *et al.* 1992) (Thanavala *et al.* 1995) (Kumar *et al.* 2003)
	Potato	Hepatitis B core antigen	AMT	(Richter *et al.* 2000)
	Tobacco		AMT	(Tsuda *et al.* 1998)
Hepatitis C virus	**Tobacco**	HVR1/CTB fusion protein	TMV	(Nemchinov *et al.* 2000)
Human immunodeficiency virus	**Tobacco / blackeyed bean**	HIV epitope (gp 120)	CPMV / AMT	(Doran, 2000) (Mushegian *et al.* 1995)
	Cowpea	HIV epitope (gp 41)	CPMV	(Brennan *et al.* 1999)
	Tobacco	p24	TBSV AMT	(Zhang *et al.* 2000) (Zhang *et al.* 2002)
	Tobacco	HIV epitope (gp 41)	PVX AIMV / TMV	(Marusic *et al.* 2001) (Yusibov *et al.* 1997)
	Tobacco	α-trichosanthin	TMV	(Kumagai *et al.* 1993)
Human papillomavirus	**Tobacco** **Potato**	HPV-16 *L1*	AMT	(Warzecha *et al.* 2003) (Varsani *et al.* 2003) (Biemelt *et al.*, 2003) (Liu *et al.* 2005)
Infectious bronchitis virus	**Potato**	S1 glycoprotein	AMT	(Zhou *et al.* 2003)
Norwalk virus	**Tobacco / potato**	Coat protein	AMT	(Dixon *et al.* 1997)
	Potato	Capsid protein	AMT AMT	(Mason *et al.* 1996) (Tacket *et al.* 2000)
Pseudomonas aeruginosa	**Cowpea**	Outer membrane (OM) protein F epitope - CPMV coat protein fusion	CPMV	(Brennan *et al.* 1999)
		Linear B-cell epitope from OM- CPMV coat protein fusion		(Brennan *et al.* 1999)
Rabbit hemorrhagic disease virus	**Tobacco** **Potato**	VP60	PPV AMT	(Fernandez-Fernandez *et al.* 2001) (Castanon *et al.* 1999)
Rabies	**Tomato**	Rabies virus glycoprotein	AMT	(McGarvey *et al.* 1995)
	Tobacco	Rabies virus B-cell epitope	AIMV / TMV	(Yusibov *et al.* 1997)
Rinderpest virus	**Tobacco**	Rinderpest virus hemagglutinin (H) protein	AMT	(Khandelwal *et al.* 2003)
Rubella (measles)	**Tobacco** **Lettuce** **Rice**	MV hemaglutinin protein		(Webster *et al.* 2002)

Target	Host	Protein	Expression system	Reference
Staphylococcus aureus	**Cowpea**	D2 peptide of fibronectin-binding protein B - CMPV coat protein fusion	CPMV	(Brennan *et al.* 1999)
		D2 peptide of fibronectin-binding protein B - CMPV & PVX coat protein fusions	CPMV / PVX	(Brennan *et al.* 1999)
Swine-transmissible gastroenteritis coronavirus	***Arabidopsis thaliana***	S glycoprotein	AMT	(Gomez *et al.* 1998)
	Maize	TEGV spike protein		(Streatfield *et al.* 2001)
	Potato			(Gomez *et al.* 2000)
HSV-2	**Soybean**	Monoclonal antibody		(Zeitlin *et al.* 1998)
Human carcinoembryonic antigen	**Tobacco**	Monoclonal antibody	AMVI	(Vaquero *et al.* 1999)
Streptococcus mutans Streptococcus sobrinus	**Tobacco**	Monoclonal antibody to streptococcal antigen (SA) I/II cell surface adhesion molecule	AMT	(Ma *et al.* 1995)
Globodera rostochiensis (nematode)	**Tobacco**	Monoclonal antibody to subventral gland proteins	AMT	(Stevens *et al.* 2000)

AMT: Agrobacterium-mediated transformation; **AMVI**: Agrobacterium-mediated vacuum infiltration; **AIMV**: Alfalfa mosaic virus; **CTB**: cholera toxin subunit B; **CPMV**: Cowpea mosaic virus; **PBT**: particle bombardment technique; **PPV**: Plum pox virus; **PVX**: Potato virus X; **TBSV**: Tomato bushy stunt virus; **TMV**: Tobacco mosaic virus

Three publications have described the production of HPV subunit antigens in transgenic plants (Table 1.4), also the focal point of this thesis. Varsani *et al.* (2003) have explored the possibility and feasibility of expressing the HPV-16 L1 protein in transgenic *Nicotiana tabacum* cv. Xanthi. Two forms of the gene under investigation for suitable expression in transgenic plants included the full-length HPV-16 *L1* gene and a truncated version lacking the nuclear localization signal (NLS) located at the carboxyl terminus (L1Δ483). Biemelt *et al.* (2003) investigated the expression of the HPV-16 *L1* gene in *N. tabacum* cv. Samsun NN and *Solanum tuberosum* cv. Solara, or potato. Plants were transformed with the wild-type HPV-16 *L1* gene and with modified versions of the *L1* gene in which the codon composition had been changed to best suit the codon preference for a plant and human cellular environment. A third publication by Warzecha *et al.* (2003) has described the expression of a plant codon optimized HPV-11 *L1* gene in transgenic potato plants.

In the quest to explore this alternative HPV subunit production strategy, all three publications have raised relevant issues associated with the expression of foreign proteins in plants.

Despite successfully regenerating transgenic plants that showed integration of the wild-type HPV-16 *L1* (full-length and truncated) genes, Varsani *et al.* (2003) could show only low levels of protein expression despite confirmation of RNA expression. Accumulation of both proteins was below detection limits so effort was made to concentrate the expressed proteins. Once concentrated by centrifugation, characterization of the plant-derived particles using characterized monoclonal antibodies revealed that the plant-produced L1 proteins retained appropriate antigenicity compared to insect cell-produced protein. Electron microscopy showed that transgenically-expressed full-length L1 assembled into particles that resembled insect cell-derived particles in both size and structure. In contrast, the truncated version of the HPV-16 *L1* gene did not result in the assembly of full-sized particles, but predominantly pentameric capsomers. This distinct difference has let Varsani *et al.* (2003) to speculate that the full-length HPV-16 L1 protein reached higher concentrations in the nucleus than the NLS^- version did outside it, thus favouring particle assembly for the former: higher concentration of free L1 protein drives assembly into capsomers which in turn results in the formation of VLPs.

Overall, their yield of transgenic plant-derived HPV-16 L1 protein was calculated to be between 2 and 4μg per kilogram. Its effectiveness as a potential vaccine antigen was evaluated by the immunization of rabbits, and the HPV-16 L1 plant-derived particles elicited a weak immune response, although in retrospect it was mentioned that the injected dose (0.1μg) was probably too low.

Biemelt *et al.* (2003) found that expression of the wild-type HPV-16 *L1* gene in transgenic tobacco plants was below the detection limit of approximately 5ng/ml of L1 protein. Their attempts to increase protein expression levels in transgenic plants led to the codon optimization of the *L1* gene to best suit the codon usage for *Solanum tuberosum*, and the codon usage in mammalian cells.

The *L1* wild-type gene, the plant codon optimized gene (*L1p*) and the humanized gene (*L1h*) were introduced into tobacco plants. *L1p* transgenic plants produced no detectable L1 protein: total RNA was isolated from transgenic lines and examined by Northern blot. *L1p*-specific mRNA could not be detected, but instead results revealed the presence of putative mRNA degradation products. In contrast, analysis of the *L1h* transgenic plants indicated that L1 protein could be detected using antibodies. Following mRNA transcript analysis of these plants it became apparent that again the RNA was subject to degradation, but not to the same degree of severity as in the *L1p* transgenic plants.

The authors attributed the failure of expression of the *L1* gene to failure of the translation machinery to recognize the *L1* transcript. In order to improve the protein translation efficiency, they altered both the *L1h* and *L1p* expression constructs by including the TMV Ω translational enhancer. Characterization of *L1p* transgenic plants revealed no changes in expression levels; however a significant increase in protein levels was detected in *L1h* plants. Biemelt *et al.* (2003) estimated the L1 protein concentration in transgenic plants to be approximately 0.5% of the total soluble protein.

While these result were achieved in transgenic tobacco lines, Biemelt *et al.* (2003) further explored the possibilities of expressing the TMV Ω – *L1h* construct in potato, a plant more suitable for their intended feeding experiments. This resulted in the accumulation of L1h protein accounting for approximately 0.2% of the total soluble tuber protein. Of particular importance is the fact that all of the plant-derived VLPs (tobacco and potato) resembled those derived from insect cells, indicating that the L1 protein was not significantly modified.

Plant-derived purified VLPs as well as insect cell-derived VLPs were injected into two groups of six mice (40ng each), followed by evaluation of the antibody response. The overall outcome according to Biemelt *et al.* (2003) was that plant-derived VLPs were as immunogenic as their insect cell-derived counterparts. From feeding experiments, the authors have concluded that oral immunization of mice with 5g of potato tuber containing about 60μg of L1 led to a transient short-lived immune response. However, this response

could be boosted by subimmunogenic vaccination with 20ng of purified VLPs, indicating that orally ingested plant-derived VLPs could prime a humoral antibody response against the L1 protein.

These authors also found that a significant portion of the plant-derived L1 protein exists in form of viral capsomeres rather than as VLPs, again indicating an equilibrium reaction such as described by Varsani *et al.* (2003), in which a significant amount of free monomeric L1 protein is required to drive the equilibrium reaction towards the assembly of VLPs.

Liu *et al.* (2005) have also recently reported on the expression of HPV-16 L1 protein in tobacco: they used a plant codon-optimized gene to obtain transgenic expression levels of around 0.05% total soluble protein. While they did not show immunogenicity of the protein, it did assemble into 55 nm particles and agglutinated erythrocytes.

Warzecha *et al.* (2003) reported on the expression of the HPV-11 *L1* gene in transgenic tobacco and potato plants. They explored the expression of full-length and truncated (NLS^-) forms of a plant-codon optimized HPV-11 *L1* gene. Using GFP-L1 fusion proteins in transient expression experiments, they confirmed that the full-length L1 protein was entirely localized within the nucleus of the plant cell, whereas the protein lacking the nuclear localization signal was found abundantly in the cytoplasm. However, their attempts to regenerate transgenic potato plants were successful only in the case of the NLS^- version, which only expressed at levels around 20ng/g of tuber. Again, 5g doses of this tissue delivered by feeding were sufficient to weakly prime an immune response in mice.

The research reviewed above has shown that the production of HPV antigens in plants is not a straightforward proposition: all the publications illuminated some of the challenges that might be involved, and indicated that it is probably a gene-dependent and strictly empirically determined process. Among the factors that might influence the expression of an introduced *L1* gene are post-transcriptional and post-translational modification effects: RNA stability and RNA gene silencing are post-transcriptional; however, if the RNA

transcript is not degraded, another set of hurdles needs to be overcome. These include factors such as the ability of the cellular machinery to recognize the transcript; correct folding of the protein, and protein turnover. Among these factors affecting the final protein production and with special emphasis on the plant cellular environment are plant proteases that potentially could severely degrade the final product.

There are a number of techniques that might help overcome some of the problems associated with expression of foreign proteins in plant systems. For example, Kapila *et al.* (1997) have described the use of recombinant *Agrobacterium* for transient protein expression in intact leaves by vacuum infiltration, and have described the advantages of transient over transgenic expression as follows: "The first and foremost advantage of transient expression is the up to a 1000-fold higher level of gene expression which can be measured shortly after DNA delivery. Gene expression is not affected by an unfavourable insertional position within the plant genome as might be in transgenic plants. Furthermore, transient gene expression can be applied to plant systems that present difficulties in the regeneration of transgenic plants and have been explored by means of electroporation, particle bombardment and microinjection" (Kapila *et al.* 1997). Not only does this technology provide an alternative, but it has successfully been combined with the more recently developed technology of targeting the expressed proteins to various organelles within plant cells.

The use of stable plant viral vectors for transient expression of various genes has been thoroughly reported on. Various examples including the use of Alfalfa mosaic virus, Cowpea mosaic virus, Plum pox virus, Tomato bushy stunt virus and Tobacco mosaic virus are listed in Table 1.4. Moreover, Varsani (2003) [AD Varsani (2003). Development Of Candidate Human Papillomavirus Vaccines. PhD Thesis, University of Cape Town, June 2003] in our laboratory has successfully used a recombinant Tobacco mosaic virus vector in to express the full-length and NLS^- versions of the wild-type HPV-16 *L1* gene in *Nicotiana benthamiana.* While expression levels were still low, they represented a 10-fold increase (to ± 40 μg/kg) over the best-case transgenic expression of

the same genes. Similar antigenicity and immunogenicity data was obtained as for the transgenically expressed L1 protein.

Another rapidly expanding field of research is the transformation of microalgae (Leon-Bañares *et al.* 2004). Stable nuclear transformations of eukaryotic chlorophytes, diatoms and dinoflagellates have been reported. Nuclear and chloroplast transformation of freshwater alga *Chlamydomonas reinhardtii* is the first and best-studied transformation system of the chlorophytes or green algae group, providing valuable information for the potential transformation of other microalgae. Transgenic microalgae display the possibility of enhancing the productivity of traditional algal compounds; however their application for the production of pharmaceutical compounds in large-scale bioreactors could have unlimited potential (Leon-Bañares *et al.* 2004).

2.2 PROJECT AIMS

HPV-11 is causally associated with genital warts and laryngeal papillomas, and infections of the upper respiratory mucosa and the upper digestive tract may be associated with oesophageal cancer. These associations as well as the increased morbidity, severity and higher incidence levels of genital warts in immunocompromised individuals has made the virus a key target for the development of an efficacious vaccine. The estimated costs for established vaccine candidates of around US$ 300 per vaccinated individual largely remain unaffordable to people in developing countries, reemphasizing the need for the development for a more cheaply produced, efficacious and safe alternative vaccine against HPV-11.

Although expression of HPV-16 and HPV-11 L1 protein in plants has been achieved, it has proven to be very difficult to obtain high protein expression levels for HPV-11 in particular. The principal aim of this study, therefore, was to help develop a safe and inexpensive plant-derived subunit vaccine against HPV-11. I used *Agrobacterium tumefaciens*-mediated transformation in two different transgenic expression systems -

Nicotiana tabacum cv. Xanthi and *Arabidopsis thaliana* - to do comparative studies on the expression of the HPV-11 *L1* gene, and to determine heritability of the transgene.

The secondary aim of this investigation was a proof-of-concept study for plant-produced HPV vaccines: this involved the transgenic expression of the CRPV *L1* gene in *Nicotiana tabacum* cv. Xanthi, and the vaccination of rabbits with plant-produced CRPV L1 protein followed by live virus challenge.

To overcome possible yield limitations in transgenic expression of HPV-11 L1 protein, I also explored another protein expression possibility: this involved the transient expression of HPV-11 and CRPV L1 proteins in a genetically engineered TMV-based vector system in *Nicotiana benthamiana*.

Altogether the work presented in this thesis describes the step-by-step developmental process of what is hoped to become an alternative procedure for the production of a potential candidate vaccine, a safe and cheap subunit vaccine that is desperately needed to combat the tide of ever increasing HPV-11 infection rates in impoverished regions of the globe.

Chapter 2

Expression of HPV-11 *L1* in transgenic *Arabidopsis thaliana* (ecotype Columbia) and *Nicotiana tabacum* cv. Xanthi

Contents

ABSTRACT

I have investigated the possibility and feasibility of producing the HPV-11 L1 major capsid protein in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* as a potential source for an inexpensive subunit vaccine.

Transformation of plants was only achieved with the HPV-11 *L1* gene which had the C-terminal nuclear localization signal (NLS^-) removed and not at all with the full-length gene. The HPV-11 *L1 NLS*$^-$ gene was stably integrated and inherited through consecutive generations of transgenic plants. Plant-derived HPV-11 L1 protein was capable of assembling into virus-like particles (VLPs), although resulting particles displayed a pleomorphic phenotype and appeared to encapsidate nucleic acid. Characterization of the plant-derived HPV-11 L1 NLS^- particles showed that neutralising surface-linear and conformation-specific monoclonal antibodies were capable of binding the *A. thaliana* plant-derived particles. Although a lesser degree of binding was observed in the *N. tabacum* protein extract, these results suggest that plant-derived and insect cell-derived VLPs displayed similar antigenic properties. A yield of up to 11mg/kg of HPV-11 L1 NLS^- protein was harvested from transgenic *A. thaliana* plants, whereas *N. tabacum* plants only yielded 2.2mg per kilogram of fresh leaf material.

Immunization of New Zealand white rabbits with plant-derived HPV-11 L1 NLS^- protein extract, calculated to contain up to approximately 50µg of HPV-11 L1 NLS^- protein, induced an antibody response that predominantly recognized insect cell-produced HPV-11 L1 NLS^- and not HPV-11 L1 NLS^+ VLPs. Evaluation of the same sera concluded that none of them were able to neutralise pseudovirus *in vitro*. This has important implications for the production of HPV-11 vaccines in plants.

2.1 INTRODUCTION

While plants have sustained human life since the beginning of time and have been altered through traditional breeding methodologies to achieve desired phenotypes, they have recently become an important resource in the field of recombinant DNA technology. Over the past twenty years the development of this technology has made a significant contribution to many facets of agriculture and lately also on biopharmaceutical industries.

Applications of plant genetic engineering processes are widespread, having an impact and making a difference in terms of how we deal with persistent and emerging problems. For example, the alteration of plants' genetic make-up has made it possible to deal with issues of waste products associated with paper-pulp, food and agricultural industries (Herbers *et al.* 1995).

Recent developments in plant biotechnology have led to an entirely new and exciting research field, involving the use of transgenic plants as bioreactors for the production of biopharmaceuticals (Giddings *et al.* 2000). To date, the production of a number of therapeutic proteins, including human somatotropin (Staub *et al.* 2000), human insulin (Arakawa *et al.* 1998), human epidermal growth factor (Higo *et al.* 1993), human serum albumin (Sijmons *et al.* 1990), interferon (De Zoeten *et al.* 1989) and a range of different monoclonal antibodies (During *et al.* 1990; Hiatt *et al.* 1989; Ma *et al.* 1995) has been reported.

These novel manufacturing systems have proven to be viable alternatives to existing fermentation or cell culture systems. The plant produced product generally out-competes traditional production formulations in terms of total product volume, product safety and cost-effectiveness. Estimates are that the costs of producing recombinant proteins in plants could be 10 to 50 times lower than production costs of the identical protein in an *Escherichia coli* fermentation system (Kusnandi *et al.* 1997), which is already far cheaper than mammalian cell production systems. Plants have predominantly been described as being free of human disease agents, although it has been found that the human

opportunistic pathogen, *Pseudomonas aeruginosa* PA14, is indeed capable of causing local and systemic infections in *Arabidopsis thaliana* (Plotnikova *et al.* 2000). Nevertheless, transgenic plants have become an attractive potential source for the production of prophylactic viral subunit vaccines. Traditionally, human viral vaccines have consisted of either live attenuated or inactivated virus suspensions. Bulk production has involved possible secondary health risks, a factor not only affecting the safety of the vaccine, but also a determinant of the final cost.

Transgenic plants have served as model systems for understanding viral assembly and replication (Zheng *et al.* 2000), for characterizing the contribution of viral genes, cellular elements and environmental cofactors to oncogenesis, and in assessing their relative importance (Brandsma, 1994).

As an example, Hepatitis B surface antigen (HBsAg) has been produced in transgenic tobacco plants, and shows similar antigenic and physical properties to human serum- and recombinant yeast-derived HBsAg (Mason *et al.* 1992). Tsuda *et al.* (1998) have shown that the hepatitis core antigen (HBcAg) can be produced in transgenic *Nicotiana tabacum* cv. SR-1 plants without loss of its antigenic determinants. Genetically engineered tomato plants have been reported to express the gene for glycoprotein G, the protein coating the outer surface of the rabies virus (McGarvey *et al.* 1995). Structural proteins from Foot-and-mouth disease virus (FMDV) (Carrillo *et al.* 1998; Wigdorovitz *et al.* 1999) and Rabbit haemorrhagic disease virus (Castanon *et al.* 1999) have been expressed in plants and have proven to protect from viral disease when used as vaccines.

Further development of transgenic plant expression systems should make plant-derived human vaccines more readily available to developing countries (Richter *et al.* 1996). The cultivation, harvesting and processing of large quantities of transgenic crops could be done at a relatively low cost and make use of existing infrastructure (Giddings *et al.* 2000).

A number of recent publications describing the use of plants as bioreactors for the production of human papillomavirus L1 proteins (Biemelt *et al.* 2003; Liu *et al.* 2005; Varsani *et al.* 2003; and Warzecha *et al.* 2003) have explored the associated difficulty and viability of this alternate expression system. In particular, although they changed the codon usage of the HPV-11 *L1* gene to reflect solanaceous plant usage, Warzecha *et al.* (2003) achieved only very low expression levels of the L1 protein. Accordingly, I report here on the genetic manipulation of *Arabidopsis thaliana* and *Nicotiana tabacum* cv. Xanthi, two well characterized plant expression systems, with the aim of expressing an unmodified HPV-11 L1 major capsid protein gene. If successful, such expression could potentially be used for the formulation of an alternative safe and cheap protein subunit vaccine applied for the prevention of HPV-11 associated disease in developing countries.

2.2 MATERIALS AND METHODS

2.2.1 Characterisation of the HPV-11 *L1* gene

Wendelin Schnippenkoetter in this laboratory in 1998 amplified via polymerase chain reaction (PCR) the HPV-11 *L1* open reading frame (ORF) from a HPV-11 DNA-positive biopsy sample obtained from Professor A-L Williamson (Institute of Infectious Disease and Molecular Medicine, University of Cape Town, South Africa) (unpublished work). The full-length HPV-11 *L1* ORF was amplified using primer pair 1 (Table 2.1), whereas primer pair 2 was used to exclude the putative bipartite nuclear localization signal (NLS; **KR**pavskpstap**KRKRtKtKK**) spanning amino acids 482 – 502 at the L1 C terminus (Table 2.1). PCR amplification products HPV-11 *L1 NLS*$^{+}$ and *NLS*$^{-}$ were cloned into the T-tailed vector pMOSBLUE (Amersham Life Science) and sequenced. They are referred to as constructs pHPL51 and pHPS51 respectively.

Table 2.1 Primers used for the amplification of the HPV-11 *L1* (502) and *L1 NLS*$^{-}$(ΔC481) ORFs

Primer Pair	Construct	Primers
1	pHPL51 (502)	Forward 5'- GGAATTCCGGAGGAATTTTTTACAG**ATG**TGGCGGC -3' Reverse 5'- GACGCCGTCGACTCCGGAACTGACACACATATA**TTA**G -3'
2	pHPS51 (ΔC481)	Forward 5'- GGAATTCCGGAGGAATTTTTTACAG**ATG**TGGCGGC -3' Reverse 5'- ACGCCGTCGACTCCGGA**TTA**TATACCTGTTACGAGCAGC -3'
3	pART27 *L1*	Forward 5'- **ATG**TGGCGGCCTAGCGAC -3' Reverse 5'- **TTA**CTTTTTGGTTTTGGTACGTTTTCG-3'
4	pART27 *L1 NLS*$^{-}$	Forward 5'- **ATG**TGGCGGCCTAGCGAC -3' Reverse 5'- **TTA**TATACCTGTACGAGCAGACG -3'
5	HPV-11 *L1 NLS*$^{-}$ internal	Forward 5'- TGATACAGGCTTTGGTGC -3' Reverse 5'- TGGCTCATTGGTGTCTTC -3'

GAATTC: *Eco*RI; TCCGGA *Mor*I, GTCGAC: *Sal*I, **ATG** = start codon, **TAA** = stop codon

2.2.2 Cloning of HPV-11 *L1 NLS*$^{+}$ and HPV-11 *L1 NLS*$^{-}$ gene into the binary vector pART27 and *Agrobacterium tumefaciens* – mediated transformation

The HPV-11 *L1 NLS*$^{+}$ and HPV-11 *L1 NLS*$^{-}$ ORFs were excised from pHPL51 and pHPS51 respectively, and cloned into pUC19 (*Eco*RI / *Sal*I). *Eco*RI / *Hind*III restriction enzyme sites facilitated directional subcloning into the plasmid pART7 (Gleave, 1992).

This placed the *L1* genes downstream of the *Cauliflower mosaic virus* 35S promoter (CaMV35S, Cabb B-JI isolate) and upstream of the octopine synthase gene terminator (*ocs* 3'). The CaMV35S promoter, HPV-11 *L1* / HPV-11 *L1 NLS*⁻ ORF and *ocs*3' cassette was excised (*Not*I) and cloned into the *Not*I site of the binary vector pART27 (Gleave, 1992) (Figure 2.1).

The binary vector pART27 includes the following features in the T-DNA segment: the neomycin phosphotranferase (*nptII*) gene for kanamycin resistance, acting as a selection marker in both *E. coli* and *Agrobacterium tumefaciens* as well as a *lacZ*' region immediately 3' of the right T-DNA border. The pART27 backbone features the ColE1 origin of replication for high copy maintenance in *E. coli*, the Tn7 spectinomycin/streptomycin resistance gene for bacterial selection, the RK2 minimal replicon for replication and maintenance in *E. coli*, and the *Agrobacterium* and RK2 derived origin of transfer (*ori*T) for conjugal transfer (Gleave, 1992).

A. tumefaciens strain C58C1, carrying the virulence plasmid pMP90, was transformed with HPV-11 *L1* / HPV-11 *L1 NLS*⁻ pART27 plasmid DNA using the freeze-thaw method described by Hooykaas (1988). Selection of transformed cells was performed on Luria agar (LA) plates containing kanamycin (40μg/ml) and rifampicin (100μg/ml) at 30°C. Transformants were screened by PCR using primer pair 3 (HPV-11 *L1*) and pair 4 (HPV-11 *L1 NLS*⁻) (Table 2.1). The PCR was performed using 25 cycles of 94°C for 30s, 55°C for 45s, and 72°C for 45s, followed by a 5min extension step at 72°C.

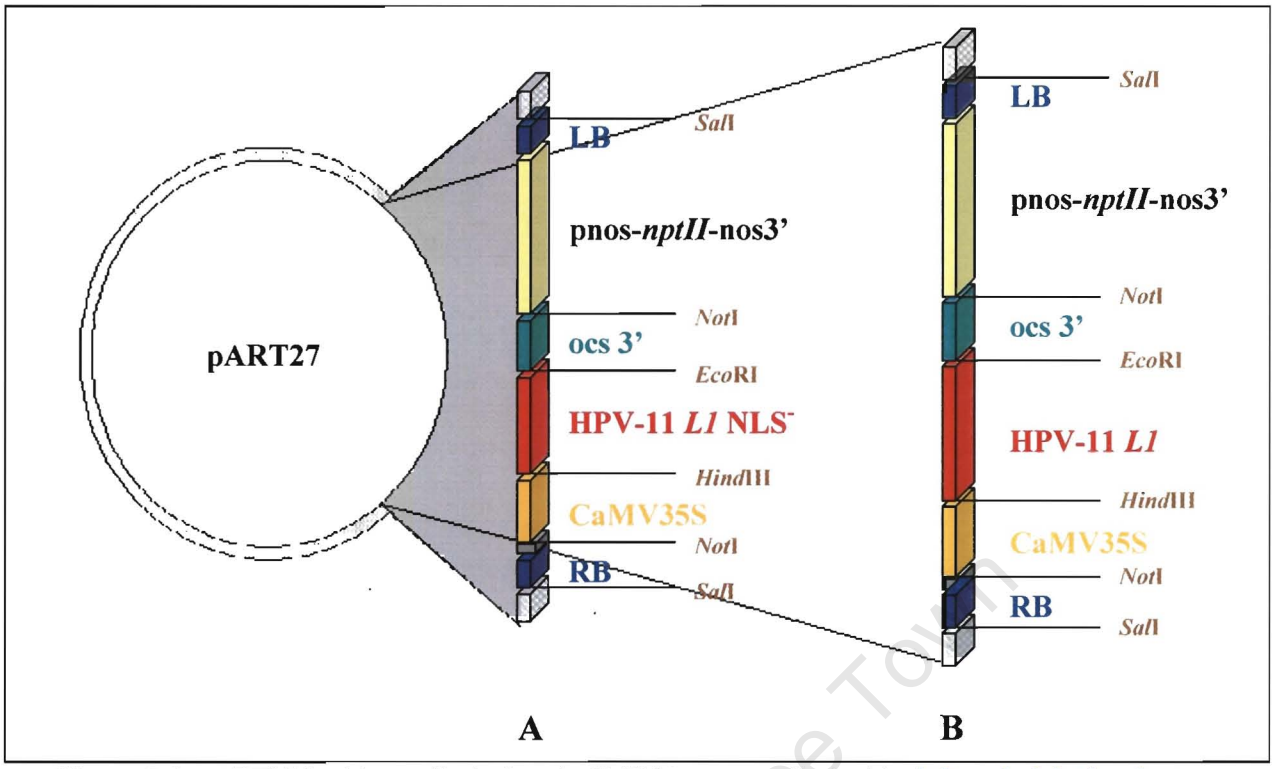


Figure 2.1: pART27 backbone displaying the T-DNA cassette flanked by **left and right borders (LB and RB)**, containing either the **HPV-11 *L1 NLS*⁻** (**A**) or the full-length **HPV-11 *L1*** (**B**) gene under the control of the **CaMV35S** promoter, followed by the ***nptII*** selection gene under the nopaline octapine synthase promoter (**pnos**)(See Appendix B for complete pART27 map).

2.2.3a Transformation of *Arabidopsis thaliana* (ecotype Columbia)

Healthy *A. thaliana* (ecotype Columbia) plants were transformed with the *Agrobacterium tumefaciens* constructs (HPV-11 *L1* / HPV-11 *L1 NLS*⁻) according to the simplified *Arabidopsis* transformation protocol described by Clough *et al.* (2000). In summary, primary bolts were cut off to induce proliferation of secondary bolts bearing a multitude of immature flower clusters. Four days after the initial clipping of the primary bolts, *A. tumefaciens* cultures containing either pART27 *L1* or pART27 *L1 NLS*⁻ were set up to grow for two days at 30°C. Bacteria were harvested by centrifugation at 4000 x g and resuspended in 500 ml freshly prepared phosphate buffered saline (PBS, 1.47mM KH_2PO_4, 10mM Na_2HPO_4, 2.7mM KCl, 137mM NaCl, pH 7.4) containing 5% sucrose (w/v). The surfactant Silwet L-77 was added to the bacterial suspension to a final concentration of 0.03%, before plants were dipped for 10 to 15s, ensuring an entire coating of all leaves, stems and inflorescence. Dipped plants were placed in plastic bags to maintain high levels of humidity and left to grow for another 48hrs under minimal

light conditions. On day 3 plastic bags were removed and transformed plants were exposed to a 16 / 8hr day/night photoperiod.

Watering times were reduced as seeds reached maturity and dry seeds were harvested. In order to determine the transformation efficiency and screen successive transformed plants, dry seeds were sterilized in 10% bleach for 5min, spun down and washed in sterile distilled H_2O for 5min. This was repeated 3 times, where after seeds were resuspended in H_2O and stored at 4°C overnight. Individual seeds were plated on plant nutrient sucrose medium [PNS, (1M KNO_3 5ml, 1M $MgSO_4.7H_2O$ 2ml, 1M $Ca(NO_3)_2$.$4H_2O$ 2ml, 20mM Fe.EDTA 2.5ml, micronutrients 1ml, agar 7.5g/l, sucrose 5g/l ; after autoclaving add 1M KPO_4 pH 5.5 2.5ml and add 10mM tryptophan 5ml (final conc. 50µM); Micronutrient stock: 70mM H_3BO_4, 14mM $MnSO_4.H_2O$, 0.5mM $CuSO_4.5H_2O$, 1mM $ZnSo_4.6H_2O$, 0.2mM Na_2MoO_4, 10mM NaCl, 0.01mM $CoCl_2.6H_2O$)] containing kanamycin (250µg/ml). Germination was allowed to take place under a 16 / 8hr day/night photoperiod, at 25°C and 80% relative humidity. Seedlings were left on PNS media until complete development of a mature 4th leaf and putative transformed seedlings could be distinguished from untransformed ones by means of kanamycin selection, before being transplanted to soil.

2.2.3b Transformation of *Nicotiana tabacum* cv. Xanthi

The transformation protocol established by Horsch *et al.* (1985) was used for the generation of transgenic *N. tabacum* cv. Xanthi plants. Wild type *N. tabacum* leaves were harvested and sterilized on either side for 5min in 10% bleach, followed by 3 successive washes in sterile distilled water. Leaves were cut into 1cm^2 squares and submerged in a 48hr *A. tumefaciens* culture carrying the binary vector pART27 HPV-11 *L1* or HPV-11 *L1 NLS* for 5min. Submerged leaf discs were dried on sterile filter paper and transferred to non-selective Murashige and Skoog (MS) co-cultivation plant tissue culture (T/C) media (4.3g of MS basal salts/litre [Gibco], 0.1mg NAA [1-naphthaleneacetic acid]/litre, 1mg BAP [benzylamino purine]/litre, 30g sucrose/litre and 8g phytagar/litre, pH 5.8) and kept in the dark at 25°C for 72hrs (Murashige & Skoog, 1962). To induce the formation

of transgenic calli and prevent further *Agrobacterium* growth, leaf discs were transferred to MS regeneration / shooting T/C media (4.3g of MS basal salts/litre [Gibco], 0.1mg NAA [1-naphthaleneacetic acid]/litre, 1mg BAP [benzylamino purine]/litre, 30g sucrose/litre and 8g phytagar/litre, pH 5.8) with kanamycin (300µg/ml) and cefotaxim (250µg/ml). Kanamycin resistant calli were allowed to differentiate into shoots with 1 to 2 leaves, before being cut off and placed on MS root-inducing plant T/C media (4.3g of MS basal salts/litre [Gibco], 0.5g MES [2-(*N*-morhpholino ethanesulfonic acid)], 30g sucrose/litre and 8g phytagar/litre, pH 5.7) with kanamycin (100µg/ml). Putative transgenic shoots were kept on root-inducing media at 22°C and were exposed to a 16/8hr day/night photoperiod to allow for root formation. Plantlets with sufficient roots were transferred to soil, covered in plastic bags and left to acclimatize under minimal light conditions for two days. Thereafter plants were grown to maturity under 16hrs of light in plant rooms maintained at 25°C and 80% relative humidity. Flowering regenerated plants (R_0 generation) were self-pollinated and the seeds collected. Dry seeds were screened on plant T/C media containing kanamycin (250µg/ml) and putative transgenic seedlings were transferred to soil, once the 4th leaves had grown to maturity.

2.2.4 Screening of transformed *A. thaliana* and *N. tabacum* plants for the HPV-11 *L1 NLS*$^+$ and HPV-11 *L1 NLS*$^-$ genes

Plant genomic DNA was isolated in extraction buffer (100mM Tris, 50mM EDTA, 500mM NaCl pH 8.0) from putative transgenic and wild type *A. thaliana* and *N. tabacum* leaves using the method outlined by Dellaporta *et al.* (1983). DNA from transformed *A. thaliana and N. tabacum* plants was screened by PCR for the HPV-11 *L1* and HPV-11 *L1 NLS*$^-$ genes using primer pairs 3 and 4 as listed in Table 2.1. The PCR was performed using 25 cycles of 94°C for 30s, 55°C for 45s, and 72°C for 45s, followed by a 5min extension step at 72°C and resulted in the amplification of a 1.5 kb fragment.

2.2.5 Southern blot analysis of PCR positive transgenic plants

Plant genomic DNA from PCR positive transgenic HPV-11 *L1 NLS* plants was digested with *Hind*III overnight, followed by further digestion with *Eco*RI for 1hr. Digested plant genomic DNA (30µg) was resolved on a 0.8% agarose gel. Gel denaturing and neutralisation steps were performed according to the manufacture's instructions (Dig DNA Labelling and Detection Kit, Roche Applied Science) before the DNA was transferred onto Hybond N^+ nitrocellulose membrane (Amersham) by capillary transfer in 20x SSC (3M NaCl, 0.3M sodium citrate, pH 7.0). Transferred genomic DNA was cross-linked to the membrane by exposure to short wavelength ultraviolet (UV) light for 2min. The membrane was pre-hybridized in DIG Easy Hyb (Roche) at 42°C for 1hr. A specific HPV-11 *L1* DIG-labelled probe was denatured at 100°C for 5min and added to pre-warmed DIG Easy Hyb to form the hybridization solution. The blot was incubated at 42°C for 16hrs. After hybridization, the blot was washed twice in low stringency buffer (2x SSC, 0.1% SDS) for 5min each. This was followed by two 15min washes in high stringency buffer (0.1x SSC, 0.1% SDS) at 68°C. To evaluate the hybridization results, the blot was briefly washed in washing buffer (DIG DNA Labelling and Detection Kit, Roche Applied Science), followed by an incubation period of 30min in blocking buffer. Anti-DIG antibody solution (1:10000 dilution) was prepared and the membrane incubated for 30min with shaking. This was followed by another two 15min washes in washing buffer, where after the membrane was equilibrated in detection buffer (Dig DNA Labelling and Detection Kit, Roche Applied Science) for 3min. CDP-*Star* was diluted 1:100 in detection buffer (100mM Tris, 100mM NaCl, 50mM $MgCl_2$ pH 9.5), added to the membrane and incubated at room temperature for 5min. Results were obtained by exposure of the membrane to high sensitivity X-ray film for 10min.

2.2.6 Analysis of total RNA extracted from transgenic plants

Total RNA was extracted from fresh leaf material using the TRIzol™ reagent (Life Technologies). Total RNA samples were pre-digested with RNase-Free DNase (Promega) at 37°C for 30min. DNase stop solution was added to the samples and the

DNase enzyme was heat inactivated at 65°C for 10min. A final volume of 5µl treated total RNA was used for the detection of the *L1 NLS* mRNA by RT-PCR amplification, using the Access RT-PCR system (Promega). The profile for the RT-PCR is listed as: 45°C for 45min, 94°C for 2min; followed by 30 cycles of 94°C for 30s, 55°C for 1min and 68°C for 2min. The final elongation step was performed at 68°C for 5min. Primer pair 5 (Table 2.1) was used to amplify an internal 300 bp fragment.

2.2.7 Processing and concentration of transgenic plant material

Healthy leaves from *N. tabacum* and entire *A. thaliana* plants were harvested and processed in 1:2 (w/v) ratio with cold phosphate buffered saline (PBS; 1.47mM KH_2PO_4, 10mM Na_2HPO_4, 2.7mM KCl, pH 7.4) containing 500mM NaCl. The protein extraction process is outlined in figure 2.2. PBS as defined above was used throughout the entire extraction process. VLPs have been described as being more stable at higher NaCl concentrations hence the salt concentration of the PBS was increased to 500mM in order to prevent VLP disintegration (Cook *et al.* 1999).

This VLP isolation and purification protocol was the end result of many attempts to successfully isolate VLPs from homogenized transgenic plant material. Early attempts by me and others in our laboratory to extract and purify HPV-16 L1 VLPs from plants, together with experiments aimed at determining the recovery rate of VLPs from non-transgenic plant extracts spiked with insect cell-derived HPV VLPs, have culminated in this final protocol. Preliminary experiments further examining the recovery of insect cell-produced VLPs from non-transgenic plant extracts have included several other variables: among these were investigations of the effects on VLP recovery after extract/VLP mixtures were exposed to heat treatment, to PBS of different pH values, and substitutions of various salts, separately or in combination of two and three. The goal was to arrive at a method which resulted in the highest concentration of L1/VLPs with the fewest processing steps. My goal was not to optimise the VLP extraction protocol. In conjunction with my efforts to extract the highest possible concentration of L1 VLPs

from transgenic plants, three colleagues worked in parallel on optimising this particular protocol and are still modifying this VLP purification method to date.

Results from these investigations allowed formulation of the protocol laid out in figure 2.4, as being most suited to extract VLPs from transgenic plant material. It is a relatively simple and short extraction protocol which ensures the extraction of the desired protein while simultaneously minimizing the loss of material. Essentially, most unwanted cellular debris is removed by initial cheesecloth filtration and low-speed centrifugation. The resulting supernatant (Number ②) is free of coarse cellular debris and wax from the plant cuticle. Oxidation of this supernatant (with tobacco extracts at least) is a rapidly occurring process during which the supernatant undergoes a colour change from dark green to dark brown. To prevent this from happening we investigated the use of antioxidants such as sodium metabisulphide or ascorbic acid, but instead decided on processing the supernatant at 4°C with the addition of complete protease inhibitor (Roche), thus keeping levels of oxidation and protein degradation to a minimum.

After the addition of coarse polyethylene glycol (PEG, molecular weight 8000, Promega) at 10% w/v, the supernatant was stirred at 4°C until all PEG was dissolved. Further low-speed centrifugation resulted in the pelleting of all remaining particulates including capsomers (Number ③); all of which were concentrated by resuspension in $1/10^{th}$ of the original volume of PBS. An additional low-speed centrifugation step ensured further removal of debris before all remaining particulate protein in the supernatant (Number ⑤) was harvested by ultracentrifugation. To the final concentrated protein extract (Number ⑥) was added additional complete protease inhibitor (Roche) and aliquots were stored at -70°C for further characterization and evaluation.

No centrifugation-based isolation and purification procedure ever prevents loss of protein. As it stands, I feel I have reduced the amount of protein lost throughout all steps of this protocol to a minimum.

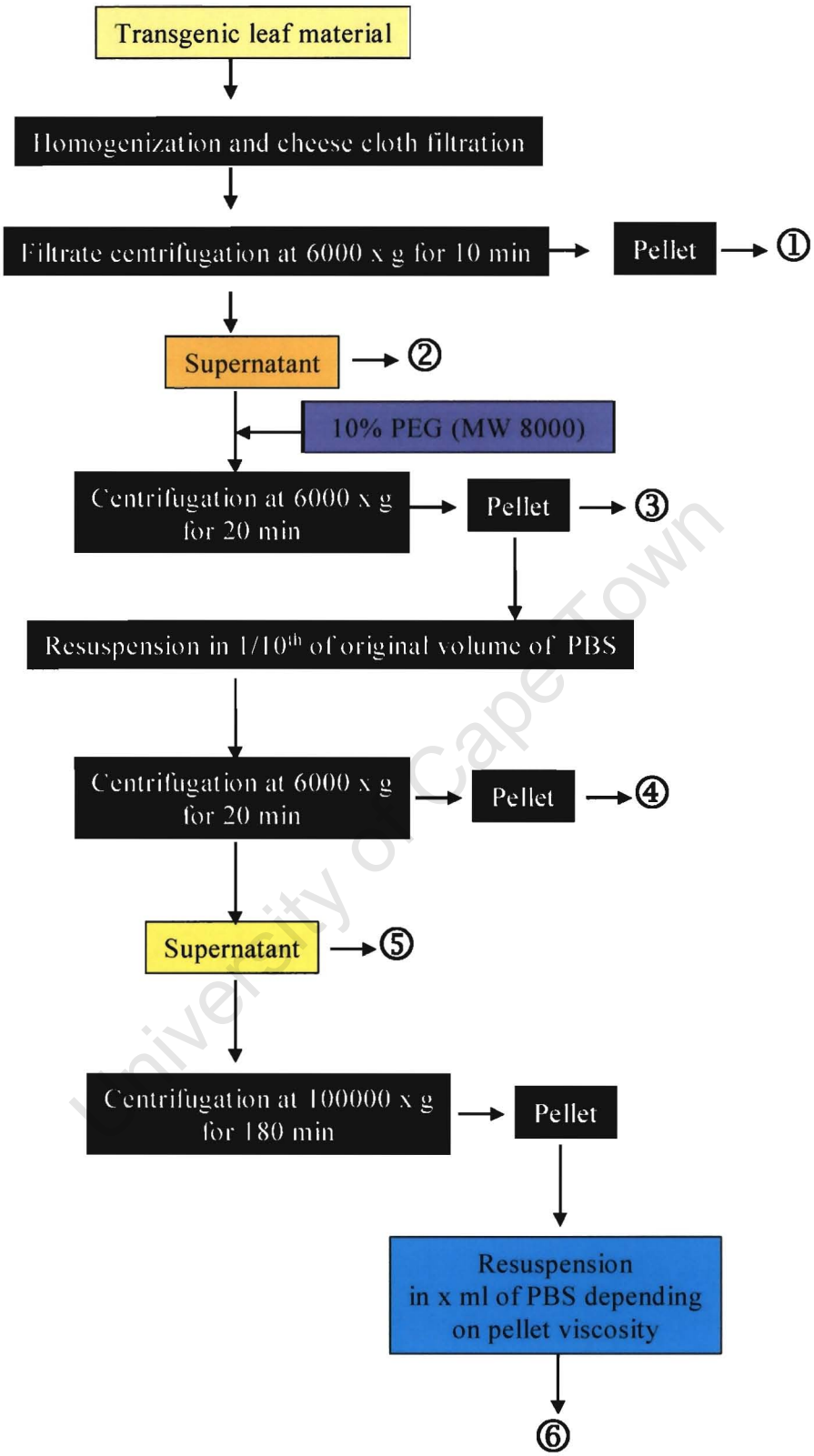


Figure 2.2: Flow chart showing the processing steps involved in the isolation and purification of total protein from transgenic leaf material using the polyethylene glycol (PEG, molecular weight 8000) precipitation protocol. Individual sampling points are indicated by numbers 1 through 6.

2.2.8 Monoclonal antibody characterization of plant-derived HPV-11 L1 NLS⁻ protein

HPV-11 L1 NLS⁻ protein-containing extracts derived from *A. thaliana* and *N. tabacum* (Number ⑥, Figure 2.2), together with non-transgenic plant protein extracts, were characterized by direct enzyme linked immunosorbent assay (ELISA) using a panel of monoclonal antibodies (MAbs) to HPV-6, HPV-11 and HPV-16 (generously provided by Associate Professor Neil Christensen, The Milton S. Hershey Medical Centre, Penn State College of Medicine, Hershey, Pennsylvania, USA). These MAbs are listed in table 2.2.

Table 2.2: MAbs used for the detection and characterization of the HPV-11 L1 NLS⁻ protein

Monoclonal antibody	Isotype	Neutralisation	Nature of Epitope	Cross-reactivity	References
H11:B2	IgG2b	Yes (HPV-11)	conformation-specific	Yes (HPV-11&6)	Christensen *et al.* 1990b Ludmerer *et al.* 1996 Christensen *et al.* 1996b Chen *et al.* 2000
H11:H3	IgG2b	Yes (HPV-11)	conformation-specific	No	
H6:C6	IgG2a	No (HPV-11)	linear (surface)	Yes (HPV-6&11)	
H6:E51	IgG1	No (HPV-11)	linear (surface)	Yes (HPV-6&11&18)	
H6:I2	IgM	Yes (HPV-11)	linear (surface)	Yes (HPV-6&11)	
H16:D9	IgG1	No (HPV-11)	linear (surface)	Yes (HPV-11 & 16)	

ELISA plates were coated with protein extracts for 1hr and then blocked in 2% (w/v) non-fat milk in PBS (1.47mM KH_2PO_4, 10mM Na_2HPO_4, 2.7mM KCl, 137mM NaCl, pH 7.4) for 1hr. MAbs (1:1000) were used to detect the plant-derived antigen for 1hr. Anti-mouse-alkaline phosphatase conjugated secondary antibody (Sigma) (1:5000) was allowed to bind the primary antibody for 1hr at 37°C. The secondary antibody was detected using p-nitrophenyl phosphate (pNPP, Sigma) and the absorbance was measured at 405nm using a Titrex ELISA plate reader. All samples were analysed in triplicate to determine the mean absorbance and calculate the respective standard deviations.

2.2.9 Western blot analysis of plant-derived HPV-11 L1 NLS⁻ protein

Trichloroacetic acid (TCA) was added to 2ml plant protein extracts to a final concentration of 5% (v/v). The mixture was centrifuged at 6000 x g for 20min at 4°C.

The pellet was washed in 100% acetone-20mM HCl and centrifuged at 6000 x g for 20min at 4°C. This was followed by another two 100% acetone-20mM HCl washing steps as described above, before the pellet was resuspended overnight in 25mM Tris, 2% SDS amounting to 1/5 of the original volume.

For analysis of plant-derived L1 protein, 150µl of TCA-precipitated and non-TCA-precipitated plant protein extract aliquots were denatured at 100°C for 10 min in SDS PAGE gel loading buffer with reducing agents. The denatured protein extracts were resolved on a 12% SDS-PAGE gel. Semi-dry electrophoresis (BioRad) at 15V for 120min ensured transfer of the separated proteins onto nitrocellulose membrane. The membrane was blocked in 5% non-fat milk in PBS (1.47mM KH_2PO_4, 10mM Na_2HPO_4, 2.7mM KCl, 137mM NaCl, pH 7.4) for 1hr, before being incubated in primary antibody at 4°C overnight. MAbs to HPV-6 L1 (H6:C6 and H6:E51; 1:1000) and polyclonal antiserum against HPV-11 L1 (1:200) were used for detection. The membrane was washed in PBS for 1hr before being probed with either goat anti-rabbit and/or goat anti-mouse-alkaline phosphatase conjugated secondary antibodies (Sigma) at dilutions of 1:5000. This was followed by another washing step in PBS for 1hr, before 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) in substrate buffer were used for the detection of the protein.

2.2.10 Expression of the HPV-11 *L1 NLS*$^+$ and *NLS*$^-$ genes in *Sf*21 cells using recombinant baculovirus

The HPV-11 *L1 NLS*$^-$ gene was PCR amplified using the primers as listed in table 2.3, cloned into pGEM®-T Easy vector (Promega) and sequenced.

Table 2.3: Primers used for the amplification of the HPV-11 *L1 NLS*$^-$ ORF

Primer Pair	Construct	Primers
6	pFAST-HPV-*11 L1 NLS*$^-$	Forward 5'- TCTAGA**ATG**TGGCGGCCTAGCGAC-3' Reverse 5'- CTCGAG**TTA**CTTTTTGGTTTTGGTACG -3'

TCTAGA *Xba*I, CTCGAG *Xho*I, **ATG** = start codon, **TAA** = stop codon

The addition of the *Xba*I and *Xho*I restriction sites allowed directional subcloning into the pFastBac™1 vector (Invitrogen). Transformation of competent DH5α *E.coli* cells, restriction digests and selection of positive clones according to the manufacture's protocol resulted in the amplification of the required pFastBac™1 HPV-11 *L1 NLS*⁻ construct. This DNA was used for the transfection of 'max efficiency®' DH10Bac™ competent *E.coli* cells (Invitrogen) for the preparation of bacmid clones. Recombinant bacmid DNA was amplified and isolated for the transfection of *Spodoptera frugiperda* (*Sf*21) cells (Invitrogen) in the presence of Cellfectin® (Invitrogen). The recombinant baculovirus production of foreign proteins in *Sf*21 rather than *Sf*9 cells has the advantage of generally allowing a higher yield of intracellular proteins.

*Sf*21 cells were transfected according to the manufacturer's Bac-to-Bac® protocol. Two millilitres of unsupplemented Grace's insect cell medium containing approximately $9x10^5$ *Sf*21 cells were pipetted into the 35mm wells of a six well T/C plate and left to attach at 27°C for 1hr. A total amount of 1μg of purified bacmid DNA (~ 5μl) was added to 100μl of unsupplemented Grace's medium. In parallel, 6μl of Cellfectin® reagent were diluted in 100μl of unsupplemented Grace's medium. The diluted bacmid DNA was combined with the diluted Cellfectin® reagent (total volume ~ 210μl), gently mixed and incubated for 15 to 45min at room temperature before the total volume was made up to 1ml by addition of unsupplemented Grace's medium. Media from attached cells was removed and the cells washed once with 2ml of fresh media. The wash medium was removed before the DNA:lipid complex was added to the wells. Plates were incubated at 27°C for 5 hrs. After 5hrs, the DNA:lipid complex was removed and 2ml of complete growth medium (TC100, 10% foetal calf serum, antibiotics) was added to the wells. Thereafter plates were incubated for 48 to 72hrs at 27°C in a humidified incubator. Seventy two hrs post-transfection, the medium containing the infectious recombinant virus (~ 2ml, 1st supernatant) was collected, centrifuged at 500 x g to removed cellular debris and stored at 4°C. To obtain the working recombinant virus stock (2nd supernatant), an additional 2ml of complete Grace's media were added to the cells and incubated for a further 48 to 72hrs.

To ensure sufficient viral stocks for further experiments, the 1st supernatant of recombinant virus was amplified accordingly. To a 25cm^2 flask seeded with 1x10^6 *Sf*21 cells (viability of >97%) were added 300µl of 1st supernatant and 300µl complete Grace's media. Flasks were incubated for 1hr at room temperature before the virus was collected and the cells incubated for another 48 to 72hrs in 5ml of fresh complete media.

Six 75cm^2 insect cell T/C flasks seeded with 1x10^6 *Sf*21 cells were infected with 100µl of amplified recombinant baculovirus stocks to produce insect cell-derived HPV-11 L1 NLS$^+$ and NLS$^-$ VLPs in a final volume of 10ml. A HPV-11 *L1 NLS*$^+$ recombinant baculovirus supernatant was obtained from Professor Robert Rose (Department of Medicine, University of Rochester Medical Centre, Rochester, NY) in 1999 and used for the expression of HPV-11 L1 NLS$^+$ VLPs. Flasks were incubated at 27°C for 48 to 72hrs. Infected cells were harvested by repeatedly washing them off the flask wall and centrifuging them at 4000 x g.

2.2.11 Extraction of *Sf*21 cell – produced HPV-11 L1 NLS$^+$ and NLS$^-$ VLPs

Pelleted *Sf*21 cells were resuspended in CsCl buffer [PBS containing 0.4g/ml CsCl and complete protease inhibitor (Roche)] and sonicated by applying 4 bursts at 10 second intervals. The sonicated suspension was centrifuged using a Beckmann SW 55.1 rotor at 100000 x g at 10°C for 24hrs. Two distinct bands were observed on the CsCl gradient: the top band was extracted by puncturing the tubes from the side with a syringe needle and dialysed against PBS (1.47mM KH_2PO_4, 10mM Na_2HPO_4, 2.7mM KCl, 500mM NaCl, pH 7.4) for 48hrs. Dialysed protein was divided into 100µl aliquots and frozen at -70°C for further use.

2.2.12 Electron microscopy of plant-derived HPV-11 L1 NLS$^-$ protein

A. thaliana and *N. tabacum* plant protein extracts and insect cell-derived HPV-11 L1 protein were viewed either directly or after immunotrapping onto carbon-coated copper grids. Protein samples were directly adsorbed onto copper grids for 30min, thoroughly

washed in sterile distilled water (2min) and then stained in 2% uranyl acetate for 2min. Immunotrapping of plant- and insect cell-derived L1 protein entailed the coating of carbon coated copper grids with rabbit-anti-HPV-11 L1 antiserum raised against insect cell-derived HPV-11 L1 VLPs diluted 1:50 in PBS (1.47mM KH_2PO_4, 10mM Na_2HPO_4, 2.7mM KCl, 137mM NaCl, pH 7.4). Grids were thoroughly washed in sterile distilled water (2min) and then blocked in 1% BSA PBS for 30min. Grids were transferred onto the plant protein extract allowing for adsorption of L1 protein for 30min. This was followed by another thorough washing step in sterile distilled water before grids were stained in 2% uranyl acetate for 2min.

Immunogold-labeling of plant-derived L1 protein involved the following preparation protocol: carbon coated copper grids were incubated overnight at 4°C with HPV-11 L1 polyclonal antiserum, diluted 1:50 in 0.1% BSA PBS. Grids were thoroughly washed in sterile distilled water (2min) and immunotrapping of transgenic and non-transgenic plant protein extracts was allowed to take place for 60min at ambient temperature. This was followed by a further 2 washing steps with sterile distilled water (2min each) before grids were blocked in 1% BSA PBS for 30min at ambient temperature. Grids were washed (2min) and probed with HPV-11 MAb H11:H3 (1:1000 in 1% BSA PBS) for 60min at room temperature. Another 2min washing step preceded the 60min incubation of grids in the gold-labelled anti-mouse-conjugated secondary antibody (30nm gold particles) diluted 1:100 in PBS. Grids were washed one final time in sterile distilled water (2 x 2min) before being stained with 2% uranyl acetate for 2min.

2.2.13 Immunization of New Zealand white rabbits with plant-derived HPV-11 L1 NLS^- protein extracts and evaluation of sera

Concentrated *A. thaliana* and *N. tabacum* plant protein extracts (Step 6, Figure 2.2) were injected into New Zealand white rabbits. Inoculum preparations for individual rabbits included the mixing of 500µl plant protein extract with 500µl of Freund's incomplete adjuvant. Rabbits were immunized by 3 subcutaneous and 1 intramuscular injection on days 1, 28 and 56. Antiserum taken on days 1, 15, 28, 42, 56 and 70 was analyzed by

direct ELISA (1:20 dilution) against HPV-11 L1 NLS^+ and HPV-11 L1 NLS^- insect cell-derived VLPs at a concentration of 0.4µg/100µl.

2.2.14 Generation of HPV-11 pseudoviruses

The plasmids required for the production of HPV-11 pseudoviruses to be used in the neutralisation assay were obtained from Dr John Schiller (Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, MD, USA). The cotransfection of 293TT cells as described by Pastrana *et al.* (2004) and the generation of pseudoviruses was performed by my colleague Mrs Debbie Stewart (Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa). In brief, 20 million 293TT cells were seeded in 182 cm^2 flasks (Cellstar Greiner) 12hrs prior to transfection. The cell transfection was performed in the presence of FUGENE 6 according to the manufacturer's instructions. A total volume of 175µl of the lipid reagent was added to 5.7ml of serum free medium and the mixture incubated for 5min at room temperature. A total amount of 80µg of plasmid DNA comprised of plasmids pYSEAP (26µg), p11L1*h* (27µg) and p11L2*h* (27µg) was added to the flask and left to incubate for 30min at ambient temperature. This mixture was used for the transfection of 293TT cells and added drop wise to the cells, allowing for the occasional gentle swirling before returning the cells to the incubator.

Cells were split into two 225 cm^2 flasks after incubation of 20 to 24hrs. Forty to 48hrs post transfection, cells were harvested, trypsinised and washed with PBS. Pellets were resuspended in PBS (with additional 9.5mM $MgCl_2$, 0.5% Brij 58, 0.5% Benzonase, 0.2% Plasmid-Safe ATP-dependant DNase) to a final concentration of 50 million cells/ml and incubated at 37°C for 45min. The NaCl concentration was adjusted to 850mM and the mixture incubated at 4°C for 10min, before being centrifuged at 1500 x g for 10min at 4°C. The supernatant was aliquoted and frozen at -70°C for future use.

2.2.15 Animal serum neutralisation assays

The HPV-11 pseudovirus neutralising antibody assay was performed by Mrs Debbie Stewart (Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa) according to the method published by Pastrana *et al.* (2004). 293TT cells were rinsed with PBS and trypsinised in 2ml of trypsin at 37°C for 5min, before being collected by centrifugation and resuspended in 5ml of neutralisation buffer [DMEN without phenol red, 10% heat-inactivated FBS, 1% glutamate, 1% nonessential amino acids, 1% penicillin-streptomycin fungizone and 10mM HEPES (Invitrogen)]. Cells were counted using the trypan blue exclusion method and adjusted to a final concentration of 3 x 10^5 cells per ml. 293TT cells were preplated 3 to 4hrs in advance in 96-well T/C flat bottom plates at 30000 cells/well in 100µl neutralisation buffer.

Rabbit-anti-HPV-11 L1 NLS$^-$ serum (pre and post-inoculation) was diluted in neutralisation buffer (1:50 to 1:12150) in 96-well non-treated sterile T/C plates. The positive control consisted of MAb H11:H3 dilutions in neutralisation buffer, ranging from 10^{-4} to 10^{-9}. A final volume of 25µl of diluted serum was transferred to another 96-well non-treated sterile T/C plates to which 100µl of diluted HPV-11 pseudovirus stock was added. The serum/pseudovirus mixture was incubated and at 37°C for 1hr before being transferred onto the preplated 293TT cells. This was allowed to incubate for a further period of 68 to 72hrs. At the end of this incubation period, 120µl of supernatant was harvested into non-treated sterile T/C plates and clarified by centrifugation at 1500 x g for 5min. A total volume of 15µl of clarified supernatant was used to determine the secreted alkaline phosphatase (SEAP) content by applying the Great ESCAPE SEAP Chemiluminescence Kit (BD Clonetech) according to the manufacturer's instructions.

Table 2.4: Summary of all characteristics of the transgenic *A. thaliana* expression system

Transgenic Plant \ Characterization	Generation (T_x) / Transgenic Line	Phenotype	PCR	RT-PCR	Southern blot	ELISA	Western blot	Electron microscopy	Animal evaluation
		1 through 6	1 through 6	1,2,3 and 6	1,2,3 and 6	3	3	3	3
Arabidopsis thaliana (ecotype Columbia)	03	Normal (healthy)	✓	✓	✓	✓	✓	✓	✓
	04	Normal (healthy)	✓	✓	✓	✓	✓	✓	✓
	05	Normal (healthy)	✓	✓	✓	✓	✓	✓	✓
	06	Normal (healthy)	✓	✓	✓	✓	✓	✓	✓
	09	Normal (healthy)	✓	✓	✓	✓	✓	✓	✓
	10	Normal (healthy)	✓	✓	✓	✓	✓	✓	✓
	12	Normal (healthy)	✓	✓	✓	✓	✓	✓	✓
	16	Normal (healthy)	✓	✓	✓	✓	✓	✓	✓
	22	Normal (healthy)	✓	✓	✓	✓	✓	✓	✓
	28	Normal (healthy)	✓	✓	✓	✓	✓	✓	✓

Table 2.5: Summary of all characteristics of the transgenic *N. tabacum* cv. Xanthi expression system

Transgenic Plant \ Characterization	Generation (T_x) / Transgenic Line	Phenotype	PCR	RT-PCR	ELISA	Western blot	Electron microscopy	Animal evaluation
		1 through 3	1 through 3	1 through 3	1 through 3	1 through 3	1 through 3	1
Nicotiana tabacum cv. Xanthi	X1	Normal (healthy)	✓	✓	✓	✓	✓	✓
	Xi	Normal (healthy)	✓	✓	✓	✓	✓	✓
	Xh	Normal (healthy)	✓	✓	✓	✓	✓	✓
	Xk	Severely stunted growth	✓ (Generation 1 &2 only)	✓ (Generation 1 & 2only)	✓ (Generation 1 & 2only)	✓ (Generation 1 & 2only)	✓ (Generation 1 & 2only)	×

2.3.1 Sequence of the *L1* gene

Figure 2.3 shows the HPV-11 *L1* nucleotide sequence obtained after PCR amplification, cloning and sequencing. With exception of a silent mutation at position 257 (C to T), this HPV-11 *L1* sequence does not differ from the prototype HPV-11 *L1* sequence (GenBank accession number M 14119).

```
1       ATGTGGCGGC CTAGCGACAG CACAGTATAT GTGCCTCCTC CCAACCCTGT
51      ATCCAAGGTT GTTGCCACGG ATGCGTATGT TAAACGCACC AACATATTTT
101     ATCATGCCAG CAGTTCTAGA CTCCTTGCTG TGGGACATCC ATATTACTCT
151     ATCAAAAAAG TTAACAAAAC AGTTGTACCA AAGGTGTCTG GATATCAATA
201     TAGAGTGTTT AAGGTAGTGT TGCCAGATCC TAACAAGTTT GCATTACCTG
251     ATTCATCTCT GTTTGACCCC ACTACACAGC GTTTAGTATG GGCGTGCACA
              C
301     GGGTTGGAGG TAGGCAGGGG TCAACCTTTA GGCGTTGGTG TTAGTGGGCA
351     TCCATTGCTA AACAAATATG ATGATGTAGA AAATAGTGGT GGGTATGGTG
401     GTAATCCTGG TCAGGATAAT AGGGTTAATG TAGGTATGGA TTATAAACAA
451     ACCCAGCTAT GTATGGTGGG CTGTGCTCCA CCGTTAGGTG AACATTGGGG
501     TAAGGGTACA CAATGTTCAA ATACCTCTGT ACAAAATGGT GACTGCCCCC
551     CGTTGGAACT TATTACCAGT GTTATACAGG ATGGGGACAT GGTTGATACA
601     GGCTTTGGTG CTATGAATTT TGCAGACTTA CAAACCAATA AATCGGATGT
651     TCCCCTTGAT ATTTGTGGAA CTGTCTGCAA ATATCCTGAT TATTTGCAAA
701     TGGCTGCAGA CCCTTATGGT GATAGGTTGT TTTTTTATTT GCGAAAGGAA
751     CAAATGTTTG CTAGACACTT TTTTAATAGG GCCGGTACTG TGGGGGAACC
801     TGTGCCTGAT GACCTGTTGG TAAAAGGGGG TAATAACAGA TCATCTGTAG
851     CTAGTAGTAT TTATGTACAT ACACCTAGTG GCTCATTGGT GTCTTCAGAG
901     GCTCAATTAT TTAATAAACC ATATTGGCTT CAAAAGGCTC AGGGACATAA
951     CAATGGTATT TGCTGGGGAA ACCACTTGTT TGTTACTGTG GTAGATACCA
1001    CACGCAGTAC AAATATGACA CTATGTGCAT CTGTGTCTAA ATCTGCTACA
1051    TACACTAATT CAGATTATAA GGAATACATG CGCCATGTGG AGGAGTTTGA
1101    TTTACAGTTT ATTTTTCAAT TGTGTAGCAT TACATTATCT GCAGAAGTCA
1151    TGGCCTATAT ACACACAATG AATCCTTCTG TTTTGGAGGA CTGGAACTTT
1201    GGTTTATCGC CTCCACCAAA TGGTACACTG GAGGATACTT ATAGATATGT
1251    ACAGTCACAG GCCATTACCT GTCAGAAACC CACACCTGAA AAAGAAAAAC
1301    AGGATCCCTA TAAGGATATG AGTTTTTGGG AGGTTAACTT AAAAGAAAAG
1351    TTTTCAAGTG AATTAGATCA GTTTCCCCTT GGACGTAAGT TTTTATTGCA
1401    AAGTGGATAT CGAGGACGGA CGTCTGCTCG TACAGGTATA AAGCGCCCAG
                                                  TAA (L1 NLS-)
1451    CTGTGTCTAA GCCCTCTACA GCCCCCAAAC GAAAACGTAC CAAAACCAAA
1501    AAGTAA
```

Figure 2.3: HPV-11 L1 and L1 NLS$^-$ sequences. The silent mutation at nucleotide position 257 (C to T) and the PCR generated stop codon TAA at position 1441-1443 (HPV-11 *L1 NLS*$^-$) are shown in boldface.

No differences in the amino acid composition are found upon translation and comparison of this sequence to the published L1 prototype or the plant codon-optimised HPV-11 L1 sequence (Warzecha *et al.* 2003), suggesting that the nucleotide changes are conservative and that no structural changes should result.

2.3.2 Plant transformation: genetic analysis of transgenic plants

Agrobacterium tumefaciens transformation of *A. thaliana* and *N. tabacum* with pART27 constructs containing the HPV-11 *L1 NLS*$^+$ and HPV-11 *L1 NLS*$^-$ were successful only in terms of transferring and integrating the HPV-11 *L1 NLS*$^-$ gene into the host's chromosome. When my work on this project first commenced in 1999 it was known to our group that *in planta* expression of the HPV-11 *L1 NLS*$^+$ gene presented great difficulties and that it was virtually impossible to do so (personal communication between Ed Rybicki and Hugh Mason). Three additional attempts to integrate the HPV-11 *L1 NLS*$^+$ gene failed in both plant systems. In contrast initial PCR screening of the putative HPV-11 *L1 NLS*$^-$ transgenic *N. tabacum* regenerated generation (R_0) and *A. thaliana* T_1 generation plants, using the specific *L1* primer pair 3 (Table 2.1), showed that 4 out of 15 regenerated *N. tabacum* lines were positive for the HPV-11 *L1 NLS*$^-$ gene. The presence of the gene was also confirmed in 10 out of 20 *A. thaliana* T_1 generation lines when screened by PCR as described above.

In order to confirm inheritance of the transgene, tobacco and *Arabidopsis* T_1 generation plants were self-pollinated and seeds screened on kanamycin-containing T/C media. Random PCR screening of plants from individual lines including up to the third (T_3) generation (*N. tabacum*) and the sixth (T_6) generation (*A. thaliana*) confirmed that the gene was stably integrated and inherited over several generations (Figures 2.4 and 2.6). This was reconfirmed for *Arabidopsis* by Southern blot using an *L1* gene specific probe (Figure 2.5). However, attempts to determine the HPV-11 *L1 NLS*$^-$ gene copy number in transgenic *A. thaliana* plants were unsuccessful. No gene specific probe was found to bind to the target DNA after plant genomic DNA was digested with *Hind*III only. Excision of the integrated gene by digestion with *Eco*RI/*Hind*III confirmed the PCR results, but unfortunately meant determination of the number of gene integration events into the plant genome was not possible.

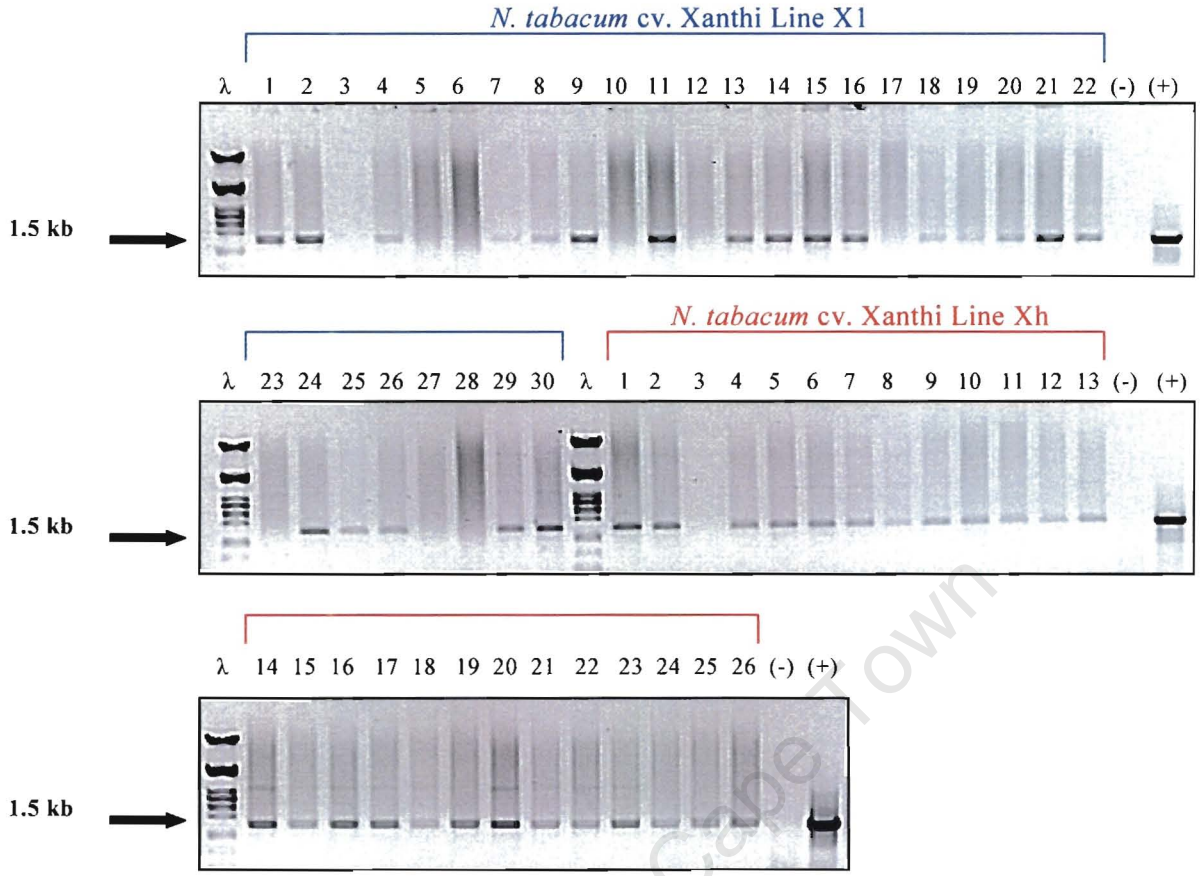


Figure 2.4: Genomic DNA analysis of two individual lines of transgenic *N. tabacum* cv. Xanthi T_1 generation plants for the presence of the HPV-11 *L1 NLS* gene. The majority of the individual plants are positive for the HPV-11 *L1 NLS* gene, as is shown by a 1.5 kb PCR amplification product. Genomic DNA extracted from a non-transgenic plant and HPV-11 *L1 NLS* plasmid DNA represent the negative and positive controls respectively.

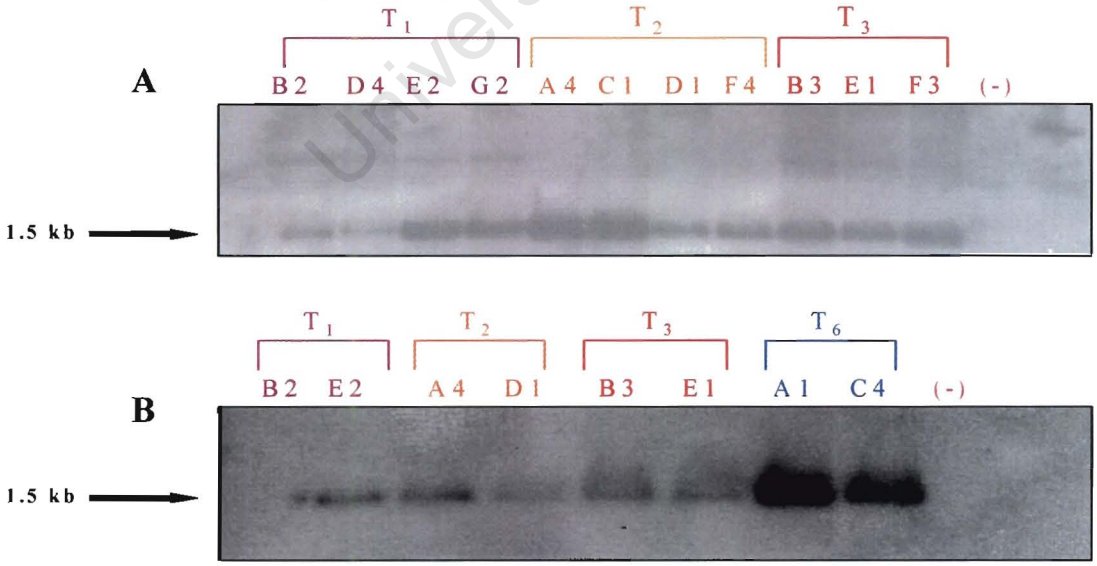


Figure 2.5: Southern blot analysis confirming the integration of the HPV-11 *L1 NLS* ORF into the *A. thaliana* genome of the T_1 generation as well as stable inheritance in the T_2, T_3 (**A**) and the T_6 generation (**B**).

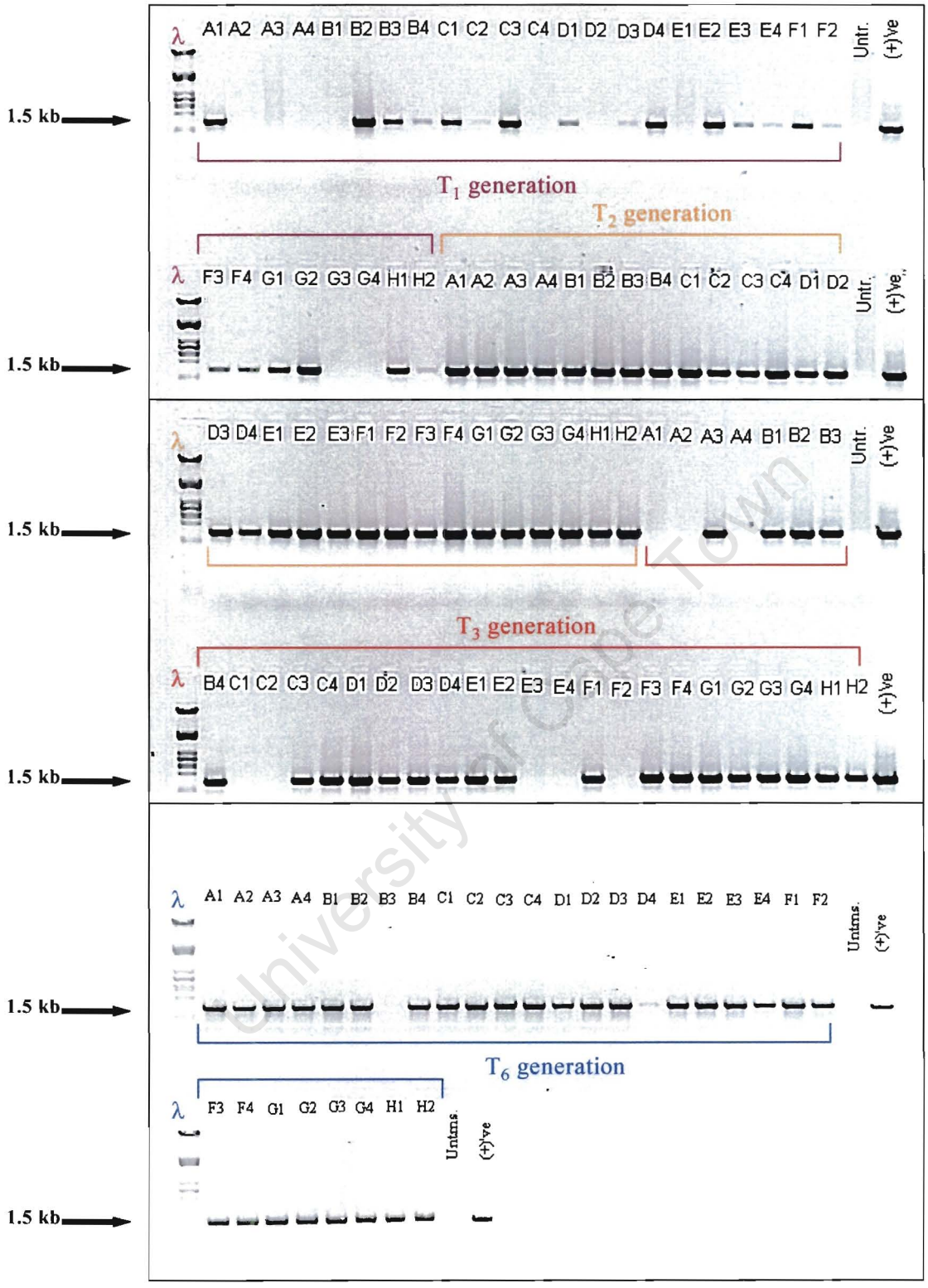


Figure 2.6: Screening of genomic DNA extracted from 10 individual transgenic *A. thaliana* lines of the T1, T2, T3 and T6 generations (A1-4 represents 4 plants of line A).The ratio of transgenic to non – transgenic increases in successive generations. Genomic DNA extracted from a non-transgenic plant and HPV-11 *L1 NLS*⁻ plasmid DNA represent the negative and positive controls respectively.

To determine whether the integrated HPV-11 *L1 NLS* gene was indeed transcribed in all successive generations of *A. thaliana* and *N. tabacum* plants, total RNA was isolated and analyzed by RT-PCR. Although not confirmed by northern blot, results indicate that the DNase treated RNA from PCR positive transgenic plants contained at least a partial fragment of the HPV-11 *L1 NLS* mRNA transcript in all generations, as shown by amplification of a 300 bp amplicon in figures 2.7 and 2.8.

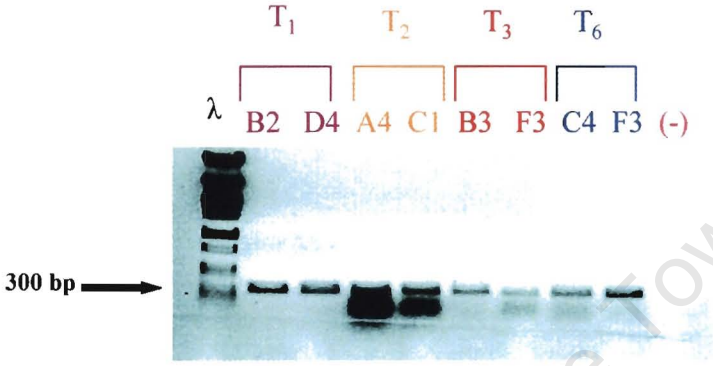


Figure 2.7: RT-PCR analysis of PCR positive transgenic *A. thaliana* plants from the T_1, T_2, T_3 and T_6 generations. A total reaction volume of 10µl is loaded in each lane and shows the amplification of a 303 bp product by RT-PCR, thus indicating the presence of the HPV-11 *L1 NLS* transcript within total plant RNA. Total RNA extracted from a non-transgenic plant represents the negative control.

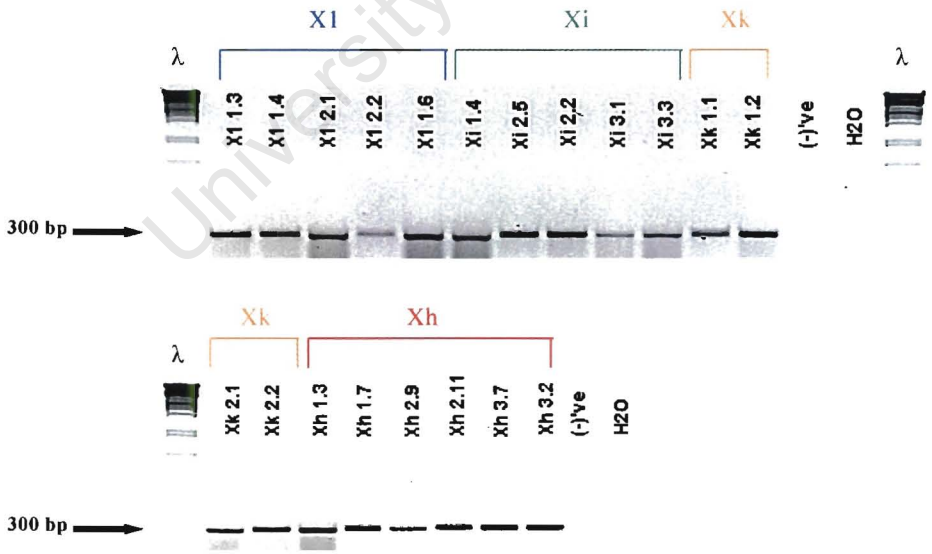


Figure 2.8: RT-PCR analysis of individual T_1, T_2 and T_3 generation *N. tabacum* cv. Xanthi plants from lines **X1**, **Xi**, **Xk** and **Xh**. (for example: **Xi 2.5** represents line **Xi**, T2 generation, plant number 5). Individual plants from the respective lines express the *L1 NLS* gene as shown by the 303 bp amplification product (total reaction volume of 10µl loaded per lane). Total RNA extracted from a non-transgenic plant represents the negative control.

2.3.3. Transgenic plants: analysis of plant-derived HPV-11 L1 NLS⁻ protein

Having confirmed gene transcription in all transgenic HPV-11 *L1 NLS⁻ N. tabacum* and *A. thaliana* lines, it was necessary to determine whether the expressed gene was translated *in planta*. In order to determine protein expression, transgenic plant material from individual first generation (T_1) *N. tabacum* lines was harvested and processed according to figure 2.2. Calculations involving all the steps in figure 2.2 have concluded that the final protein extract (Number ⑥) was 100-fold more concentrated than the initial supernatant (Number ②).

The final product resulting from homogenization, filtration and centrifugation of the transgenic *N. tabacum* leaf material (Number ⑥, Figure 2.2) was analyzed by direct ELISA and immunogold-labelling, and showed that HPV-11 L1 NLS⁻ protein was indeed present in the concentrated plant protein extract (Figure 2.9).

Monoclonal antibodies to HPV-6 (H6:C6, H6:E51 and H6:I2); HPV-11 (H11:B2 and H11:H3) and to HPV-16 (H16:D9) (Table 2.2) were used to detect and characterize the antigenicity of the plant-derived HPV-11 L1 NLS⁻ protein in the final product of the processed *N. tabacum* leaf material. Analysis of all generations of the four *N. tabacum* lines showed that the best binding to *N. tabacum*-derived protein was achieved by the surface linear and HPV-11 neutralising antibody H6:I2. Detection of the protein was also confirmed by two other MAbs (H6:C6 and H6:E51); however, these antibodies recognize surface exposed linear epitopes and are not neutralising (Figure 2.9).

Conformation-specific neutralising, MAbs H11:B2 and H11:H3, which only bind intact VLPs, did not react, suggesting that most of the plant-derived HPV-11 L1 NLS⁻ protein exists in an unassembled state. HPV-11 and HPV-16 cross-reactive MAb H16:D9 primarily binds denatured protein and should have therefore reacted strongly in the presence of unassembled HPV-11 L1 NLS⁻ protein in the plant extract.

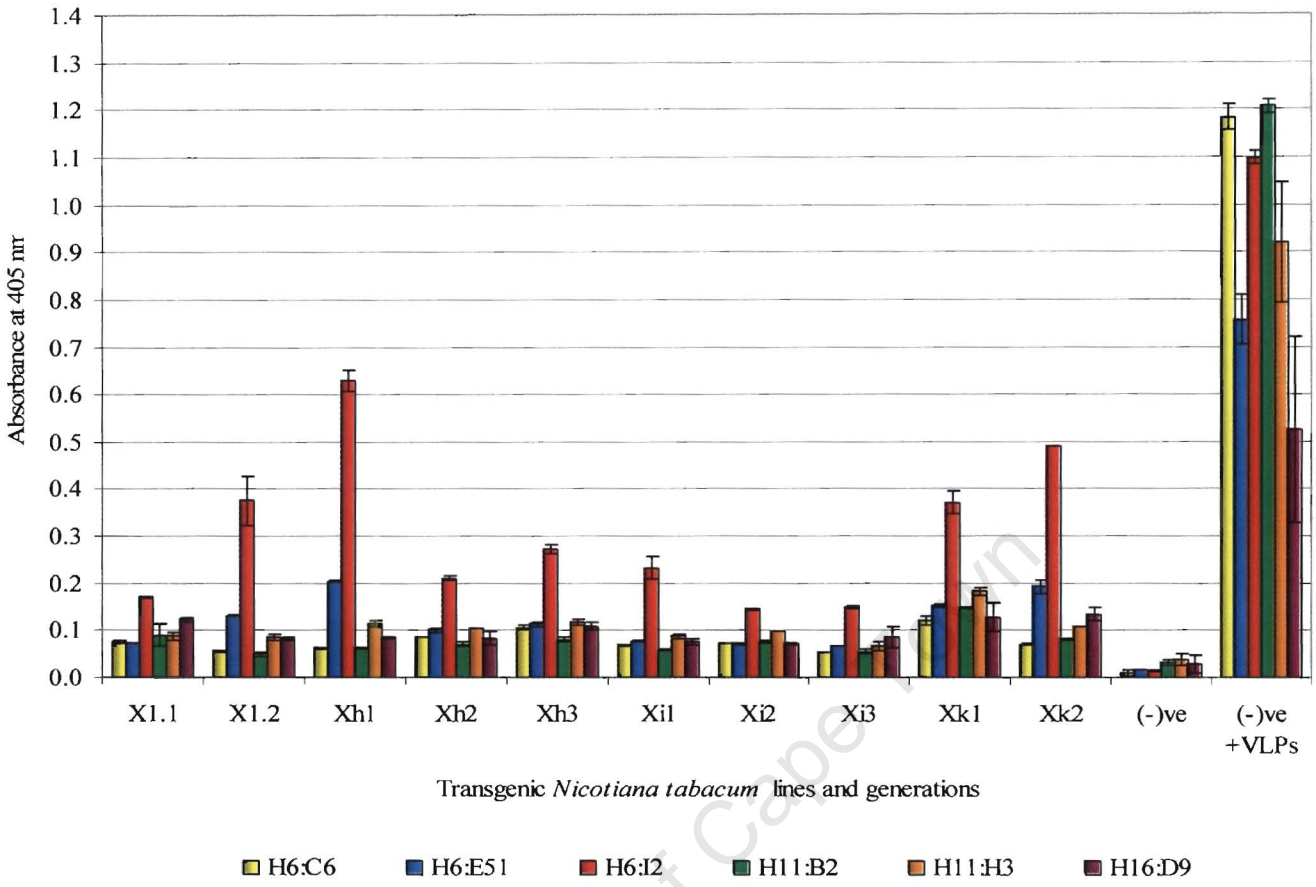


Figure 2.9: MAb characterization of HPV-11 L1 NLS⁻ transgenic *N. tabacum* protein extract from individual lines and generations by direct ELISA. (for example: X1.2 represents line **X1**, T_2 generation). Non-transgenic *N. tabacum* protein extract was used as negative control; the positive control consisted of non-transgenic protein extract spiked with 4µg/ml (0.4µg/well) insect cell-derived HPV-11 L1 NLS⁻ VLPs. Error bars represent the standard deviation calculated from triplicate analysis of samples.

Results shown in figure 2.9 reflect the opposite, however, indicating some degree of assembly. The rather weak detection of the HPV-11 L1 NLS⁻ VLP spiked positive control by MAb H16:D9 was anticipated, as it is not suitable for detecting intact VLPs.

To characterize the antigenicity of *A. thaliana*-derived HPV-11 L1 NLS⁻ protein, T_3 generation plants from all ten lines were harvested and pooled. My initial interest was in determining whether or not the HPV-11 L1 NLS⁻ protein was at all expressed in *A. thaliana*, rather than selecting high or low expressers. The limited biomass obtainable from these plants and the fact that at least 100 plants from each individual line would have been required to obtain enough leaf material for analysis necessitated the pooling of

the leaf material from all transgenic *A. thaliana* lines. The resulting protein extract (Number ⑥, Figure 2.2) was characterized with all the above listed MAbs. The results (Figure 2.10) show that all MAbs were capable of binding the *A. thaliana*-derived protein extract. Binding of neutralising conformation-specific antibodies H11:B2 and H11:H3 to antigen in the final extract was essentially equivalent to the binding displayed by the positive control, suggesting that *A. thaliana*-derived HPV-11 L1 NLS$^-$ protein could potentially elicit a neutralising antibody response once administered to animals.

Figure 2.10 shows the binding of the surface linear MAbs H6:C6, H6:E51 and H6:I2 to the *A. thaliana*-derived protein extract: these bind intact VLPs as well as denatured L1 protein. Binding of these 3 antibodies to the plant-derived protein is not of the same order of magnitude as binding to the positive control, but together these results suggest that most of the HPV-11 L1 NLS$^-$ protein extracted from *A. thaliana* plants is in the form of assembled pentamers and possibly also VLPs.

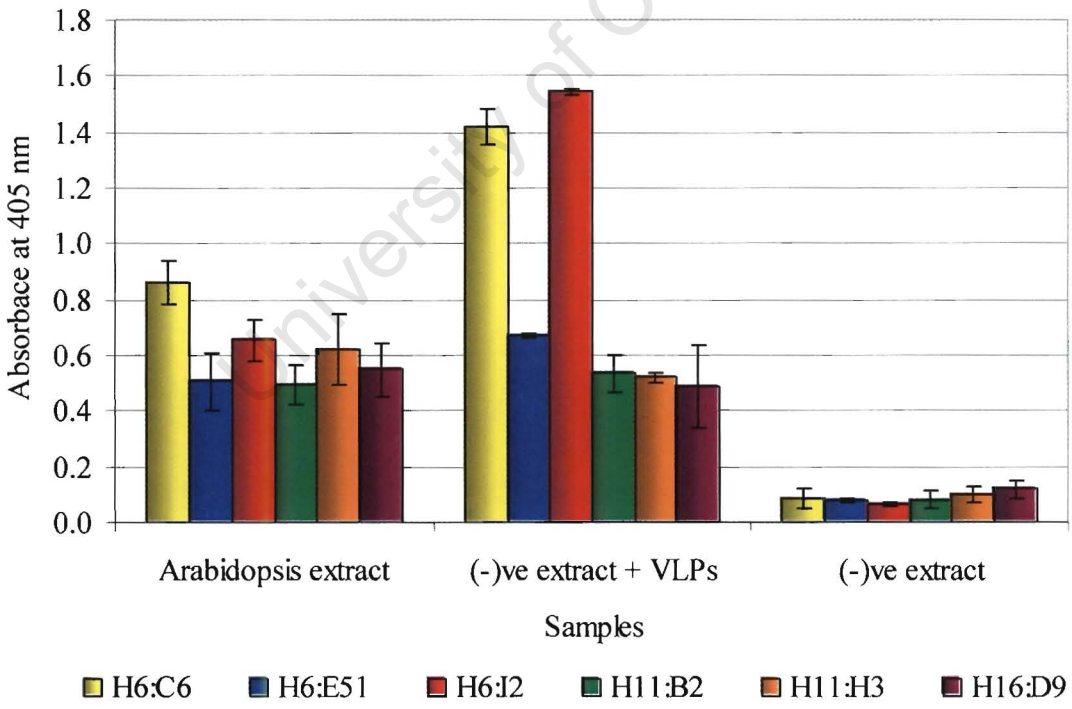


Figure 2.10: Monoclonal antibody characterization of HPV-11 L1 NLS$^-$ transgenic T_3 generation *A. thaliana* protein extract from pooled lines by direct ELISA. Non-transgenic *A. thaliana* protein extract was used as negative control; the positive control was non-transgenic protein extract spiked with 4µg/ml (0.4µg/well) insect cell-derived HPV-11 L1 NLS$^-$ VLPs. Error bars represent the standard deviation calculated from triplicate analysis of samples.

An insect cell-derived HPV-11 VLP only positive control was included in the characterization analysis (data not shown) and showed that VLPs could be detected to the same degree and quality as in the reactions shown above (Figure 2.10), thus indicating that addition of plant sap does not change their properties.

2.3.4 Determination of the HPV-11 L1 NLS⁻ protein concentration

To determine how much antigenically and morphologically correct protein could be extracted from one kilogram of transgenic leaf material, the amount of transgenic plant-derived HPV-11 L1 NLS⁻ protein was compared to a standard curve of known concentrations of authentic insect cell-derived HPV-11 L1 NLS⁻ VLPs. Non-transgenic plant protein extracts were spiked with insect cell-derived HPV-11 L1 NLS⁻ VLPs resulting in known concentrations. These represented the positive control and $O.D._{405nm}$ comparisons allowed for the calculation of the total amount of HPV-11 L1 NLS⁻ protein from sample extracts (Number ⑥, Figure 2.2) of transgenic *A. thaliana* and *N. tabacum* batches for which the total weight and homogenization buffer volume was known.

The respective yields of HPV-11 L1 NLS⁻ protein per kilogram of fresh leaf material were calculated by means of a standard curve constructed using insect cell-derived VLPs spiked into non-transgenic plant sap and are presented in table 2.6. The lowest and highest yield per kilogram according to plant species and generation are shown in boldface. In summary, total yields of HPV-11 L1 NLS⁻ protein harvested from *A. thaliana* fell into a range between 3 and 11mg/kg of leaf material, whereas protein from *N. tabacum* only yielded between 0.2 and 2.2mg/kg.

Table 2.6: Calculated yields of HPV-11 L1 NLS⁻ protein derived from 1 kg of transgenic leaf material

Samples		Concentration of HPV-11 L1 NLS⁻ protein per kilogram of fresh leaf material (mg/kg)					
		H6:C6	**H6:E51**	**H6:I2**	**H11:B2**	**H11:H3**	**H16:D9**
A. thaliana	Gen. 3	4.86±0.45	6.07±1.23	**3.39**±0.39	7.40±1.02	**9.58**±1.97	9.02±1.56
N. tabacum transgenic lines	X1 Gen.1	**0.26**±0.01	0.37±0.00	0.62±0.01	0.30±0.08	0.38±0.03	**0.93**±0.04
	X1 Gen.2	0.19±0.01	0.69±0.01	**1.37**±0.19	**0.16**±0.02	0.37±0.03	0.63±0.03
	Xh Gen.1	0.21±0.00	1.07±0.01	**2.29**±0.08	**0.21**±0.01	0.49±0.03	0.64±0.02
	Xh Gen.2	0.29±0.00	0.53±0.03	**0.77**±0.02	**0.23**±0.02	0.45±0.00	0.63±0.10
	Xh Gen.3	0.35±0.02	0.60±0.03	**0.99**±0.03	**0.27**±0.01	0.51±0.03	0.83±0.05
	Xi Gen.1	0.23±0.00	0.41±0.01	**0.85**±0.08	**0.19**±0.01	0.38±0.02	0.58±0.05
	Xi Gen.2	**0.24**±0.00	0.38±0.01	0.53±0.01	0.25±0.01	0.42±0.00	**0.55**±0.02
	Xi Gen.3	**0.18**±0.00	0.35±0.01	0.54±0.01	0.18±0.01	0.29±0.04	**0.65**±0.17
	Xk Gen.1	**0.40**±0.03	0.80±0.01	**1.35**±0.08	0.49±0.01	0.79±0.03	0.97±0.23
	Xk Gen.2	**0.24**±0.00	1.02±0.08	**1.78**±0.00	0.27±0.01	0.47±0.00	1.02±0.11

Gen. = Transgenic generation, Standard error calculated from triplicate analysis of samples.

2.3.5 Plant protein extracts: Analysis by electron microscopy and western blotting

The protein extracts derived from homogenized transgenic *A. thaliana* and *N. tabacum* plant material were trapped onto carbon coated copper grids using anti-HPV-11 rabbit polyclonal antiserum (1:50 dilution), decorated with conformational monoclonal antibody H11:H3 (1:1000 dilution) and detected with an anti-mouse-gold-conjugated secondary antibody (1:100 dilution, 30nm gold particles). Gold-labelling was observed in both transgenic *N. tabacum* (Figure 2.11 **A** and **B**) and transgenic *A. thaliana* (**C** and **D**) extracts. No gold-labelling was observed in the non-transgenic *A. thaliana* protein extracts as shown in figure 2.11 **E**.

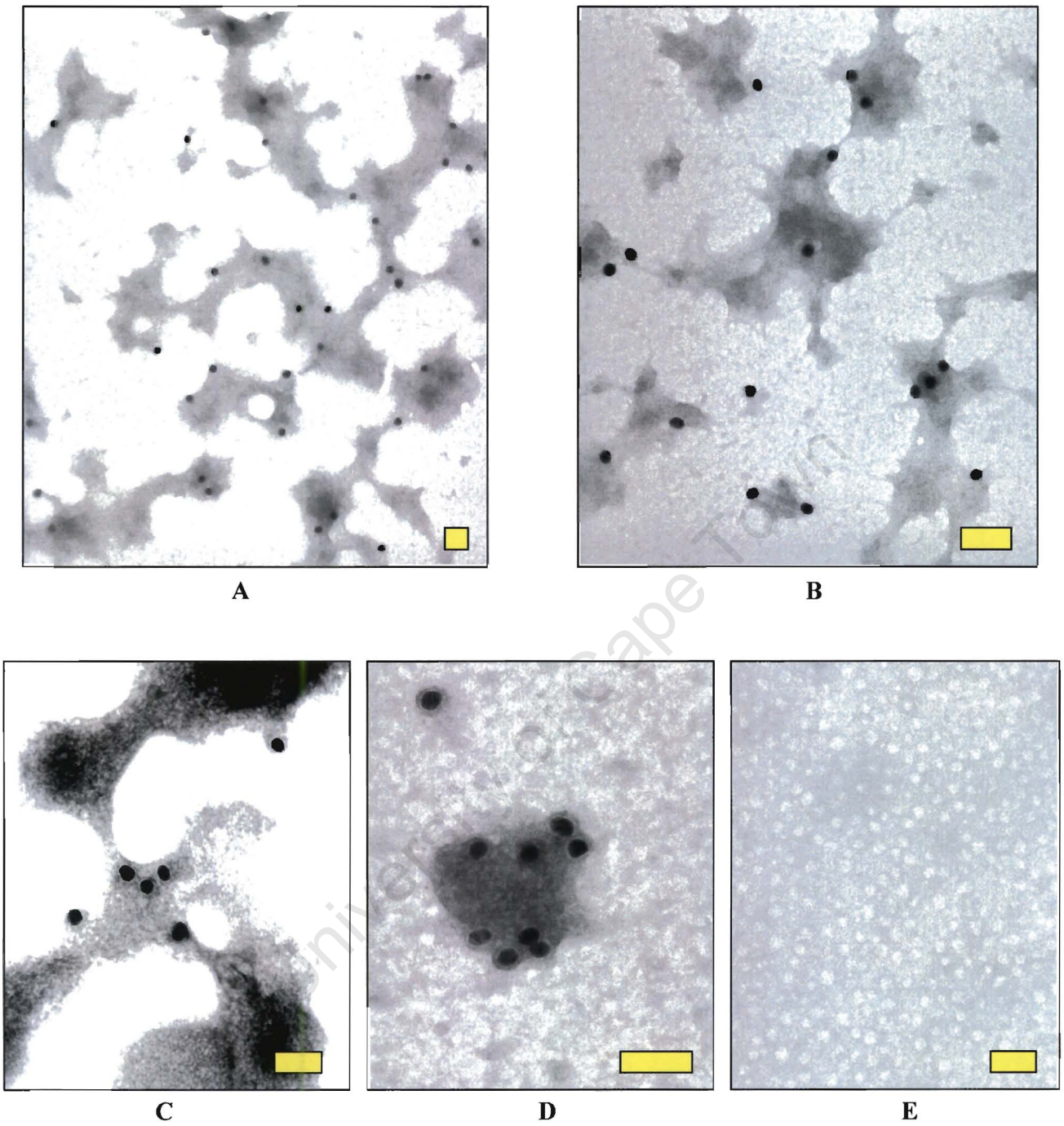


Figure 2.11: Electron micrographs of immunogold labelled HPV-11 L1 NLS$^-$ protein extracted from *N. tabacum* (**A** and **B**) and *A. thaliana* (**C** and **D**). Plant protein extract was trapped onto carbon coated copper grids with HPV-11 rabbit polyclonal antiserum, decorated with monoclonal antibody H11:H3 and detected with an anti-mouse-gold-conjugated secondary antibody. Gold particles are 30 nm in diameter. Non-transformed *A. thaliana* plant protein extract is shown in **E**. (**Bar = 100nm**).

A. thaliana and *N. tabacum* protein extracts were immunotrapped onto carbon coated copper grids using anti-HPV-11 polyclonal antiserum and MAb H11:H3 respectively and negatively stained with 2% uranyl acetate. A range of different particle sizes, varying from 20 to 60nm in diameter, were observed in both protein extracts as is shown in figures 2.12 and 2.13. None of the particles, except for number III (Figure 2.12), were heavily stained with uranyl acetate, suggesting that HPV-11 L1 NLS^- plant and insect cell-derived particles (Figure 2.13 C) all encapsidated nucleic acid to a certain extent. Similar findings had been described by Varsani *et al* (2003) who found that HPV-16 L1 particles derived from transgenic tobacco plants also encapsidated nucleic acids to varying degrees.

Insect cell-derived HPV-11 L1 NLS^- particles also apparently displayed some degree of nucleic acid packaging (Figure 2.13 C). They displayed a more uniform morphology than their plant-derived counterparts (Figure 2.12 and 2.13 A and B) with an average diameter of 50nm, yet they did not resemble their insect cell-derived HPV-11 L1 NLS^+ counterparts: HPV-11 L1 NLS^+ particles as seen in figure 2.14 (A to E) show the typically characteristic "doughnut" shape and are heavily stained with uranyl acetate.

The negative controls were plant protein extracts from both non-transgenic *A. thaliana* (data not shown) and *N. tabacum* (Figure 2.13 D): these showed no HPV-like particles or any other upon examination by electron microscopy.

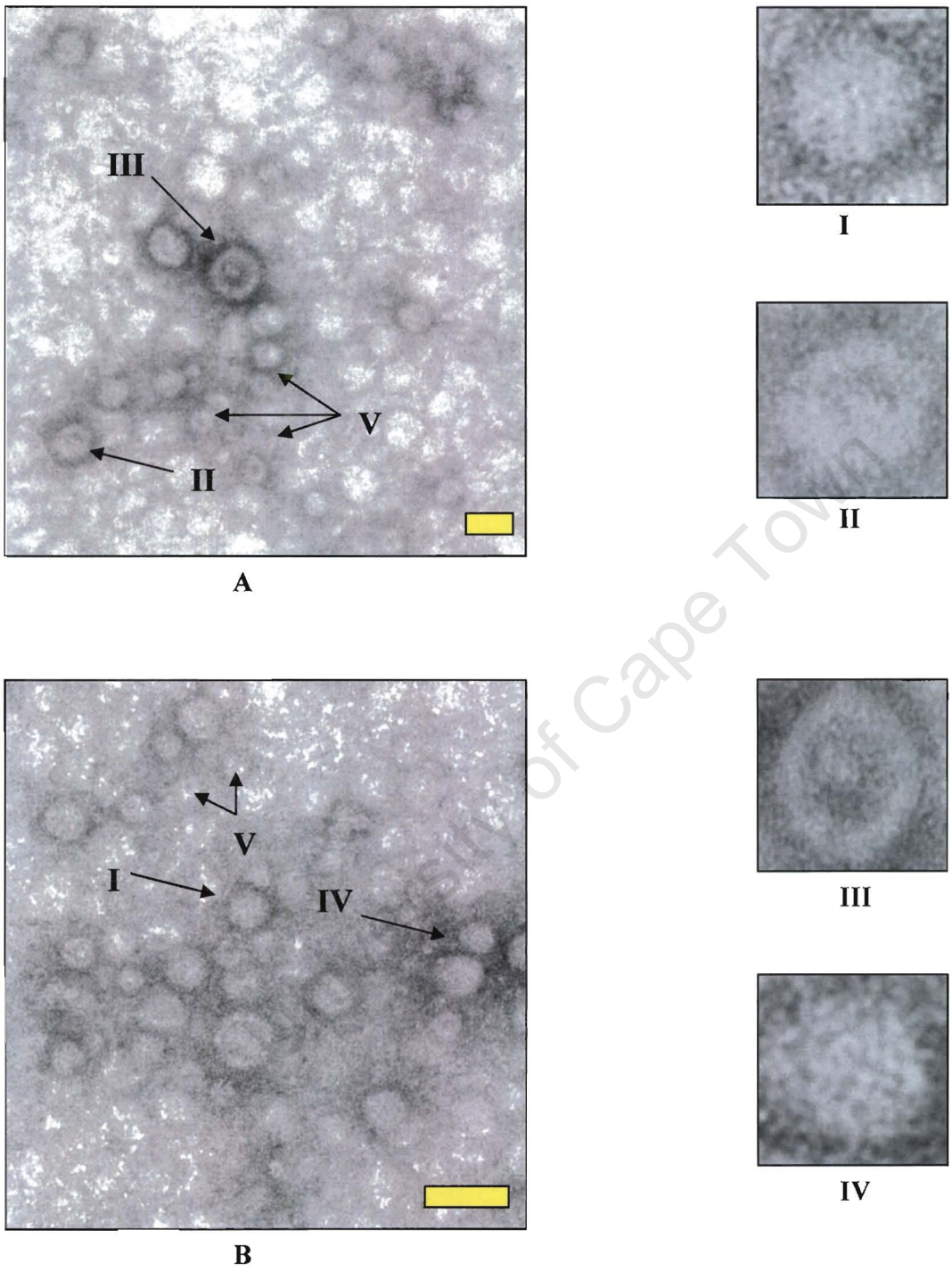


Figure 2.12: Electron micrographs of T_3 generation *A. thaliana*-derived HPV-11 L1 NLS$^-$ VLPs. Particles were immunotrapped using polyclonal antiserum at a dilution of 1:50 and range in size from 22 to 55 nm in diameter. Particles indicated by **V** could possibly be stain filled capsomers. Images **I - IV** represent enlargements of individual particles observed in **A** and **B.** (**Bar = 50nm**)

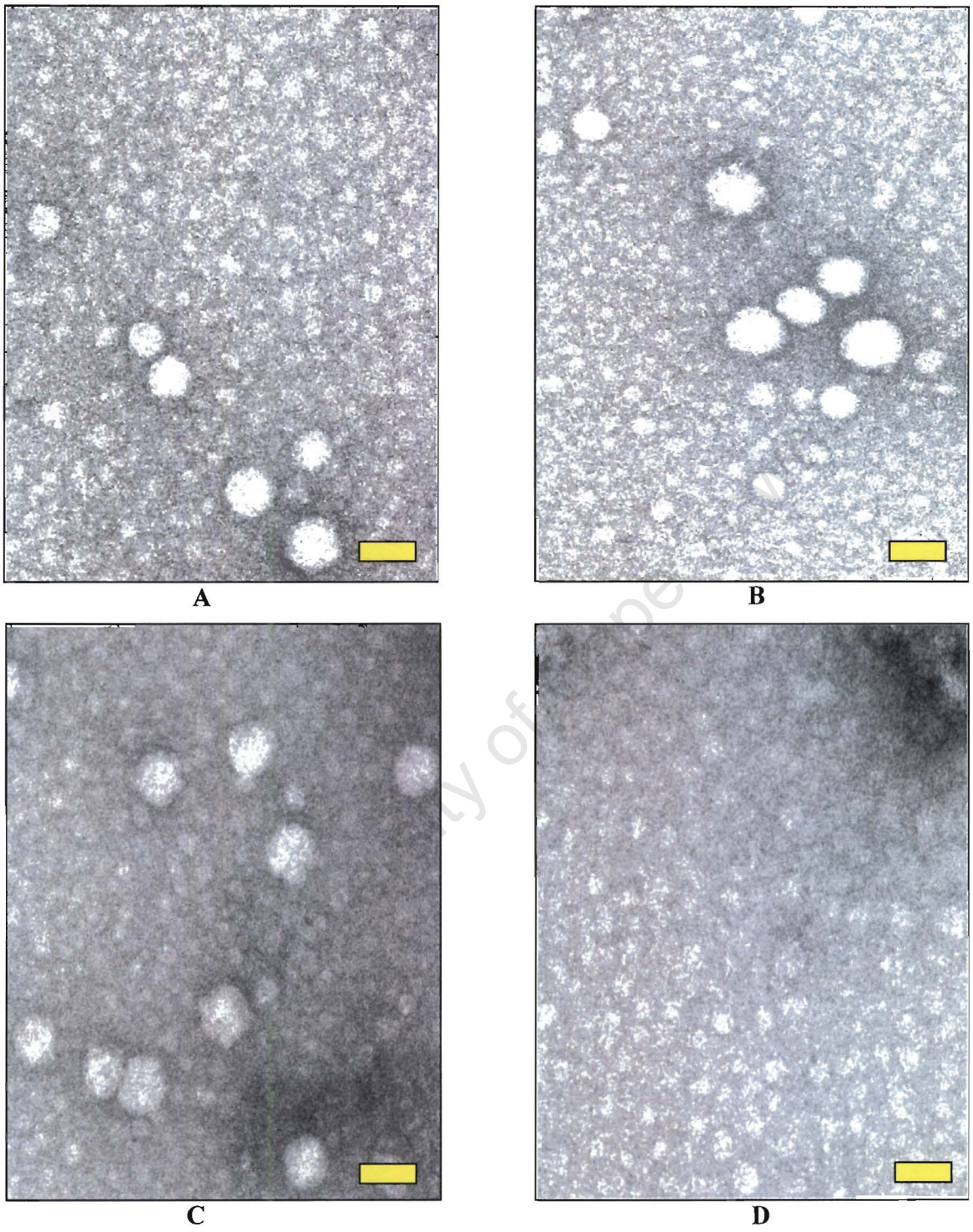


Figure 2.13: Electron micrographs of *N. tabacum*-derived HPV-11 L1 NLS$^-$ VLPs (**A** and **B**). Particles were immunotrapped using H11:H3 at a dilution of 1:1000. The positive control (**C**) shows immunotrapped insect cell-derived HPV-11 L1 NLS$^-$ VLPs, whereas immunotrapped non-transgenic plant protein extract is represented in **D**. VLPs range from 30 to 60 nm in diameter. (**Bar = 50nm**)

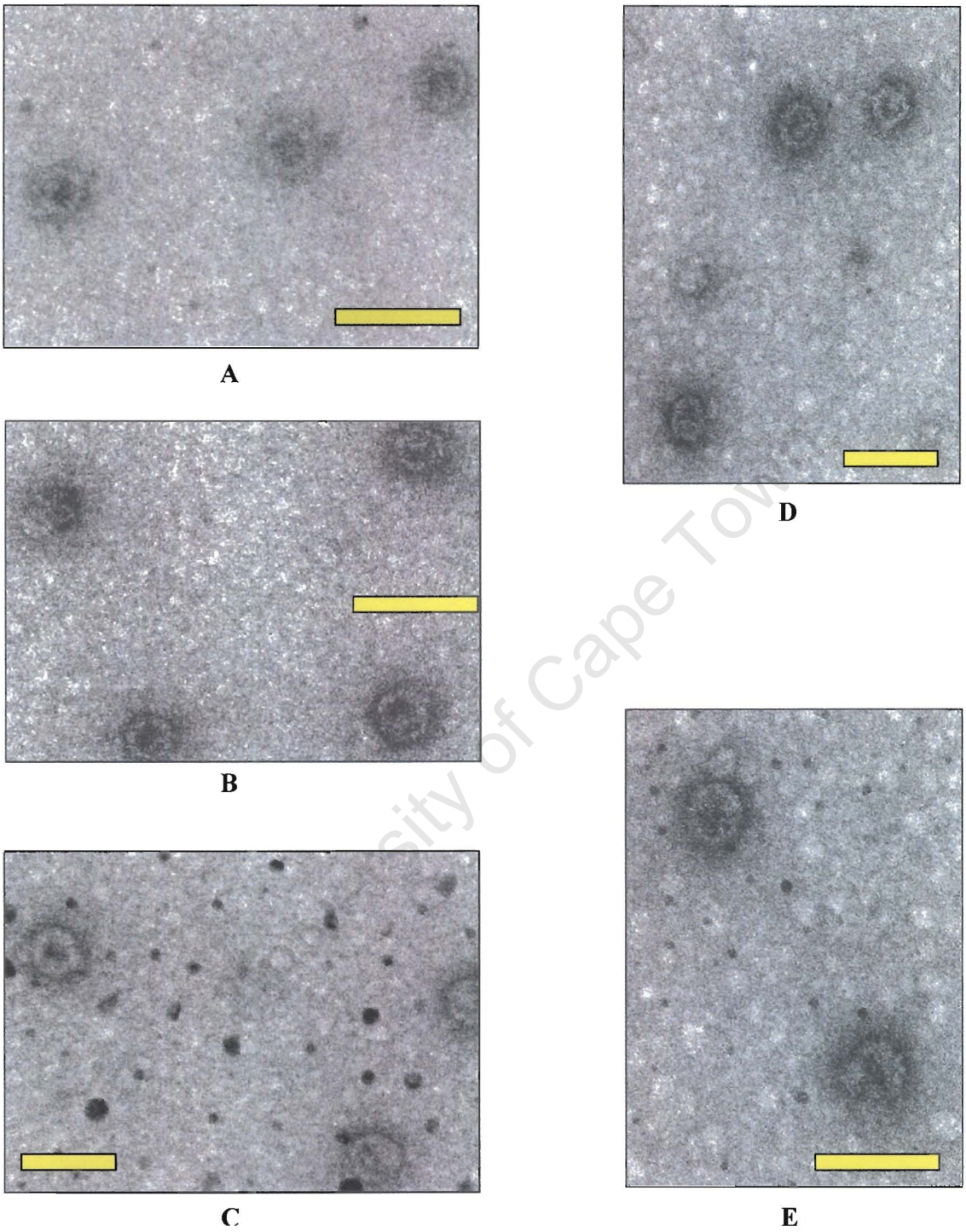


Figure 2.14: Electron micrographs showing insect cell-derived HPV-11 L1 NLS$^+$ VLPs trapped with monoclonal antibody H11:H3 (**A** and **B**) and HPV polyclonal antibody (**C**, **D** and **E**). Particles appear to be 'doughnut' shaped when stained with 2% uranyl acetate, are 55 nm in diameter and serve as reference to figures 2.12 and 2.13. **(Bar = 100nm)**

Western blot analysis of the protein extracted from individual transgenic *N. tabacum* lines revealed that the L1 protein continued to be translated over several generations. However, as indicated in figures 2.15a (A and B), the protein was severely degraded and was only present in the form of an approximately 36 kDa protein. Detection of HPV-11 L1 NLS$^-$ protein was only achieved once total plant protein was precipitated in 5% TCA (v/v) as described in 2.2.8 and resolved on a 12% SDS-PAGE gel.

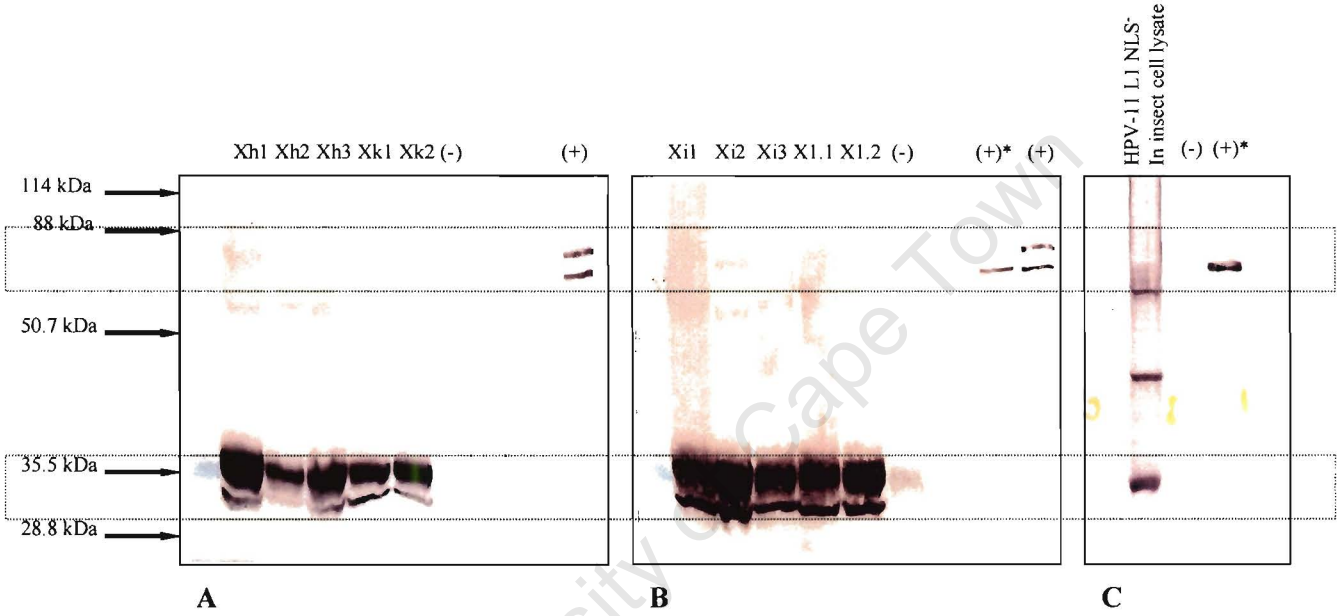


Figure 2.15a: Western blot analysis of TCA precipitated *N. tabacum* protein extract detected with H6:I2 (**A** and **B**). The HPV-11 L1 NLS$^-$ protein is present in all generations of the four transgenic lines and is approximately 36 kDa in size. No HPV-11 L1 NLS$^-$ protein was detected in the TCA precipitated non-transgenic control [**(-)** in **A** and **B**]. The positive control (+) and (+)* [**A, B** and **C**], represent insect cell-derived CsCl-purified HPV-11 L1 NLS$^+$ and HPV-11 L1 NLS$^-$ VLPs respectively.
Western blot analysis of non-concentrated, non-purified HPV-11 L1 NLS$^-$ VLPs in insect cell lysate (**C**) detected with H6:C6 shows that the protein is cleaved twice, resulting in three proteolytic products one of which is approximately 36 kDa in size. Non-infected insect cell lysate was used as negative control (-) in **C**.

As a control experiment I used insect cell lysate from cells infected with an HPV-11 L1 NLS$^-$ recombinant baculovirus, which was left standing for 24 hrs before an aliquot was analyzed by western blot. The results as shown in figure 2.15a (C) indicate that purification of the recombinant protein has to be completed directly after harvesting of infected insect cells. If left unattended, the protein is also subject to proteolytic degradation as indicated by the presence of three protein species of different sizes. More

importantly however is the fact that only the smallest L1 protein species (approximately 36 kDa) is present in the *N. tabacum* extracts, indicating that the protein is subject to rapid and severe degradation even in the presence of complete protease inhibitor.

The same degree of HPV-11 L1 NLS$^-$ protein proteolysis was expected before western blot analysis of the protein extract from the pooled T_3 generation transgenic *A. thaliana* leaf material. However, no extensive protein degradation was observed. The detected protein [Figure 2.15b (D and E)] was of the same size (55 kDa) as the insect cell-derived positive control [Figure 2.15b (F)], indicating that cellular modifications including proteases activities differ markedly between the two plant systems.

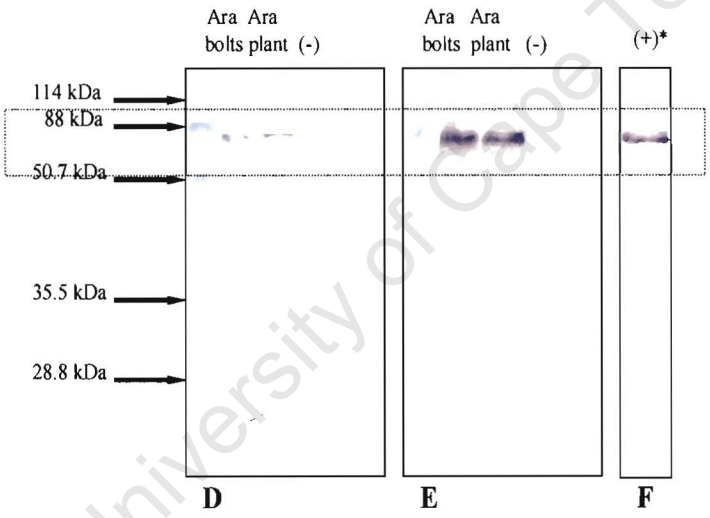


Figure 2.15b: Western blot analysis of TCA precipitated *A. thaliana* protein extract detected with H6:I2 (**D**) and H6:E51 (**E**). HPV-11 L1 NLS$^-$ protein is present in both protein extracts from primary bolts and complete plants (Ara bolts and Ara plant). No HPV-11 L1 NLS$^-$ protein was detected in the TCA precipitated non-transgenic control [**(-)** in **D** and **E**]. The detected protein runs at the same position as the positive control in figure 2.15b (**F**).

2.3.6 Analysis of the immune response to transgenic plant-derived HPV-11 L1 protein

A successful vaccine against any virus disease usually elicits neutralising antibodies. Results already shown indicate that the HPV-11 L1 NLS^- protein extracted from transgenic tobacco could be detected by a neutralising conformation-specific MAb used in the immunogold-labelling procedure (Figure 2.11), but that manipulation of the already severely degraded protein in an ELISA resulted in further denaturation (Figure 2.9), evident by failure of the conformation-specific MAb to bind the immobilized protein. These problems were not encountered in the ELISA evaluation of the HPV-11 L1 NLS^- protein extracted from *A. thaliana*, where neutralising conformation-specific MAbs were capable of detecting the protein. With this in mind I did a preliminary experiment to establish whether the plant-derived protein was capable of eliciting a neutralising antibody response in naïve New Zealand white rabbits.

Two New Zealand white rabbits were used to evaluate each of the 4 HPV-11 L1 NLS^- plant-derived antigens (1 *A. thaliana* and 3 *N. tabacum*) and an insect cell-derived HPV-11 L1 protein in this preliminary trial. The inclusion of 2 rabbits receiving the insect cell-derived HPV-11 L1 NLS^+ protein served both as control in this experiment and for further generation of reagents. The antibody response of these two animals was as expected, resulting in a high anti-HPV-11 L1 NLS^+ antibody titre. The ELISA analysis of this serum is not included in the following data (Figures 2.16 and 2.17 a and b), but its virus neutralising capabilities are recorded in the neutralisation assay section.

I did not include in this experiment a placebo (PBS, non-transgenic material) group, nor an adjuvant-only group: the reasoning for this is that within our group we have never found HPV-reactive antibodies under these conditions using a variety of L1 antigens, and have thus dispensed with these controls. Furthermore, no antigenicity experiments were undertaken to evaluate transgenic HPV-11 L1 NLS^- *N. tabacum* line Xk. As noted in table 2.5, growth of this particular transgenic line was severely stunted and not suitable for sufficient growth of biomass and protein extraction

Table 2.7: Layout of preliminary antigenicity evaluation animal experiment

Animal	Antigen source	Extract from respective transgenic line	Assigned animal number	Dosage (μg)	Adjuvant	Administration
New Zealand white rabbits	*A. thaliana*	Pooled T_3 generation	#11 and #12	21-43	Freund's incomplete	3 subcutaneous sites, 1 intramuscular site
	N. tabacum	X1	#13 and #14	13-47		
		Xh	#15 and #16	10-114		
		Xi	#17 and #18	9.5-42		
	Insect cells	n/a	#19 and #20	30		

A total of 10 New Zealand white rabbits were immunized with plant-derived antigen as laid out in table 2.7. The concentration of the plant-derived protein was achieved by centrifugation as shown in figure 2.2. This resulted in the respective 25-fold and 100-fold concentration of the *A. thaliana* and *N. tabacum* inoculum.

Rabbits #11 and #12 were immunized with T_3 generation transgenic *A. thaliana*-derived protein extract calculated to contain between 21μg and 43μg (according to Table 2.7) in 1ml of inoculum containing Freund's incomplete adjuvant. The serum analysis is presented in figure 2.16.

Results presented in figure 2.16 show that rabbit #11 gave the best antibody response against HPV-11 L1 NLS^- VLPs. I observed a steady rise in antibody titre over the background absorbance levels of the prebleed serum taken on day 1 after the first booster injection on day 28. A similar rise was expected to occur after the second booster on day 56, but was not seen. Rabbit #12 showed no significant antibody response when serum was tested against HPV-11 L1 NLS^- VLPs, and can hence be classified as a non-responder.

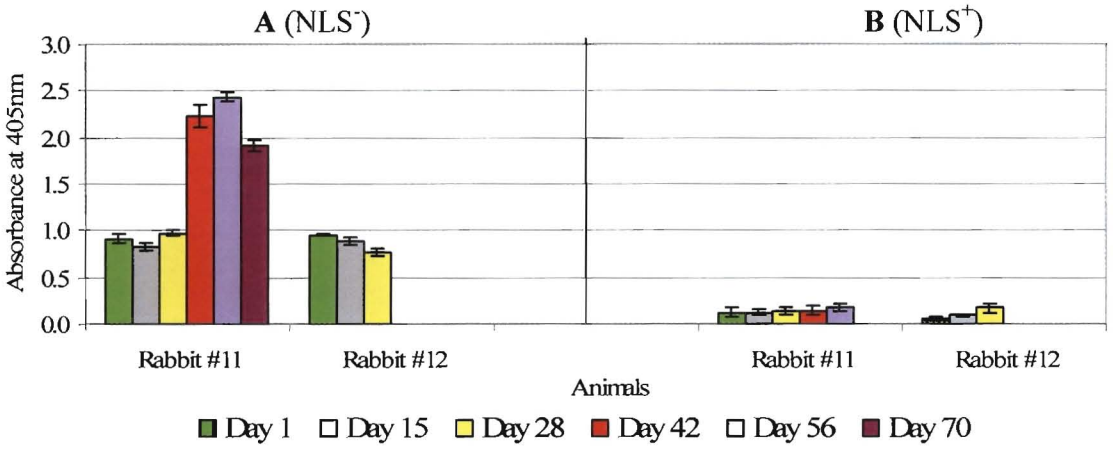


Figure 2.16: Analysis of antisera from New Zealand white rabbits #11 and #12 immunized with *A. thaliana*-derived HPV-11 L1 NLS$^-$ antigen. Rabbits were immunized on days 1, 28 and 56 and antisera from days 1, 15, 28, 42, 56 and 70 post injection was tested by direct ELISA in wells coated with 0.4µg of insect cell-derived HPV-11 L1 NLS$^-$ (**A**) and HPV-11 L1 NLS$^+$ (**B**) VLPs. Data from rabbit #12 is incomplete as it had to be euthanized due to growth of an abscess on the neck. Not enough blood was collected from rabbit #11 on day 70 to complete the evaluation of the serum against HPV-11 L1 NLS$^+$ (**B**). Error bars represent the standard deviation calculated from triplicate analysis of samples.

The most interesting result was obtained by evaluating the serum reactivity against HPV-11 L1 NLS$^+$ VLPs: Figure 2.16 indicates that serum failed to react when tested against HPV-11 L1 NLS$^+$ VLPs (Figure 2.16 B). This lack of recognition of the HPV-11 L1 NLS$^+$ VLPs is of great concern because these VLPs most closely resemble the actual virus: implications will be discussed later.

Of the sera collected from rabbits #13 to #18, only the last bleed (Day 70) from rabbit #14, but all post-bleed sera from rabbit #17, reacted with HPV-11 L1 NLS$^-$ VLPs (Figure 2.17 a) This did not correlate with inoculum dose, as responder rabbit #14 received 13-47µg, non-responders #15 and #16 received 10-114µg, and responder #17 received 9.5-42µg. Reactions of rabbits #14, #16 and #17 are shown in figure 2.17b: #14 was a non-responder for this antigen, while both #16 and #17 appeared to bind this antigen, as shown by rising absorbances of successive bleeds.

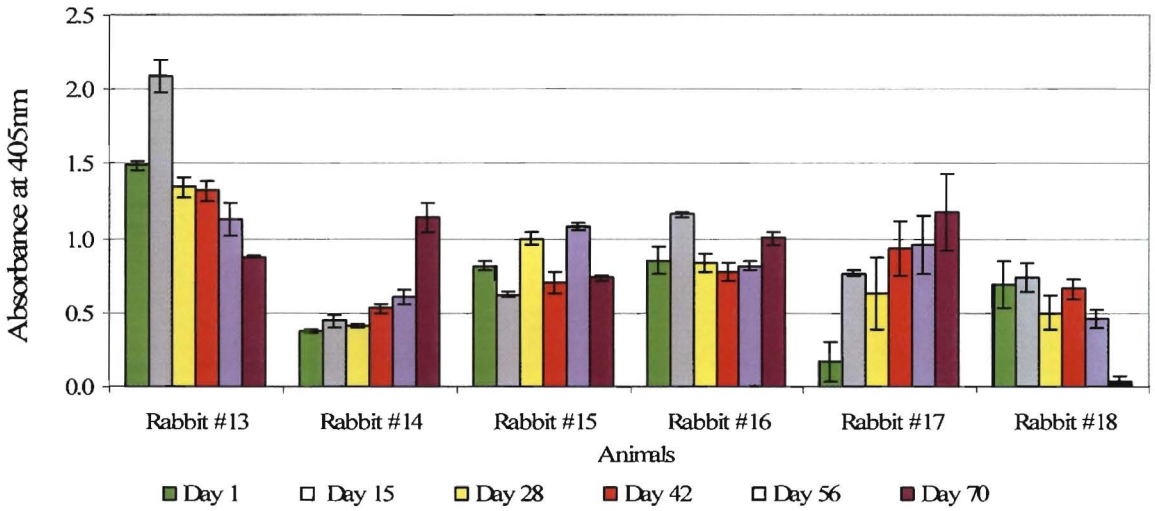


Figure 2.17a: Analysis of antisera from New Zealand white rabbits #13 to #18 immunized with *N. tabacum*-derived HPV-11 L1 NLS$^-$ antigen. Antiserum from days 1, 15, 28, 42, 56 and 70 was tested by direct ELISA with wells coated with 0.4µg of insect cell-derived HPV-11 L1 NLS$^-$ VLPs. The initial immunization was administered on day 1 was followed by subsequent booster inoculations on day 28 and 56. Error bars represent the standard deviation calculated from triplicate analysis of samples.

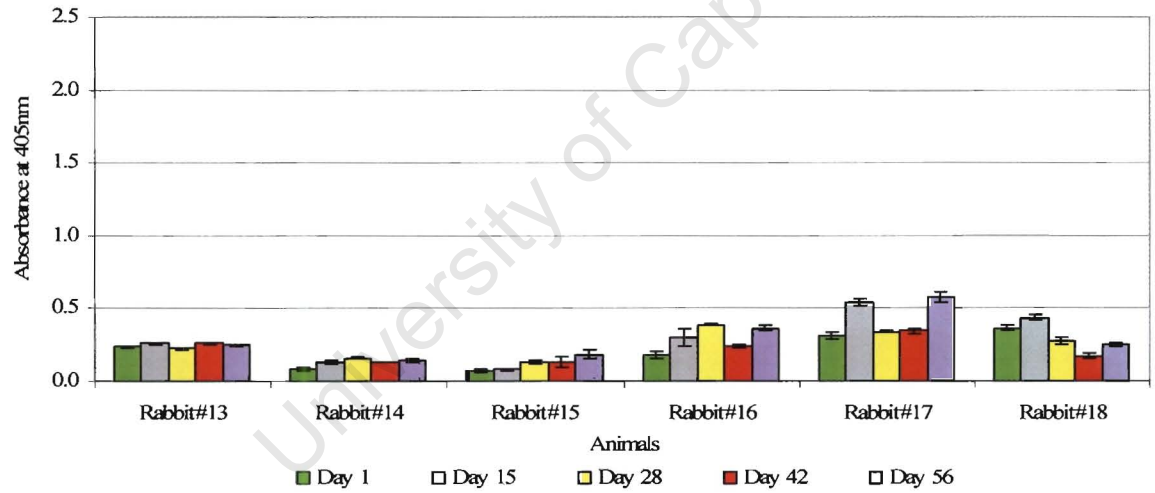


Figure 2.17b: Analysis of antisera from New Zealand white rabbits #13 to #18 immunized with *N. tabacum*-derived HPV-11 L1 NLS$^-$ antigen. Antisera collected on days 1, 15, 28, 42, 56 and 70 was tested by direct ELISA with wells coated with 0.4µg of insect cell-derived HPV-11 L1 NLS$^+$ VLPs. Error bars represent the standard deviation calculated from triplicate analysis of samples.

Given that antibody responses seemed specific for HPV-11 L1 NLS$^-$, the last bleed (Day 56) rabbit antiserum was used to determine whether insect cell-derived HPV-11 L1 NLS$^+$ and NLS$^-$ VLPs could be detected by western blot. While very strong reactions were seen with baculovirus and *Sf*21 cell proteins in both prebleed and last bleed sera, neither L1

protein was detected (data not shown). This suggested that the predominant antibody response was most probably not targeted against any linear epitopes.

To further investigate this, Day 56 antisera from *A. thaliana* and *N. tabacum* immunized rabbits were tested against denatured and non-denatured insect cell-derived HPV-11 L1 NLS^+ and NLS^- VLPs by direct ELISA. The results are shown in figure 2.18: these essentially reconfirm results presented in figures 2.16 and 2.17 a and b. With the exception of rabbit #11, the antisera from all remaining rabbits immunized with either plant-derived protein extract reacted predominantly with non-denatured insect cell-derived HPV-11 L1 NLS^- VLPs, and not with denatured protein from either type of VLPs. This could explain the lack of reactivity in western blots.

Furthermore, I note the variability of direct ELISA results as presented in figures 2.17 a and b. Although ELISA microtitre plate wells were coated with the same amount of antigen, one example of the most profound differences is displayed by rabbit #13. When prebleed serum (day 1) was tested against insect cell-derived HPV-11 L1 NLS^- VLPs, the measured absorbance reached a value of approximately 1.5. However, when the same serum was tested against HPV-11 L1 NLS^+ VLPs, the maximum absorbance reading was around 0.25 (Figure 2.17 b). This represents a 6-fold difference and highlights the variability between individual experiments.

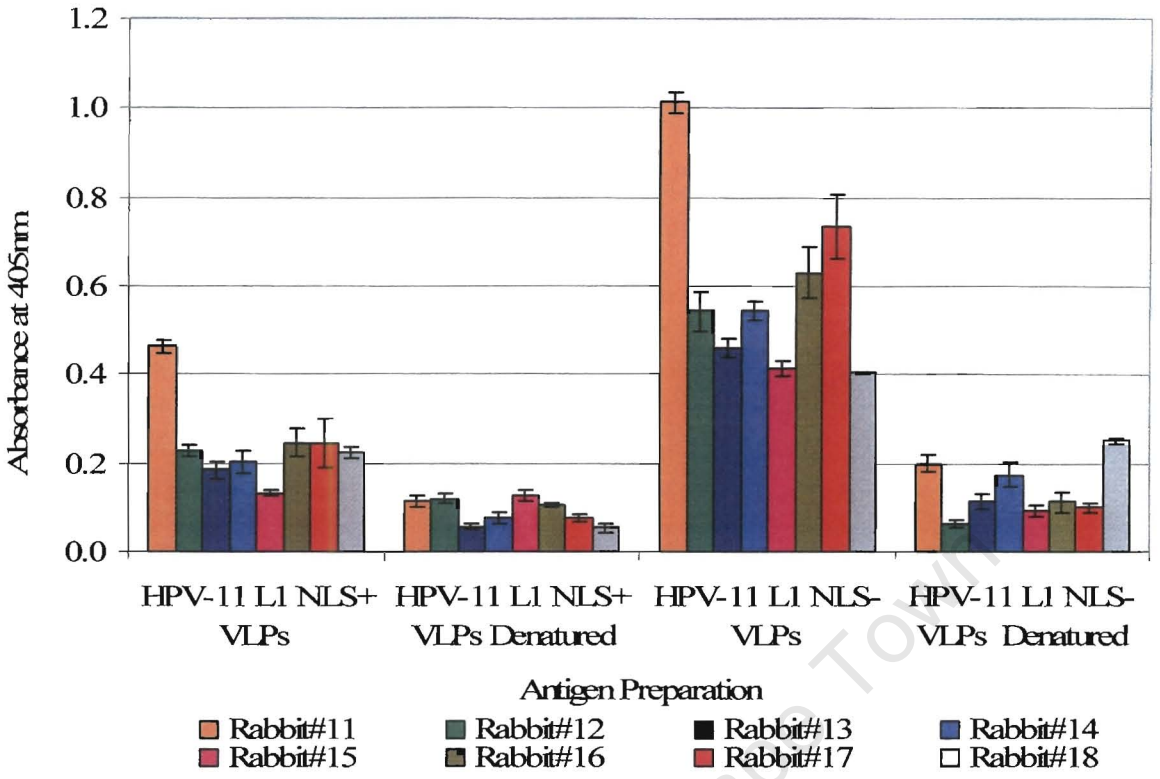
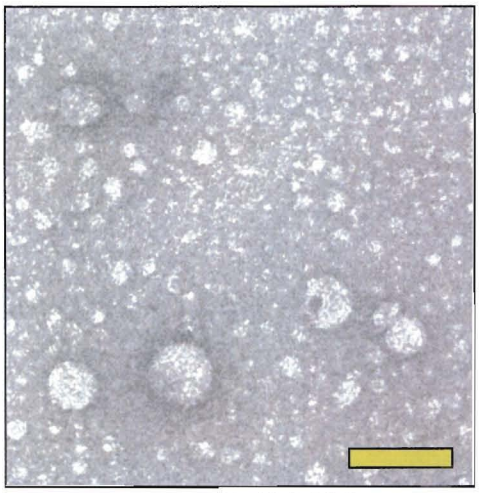
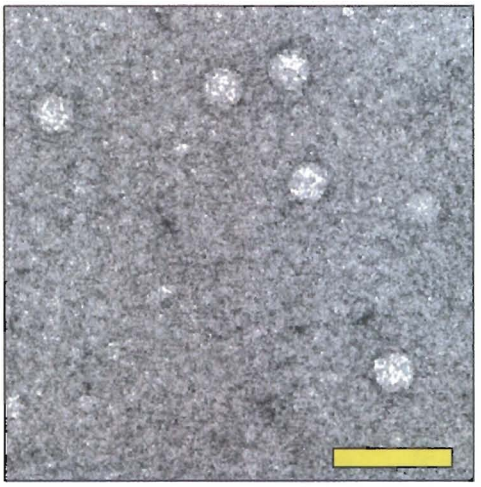


Figure 2.18: Analysis of the last bleed antisera from individual New Zealand white rabbits immunized with HPV-11 L1 NLS$^-$ plant protein extract. Sera were tested against equal concentrations of denatured and non-denatured insect cell-derived HPV-11 L1 NLS$^+$ and NLS$^-$ VLPs at a concentration of 0.4μg/well. Error bars represent the standard deviation calculated from triplicate analysis of samples. Insect cell-derived VLPs were denatured at 100°C for 10 min and complete denaturation was confirmed by SDS-PAGE electrophoresis. Rabbits #11 and #12 were immunized with 21μg to 43μg *A. thaliana*-derived HPV-11 L1 NLS$^-$ antigen. Rabbits #13 and #14, #15 and #16, and #17 and #18 were inoculated with *N. tabacum*-derived protein extract from T_1 generation transgenic plant lines X1, Xh and Xi respectively. Initial immunizations were followed by two additional booster inoculations on days 28 and 56. Inoculum preparations for individual rabbits included the dilution of 500μl plant protein extract with Freund's incomplete adjuvant in a ratio of 1:1. Antiserum was collected on days 1, 15, 28, 42, 56 and 70, the last of which is evaluated in the above figure.

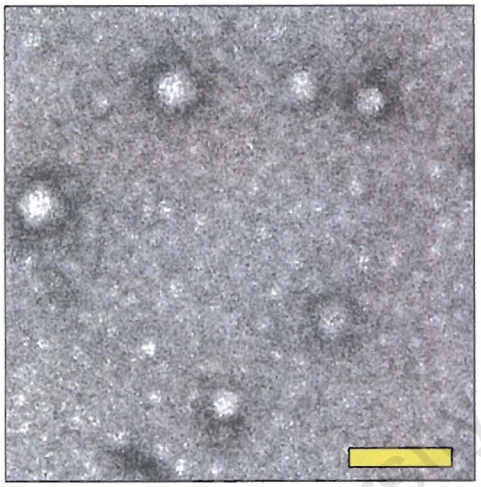
All day 56 rabbit antisera were used to immunotrap insect cell-derived HPV-11 L1 NLS$^-$ VLPs which were then examined by electron microscopy. All electron micrographs are shown in figure 2.19: these further indicate that insect cell-derived HPV-11 L1 NLS$^-$ VLPs differ in morphology to HPV-11 L1 NLS$^+$ VLPs, as discussed in section 2.3.4. It is further evident that all sera apparently bound VLPs: this could reflect differences in sensitivity of detection between ELISA and immuno-electron microscopy (see discussion).



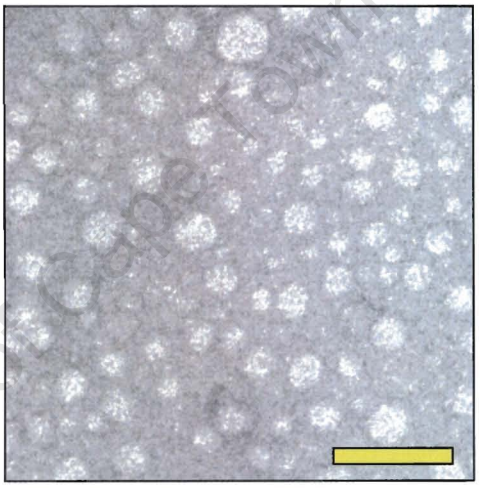
A (Rabbit#11)



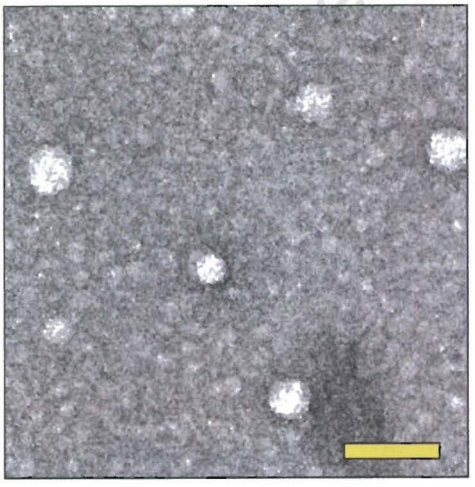
B (Rabbit#12)



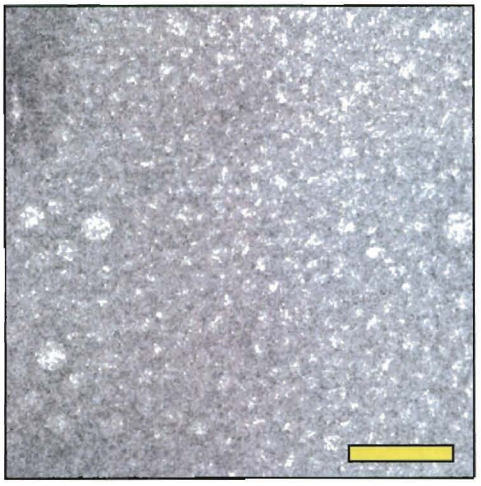
C (Rabbit#13)



D (Rabbit#14)



E (Rabbit#15)



F (Rabbit#16)

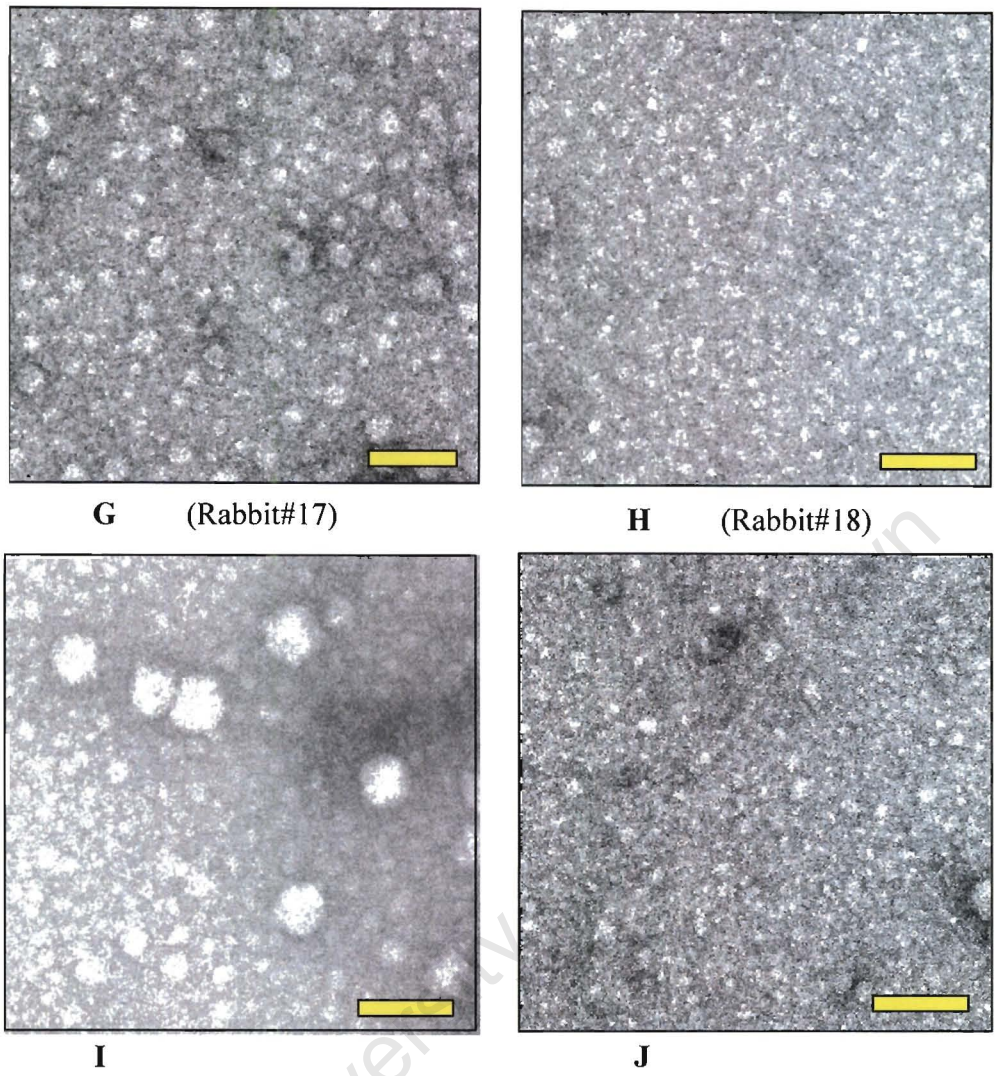


Figure 2.19: Electron micrographs showing insect cell-derived HPV-11 L1 NLS$^-$ VLPs, immunotrapped with polyclonal antisera from rabbits immunized with transgenic *A. thaliana* plant protein extract (**A** and **B**) and *N. tabacum* cv Xanthi plant protein extract from transgenic lines X1, Xh and Xi (**C** and **D**; **E** and **F**; **G** and **H** respectively).The positive control shows HPV-11 L1 NLS$^-$ VLPs immunotrapped with HPV-11 polyclonal antiserum (**I**).The HPV polyclonal antibody is replaced by an anti-TMV polyclonal antibody in the negative control (**J**). Particle diameters vary from 30 to 60 nm. **(Bar = 100 nm)**

2.3.7 Pseudovirus neutralisation assays

All rabbit sera taken on days 1 and 56 were analyzed for their capacity to neutralise HPV-11 pseudoviruses. Serum sample dilutions ranging from 1:50 to 1:12150 were incubated together with HPV-11 L1 pseudoviruses before being assayed as described in section

2.2.14 of the Materials and Methods (Figure 2.20). One of the two internal controls was the serum collected from rabbit #19, which was inoculated with insect cell-derived HPV-11 L1 NLS$^+$ VLPs. The other was a serum blank (Figure 2.20 A and B). Furthermore, an additional positive control was included: HPV-11 L1 pseudoviruses were incubated with dilutions of the conformation-specific and neutralising MAb H11:H3 ranging from 10^{-4} to 10^{-9} (Figure 2.21).

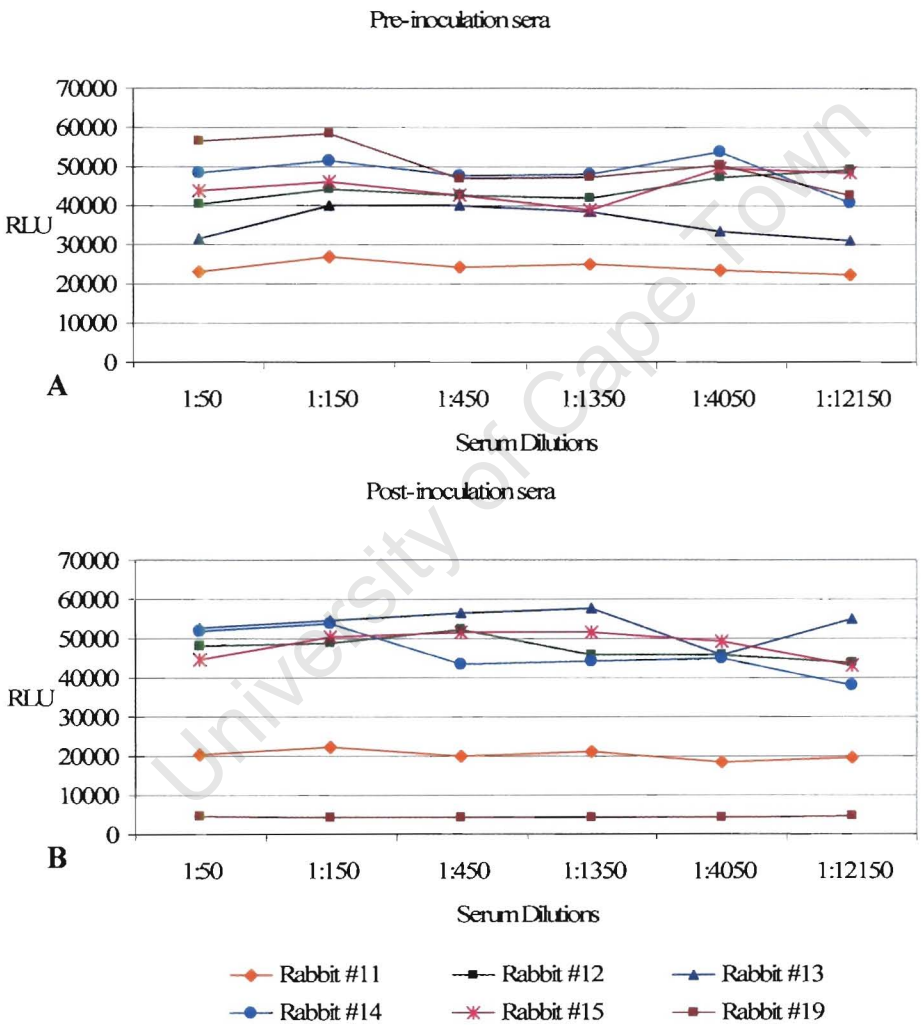


Figure 2.20: HPV-11 pseudovirus neutralising assay using the pre-inoculation serum (Day 1) collected from all rabbits immunized with either transgenic plant-derived *A. thaliana* and *N. tabacum* protein extracts (A). The evaluation of the post-inoculation sera (Day 56) from all animals is shown in B. Included in the data set is the serum collected from rabbit #19 which was inoculated with insect cell-derived HPV-11 L1 NLS$^+$ VLPs. The data is presented as relative light units (RLU) observed at various dilutions of the tested serum.

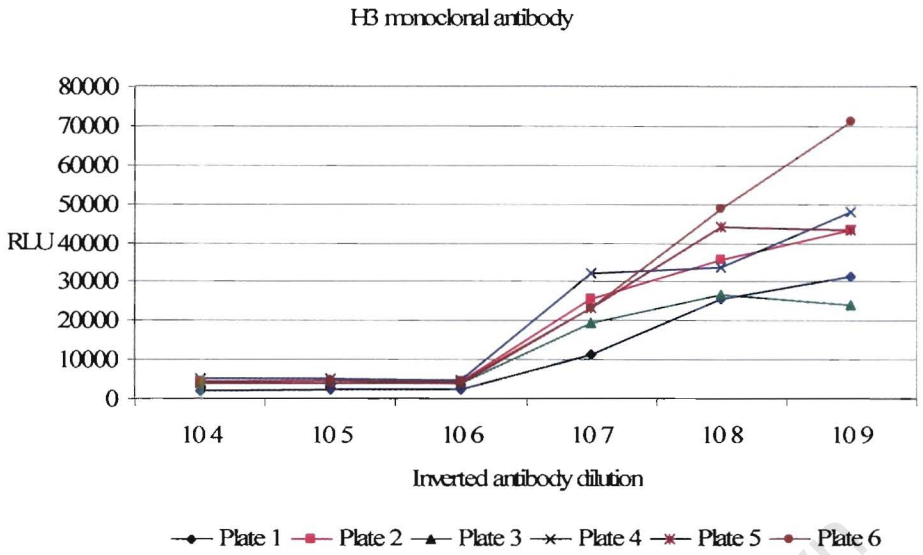


Figure 2.21: Neutralisation assay results obtained from incubation of HPV-11 pseudoviruses with known conformation-specific and neutralising MAb H11:H3. Data is presented as a collection from six individual experiments.

The results obtained from the evaluation of the pre-inoculation serum were as expected (Figure 2.20 A): no specific neutralising capacity was observed for any serum. However, in comparison to the serum collected from rabbit #19 (internal positive control) which still neutralised the pseudovirus at the highest dilution tested (1:12150), none of the post-inoculation day 56 sera were neutralising; even at the lowest dilution of 1:50. The virus neutralising capability at a dilution of $1:10^6$ of the known conformation-specific and neutralising MAB H11:H3 extends far beyond that achieved by the serum collected from rabbit #19, and serves as an excellent additional control (Figure 2.21).

Taken together, these serological indicate that plant-derived HPV-11 L1 NLS^- VLPs are modified to some extent, yet still retain certain overall virion morphological features as seen by electron microscopy. Immunization of animals, however, results in an antibody response that is not capable of recognizing or neutralising HPV-11 L1 NLS^+ VLPs, the closest counterpart to the actual virus.

2.4 DISCUSSION

The overall aim of this study was to gain some insight as to the feasibility of developing a plant-derived vaccine against the human papillomavirus type 11. This is against a background of the scheduled release in 2006 of the yeast-derived quadrivalent vaccine against HPV types 6, 11, 16 and 18 by Merck: this is a vaccine that has shown great effectiveness, but at the same time will remain largely non-affordable to people in disadvantaged regions of the globe for the foreseeable future.

When work on this project first commenced in 1999 it soon became apparent that *in planta* expression of the wild-type HPV-11 *L1* gene presented great difficulties and that it was effectively impossible to transform and regenerate either *A. thaliana* (ecotype Columbia) or *N. tabacum* cv. Xanthi. It was possible, however, to generate transgenic plants of both types expressing the NLS^- version of the gene. Through collaborations between Professors Ed Rybicki and Hugh Mason it became known to me that identical observations had been made within Professor Mason's research group. There is still no explanation as to why this is the case; only the speculations that presence of the NLS would lead to an accumulation of the L1 protein in the plant nucleus, which might reach toxic levels, or interfere with the cell cycle so as to block regeneration. In any case, as the objective was to make a candidate vaccine rather than to investigate the vagaries of plant expression, efforts to use the wild type *L1* gene were abandoned.

In 2003 Warzecha *et al.* published findings relating to HPV-11 *L1* expression in plants that were of great importance to this work. Not only did they confirm the interference of the NLS sequence, but also recapitulated other observations from my research. Amongst their observations are the few transgenic plant codon-optimized HPV-11 *L1* potato plants they have managed to regenerate. Out of 100 potato internode segments, only 3 transgenic HPV-11 L1 NLS^+ and 7 HPV-11 L1 NLS^- plants were successfully regenerated. Significantly, they found no expression of the protein in HPV-11 L1 NLS^+ transformed plants. I found that out of a total of 15 putative transgenic *Nicotiana tabacum* cv. Xanthi plants regenerated in this study, only 4 had shown integration of the

HPV-11 *L1 NLS*⁻ gene. In *Arabidopsis thaliana*, transformation leads to the production of approximately 40000 seeds, thereby increasing the number of putative transgenic offspring: however, selective screening of putative transgenic seeds resulted in the selection of only 10 individual transgenic lines. Warzecha *et al* (2003) did not achieve high expression levels in HPV-11 L1 NLS⁻ transgenics: although they used a plant codon-optimised gene, their L1 levels in potato tubers were only around 20µg/kg. This is probably far too low for a viable vaccine production vehicle.

These results for HPV-11 *L1* are not similar to results published in other studies on the expression of the HPV-16 *L1* gene in transgenic plants. Biemelt *et al.* (2003) and Varsani *et al.* (2003) both managed to transform either tobacco or potato plants with constructs expressing both the HPV-16 *L1 NLS*⁺ and *L1 NLS*⁻ genes, resulting in the regeneration of up to 75 individual lines. While successful integration does not necessarily result in expression of the protein, both groups also reported expression and assembly of the L1 protein, despite very different expression levels (12mg/kg and 4µg/kg respectively). The HPV-11 and 16 *L1* genes are significantly different, sharing only 66% nucleotide and 68% amino acid identity, but the proteins are functionally very similar. The reasons for the difference in transformability of the HPV-16 and HPV-11 *L1* genes remain obscure.

Biemelt *et al.* (2003) found that the 'humanized' *L1* gene expressed better than the 'plantised' L1 gene, which was better than the wild-type: Varsani *et al.* (2003) achieved expression with wild-type *L1*. Given the very difficult and unpredictable expression levels achieved with a spectrum of HPV *L1* genes in potato and tobacco - 'plantised' HPV-11 *L1 NLS*⁻; wild-type, 'humanised' and 'plantised' HPV-16 *L1* and *L1 NLS*⁻ - it was decided that I should concentrate on trying to maximize expression of a wild-type HPV-11 *L1 NLS*⁻ gene in two expression hosts. The lack of funds to experiment with codon optimization was also an important consideration in this regard.

In this study, therefore, both *A. thaliana* and *N. tabacum* were transformed with the HPV-11 *L1 NLS*⁻ gene, missing the last 21 carboxyl terminal amino acids, and thus presumably preventing the translocation of the protein into the nucleus. This form of the gene was

successfully integrated into the host's genome as shown by PCR screening of genomic DNA extracted from plants of consecutive generations. Furthermore, the presence of the gene in the 6th generation *A. thaliana* plants indicates the stable inheritance of the transgene. Although initial attempts to detect and determine the copy number of the integrated transgene were not successful, detection in *A. thaliana* genomic DNA by Southern blot was achieved once the transgene had been excised from the host's genome.

Whether or not the gene was transcribed by the host's cellular machinery was investigated by analysis of extracted total RNA using an RT-PCR approach: active transcription of the gene was shown throughout successive generations of both plant systems. One possible shortcoming of this approach, given that Biemelt *et al.* (2003) and Warzecha *et al.* (2003) found by northern blot that their respective *L1* mRNAs were degraded to some extent, was that such degradation was not detectable in my system. It would perhaps have been more appropriate to have examined the *L1* mRNA integrity by northern blot in order to determine if such degradation occurred in my plants. However, given the levels of expression found in my plants – around 11mg/kg in *A. thaliana*, and 2.3mg/kg in tobacco – this was probably not a limiting factor in my work.

I note that these expression levels are very significantly higher than found by Warzecha *et al.* (2003) in transgenic potato, despite my having used a wild-type gene. These levels were comparable to those of Biemelt *et al.* (2003) with their 'humanised' HPV-16 *L1* gene in potato or tobacco. Thus, this study is already the most successful by several orders of magnitude in terms of simply expressing an HPV-11 *L1* gene in plants.

I have calculated the final amounts of HPV-11 L1 NLS^- protein harvested from 1 kg of fresh transgenic *A. thaliana* or *N. tabacum* leaf material. Overall a final yield of 11mg/kg and 2.3mg/kg were calculated to be obtained form *A. thaliana* and *N. tabacum* plants respectively. Considering the report by Warzecha *et al.* (2003) who after expression of a plant-codon optimized HPV-11 L1 NLS^- gene in transgenic potatoes only obtained a yield of 20µg/kg, these figures resulting from the expression of a non-codon-optimized HPV-11 *L1* gene are quite staggering.

Although it is stated in the literature that transgenic plants often express incorporated transgenes at low levels, several reports including the following by Lauterslager *et al.* (2001) have reported on harvesting a comparable 13mg/kg of the *E. coli* enterotoxin B subunit from transgenic potato plants expressing the codon optimized *recLT-B* gene. In addition, but on a lower scale, Tsuda *et al.* (1998) have approximated the yield of Hepatitis B virus core antigen from transgenic tobacco plants to be around 1.3mg/kg. Although levels of foreign proteins expressed in transgenic plants are highly variable, these reported yields stand to reason and justify that higher expression levels of desired antigens can be obtained from transgenic plants.

While I did not systematically examine the integrity of the mRNA resulting from transcription of the integrated HPV-11 *L1 NLS*⁻ genes, and L1 expression levels seemed high, throughout this study I uncovered evidence that indicated that post-translational effects were significantly affecting the production of vaccine appropriate L1 protein in transgenic plants.

Native HPV virions have been described as a T = 7 icosahedral capsid. Five copies of the L1 major capsid protein form a pentamer, also termed a capsomer or capsomere, 72 of which come together to form the viral capsid. Capsids are generally 55 nm in diameter (Baker *et al.* 1991 and others). Rose *et al.* (1993) originally described the expression of the HPV-11 *L1* gene in *Sf*9 insect cells by recombinant baculovirus. This resulted in the formation of empty virus capsids better known as virus-like particles (VLPs). Close examination of these VLPs revealed that they were morphologically and immunologically identical to native HPV-11 virions. In addition, it had been found that the nuclear localization signal of the HPV-11 *L1* gene was not required for correct assembly of VLPs (Merle *et al.*1999) and that such particles remain morphologically and antigenically identical to native virions despite removal of several amino acids at the C-terminus (Rose *et al.* 1993, 1998).

I have found that the expression of my HPV-11 *L1 NLS*⁻ gene in *Sf*21 cells by recombinant baculovirus resulted in a range of different sized particles that did not

resemble the classical "doughnut" shape, and appeared not to be empty. Furthermore, electron microscopy of particles extracted from both *A. thaliana* and *N. tabacum* plants has shown the same pleomorphic range of the particles, ranging from 20 to 60 nm in diameter and which appear to contain packaged nucleic acid. This agrees with the results published by Li *et al.* (1997), who found that removal of 11 carboxyl-terminal amino acids still allowed for VLP assembly, but that particles were less uniform than those resulting from expression of the full-length *L1* gene. With regard to the packaging of foreign nucleic acids by the resulting particles, Varsani *et al.* (2003) found that plant-derived HPV-16 *L1* particles also apparently incorporate foreign nucleic acid to a significant extent.

Another difference to the insect cell-derived 'gold standard' VLPs that has been described by Li *et al.* (1997), but not by Varsani *et al.* (2003) or more importantly by Warzecha *et al.* (2003), was the pleomorphic nature of the HPV L1 NLS$^-$ particles. Warzecha's potato-derived HPV-11 L1 NLS$^-$ particles were apparently found to be 55 nm diameter particles which displayed the perfect 'doughnut' shape. Varsani *et al.* (2003) found some pleomorphism for HPV-16 L1 VLPs made in plants, but these still largely resembled insect cell-derived VLPs. However, Biemelt *et al.* (2003) found that HPV-16 L1 in plants predominantly assembled into 60S particles and capsomeres rather than VLPs. Their electron microscopy of 'classical' VLPs purified via Caesium Chloride (CsCl) gradient centrifugation from plants also appear different to insect cell-derived VLPs in that particles do not appear empty.

However, several other observations indicating post-translational modifications of the HPV-11 L1 NLS$^-$ were recorded in this work. To determine whether protein degradation occurred for insect cell-derived L1 VLPs, I set up a trial experiment where crude insect cell lysate containing HPV-11 L1 NLS$^-$ was left standing at 4°C for several hours before being examined by western blot. This analysis revealed three distinct L1 proteolysis products [Figure 2.15a (**C**)], approximately 53, 45 and 36 kDa in size relative to the purified 55 kDa positive control. Li *et al.* (1997) had reported that HPV-11 L1 capsids are susceptible to trypsin digestion: this resulted in two distinctive cleavage products

approximately 42 kDa and 48 kDa in size. It was suggested that the amino acid arginine at position 415 (R415) is the trypsin cleavage site resulting in the 42 kDa species, whereas cleavage at residues K450 or R471 would result in ~45 or ~47 kDa species respectively.

The western blot analyses of the insect cell and plant-derived HPV-11 L1 NLS$^-$ proteins were conducted in parallel, and showed that the *N. tabacum*-expressed HPV-11 L1 NLS$^-$ protein is subject to severe degradation: digestion by a presumably trypsin-like protease resulted in a protein product that is approximately 36 kDa in size [Figure 2.15a (**A** and **B**)]. Unlike the degradation pattern shown by the crude insect cell lysate [Figure 2.15a (**C**)], no intermediate protein species were observed in the *N. tabacum* protein extract, suggesting complete digestion of the protein. Although analysis of the *A. thaliana* protein extract was anticipated to yield similar results, surprisingly protein degradation was not observed in the HPV-11 L1 NLS$^-$ protein extracted from *A. thaliana* [Figure 2.15b (**D** and **E**)]. Western blot analysis indicated that the plant-derived protein was of identical size to the purified insect cell-derived 55 kDa HPV L1 NLS$^-$ VLP positive control. One could speculate on the differences in protease content of *A. thaliana* and *N. tabacum* plants; however, it remains quite clear that the protein from *A. thaliana* was not degraded. No severe post-translational modifications of the *N. tabacum*-derived HPV-16 or the HPV-11 L1 protein had been reported by any of Biemelt *et al.* (2003), Varsani *et al.* (2003) or Warzecha *et al.* (2003). However, mine was the only study with HPV-11 L1 expressed in tobacco – and given the already – proven unpredictability of L1 expression in plants, perhaps this is just more proof that empiricism is the only way to approach this subject.

The phenomenon of the HPV-11 L1 NLS$^-$ protein being severely degraded in *N. tabacum* plants, but not in *A. thaliana* is interesting, and would undoubtedly impact on the vaccine potential of such proteins. In this regard, it is known that severely truncated PV L1 proteins (86 aa deletions) are still capable of assembling into VLPs (Chen *et al.* 1998; Rose *et al.* 1998); here I have shown that both the degraded and the non-degraded protein – made in plants and insect cells – resulted in the formation of pleomorphic VLPs. It is interesting that Biemelt *et al.* (2003) also addressed the problem of VLP assembly in

plants, and like Varsani *et al.* (2003), linked this to the concentration of the L1 protein in the cytoplasm. The greater the concentration of free L1 protein the more capsomeres are being formed which eventually result in the assembly of complete VLPs. However, virus assembly takes place in the nucleus, and the L1 NLS is responsible for translocating the L1 and L2 proteins into the nucleus, thereby increasing their respective concentrations and allowing for capsid assembly together with DNA packaging. Theoretically my HPV-11 *L1 NLS*$^{-}$ gene is not capable of translocating to the nucleus; therefore it is perhaps justified to assume that the L1 concentration in the cytoplasm is not high enough to favour the formation of homogeneous HPV-11 L1 NLS^{-} VLPs. In this regard, Biemelt *et al.* (2003) speculated that, sedimentation analysis of soluble proteins derived from crude extracts of leaves and tubers revealed that a significant portion of the L1 antigen is in the form of viral capsomeres but also of higher-order structures with a lower sedimentation coefficient compared to intact VLPs and which are not easily detectable by electron microscopy. The reason for preferential formation of these structures compared to VLPs needs to be further investigated. From our observation in a mammalian expression system, we believe that relatively high concentrations of L1 protein are required for formation of VLP structures. Under such conditions, the same *L1* open reading frame used to generate the transgenic plants leads to the formation of capsomeres, intact VLPs as well as smaller quantities of the 60 to 70S assembly forms, which might represent assembly intermediates or assembly by-products (unpublished observations). Subcellular localisation analysis studies in a transient viral plant expression system suggest that the L1 protein fails to enter the nucleus but it remains to be investigated whether this is also the case for the transgenic lines. This would be consistent with our observation that in mammalian cells the 60 to 70S intermediates are predominantly localised within the cytoplasm (unpublished observations).

With the overall goal of developing an HPV-11 vaccine of plant origin in mind, and having shown that VLPs were made in my transgenics, I focused on the antigenic properties of my plant-produced protein. Another important question to ask was, given the evidence of proteolysis and particle pleomorphism, whether the antigenic properties of the plant-derived VLPs had been retained. It has been reported in the literature that

removal of 86 amino acids from the HPV-11 L1 carboxyl-terminal end resulted in the formation of pentameric capsomers that were no longer able to establish inter-capsomeric contacts: however, the monoclonal antibody H11:H3 capsid-neutralising domain was entirely contained within pentameric L1 capsomers and interpentamer associations were not required for the induction of virus-neutralising antibodies (Rose *et al.* 1998). In contrast, Chen *et al.* (1998) generated COPV L1 mutants lacking 67 amino acids from the C-terminal end which were capable of assembling into VLPs, but failed to express conformation-specific epitopes recognizable by MAbs.

It was an interesting question as to whose findings were more applicable to my study. Given that HPV-11 L1 NLS$^-$ protein extracted from *N. tabacum* is subject to extensive proteolysis, the cleavage of 86 amino acids from the carboxyl terminus should still favour the formation of pentameric capsomers. That this was true was seen by the detection by electron microscopy of pleomorphic VLPs in my study plants. However, these did not bind the conformation-specific MAb H11:H3 in the direct ELISA shown in figure 2.19. An additional conformation-specific and neutralising MAb (H11:B2) also failed to bind the tobacco plant-derived protein in the ELISA, making it probable that this is similar to the case of Chen *et al.* (1998), where the formation of VLPs from severely truncated COPV L1 protein did not necessarily preserve conformation-specific epitopes on the surface of the capsid. Analysis of the non-degraded *A. thaliana*-produced HPV-11 L1 NLS$^-$ protein shown in figure 2.10, however, indicated that all MAbs, including the conformation-specific and neutralising MAbs, were capable of binding to the protein.

Attempts to detect freshly harvested and processed *N. tabacum*-derived HPV-11 L1 protein by double-antibody-sandwich (DAS) ELISA were also not successful, despite considerable efforts in substituting and swapping the capturing and detecting antibodies from the range of different available MAbs and polyclonal antiserum (results not shown). This ruled out denaturation of a more labile truncated protein by adsorption to the microtitre plate wells as a reason for the loss of reactivity with conformation-specific MAbs. It is worthy noting that protease degradation of the tobacco L1 NLS$^-$ protein

occurred despite addition of complete protease inhibitor according to the manufacture's instruction during the extraction procedure from transgenic leaf material.

With any potential vaccine preparation it is of utmost importance to establish whether the developed product is indeed capable of inducing an antibody response. Thus far, my results have indicated that neutralising conformation-specific MAbs could detect the *A. thaliana*-derived HPV-11 L1 NLS^- protein, but not the protein extract derived from *N. tabacum*, possibly due to loss of epitope(s) despite VLP assembly. Despite this I evaluated the ability of the protein extract to induce an antibody response after inoculations by injection of New Zealand white rabbits. With perhaps the exception of the serum collected from rabbit #17 (Figure 2.17b) all other sera predominantly reacted with NLS^- VLPs (Figures 2.16 and 2.17a), and not with NLS^+ VLPs: this was completely unexpected, given that especially the *A. thaliana*-derived protein reacted with MAbs specific for conformation-dependent epitopes. All sera were accordingly retested, with the same results. Additionally, serum collected on day 56 reacted exclusively with non-denatured insect cell-derived NLS^- VLPs (Figure 2.18). Taken together, these results present a major problem – both for interpretation and potentially for the prospect of HPV-11 plant-produced vaccines. I note that Warzecha et al (2003) found that their plant-derived material reacted with appropriate antibodies, but that no reaction could be shown by sera from even multiply - orally immunized mice with insect cell-derived VLPs – presumably NLS^+ - unless the mice had been orally boosted with those VLPs. In contrast, both Varsani *et al.* (2003) and Biemelt *et al.* (2003) showed that injection of plant-produced HPV-16 L1 was capable of eliciting antibodies reactive with native VLPs produced in insect cells. Thus, this phenomenon is apparently limited to the HPV-11 L1 NLS^- protein.

With regard especially to the results shown in figure 2.18, my suggestion is that an immune response is primarily raised against an immunodominant epitope that appears to be surface exposed in VLPs formed by the plant-derived L1 NLS^- protein. As to what kind of structural changes occur within the capsid that allow for the recognition of this immunodominant epitope, which is otherwise not surface exposed on the actual HPV-11

virion, this needs further investigation. Nevertheless, the lack of recognition by immune sera of the HPV-11 L1 NLS^+ VLPs, the closest counterpart to the actual virus, implies that antibodies generated from injection of this particular plant-derived HPV-11 L1 NLS^- protein will not be able to prevent infection and disease, which completely negates their potential use for prophylactic vaccination.

Thanks to my colleague Debbie Stewart, we have been able to evaluate all sera collected from immunized rabbits for their capacity to neutralise the virus *in vitro*. The results presented in figures 2.20 and 2.21 showed that in comparison to the known HPV-11 neutralising monoclonal antibody H11:H3 (Figure 2.21), none of the sera collected on day 56 were able to neutralise pseudoviruses. I note that two publications by Embers *et al.* (2002, 2004) described detection of papillomavirus neutralising antibodies at a serum dilution of 1:5, nevertheless we have found that results obtained with >1:50 dilutions of sera were very hard to duplicate due to non-specific activity of certain sera. Nonetheless, our findings support the conclusion that this plant-derived HPV-11 L1 NLS^- protein is not suitable for application as a potential vaccine for the prevention of HPV-11 infection. I note that none of the earlier L1 plant expression workers reported any neutralisation data, thus, this is the first published report of this sort of data for a plant-produced HPV L1 protein.

The question as to what changes occur within the plant-derived L1 protein that prevent recognition of VLPs formed from non-truncated L1 protein, warrant further investigation. It is of interest that the work in our laboratory (J. Maclean, unpublished) has shown that HPV-16 L1 protein produced at high yield in tobacco via *Agrobacterium tumefaciens* is capable of eliciting high-titre neutralising sera in mice – however, use of Freund's incomplete adjuvant, while enhancing ELISA titres relative to protein alone, also apparently reduced neutralisation titres. This may have had an effect in this work as well, if the plant-produced HPV-11 L1 VLPs/capsomeres were more labile than their HPV-16 counterparts, and were thus more easily denatured. Unfortunately, the pseudovirus neutralisation assay was only recently established in our laboratory, so further investigations of this possibility were beyond the scope of this project.

It would be a shame to completely neglect further investigations into the development of an HPV-11 recombinant protein in plants. Even with the announced release of the Merck HPV vaccine in 2006, the quests for cheaper and safer alternatives are a market-driven phenomenon. With promising speculations as to the use of plant-derived HPV vaccines as cost-effective vaccine boosters, the ultimate requirement would be to make it work effectively. It might be possible to do this by engineering the protein in a different way, or making HPV-11:HPV-16 hybrid L1s, for example. In summary, this work showed that yield of HPV-11 L1 protein in plants could be increased by between 2000 and 10000-fold compared to the only previous report (Warzecha *et al.* 2003), despite my using a native HPV gene. The protein appeared antigenically appropriate; however, it was not particularly immunogenic, and antisera raised against it did not react with native HPV-11 L1 VLPs, nor did they neutralise HPV-11 pseudovirus infectivity. This has serious implications for plant-derived HPV-11 vaccines.

Chapter 3

Expression of CRPV *L1* in transgenic *Nicotiana tabacum* cv. Xanthi and its use as a vaccine: A proof-of-concept study

Contents

ABSTRACT

I transformed *N. tabacum* cv. Xanthi with the CRPV *L1* gene. Stable integration of the gene was shown by PCR in the R_0 and consecutive T_1 generation. Transgenic plants showed transcription of the integrated gene when total RNA was screened by RT-PCR using gene-specific internal primers. Transgenic plant material was harvested and processed: the resulting protein extract bound both neutralising conformation-specific and surface linear epitope recognizing monoclonal antibodies in ELISA tests as well as by immuno-electron microscopy.

CRPV L1 protein was found not to assemble into VLPs; however, immunotrapped preparations of the protein showed the presence of pentameric aggregates. The yield of transgenic-derived CRPV L1 protein was calculated to range between 0.4 mg to 1 mg per kilogram of fresh leaf material. Three New Zealand white rabbits were inoculated with concentrated protein extract containing between 22μg and 50μg of L1 protein, and two additional booster inoculations of half the initial dose. Immune sera were tested by ELISA for type-specific antibodies against insect cell-derived CRPV VLPs. Serum from all rabbits showed a type-specific antibody response, indicating that plant-derived CRPV L1 protein is capable of inducing an immune response in rabbits.

In vitro CRPV pseudovirus neutralisation assays of all sera collected from animals indicated none of the sera contained neutralising antibodies. However, live virus challenge of all vaccinated rabbits with infectious CRPV resulted in 2 out of 3 rabbits showing no papillomas on day 63 of the study, and 1 rabbit developing papillomas that were 6.5-fold smaller than papillomas in the control group. This suggests that, despite the lack of serum neutralising response immunization with plant-derived CRPV L1 protein is protective in this animal model. This is the first proof of principle of a prophylactic papillomavirus L1 vaccine derived from plants.

3.1 INTRODUCTION

Cottontail rabbit papillomavirus (CRPV) together with the Rabbit oral papillomavirus (ROPV) constitute the genus *Kappa papillomavirus* within the new classification of papillomaviruses (de Villiers *et al.* 2004) (see Figure 1.2, Chapter 1). In 1961, Ito & Evans were the first to show that the application of CRPV DNA to the skin of naïve domestic rabbits resulted in the developmental progression of CRPV induced warts or papillomas. The formation of cutaneous lesions as a direct result of infection by CRPV has resulted in concerted efforts to study and understand the oncogenic nature of various cutaneous and mucosa-infecting papillomaviruses (PVs).

Various PVs have been found to infect numerous animals (Table 1.1, Chapter 1). Apart from CRPV, selective PVs of the *Epsilon-*, *Lambda-* and *Xi papillomavirus* genera have been studied extensively. The developmental efforts for several candidate human PV vaccines have been made more difficult by the fact that their protective efficacy cannot be evaluated in animals. In contrast, however, development of animal PV vaccines, either in the form of DNA or protein subunit, allows for the evaluation of such a vaccine by immunization of the respective hosts followed by an experimental challenge with live virus. There have been several studies focusing on the evaluation of candidate PV vaccines designed to protect rabbits, cattle or dogs; the outcome of these forms a foundation on which various prototype vaccines are evaluated (Breitburd *et al.* 1995; Christensen *et al.* 1996, 2001; Kirnbauer *et al.* 1996; Lin *et al.* 1992; Suzich *et al.* 1995).

Developmental efforts have shown that naïve domestic rabbits could be protected from experimental challenge with live CRPV virus, after they had been vaccinated with an equivalent protein subunit vaccine. The efficacy of such a candidate CRPV subunit vaccine has been described by Breitburd *et al.* (1995) and Christensen *et al.* (1996). Both groups have shown that inoculation of domestic rabbits with either CRPV L1 or CRPV L1/L2 VLPs resulted in the induction of presumably antibody-mediated, type-specific protection of New Zealand white rabbits against experimental CRPV infection. The common denominator in both studies was the production of the CRPV VLP vaccine in

insect cells via recombinant baculovirus technology. The same technology was applied in the production of equivalent canine and bovine PV VLP vaccines, shown to protect their respective hosts from challenge with live virus (Kirnbauer *et al.* 1996; Suzich *et al.* 1995).

This chapter focuses on the evaluation of a new potential candidate CRPV VLP vaccine of plant origin. Especially after the results of the previous chapter, the question remains whether a plant-derived PV vaccine is indeed capable of eliciting an immune response similar to that achieved by vaccination of animals with recombinant baculovirus-produced VLPs. It expands on the efforts described in chapters 2 and 4 in which the developmental stages of an HPV-11 plant-derived candidate vaccine are described. The elicitation of an antibody response by a papillomavirus vaccine is generally seen to be of primary importance, and I wished to see whether induction of the immune system by vaccination with plant-derived PV VLPs would be sufficient to neutralise the invading virus and thus protect the host. Accordingly, I investigated whether protective immunity was established after vaccination of New Zealand white rabbits with plant-derived CRPV VLPs by experimental challenge of vaccinated animals with live virus.

This research presents part of a proof-of-concept study, the outcome of which is of significant importance. Successful stimulation of the rabbits' immune systems and induction of the host's capability to combat the invading virus would be a boost for the concept of safe and cost-effective production of human counterpart PV vaccines in plants. Although various HPV antigens have already been produced in plants (Biemelt *et al.* 2003; Varsani *et al.* 2003; Warzecha *et al.* 2003), I believe this to be the first report on the production and evaluation in an animal challenge model of a plant-derived animal PV L1 protein vaccine.

3.2 MATERIALS AND METHODS

3.2.1 Cloning of CRPV *L1* gene into the binary vector pART27 and *Agrobacterium tumefaciens* – mediated transformation

The full-length CRPV *L1* gene was amplified by PCR using primer pair 7 (Table 3.1) from a full-length CRPV virus clone provided by Associate Professor Neil Christensen (The Milton S. Hershey Medical Centre, Penn State College of Medicine, Hershey, Pennsylvania, USA). The PCR was performed using 25 cycles of 94°C for 30s, 55°C for 45s, and 72°C for 45s, followed by a 5min extension step at 72°C. Dr Arvind Varsani (this laboratory) cloned the PCR product into the pGEM®-T Easy vector (Promega), whereafter respective clones were sequenced.

Table 3.1 Primers used for the amplification of the CRPV *L1* ORF

Primer Pair	Construct	Primers
7	pART27 CRPV *L1*	Forward 5'-TTAATTAA**ATG**GCAGTGTGGCTGTCTACG -3' Reverse 5'-CTCGAG**TTA**AGTTCTCTTGCGTTTAGATGATTTC-3'
8	CRPV *L1* internal	Forward 5'- AAAGCATGGCGTTCGACC-3' Reverse 5'- GCACACAGATGCAGGGAGAG-3'

TTAATTAA *Pac*I, CTCGAG *Xho*I, **ATG** = start codon, **TAA** = stop codon

The CRPV *L1* gene was excised from confirmed pGEM®-T Easy clones by digestion with *Eco*RI and cloned into pART7 (*Eco*RI). The orientation of the inserted CRPV *L1* gene was determined by *Xho*I restriction analysis, before the cassette containing the CRPV *L1* gene under the control of the constitutively active CaMV35S promoter and followed by the octopine synthase gene terminator (*ocs* 3'), was excised by *Not*I digestion and subcloned into the binary vector pART27 (*Not*I) (Figure 3.1).

A. tumefaciens strain C58C1, carrying the virulence plasmid pMP90 was transformed with the pART27 CRPV *L1* plasmid DNA as described in section 2.2.1 of Chapter 2.

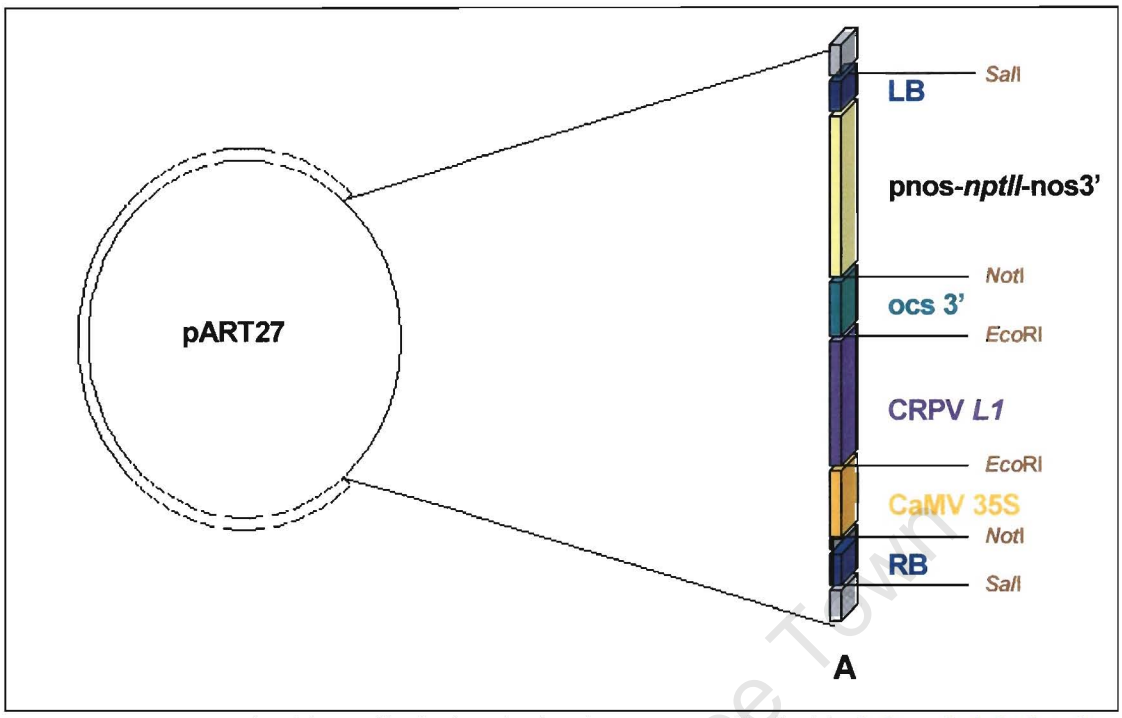


Figure 3.1: pART27 backbone displaying the T-DNA cassette flanked by **left and right borders**, containing the CRPV *L1* gene (**A**) under the control of the CaMV35S promoter, followed by the ***nptII*** selection gene under the nopaline octapine synthase promoter (**pnos**) (See Appendix B for complete pART27 map).

3.2.2 Transformation of *Nicotiana tabacum* cv. Xanthi

Wild type *N. tabacum* cv. Xanthi leaf discs were transformed with *A. tumefaciens* carrying the pART27 CRPV *L1* binary vector and transgenic plants were regenerated according to the protocol described in section 2.2.2b of chapter 2.

3.2.3 Screening of transformed *N. tabacum* plants for the CRPV *L1* gene

Plant genomic DNA was extracted in extraction buffer (100mM Tris, 50mM EDTA, 500mM NaCl pH 8.0) from putative transgenic and wild type *N. tabacum* leaves using the method outlined by Dellaporta *et al.* (1983). DNA from transformed *N. tabacum* plants was screened by PCR for the CRPV *L1* gene using primer pair 7, as listed in Table 3.1. The PCR was performed using 25 cycles of 94°C for 30s, 55°C for 45s, and 72°C for 45s, followed by a 5min extension step at 72°C and resulted in the amplification of a 1.5kb fragment.

3.2.4 Analysis of total RNA extracted from transgenic plants

Total RNA was extracted from fresh leaf material using the TRIzol™ reagent (Life Technologies). Total RNA samples were pre-digested with RNase-Free DNase (Promega) at 37°C for 30min. DNase stop solution was added to the samples and the DNase enzyme was heat inactivated at 65°C for 10min. A final volume of 5µl treated total RNA was used for the detection of the CRPV *L1* RNA by RT-PCR amplification using the Access RT-PCR system (Promega). The profile for the RT-PCR is listed as the following: 45°C for 45min, 94°C for 2min; followed by 30 cycles of 94°C for 30s, 55°C for 1min and 68°C for 2min. The final elongation step was performed at 68°C for 5min. Primer pair 8, as listed in table 3.1, was used to amplify an internal, 421 base pair fragment situated between nucleotides 433 and 854.

3.2.5 Processing and concentration of transgenic plant material

Transgenic leaf material was harvested and processed in 1:2 (w/v) cold PBS (1.47mM KH_2PO_4, 10mM Na_2HPO_4, 2.7mM KCl, 500mM NaCl, pH 7.4) according to the flow chart presented in figure 2.2 of chapter 2.

3.2.6 Monoclonal antibody characterization of plant-derived CRPV L1 protein

CRPV L1 protein containing extracts derived from *N. tabacum,* together with non-transgenic plant protein extracts, were characterized by direct ELISA using two monoclonal antibodies (MAbs) against CRPV (generously provided by Neil Christensen). These monoclonal antibodies and their properties are listed in table 3.2.

Table 3.2: Monoclonal antibodies used to detect and characterize the CRPV L1 protein

Monoclonal antibody	Isotype	Neutralization	Nature of Epitope	Cross-reactivity to BPV-1	References
CRPV:5A	IgG2a	Yes (CRPV)	conformation-specific	No	Christensen & Kreider (1991) (Christensen *et al.* 1990)
CRPV:10B	IgG2a	No	linear (surface)	unknown	

ELISA plates were coated with protein extracts for 1 hr and then blocked in 2% non-fat milk in PBS (1.47mM KH_2PO_4, 10mM Na_2HPO_4, 2.7mM KCl, 137mM NaCl, pH 7.4) for 1 hr. MAbs diluted to 1:1000 were used to detect the plant-derived antigen for 1 hr. Anti-mouse-alkaline phosphatase conjugated secondary antibody (1:5000) (Sigma) was allowed to bind the primary antibody for 1 hr at 37°C. The secondary antibody was detected using p-nitrophenyl phosphate (pNPP, Sigma) and the absorbance was measured using a Titrex ELISA plate reader at 405nm. All samples were analyzed in triplicate to determine the mean absorbance and calculate the respective standard deviation.

3.2.7 Western blot analysis of plant-derived CRPV L1 protein

For analysis of plant-derived L1 protein, all protein contained in a 2 ml plant protein extract aliquot (Number ⑥, Figure 2.2, Chapter 2) was precipitated using 5% TCA (v/v) and the western blot performed as outlined in section 2.2.9 of chapter 2. The monoclonal antibody CRPV:10B was used for detection of the protein at a dilution of 1:1000, before being probed with a goat anti-mouse-alkaline phosphatase conjugated secondary antibody (Sigma) at a dilution of 1:5000. Substrate buffer containing 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) was applied for colorimetric detection.

3.2.8 Expression of the CRPV *L1* gene in *Sf*21 cells using recombinant baculovirus

Dr Arvind Varsani (this laboratory) amplified the CRPV *L1* gene from a CRPV clone (see 3.2.1) by PCR under the following conditions: 25 cycles of 94°C for 30s, 55°C for 45s, and 72°C for 45s, followed by a 5min extension step at 72°C. Primer pair 7 as listed

in table 3.1 was used for the amplification of the *L1* gene and the gene was cloned into the pGEM®-T Easy vector and sequenced. The CRPV *L1* gene was directionally cloned into the pFastBac™1 vector (Invitrogen) using the *Eco*RI restriction enzyme sites. The protocols as described in sections 2.2.9 and 2.2.10 (Chapter 2) were followed to amplify the recombinant baculovirus and purify the CRPV L1 VLPs.

3.2.9 Electron microscopy of plant-derived CRPV L1 protein

Transgenic CRPV L1 protein extract and insect cell-derived CRPV L1 protein were viewed under the electron microscope after absorption of the respective preparations onto carbon coated copper grids for direct viewing or protein detection by immunogold-labelling.

Protein samples were directly adsorbed onto copper grids for 30min, thoroughly washed in sterile, distilled water (2min) and then stained in 2% uranyl acetate for 2min. Immunotrapping of plant- and insect cell-derived L1 protein entailed the coating of carbon coated copper grids with rabbit-anti-CRPV L1 antiserum raised against insect cell-derived CRPV L1 VLPs diluted 1:50 in PBS (1.47mM KH_2PO_4, 10mM Na_2HPO_4, 2.7mM KCl, 137mM NaCl, pH 7.4). Grids were thoroughly washed in sterile distilled water (2min) and then blocked in 1% BSA PBS for 30min. Grids were transferred onto the plant protein extract allowing for adsorption of L1 protein for 30min. This was followed by another washing step in sterile distilled water before grids were stained in 2% uranyl acetate for 2min.

For immunogold-labelling purposes, the identical protocol as described in section 2.2.12 of chapter 2 was followed, with the substitution of the HPV-11 L1 polyclonal antiserum and MAb H11:H3 for CRPV L1 polyclonal antiserum (generously provided by Dr Vandana Govan, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa) and MAbs CRPV:5A and CRPV:10B respectively.

3.2.10 Immunization of New Zealand white rabbits with plant-derived CRPV L1 protein extract and evaluation of sera

A group of three New Zealand white rabbits was inoculated with concentrated transgenic CRPV L1 *N. tabacum* protein extract (see 3.3.2). The first inoculum (1ml of extract) was administered by subcutaneous (3 sites) and intramuscular (1 site) injection. An additional two booster inoculations consisting of 500µl protein extract mixed in a ratio of 1:1 with Freund's incomplete adjuvant were administered, as described above on days 23 and 41. Serum was collected on days 1, 23, 41 and 51, pre-absorbed against non-transgenic plant protein extract and non-recombinant baculovirus insect cell debris, before being analyzed by direct ELISA and western blotting (1:20 dilution) against CRPV L1 insect cell-derived VLPs at a concentration of 1.2µg/ml.

Data is presented in the form of a ratio. The prebleed serum absorbance reading is divided by itself, resulting in a value of 1. Absorbance readings of the antiserum taken on the days, as listed above, are divided by the respective prebleed serum absorbance reading, thus presenting the data in the form of a ratio to prebleed.

3.2.11 Challenge of New Zealand white rabbits with infectious CRPV

Challenge of immunized rabbits with infectious CRPV virus stock was performed by Dr Vandana Govan according to the publications by Christensen *et al.* (1990) and (1996). At 7 weeks after the final immunization, the backs of the rabbits (3 transgenic plant-derived CRPV L1 protein immunized, 4 PBS immunized rabbits and 5 insect cell-derived CRPV VLP immunized rabbits) were shaved and divided into four identical squares (area, 2 x 2 cm) using a permanent marker. To ensure complete separation of the marked individual sites, dye was injected underneath the skin in between adjacent squares. The shaved backs were wiped down using ethanol swabs and the skin of individual sites was scarified by crosshatching using a sterile 18 gauge needle. All rabbits were challenged with 2 dilutions of infectious CRPV stock (generously provided by Neil Christensen) as described by Christensen and Kreider (1990) and Christensen *et al.* (1996). Each rabbit

was challenged at two sites for each dilution of the infectious virus stock (10^{-2} and 10^{-3}). Papilloma size was measured as length x width x height in millimetres starting 14 days post challenge. The geometric mean diameter (GMD) was calculated for each papilloma. The means and standard deviations for the GMDs in each treatment group were plotted against time after challenge with virus.

3.2.12 Generation of CRPV pseudoviruses and virus neutralisation experiments

CRPV L1 pseudoviruses were generated by my colleague Mrs Debbie Stewart according to the protocol described in section 2.2.14 of chapter 2, by the substitution of the plasmids p11L1*h* and p11L2*h* with the equivalent plasmids required for the generation of CRPV pseudoviruses.

Sera from inoculated rabbits collected on the last day of the experiment (Day 41) as well as the pre-inoculation sera (Day 1) were evaluated by Mrs Stewart for their capability to neutralise the CRPV pseudovirus *in vitro* as described in section 2.2.15 of chapter 2.

3.3 RESULTS

3.3.1 Genetic analysis of transgenic plants

Wild-type *N. tabacum* cv. Xanthi leaf discs were transformed with *A. tumefaciens* carrying the pART27 CRPV *L1* binary vector. Putative transgenic plants were regenerated and screened for the CRPV *L1* gene by PCR. Integration of the CRPV *L1* gene was confirmed in 5 out of 15 regenerated *N. tabacum* lines. R_0 generation plants were allowed to grow until reaching sexual maturity, self-pollinated under controlled conditions and the resulting T_1 generation seeds harvested. T_1 generation seeds were germinated on kanamycin-containing tissue culture media. Total genomic DNA was extracted from T_1 generation plantlets of respective transgenic lines and again screened by PCR. Figure 3.2 shows the presence of a 1.5 kb amplicon, indicating the integration and confirming the inheritance of the CRPV *L1* gene in all 5 transgenic lines.

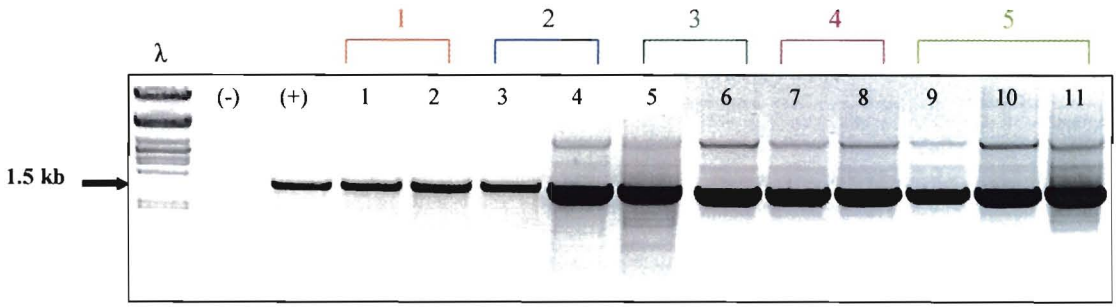


Figure 3.2: Genomic DNA analysis extracted from plants of five T_1 generation transgenic *N. tabacum* lines (analyzed plants from respective lines are shown by colour coded brackets). The presence of a 1.5 kb amplification product confirms the integration of the CRPV *L1* gene into the plant genome. The positive and negative controls are represented by CRPV *L1* plasmid DNA and genomic DNA extracted from a non-transgenic *N. tabacum* plant respectively.

Upon confirmation of the integrated CRPV *L1* gene into the plant chromosome, total RNA was extracted from individual plants and analyzed for CRPV *L1* gene transcription. RT-PCR products resulting from the amplification of an internal gene fragment indicated that the integrated gene is transcribed in all plants (Figure 3.3).

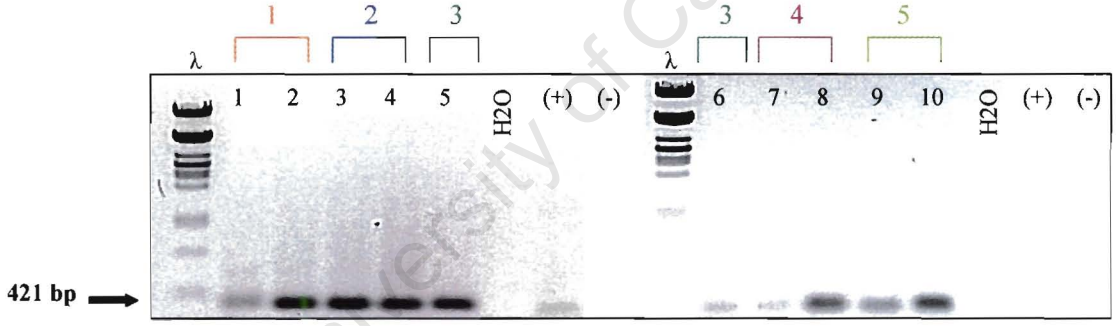


Figure 3.3: RT-PCR RNA analysis of PCR positive transgenic *N. tabacum* plants from the T_1 generation. The amplification of a 421 bp product by RT-PCR indicates the presence of the CRPV *L1* transcript within total plant RNA (total reaction volume of 10µl loaded per lane). Total RNA extracted from a non-transgenic plant and synthesized CRPV *L1* RNA were used as the negative and positive controls respectively. Synthesized RNA rapidly degrades, accounting for the faint amplification product of the positive control.

We have not determined whether the *L1* mRNA is degraded. In hindsight this should have been confirmed by northern blot in order to establish the possible presence of multiple *L1* mRNA species, thus indicating that post-transcriptional factors could influence the final product formation.

3.3.2 Analysis of plant-derived CRPV L1 protein

Transgenic plant material was harvested and processed according to figure 2.2 in chapter 2. The protein pellet resulting from ultracentrifugation of the supernatant (Number ⑥, Figure 2.2) was resuspended in 10 ml PBS (1.47mM KH_2PO_4, 10mM Na_2HPO_4, 2.7mM KCl, 500mM NaCl, pH 7.4) before being characterized by direct ELISA using MAbs CRPV:5A and CRPV:10B. Due to the high viscosity of the protein extract, a two-fold dilution series was prepared before samples were absorbed onto an ELISA microtitre plate. Characterization of the protein extract revealed a strong binding of the CRPV L1 protein by conformation-specific and neutralising MAb CRPV:5A in the undiluted sample (Figure 3.4). Detection of the same protein in the undiluted sample by surface linear MAb CRPV:10B was not as strong compared to MAb CRPV:5A, however detection of the CRPV L1 protein by the same MAb improved in the diluted protein samples (Figure 3.4).

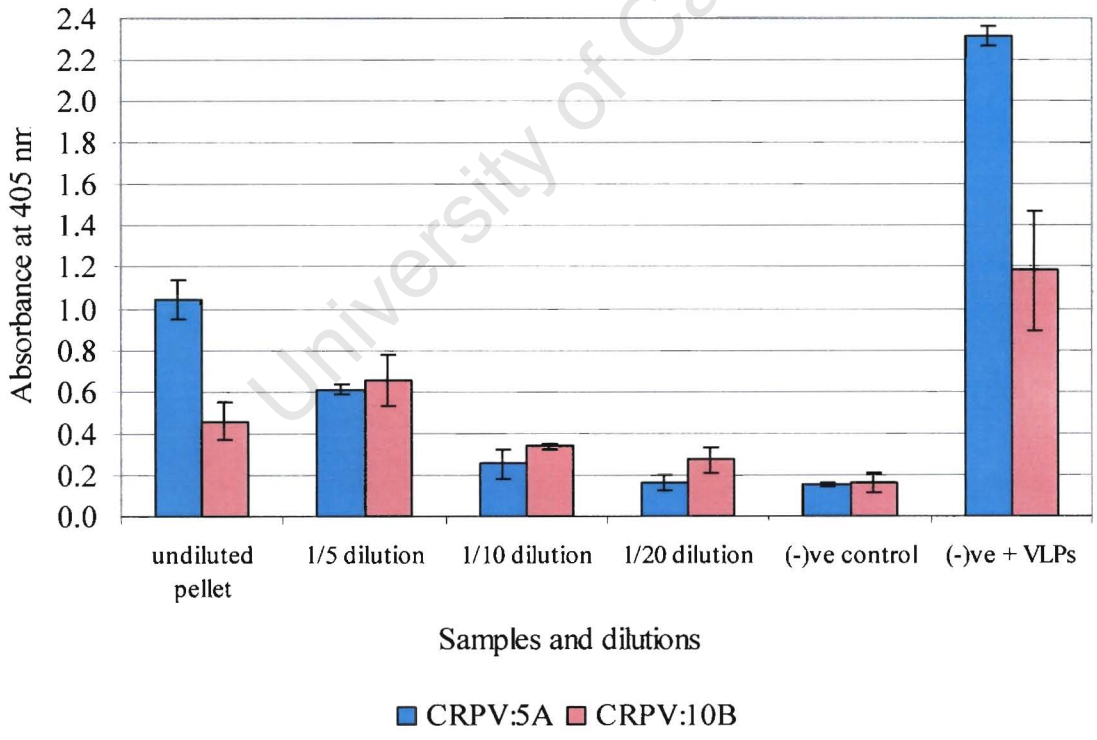


Figure 3.4: Monoclonal antibody characterization of the CRPV L1 transgenic *N. tabacum* protein extract. Non-transgenic *N. tabacum* protein extract was used as negative control, whereas the positive control consisted of non-transgenic protein extract spiked with 1.2µg/ml (0.12µg/100µl) insect cell-derived CRPV L1 VLPs. Error bars represent the standard deviation calculated from triplicate analysis of samples.

The strong detection of the CRPV L1 protein within the plant extract by the conformation-specific MAb CRPV:5A suggests that some of the plant-derived protein is assembling into pentameric or higher order structures (VLPs). However, the equally strong recognition in most dilutions of the CRPV L1 protein by surface linear epitope-binding MAb CRPV:10B indicates the presence of significant amounts of free L1 protein.

3.3.3 Quantification of the CRPV L1 protein concentration

The amount of transgenic plant-derived CRPV L1 protein was measured by comparison to a standard curve of the $O.D._{405nm}$ values plotted against known concentrations of insect cell-derived CRPV L1 protein in the colorimetric ELISA. Non-transgenic plant protein extract was spiked with insect cell-derived CRPV L1 VLPs resulting in a known concentration of 0.12µg per well (100µl). This represented the positive control and $O.D._{405nm}$ comparisons allowed for the calculation of the total amount of CRPV L1 protein from transgenic *N. tabacum* protein samples of which the total weight and homogenization buffer volume was known.

Table 3.3: Calculated yields of CRPV L1 protein derived from 1 kg of fresh transgenic leaf material

Samples and dilutions	**Concentration of CRPV L1 protein per kilogram of fresh leaf material (mg/kg)**	
	CRPV:5A	**CRPV:10B**
Undiluted	**1.02**±0.09	**0.45**±0.09
1/5 dilution	2.95±0.10	3.20±0.35
1/10 dilution	2.50±0.70	3.30±0.20
1/20 dilution	3.20±0.80	5.40±1.20

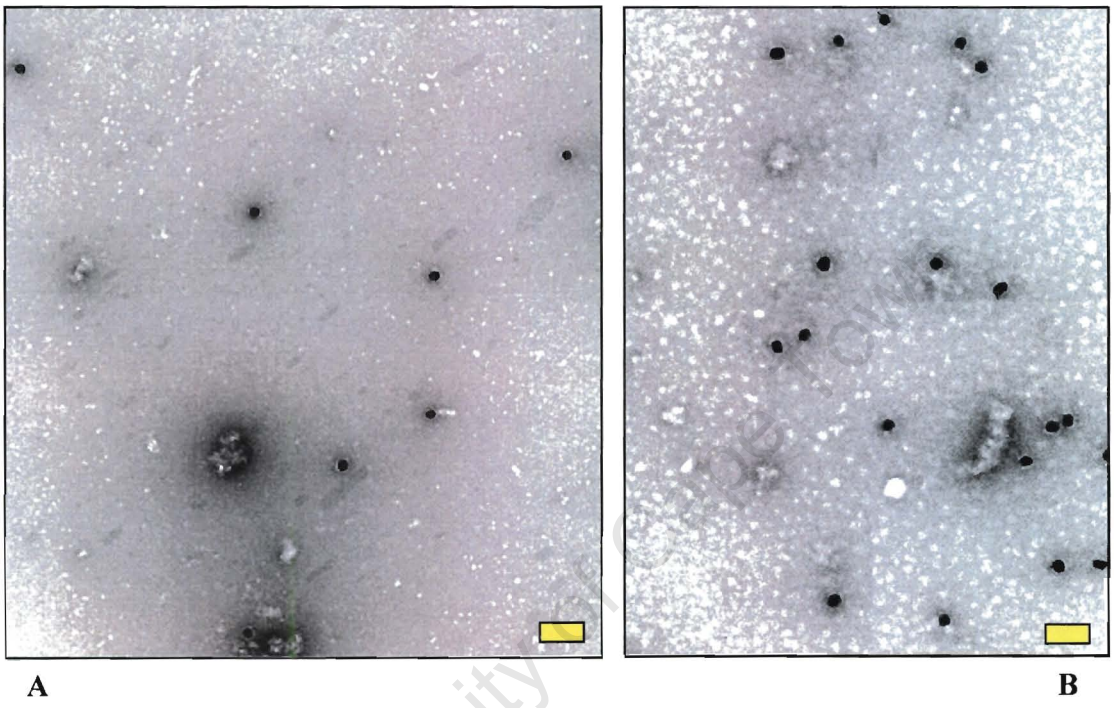
Standard error calculated from triplicate analysis of samples.

The yield of transgenic CRPV L1 protein was calculated to range from approximately 0.4mg to 1mg per kilogram of fresh leaf material and values are highlighted in table 3.3.

3.3.4 Analysis of protein extracts by electron microscopy and western blotting

The final protein extract was prepared for detection of the CRPV L1 protein by immunogold-labelling. Individual grids were coated with anti-CRPV L1 polyclonal

antiserum, onto which the protein extract was absorbed. Immunotrapped CRPV L1 protein was decorated with MAbs CRPV:5A or CRPV:10B before detection with a gold conjugated anti-mouse antibody. Results are presented in figure 3.5 and conclusively show that both MAbs were able to detect the presence of CRPV L1 protein, albeit not in the form of recognisable VLPs or other large aggregates.



A B

C

Figure 3.5: Composite electron micrograph images of immunogold-labelled CRPV L1 protein extracted from *N. tabacum* (**A** and **B**). Plant protein extract was trapped onto carbon coated copper grids using anti-CRPV L1 rabbit polyclonal antiserum; decorated with monoclonal antibodies CRPV:5A (**A**) and CRPV:10B (**B**) and detected with an anti-mouse-gold-conjugated secondary antibody. Gold particles are 30 nm in diameter. Non-transformed *N. tabacum* plant protein extract is shown in **C**. (**Bar = 100nm**)

Figure 3.5 however shows that plant-derived CRPV L1 protein is more readily detected by the surface linear MAb CRPV:10B (Figure 3.5 **B**) than by the conformation-specific MAb CRPV:5A (Figure 3.5 **A**), possibly indicating that the majority of the CRPV L1 protein exists in the monomeric form, rather than in an assembled state.

This was confirmed by viewing of plant protein extract after direct immunotrapping and staining with 2% uranyl acetate. In comparison to insect cell-derived CRPV L1 VLPs (Figure 3.6 **B** to **D**), displaying the typical "doughnut" shape when assembled, only possibly pentameric aggregates rather than VLPs were observed in the plant protein extract presented in figure 3.6 **A**.

Although the ELISA results combined with the results shown by electron microscopy confirm the presence of the plant-derived CRPV L1 protein, attempts to detect the protein by western blot were not successful. No detection was possible, even though the total protein content of an already concentrated plant protein extract had been completely precipitated in a final concentration of 5% TCA (v/v). Two plausible explanations exist: the final concentration of plant-derived CRPV L1 protein in the aliquot destined for western blot analysis was below the threshold of detection (unlikely given the results in chapter 2); or the protein and its MAb binding sites are completely destroyed in the presence of TCA, resulting in the inability of even the supposedly linear surface epitope recognizing MAb CRPV:10B to detect the protein.

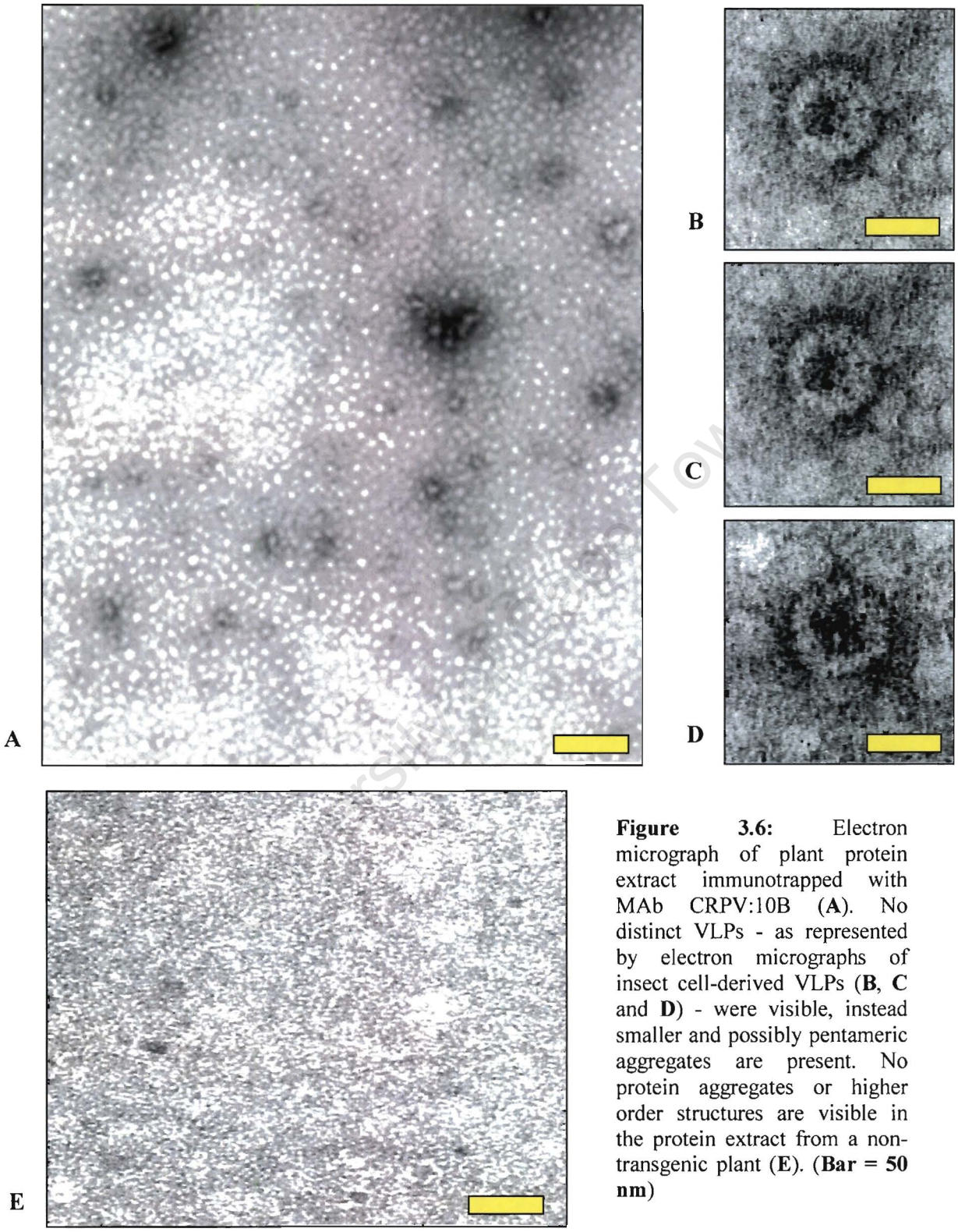


Figure 3.6: Electron micrograph of plant protein extract immunotrapped with MAb CRPV:10B (**A**). No distinct VLPs - as represented by electron micrographs of insect cell-derived VLPs (**B**, **C** and **D**) - were visible, instead smaller and possibly pentameric aggregates are present. No protein aggregates or higher order structures are visible in the protein extract from a non-transgenic plant (**E**). (**Bar = 50 nm**)

3.3.5 Analysis of the immune response of animals immunized with transgenic plant-derived CRPV L1 protein extract

Three New Zealand white rabbits were immunized with transgenic plant-derived CRPV L1 protein extract. The initial 1ml inoculum of extract, containing between 22μg and 50μg, was administered intramuscularly (1 site) and subcutaneously (3 sites). Two additional booster inoculations were injected on days 23 and 41. These consisted of 500μl CRPV L1 protein extract mixed in a ratio of 1:1 with Freund's incomplete adjuvant, thereby halving the initial concentration of CRPV L1 protein to range from 11μg to 25μg. Serum was collected on days 1, 23, 41 and 10 days after the last inoculation and evaluated by direct ELISA (1:20 dilution) against insect cell-derived CRPV L1 VLPs at a concentration of 0.12μg/well. Figure 3.7 shows the analysis of the collected serum as represented by a ratio of serum reactivity measured against the prebleed serum.

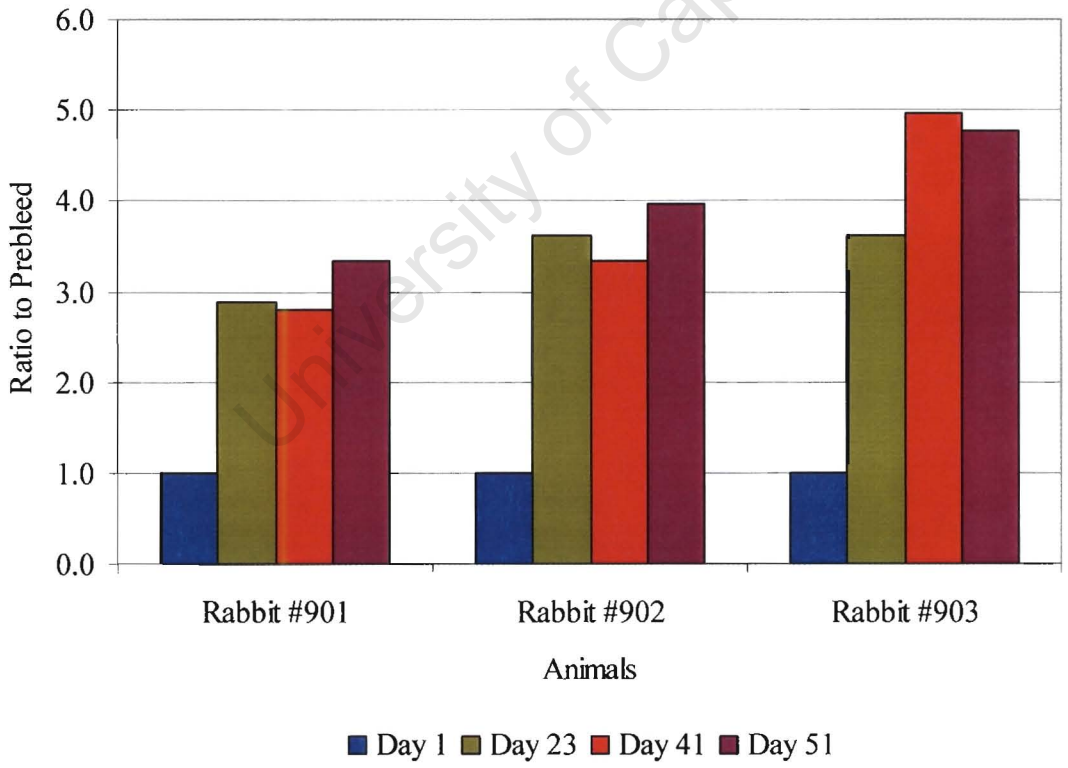


Figure 3.7: Analysis of serum collected from three New Zealand white rabbits inoculated with transgenic-derived CRPV L1 protein extract. Serum collected on days 1, 23, 41 and 51 was tested against insect cell-derived CRPV L1 VLPs at a concentration of 0.12μg/well.

The sera collected from all three rabbits were also tested by western blot. For logistical reasons, only pre-absorbed prebleed (Day 1) and last bleed (Day 51) sera were tested for their ability to detect denatured insect cell-derived CRPV L1 protein. Results indicate that all the last bleed sera collected from rabbits #901, #902 and #903 were capable of specifically detecting denatured CRPV L1 protein (Figure 3.8).

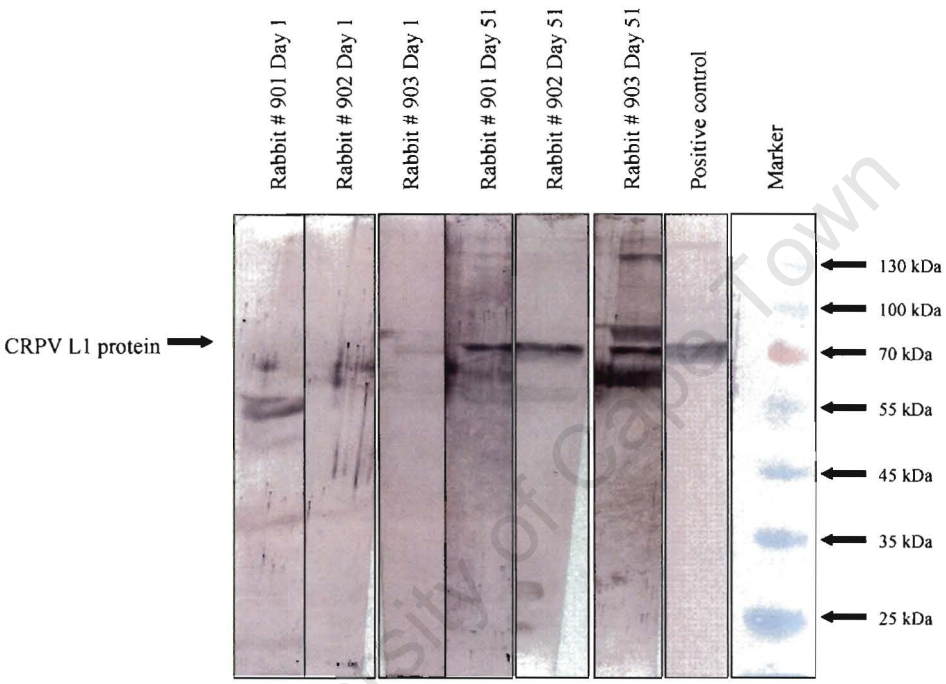


Figure 3.8: Analysis of serum collected from New Zealand white rabbits immunized with transgenic-derived CRPV L1 protein extract. Serum was tested against denatured insect cell-derived CRPV L1 protein at a final concentration of 2µg per lane. Day 1 represents the prebleed serum, whereas Day 51 represents the last bleed serum. The presence of a band in the last three lanes indicates the detection of CRPV L1 protein by the day 51 serum collected from individual rabbits. Although all sera were pre-absorbed, insect cell debris and residual baculovirus proteins are still detected by some of the sera. Detection of denatured CRPV L1 protein using MAb CRPV:10B is represented as the positive control.

3.3.6 Rabbit challenge experiment and *in vitro* virus neutralisation

Thanks to the help of my colleagues Drs Becker and Govan and Mrs Debbie Stewart, we have completed the rabbit challenge experiment and have evaluated the resulting antibody response in an *in vitro* neutralisation assay since the completion of my project work. Included in the challenge experiment are rabbits inoculated with PBS and with insect cell-derived CRPV VLPs.

Rabbits were challenged with two different infectious virus dosages of which the lower dosage (10^{-3} dilution) was known to consistently result in the formation of papillomas at 50% of inoculated sites (Christensen *et al.* 1996). In addition two sites on the rabbit's back were inoculated with a 10^{-2} dilution of the virus. The growth of papillomas was monitored on a weekly basis with measurements (length x width x height in mm) taken 14 days post challenge over a period of 63 days. The measurements from all animals in a group were compiled and are plotted as geometric mean diameter (GMD) against observation time after challenge. This is presented in figure 3.9.

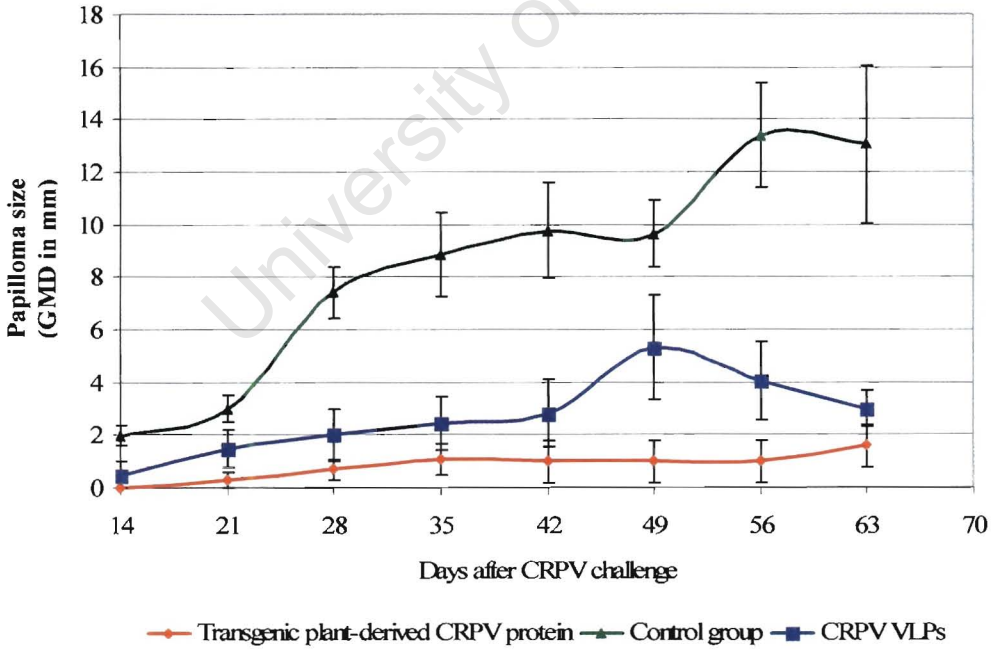


Figure 3.9: Papilloma growth on the backs of rabbits following challenge with infectious CRPV. Rabbits were challenged with 10-fold dilutions of infectious CRPV (two sites per dilution). Papilloma sizes were measured weekly beginning on day 14 and the geometric mean diameter (GMDs) calculated. The GMDs and standard error of measurement (SEM) of papillomas were plotted against time for the sites challenged with 10^{-2} dilution of infectious CRPV.

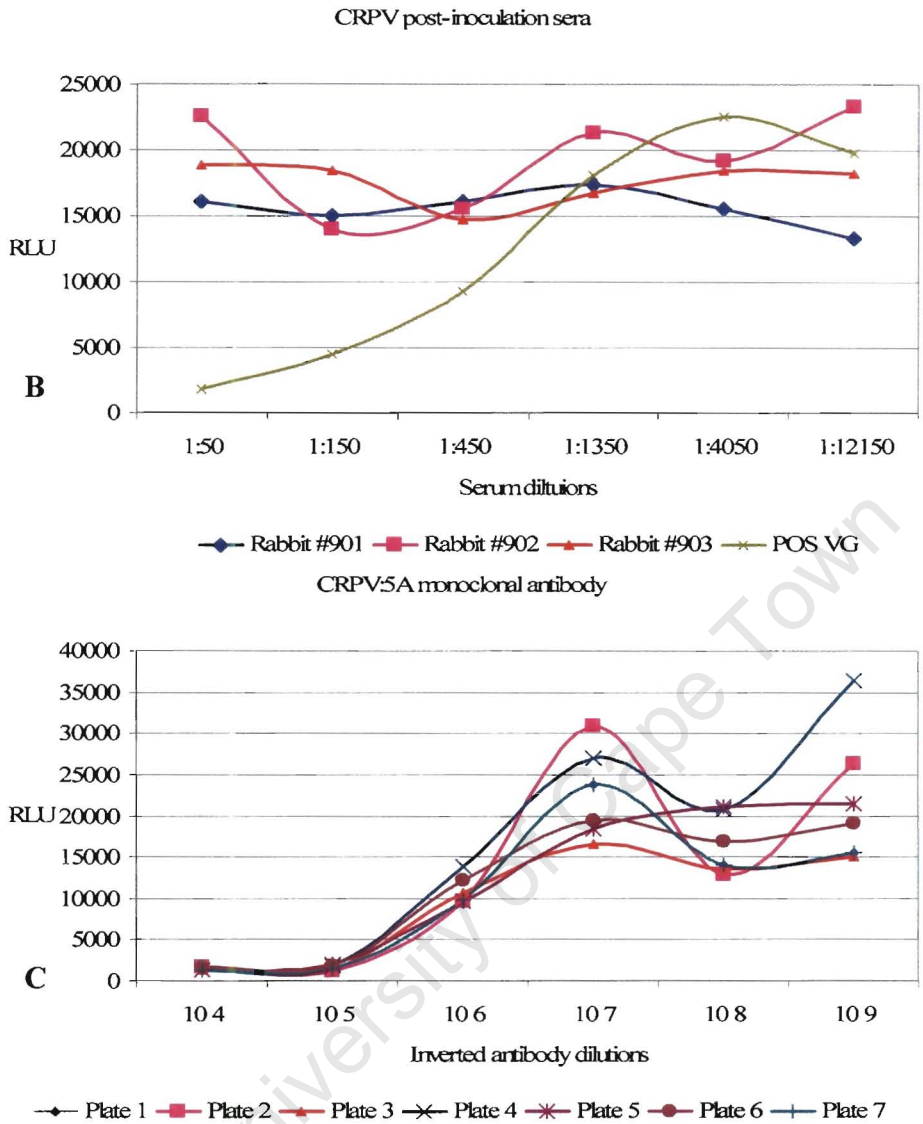


Figure 3.10: CRPV pseudovirus neutralising assay using the pre-inoculation serum (Day 1) collected from all 3 rabbits immunized with the transgenic plant-derived *N. tabacum* protein extract (**A**). The evaluation of the post-inoculation sera (Day 51) from all animals is shown in **B**. Included in the data set is the anti-CRPV serum provided by Dr Vandana Govan (pos GV). The data is presented as relative light units (RLU) observed at various dilutions of the tested serum. Neutralization assay results obtained from incubation of CRPV pseudoviruses with known conformation-specific and neutralising MAb CRPV:5A is shown in **C**. Data is presented as a collection from six individual experiments

The results shown in figure 3.10 **A** were expected; none of the sera collected from naïve animals on the day of the first inoculation (Day 1) should be capable of neutralising the CRPV pseudovirus *in vitro*. However, the results obtained from the evaluation of the last day (Day 51) serum in the neutralisation assay are surprising (Figure 3.10 **B**). The

included internal positive control serum collected from rabbits that were previously immunized with insect cell-derived CRPV VLPs shows neutralisation of the virus up to and including the 1:450 dilution. The known CRPV conformation-specific and neutralising MAb CRPV:5A derived from a hybridoma is capable of neutralising the CRPV pseudovirus at a much higher dilution of 10^{-5} (Figure 3.10 **C**). A possible explanation for these controversial results will be addressed in the discussion.

3.4 DISCUSSION

This study presents the first steps in what is to become a proof-of-concept study. The overall aim was to determine whether a plant produced papillomavirus L1 protein was in fact capable of eliciting an effective type-specific immune response.

Here I report on the expression of the wild-type CRPV *L1* gene in transgenic *N. tabacum*. The exclusion of the *L1* nuclear localization signal (NLS) had not been addressed at the point this project was initiated, as the work described in this chapter was taken over from Dr Arvind Varsani.

In summary, five confirmed transgenic tobacco lines were followed to the T_1 generation, when mRNA and protein expression were confirmed. Concentrated plant extracts bound conformation-specific and linear epitope-specific monoclonal antibodies in the ELISA; however, the electron microscopy results indicated that much of the protein was not assembled, and there were no VLPs visible. Injection of concentrated extracts into rabbits elicited antibodies which bound insect cell-produced VLPs, and animals were protected against wild-type virus challenge – but in absence of detectable neutralising antibodies.

The production of CRPV L1 in plants is novel. Moreover, the yield of 0.7 mg/kg was reasonable, given the much lower yields of HPV-11 L1 of Warzecha *et al.* (2003) and of HPV-16 L1 of Varsani *et al.* (2003) and Liu *et al.* (2005) and was comparable to the yield I obtained for HPV-11 L1 (Chapter 2), and that obtained for HPV-16 L1 by Biemelt *et al.* (2003).

The lack of higher-order assembly of the protein was interesting, given the same protein assembled in insect cells when expressed via recombinant baculovirus. However, a conformation-specific MAb did bind the plant-produced protein, indicating some degree of assembly. Thus, the possibility that the plant-derived CRPV L1 protein does assemble into pentamers cannot be excluded. It is possible that higher-order CRPV L1 structures are not very stable, as was found with transgenic *N. tabacum* HPV-11 L1 NLS^- protein

(Chapter 2). Whether plant-derived CRPV L1 protein is subject to extensive trypsin-like protease digestion, as has been shown in the case of the HPV-11 L1 NLS$^-$ protein, was shown. One explanation for the lack of VLPs is that the expressed protein does not accumulate to levels high enough to favour the formation of homogenous VLPs, as perhaps the association constant in the assembly reaction is not as high as with HPV-11 L1 and especially HPV-16 L1, which formed VLPs at very low concentrations (Varsani *et al.* 2003). Although this particular L1 gene is presumably translocated into the nucleus with the help of the still existing nuclear localization signal, protein levels in the nucleus still might not be sufficient to result in the assembly of VLPs. It needs to be noted that this particular gene has not been codon-optimized for the use *in planta*: plant preferential codon usage might significantly affect protein expression levels at the post-translational level. In general protein expression levels have been found to be extremely low in plants, however, there are some exceptions as mentioned in the discussion of Chapter 2.

It was interesting that last bleed antisera from all three rabbits were found to be able to detect native CRPV L1 VLPS in ELISA, and denatured insect cell-derived CRPV L1 protein when tested by western blot (Figure 3.8): this is in contrast to results obtained using equivalent amounts of HPV-11 L1 NLS$^-$ plant-produced protein. Protective immunity to PVs is usually directly correlated with the production of virus-neutralising antibodies. Table 3.4 summarizes various animal immunization and subsequent challenge experiments performed on rabbits, cattle and dogs to test the efficacy of developed rabbit, cattle and dog PV subunit vaccines. All were found to protect their respective hosts from challenge with live virus. Suzich *et al.* (1995) evaluated a candidate COPV subunit vaccine and reported a relatively low anti-COPV antibody titre in the serum of dogs vaccinated with COPV L1 VLPs, but cautioned that the levels of virus-neutralising antibodies might be underestimated.

The results in Table 3.4 indicate that while native L1 protein or VLPs – with or without L2 – protect against virus challenge in all systems tested, and L2 protein is also protective in cows against BPV infection, neither denatured L1 protein nor truncated L2 is protective. It is also interesting that while Jansen *et al.* (1995) and Christensen *et al.*

Table 3.4: Examples of various animal prophylactic vaccination and challenge strategies

Host	Vaccine type	Production	Dosage	Adjuvant	Timetable (days)	Challenge dosage	Protection	Neutralising antibodies	References
Rabbit	CRPV L1 or CRPV L1/L2 VLPs Native or denatured	Recombinant baculovirus	50µg subcutaneous	Freund's (complete) or 2% aluminium hydroxide	1, 14 and 28	CRPV wart extract $5x10^{10}$ (lower dose) $2x10^{11}$ (higher dose) (day 42)	Native VLPs **Yes** 62% (lower dose) 17% (higher dose) Denatured VLPs **No**	**Passive transfer serum or IgG**	Breitburd *et al.* 1995
Rabbit	CRPV L1 VLPs	Recombinant baculovirus	50µg intramuscular	None	1, 21 and 35	**10^{X} CRPV particles** 10^{-1} 10^{-4} dilution (day 46)	**Yes**	**Yes**	Christensen *et al.* 1996
						10^{-1} 10^{-4} dilution (day 213)	**Yes**		
						10^{-1} 10^{-4} dilution (day 403)	**Yes**		
Rabbit	CRPV L1 or CRPV L1/L2 VLPs	*Saccharomyces cerevisiae*	1, 10, 25, 50 or 100µg (L1) intramuscular	Aluminium hydroxide	1, 28 and 56	Condyloma extract	**Yes**	**Yes**	Jansen *et al.* 1995
Cattle	BPV-4 L1 or BPV-4 L1/L2 VLPs	Recombinant baculovirus	150µg (L1) intramuscular	Aluminium hydroxide	1 and 28	BPV-4 particles 10^{11} particles (day 42)	**Yes**	**No**	Kirnbauer *et al.* 1996
			200µg (L1/L2) intramuscular				**Yes**		
Cattle	GST or βGal fusion protein **A** BPV-4 L2 (wild type)	*Escherichia coli*	50µg of A+B+C+D in a ratio of 1:5:5:5	Aluminium hydroxide	1 and 28	BPV-4 particles 10^{11} particles (day 42)	**Yes**	**No**	Chandrachud *et al.* 1995
	B BPV-4 L2 (aa 11-200)		330µg				**Yes**		
	C BPV-4 L2 (aa 201-326)		Not determined				Not determined		
	D BPV-4 L2 (aa 327-524)		330µg				**No**		
Dog	COPV L1 VLPs	Recombinant baculovirus		None	1 and 14	(day 28)		**No**	Suzich *et al.* 1995
	Native		860µg (crude) 120µg (enriched) intradermal				**Yes**		
	Denatured		120µg				**No**		

Christensen *et al.* (1996) addressed the topic of differences in antigenic properties, a very interesting fact that needs to be taken into consideration as it is directly applicable to this work. They found that CRPV virions and CRPV L1 VLPs are not antigenically identical. It is interesting that CRPV L1 capsomers have not been investigated as vaccines. My thesis reports on the transformation of *N. tabacum* with the CRPV *L1* gene, resulting in the production of the CRPV L1 protein that is detectable by neutralising conformation-specific and surface linear epitope recognizing MAbs (Figure 3.4 and 3.5 **A** and **B**), and protective as a vaccine. Transgenic-derived CRPV L1 protein does not appear to assemble into VLPs; instead it predominantly remains in smaller, possibly pentameric aggregates (Figure 3.6 **A**). Although pentameric structures are sufficient for the induction of conformation-specific antibodies capable of virus-neutralisation with HPV-11 and HPV-16, it remains to be determined whether the concentration of pentameric transgenic CRPV L1 protein in my plants is high enough to induce such an antibody response in rabbits. It would be interesting to further investigate the assembly kinetics of CRPV L1 and to determine whether sub-VLP assemblies are capable of eliciting neutralising antibodies.

Given the fact that an antibody response was observed in all three test animals, but detectable levels of neutralising antibodies were not elicited, a question that presents itself is how the animals then resisted the challenge virus. While it is almost a dogma that neutralising antibodies are the correlate of protection against PV infections, Passmore *et al.* (2005) have pointed out that cellular immunity is a significantly under-estimated factor in protection against, and especially clearance, of PV infections.

However, there were some indications that antibodies were implicated in protection against CRPV in this work. One rabbit in the challenged group showed a progressive growth of papillomas starting at 1 mm and ending at 4.9 mm in diameter (Day 63). This animal also showed the weakest induction of CRPV L1-specific antibodies (Rabbit #901, Figure 3.7), thus indicating that protection could be associated with a high antibody titre. As already mentioned in the discussion of Chapter 2, our neutralisation assays were performed using the sera from vaccinated animals at a dilution of 1:50. Although Embers *et al.* (2002, 2004) have shown that CRPV virus neutralising antibodies have been

detected at a serum dilution of 1:5, application of lower titre dilutions in our pseudovirus neutralisation assay does present difficulties with regards to the experimental repeatability due to non-specific activity of various sera. However, if repeated, such assays could potentially indicated the presence of pseudovirus neutralising antibodies and thus confirm that the presence of these virus neutralising antibodies has thus far been underestimated.

However, there is another possible explanation for the protection observed in this study in the absence of pre-challenge neutralising antibodies: this is self-immunization. The term "self-immunization" is used to describe the following scenario. Over many years of experience and experimentation with a potential Canine oral papillomavirus (COPV) vaccine, Bennett Jenson has observed the following phenomena. The antibody response of vaccinated animals declines over time, yet after dogs have been challenged with infectious COPV, a second more steady increase in antibody titre occurs as a result of the "self-immunization" effect. This increase in antibody levels results in complete protection of dogs from further infection by the virus, suggesting that all antibodies are virus-neutralising (personal communication). Could the same be applicable to my research?

According to my results shown in figure 3.10, all post-inoculation sera (Day 51) collected from rabbits before challenge were unable to neutralise the CRPV pseudovirus *in vitro*. In turn and given the above described scenario, this prompts the following questions. Does the application of infectious CRPV induce an additional immune response in an already primed animal that results in the generation of virus neutralising antibodies, given the fact that antigenic differences could exist between CRPV virions and aggregates/VLPs? And further, can such a neutralising antibody response be generated within 14 days? Unfortunately, no sera were taken post-infection, so the possible appearance of neutralising antibodies cannot be shown. This should be done in a follow-up to this work, however, whatever its shortcomings, this remains a ground-breaking study: I believe this to be among the first in providing evidence that a plant-derived PV vaccine is capable of protecting against papillomavirus infection.

Chapter 4

Transient expression of HPV-11 *L1 NLS*$^-$ and CRPV *L1* in *Nicotiana benthamiana* via a recombinant Tobacco mosaic virus vector

Contents

ABSTRACT

To attempt to overcome the apparent limitations of protein production by means of transgenic plant expression systems as shown in previous chapters, I engineered the commercially available Tobacco mosaic virus (TMV)-based vector pBSG1057 to express the HPV-11 *L1 NLS*$^-$ and CRPV *L1* genes. Mechanical infection of *N. benthamiana* plants with synthesized vector RNA resulted in the systemic spread of recombinant TMV. Analysis by RT-PCR revealed delayed expression of the HPV-11 *L1 NLS*$^-$ gene in contrast to early expression of the CRPV *L1* gene whose transcription was soon terminated. Protein production was directly influenced by transcription of the genes and resulted in protein yields of varying degrees. Expression of the HPV-11 *L1 NLS*$^-$ gene resulted in a total yield of 10.6mg per kilogram of fresh leaf material, whereas partial expression of the CRPV *L1* gene only resulted in the harvest of 600µg per kilogram. Transiently expressed protein was detected by a panel of monoclonal antibodies against HPV-11 L1 and CRPV L1, recognizing both neutralising conformation-specific and surface linear epitopes. Direct and immunogold-labelling electron microscopy indicated the presence of HPV-11 L1 NLS$^-$ and CRPV L1 protein, but only revealed the assembly of HPV-11 L1 NLS$^-$ VLPs.

Inoculation of guinea pigs and New Zealand white rabbits with between 14 and 53µg of transiently-derived HPV-11 L1 NLS$^-$ and 2 to 8µg of transiently-derived CRPV-L1 protein respectively resulted in a type-specific antibody-mediated immune response, that in the case of HPV-11 *L1* NLS$^-$ immunization revealed the predominant recognition of a novel immunodominant epitope(s) exposed on plant-derived HPV-11 L1 NLS$^-$ VLPs, compared to insect cell-derived L1 and L1 NLS$^-$ VLPs.

Rabbits were challenged with infectious CRPV and the formation of papillomas monitored over a period of 63 days. Results indicate that vaccinated rabbits are partially protected from challenge showing an average growth of papillomas 6 times smaller than the control group. However, results obtained from *in vitro* neutralisation results indicate that none of the post-inoculation sera collected from all animals in this study were able to

neutralise either the HPV-11 or the CRPV pseudoviruses, thus putting into question the viability of these plant-produced PV antigens as potential vaccine candidates.

4.1 INTRODUCTION

Extensive research has determined that a variety of different plant viruses can tolerate the insertion of foreign genes into their genome without jeopardizing their infectious nature (Shivprasad *et al.* 1999). This has led to the development of a number of plant virus-derived vectors for the transient expression of foreign genes in plants (see Chapter 1, Table 1.5). The development of plant virus vectors has become a powerful tool for the rapid production of foreign proteins in plants and displays several advantages over the use of transgenic plant production systems.

In their review, Fischer *et al.* (1999) compare three different transient expression systems for the delivery of foreign genes into plants: these are virus vectors, agroinfiltration and biolistic bombardment. The application of the last is limited to individual plant components, the leaves for example, whereas the use of plant viruses and agroinfiltration potentially allows systemic expression in the entire plant. Only plant viruses, however allow the expression of protein in tissue which develops after inoculation with the vector. The foremost advantage displayed by the use of virus vectors is the rapidly obtainable yield of the target protein. Like agroinfiltration, protein expression levels are not subject to unfavourable gene insertion into the plant genome, nor do they display any negative growth effects on the plant itself (Fischer *et al.* 1999).

The time required to achieve the production of a target protein in a transient virus vector expression system might possibly extend beyond a month; this shortens the time required to generate a transgenic plant by a factor of 4 to 10.

Tobacco mosaic virus (TMV) (Figure 4.1) has been the focus of research for more than a century (Harrison *et al.* 1998). TMV, known as one of the simplest viruses, is a member of the alphavirus-like superfamily and was the first plant virus to be purified and have its structure as well as its genome sequence and gene function resolved. (Heinlein, 2002).

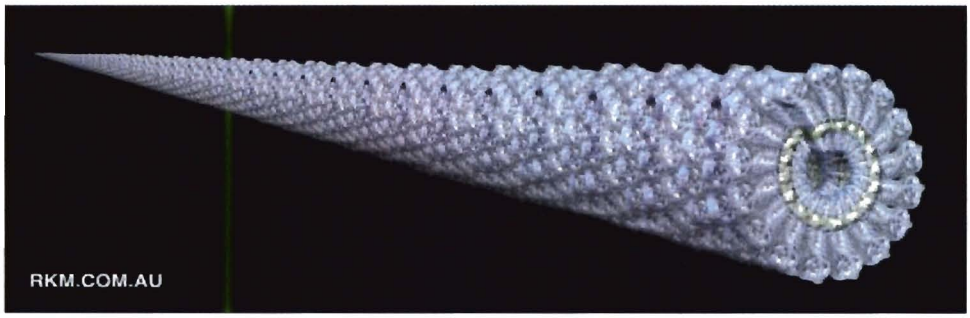


Figure 4.1: Image of a TMV particle (courtesy of Russell Kightley, www.rkm.com.au)

TMV belongs to the class of positive sense, single-stranded RNA viruses. The genome is 6395 nucleotides in length and encodes a total of four open reading frames (ORFs), three of which encode non-structural proteins. The viral RNA is encapsidated in a helically-arranged coat of 2160 copies of the only structural protein, the coat protein (CP, Figure 4.1) (Heinlein, 2002).

The 5' terminal end of the viral RNA genome is capped, and the 3' end displays a transfer RNA (t-RNA)-like structure that is capable of accepting the amino acid histidine. The first ORF encodes the 126 kDa replicase subunit and is directly translated from the viral genome. An amber stop codon (UAG) terminates the translation of the 126 kDa replicase subunit but allows for approximately 10% read-through, resulting in the synthesis of the second and much larger (183 kDa) replicase subunit (Lewandowski *et al.* 2000). Together these two proteins initiate replication of the viral RNA inside the infected plant cell (Figure 4.2).

The 126 kDa replicase subunit contains two distinct functional domains: these are the methyltransferase (MT) activity domain and the helicase (HEL)-like domain. Both domains are duplicated upon synthesis of the 183 kDa subunit, which in addition contains the RNA-dependent RNA polymerase (POL) domain (Lewandowski *et al.* 2000).

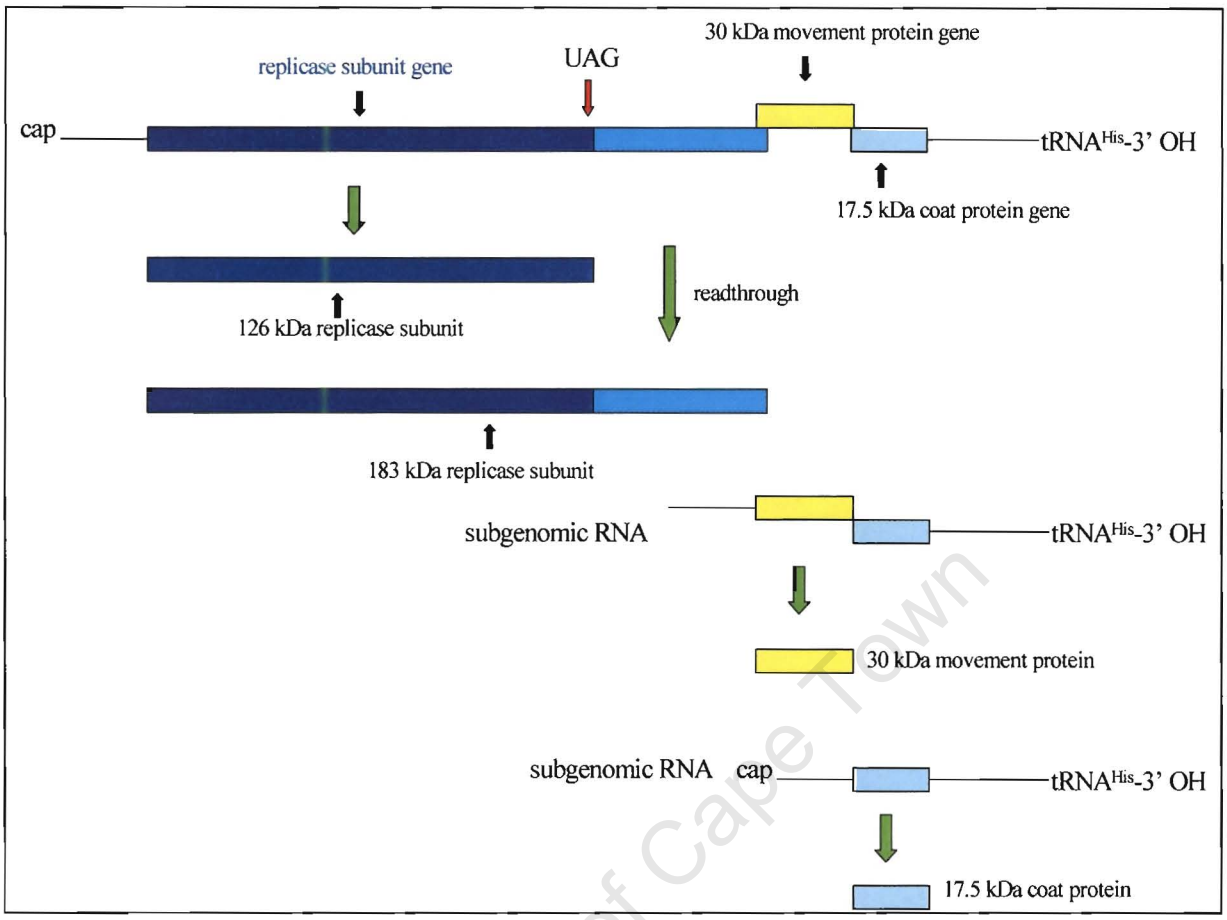


Figure 4.2: Schematic representation of the TMV genome (adapted from Heinlein, 2002). Two of the three non-structural proteins, the 126kDa and 183kDa replicase subunits, are directly translated from the viral genomic RNA. The amber stop codon (UAG) allows for read-through and synthesis of the 183 kDa replicase subunit. During replication, the third non-structural 30kDa movement protein and the structural 17.5kDa coat protein are translated from subgenomic RNA species. Only the subgenomic movement protein RNA remains uncapped in comparison to the capped genomic RNA and subgenomic coat protein RNA. Termination of the viral RNA is facilitated by the presence of a t-RNA-like structure at the 3' end, capable of accepting histidine.

During replication of the RNA genome, the two remaining ORFs encoding the 30 kDa movement protein and the 17.5 kDa coat protein are expressed by translation from the negative sense RNA of two distinct subgenomic RNAs (Figure 4.2) (Heinlein, 2002). Naturally occurring infections of plants by TMV do not require a vector. Transmission of the virus occurs at a point of contact between plants or virus contaminated surfaces (Heinlein *et al.* 1998, 2002).

TMV has been engineered for the efficient transient expression of target proteins in one of its natural host plants, *Nicotiana benthamiana*. First generation TMV vectors in which

the coat protein sequence was replaced by a foreign gene sequence did not express the target protein throughout the entire plant. Removal of the coat protein disabled movement of the virus, thereby preventing the systemic spread throughout the plant (Shivprasad *et al.* 1999). A series of second generation TMV-based expression vectors with an extra ORF under the control of a repeated subgenomic RNA promoter displayed greater stability; however factors including homologous recombination and reversion back to the original wild type virus still persisted (Donson *et al.* 1991). Shivprasad *et al.* (1999) described the construction of various TMV-based hybrid vectors, all of which contain the 5' NTR, the replicase and movement genes from TMV, but differ in the coat protein subgenomic RNA promoter and the coat protein gene. Replacement of these two components by their heterologous counterparts from various closely related tobamoviruses including Tobacco mild green mosaic virus (TMGMV) variants U2 and U5, Tomato mosaic virus (ToMV) and Sunn-hemp mosaic virus (SHMV) appeared to alleviate these previously encountered instability and recombination problems.

A TMV-based hybrid transient expression vector consisting of the replicase and movement protein genes from the TMV isolate U1, together with the coat protein subgenomic RNA promoter and coat protein gene from TMGMV isolate U5 has been found to be one of the most stable (Shivprasad *et al.* 1999). This vector is commercially known as the Geneware^{SM} vector pBSG1057 (Large Scale Biology Corporation, Vacaville, CA, USA) (see Appendix B for pBSG1057 map) and expresses the enhanced 30B-GFPC3 mutant gene (Crameri *et al.* 1996) under the control of the subgenomic coat protein promoter of TMGMV U5 (Figure 4.3). Replacement of the 30B-GFPC3 mutant gene by various other genes has led to the transient expression of various pharmaceutical proteins and peptides in plants (Hamamoto *et al.* 1993; Koo *et al.* 1999; Nemchinov *et al.* 2000a; Takamatsu *et al.* 1990; Verch *et al.* 1998; Wigdorovitz *et al.* 1999).

This chapter focuses on the transient expression of the native HPV-11 L1 NLS$^-$ and CRPV L1 proteins in *N. benthamiana* using the TMV-based expression vector pBSG1057. This study was intended to explore the possibilities of generating more protein, protein that could potentially be applied as an efficacious subunit vaccine to

prevent type-specific infection. Furthermore this study compares various aspects of both transient and transgenic plant expression systems, including the production of VLPs, their antigenic properties, the respective yields and the efficacy of the plant-derived protein in stimulating the immune system, with 'gold-standard' proteins derived from insect cells via recombinant baculovirus.

4.2 MATERIALS AND METHODS

4.2.1 Cloning of HPV-11 *L1 NLS⁻* and CRPV *L1* into the tobamovirus vector pBSG1057

The HPV-11 *L1 NLS⁻* and CRPV *L1* genes were amplified by PCR using primer pairs 9 and 10 respectively as listed in table 4.1. The PCR was performed using 25 cycles of 94°C for 30s, 55°C for 45s, and 72°C for 45s, followed by a 5min extension step at 72°C. The forward primers had been designed to include a 5' *Pac*I site, whereas the reverse primers included a 3' *Xho*I restriction enzyme site. PCR products were cloned into the pGEM®-T Easy vector (Promega) and sequenced.

Table 4.1: Primers used for the amplification of the HPV-11 *L1 NLS⁻* and CRPV *L1* ORFs

Primer Pair	Construct	Primers
9	HPV-11 *L1* NLS⁻ internal	Forward 5'- TGATACAGGCTTTGGTGC -3' Reverse 5'- TGGCTCATTGGTGTCTTC -3'
10	pBSG-11-*L1* NLS⁻	Forward 5'- TTAATTAA**ATG**TGGCGGCCTAGCGAC-3' Reverse 5'- CTCGAG**TTA**CTTTTTGGTTTTGGTACG-3'
11	pBSG-CRPV-*L1*	Forward 5'- TTAATTAA**ATG**GCAGTGTGGCTGTCTACG-3' Reverse 5'- CTCGAG**TTA**AGTACGTCTCTTGCGTTTAGATGATT-3'
12	CRPV *L1* internal	Forward 5'- AAAGCATGGCGTTCGACC-3' Reverse 5'- GCACACAGATGCAGGGAGAG-3'
13	TMV Coat Protein	Forward 5'- CATTAGCGCTGCGGCCGCCCTTATACAATCAACTCTCCG -3' Reverse 5'- ATAAGAATGCGGCGGCTCGCGAAGTAGCCGGAGTTGTTGTC-3'

TTAATTAA *Pac*I, CTCGAG *Xho*I, GCGGCCGC *Not*I, TCGCGA *Nru*I, **ATG** = start codon, **TAA** = stop codon

The introduced *Pac*I and *Xho*I restriction enzyme sites facilitated excision of the sequenced HPV-11 *L1 NLS*⁻ and CRPV *L1* genes from the pGEM®-T Easy vector, where after the genes were directionally sub-cloned into the Geneware℠ vector pBSG1057 replacing the 30B-GFPC3 mutant gene (Figure 4.3).

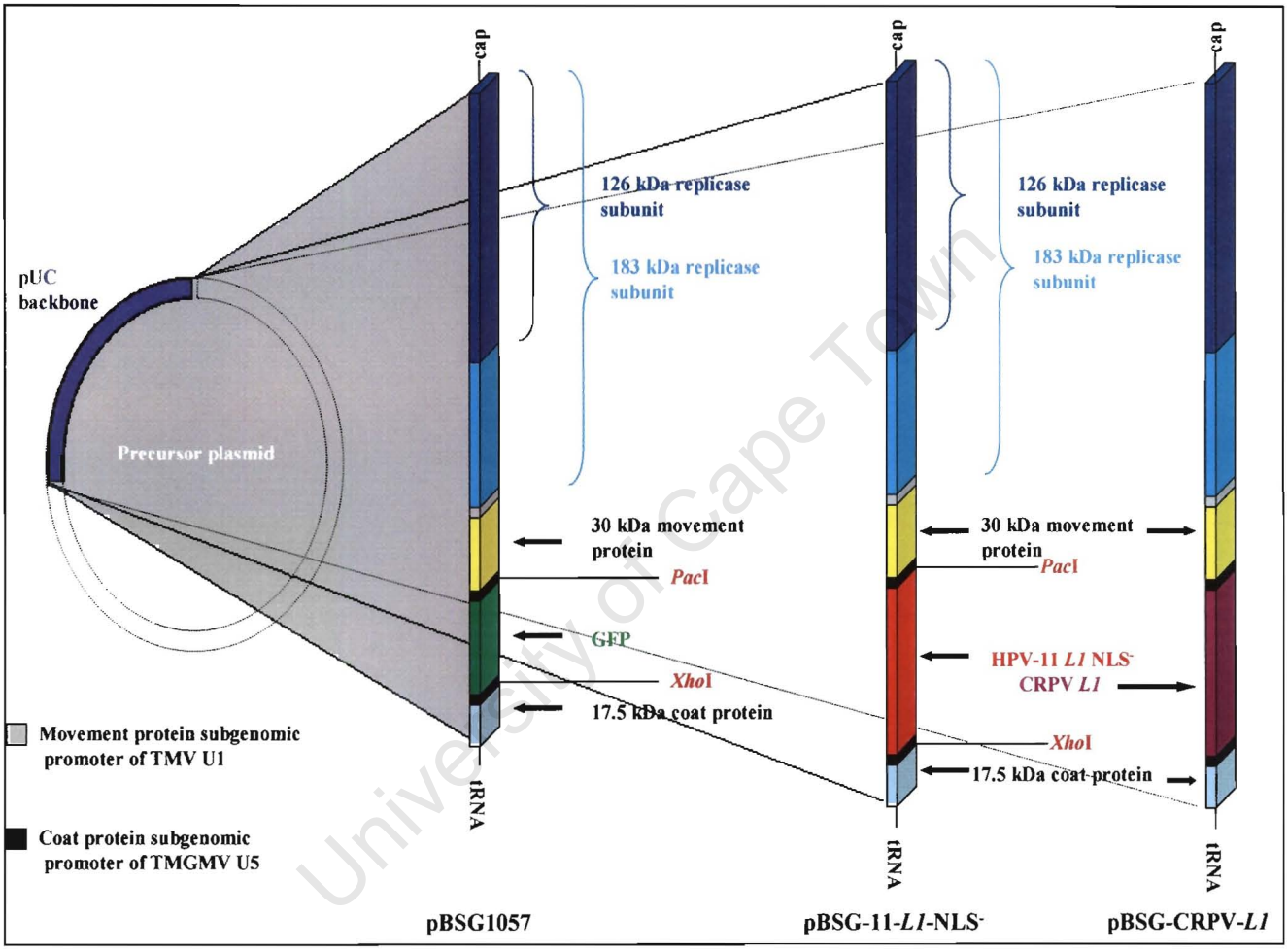


Figure 4.3: Schematic representation of the pBSG1057 vector-derived constructs pBSG-11-*L1* NLS⁻ (red) and pBSG-CRPV-*L1* (purple). The 126 and 183 kDa replicase subunit genes as well as the 30 kDa movement protein gene are derived from the TMV isolate U1. The subgenomic coat protein promoter and the 17.5 kDa coat protein originate from the TMGMV isolate U5. *Pac*I and *Xho*I restriction enzyme sites facilitate the replacement of the 30B-GFPC3 mutant gene, placing either *L1* gene under the control of the subgenomic coat protein promoter. The pUC backbone is highlighted in light purple and facilitates replication in *E. coli*. The complete pBSG1057 vector map is shown in Appendix B, figure B.3.

4.2.2 *In vitro* transcription of pBSG1057, pBSG-11-*L1 NLS*⁻ and pBSG-CRPV-*L1* constructs

Transcripts were synthesized *in vitro* using the T7 RNA polymerase (RiboMAX™ Large Scale RNA Production System-T7, Promega) and capped by addition of the RNA cap-structure analogue m7G(5)ppp(5)G (New England Biolabs). 5µg of pBSG1057, pBSG-11-*L1 NLS*⁻ and pBSG-CRPV-*L1* DNA was used for the synthesis of the RNA transcripts. The reaction was carried out in a total volume of 50µl, of which 5µl were used for confirmation of transcription by gel electrophoresis.

4.2.3 Inoculation of *Nicotiana benthamiana* plants

Three week old *N. benthamiana* plants were mechanically inoculated with 15µl of either the pBSG1057 (expressing GFP), the pBSG-11-*L1* NLS⁻ or the pBSG-CRPV-*L1* synthesized transcripts by abrasion of the fourth leaf in the presence of Celite. Plants were grown at 25°C with a 16/8 hr day/night photoperiod, and monitored for the appearance of signs signalling systemic infection. Plants inoculated with the pBSG1057 transcript served as control to indirectly monitor the movement of the virus by illumination of plants with a handheld long wavelength (366nm) ultraviolet lamp.

4.2.4. Total RNA analysis and confirmation of systemic spread throughout *N. benthamiana* plants

Depending on the rate of growth, total RNA was extracted from the inoculated leaf through to the 9th or 11th leaf of individual plants using TRIzol™ reagent (Life Technologies). A final volume of 5µl total RNA was used for the detection of the HPV-11 *L1* NLS⁻ or CRPV *L1* mRNA, together with the TMV coat protein mRNA by RT-PCR amplification using the Access RT-PCR system (Promega). The profile for the RT-PCR is listed as: 45°C for 45 min, 94°C for 2 min; followed by 30 cycles of 94°C for 30s, 55°C for 1 min and 68°C for 2 min. The final elongation step was performed at 68°C for 5 min. The reactions incorporated the respective primer pairs 9, 12 and 13 as listed in table 4.1.

4.2.5 Protein analysis of inoculated *N. benthamiana* plants

Infected plants were harvested 14 days post inoculation (dpi) and homogenized in 1:2 (w/v) cold PBS according to the flow diagram in chapter 2 (Figure 2.2). PBS (1.47mM KH_2PO_4, 10mM Na_2HPO_4, 2.7mM KCl, pH 7.4) containing 500mM NaCl was used throughout the entire extraction process. In comparison to the processing diagram presented in figure 2.2 of chapter 2, I have included an additional step in the processing protocol. The initial addition of 4% (w/v) coarse polyethylene glycol (molecular weight 8000, PEG 8000) was aimed at removing most of the TMV particles (Figure 4.4) before all remaining proteins were precipitated by the addition of an additional 6% coarse PEG 8000 (w/v). The additional 6% PEG 8000 (w/v) was completely dissolved in the supernatant (Number ④) before final protein extraction was commenced. Total isolated protein was resuspended in a final volume of PBS as indicated in figure 4.4 and analyzed for the presence of the HPV-11 L1 NLS^- and CRPV L1 proteins.

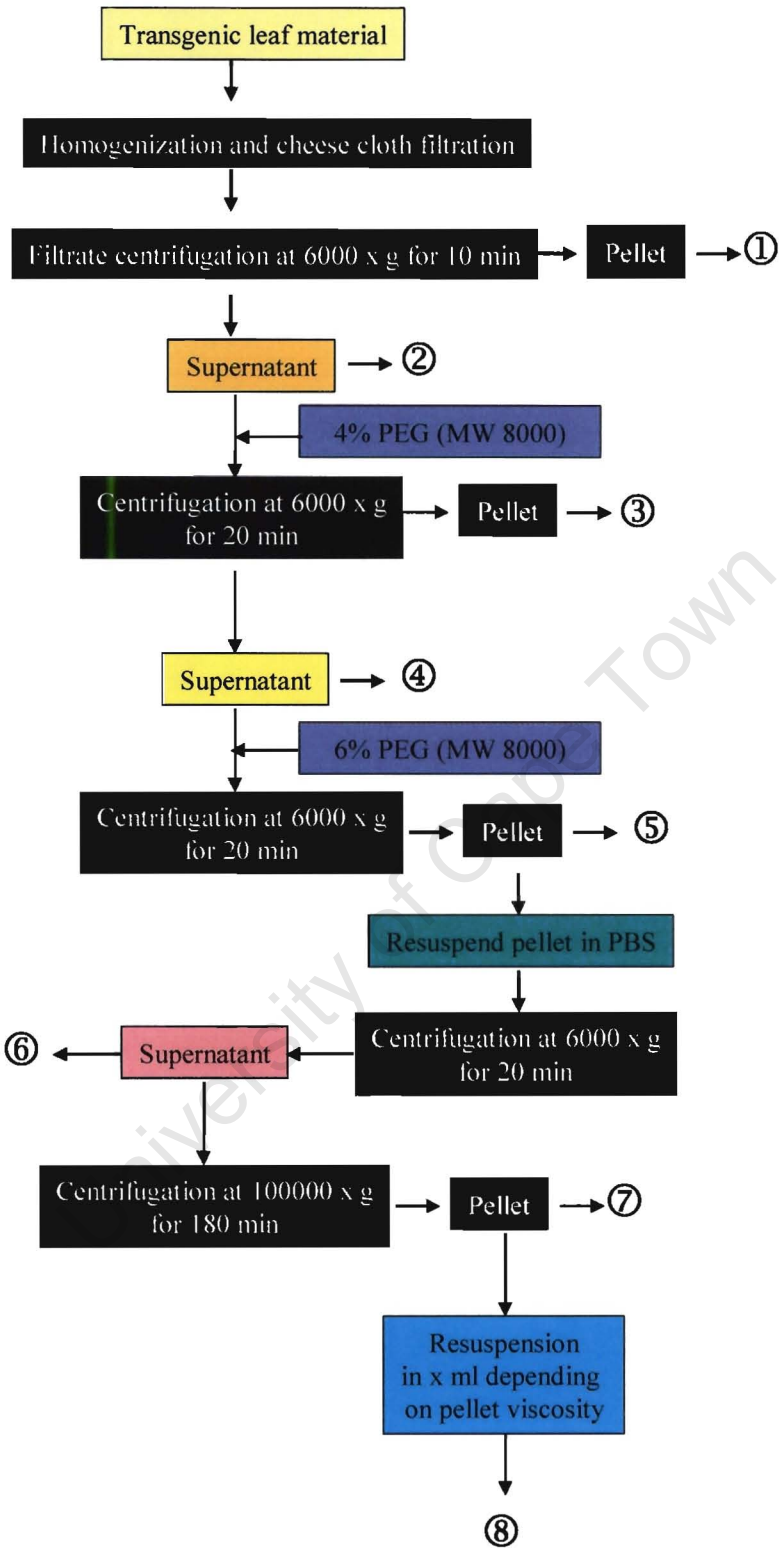


Figure 4.4: Diagram describing the processing of *N. benthamiana* plant material, isolation and purification of total plant protein. The addition of 4% PEG to supernatant number 2 is aimed at precipitation and removal of most of the TMV particles before further extraction of the HPV-11 L1 NLS^- and CRPV L1 proteins.

4.2.6 Generation of HPV-11 L1 NLS$^-$/NLS$^+$ and CRPV L1 VLPs

HPV-11 L1 and CRPV L1 VLPs were generated and harvested after infection of insect cells with the respective recombinant baculovirus stocks as described in sections 2.2.9 / 2.2.10 and 3.2.8 of chapters 2 and 3 respectively.

4.2.7 Monoclonal antibody characterization of transiently expressed L1 protein extracts

HPV-11 L1 NLS$^-$ and CRPV L1 proteins (Number ⑧, Figure 4.4) harvested from the transient expression in *N. benthamiana* plants, together with non-infected *N. benthamiana* plant protein extracts were characterized by direct ELISA using a panel of monoclonal antibodies (MAbs) to HPV-6, HPV-11, HPV-16 and CRPV respectively (generously provided by Neil Christensen). ELISA plates were coated with protein extracts for 1 hr and then blocked in 0.5% PVA in PBS for 1 hr. Monoclonal antibodies, diluted 1:1000 in 0.5% PVA in PBS, were used to detect the transiently expressed, plant-derived antigen for 1 hr. Anti-mouse-alkaline phosphatase conjugated secondary antibody (Sigma), diluted 1:5000 in 0.5% PVA and 0.8% PVP in PBS was allowed to bind the primary antibody for 1 hr at 37°C. The secondary antibody was detected using p-nitrophenyl phosphate (pNPP, Sigma) and the absorbance was measured using a Titrex ELISA plate reader at 405nm. All samples were analyzed in triplicate to determine the mean absorbance and calculate the respective standard deviation.

4.2.8 Electron microscopy of transiently expressed L1 protein extracts

Transiently expressed HPV-11 L1 NLS$^-$ and CRPV L1 protein extracts were prepared for electron microscopy as outlined in chapter 2 (section 2.2.12). *N. benthamiana* plant protein extracts, together with insect cell-derived HPV-11 L1 NLS$^-$ and CRPV L1 VLPs, were viewed after preparation of carbon-coated copper grids. HPV-11-specific MAbs H11:H3, H6:I2 and CRPV-specific MAb CRPV:5A were used in respective immunotrapping and immunogold-labelling preparations.

4.2.9 Animal immunization with transiently expressed L1 protein extracts and evaluation of sera

The antigenicity of the two transiently expressed antigen extracts was evaluated in guinea pigs and New Zealand white rabbits. A group of four guinea pigs were immunized intramuscularly with 100µl (2 x 50µl) of transiently expressed HPV-11 L1 NLS^- protein extract. The initial immunization was followed by two intramuscularly administered booster inoculations on days 23 and 41. Serum was collected on days 1, 41 and 51 and analyzed by direct ELISA and western blotting (1/20 dilution) against HPV-11 L1 NLS^+ and HPV-11 L1 NLS^- insect cell-derived VLPs at a concentration of 0.4µg/100µl.

The transiently expressed CRPV L1 protein extract was administered to a group of three New Zealand white rabbits. The first inoculum (1ml of extract) was administered by subcutaneous (3 sites) and intramuscular (1 site) injection. An additional two booster inoculations consisting of 500µl protein extract mixed in a ratio of 1:1 with Freund's incomplete adjuvant were administered on days 23 and 41. Serum was collected on days 1, 23, 41 and 51, pre-absorbed against non-transgenic plant protein extract and non-recombinant baculovirus insect cell debris, before being analyzed by direct ELISA and western blotting (1/20 dilution) against CRPV L1 insect cell-derived VLPs at a concentration of 0.12µg/100µl. All samples were analyzed in triplicate to determine the mean absorbance and calculate the respective standard deviation.

4.2.10 Animal immunization with insect cell-derived HPV-11 L1 NLS^- and NLS^+ VLPs and evaluation of sera

Two groups of four guinea pigs were each immunized with either 20µg of insect cell-derived HPV-11 L1 NLS^- or NLS^+ VLPs. Two booster inoculations were administered on days 21 and 32, before serum was collected on day 42. An endpoint titration by direct ELISA was set up to determine the optimal dilution of the guinea pig antiserum. Thereafter all sera were analyzed by direct ELISA. Plates were coated for 1 hr with non-denatured and denatured (boiled for 10 min) insect cell-derived HPV-11 L1 NLS^- and

NLS^+ VLPs at concentrations of 0.4µg/well. Wells were blocked with 2% non-fat milk in PBS for 1 hr, before the guinea pig antiserum (1/2000) was used to detect the protein. Sheep-anti-guinea pig horseradish peroxidase conjugated secondary antibody (1/5000, Sigma) was allowed to bind the primary antibody for 1 hr at 37°C. The secondary antibody was detected using 3,3',5,5'-tetra-methyl-bezidine (TMB) in an acid buffer containing 0.01% H_2O_2. The absorbance was measured using a Titrex ELISA plate reader at 450nm.

4.2.11 Generation of HPV-11 and CRPV pseudoviruses

HPV-11 and CRPV pseudoviruses for the use in *in vitro* virus neutralisation experiments were generated by Mrs Debbie Stewart according to the protocols described in sections 2.2.14 and 3.2.12 of chapters 2 and 3 respectively.

4.2.12 Rabbit challenge with infectious CRPV

Three New Zealand white rabbits were challenged by Dr Vandana Govan with two different dosages of infectious CRPV. Virus stock dilutions (10^{-2} and 10^{-3}) were applied to the backs of TMV-derived HPV-11 L1 NLS^- immunized rabbits as has been described in section 3.2.11 of chapter 3. All control animals inoculated with either insect cell-derived CRPV VLPs or PBS were treated in exactly the same manner. Throughout the course of 63 days, the size of papillomas was measured as length x width x height in millimetres starting 14 days post challenge. The geometric mean diameter (GMD) was calculated for each papilloma. The means and standard deviations for the GMDs in each treatment group were plotted against time after challenge with virus.

4.2.13 Animal serum neutralization assays

The serum collected from all animals in this study was analyzed by Mrs Debbie Stewart for the capacity to neutralise respective HPV-11 and CRPV pseudoviruses *in vitro*.

Individual experiments were conducted to evaluate the serum neutralisation capability of the following:

1) the pre-(Day 1) and post-inoculation (Day 51) serum collected from guinea pigs inoculated with transiently expressed HPV-11 L1 NLS^- protein,

2) the pre-(Day 1) and post-inoculation (Day 51) serum collected from New Zealand white rabbits inoculated with transiently expressed CRPV L1 protein,

3) the pre-(Day 1) and post-inoculation (Day 42) serum harvested from guinea pigs immunized with 20μg of insect cell-derived HPV-11 L1 NLS^- and NLS^+ VLPs.

Three-fold dilutions of all pre and post-inoculation sera ranging from 1:50 to 1:12150 were prepared in neutralisation buffer as described in section 2.2.14 of chapter 2. Sera were incubated with their respective pseudoviruses as described in section 2.2.14 and the resulting secreted alkaline phosphatase (SEAP) content was determined by application of the SEAP Chemiluminescence Kit (BD Clonetech) according to the manufacturer's instructions.

4.3 RESULTS

4.3.1 Signs and symptoms of systemic recombinant TMV infection

In order to compare the already established transgenic expression levels of the HPV-11 L1 NLS$^-$ and CRPV L1 proteins (Chapters 2 and 3 respectively), the transient expression of both the above mentioned proteins was investigated using the TMV-based vector pBSG1057. My primary interests were to establish whether a greater yield could be obtained, a yield that would surpass the production of the identical proteins in transgenic plants.

Plants were mechanically inoculated with synthesized transcripts of the respective recombinant TMV vectors and closely monitored for a period of 14 days. During the observation period, control plants infected with the pBSG1057 vector expressing the GFP protein were closely monitored using a handheld long wavelength (366nm) UV lamp. GFP expression throughout all leaves of control plants was synonymous with the appearance of leaf curling and mosaic patterns. Identical symptoms, some of which are shown in figure 4.5, were displayed by all plants infected with either the HPV-11 *L1 NLS*$^-$ or the CRPV *L1* expressing TMV construct. On day 14, entire plants were harvested and processed according to figure 4.4.

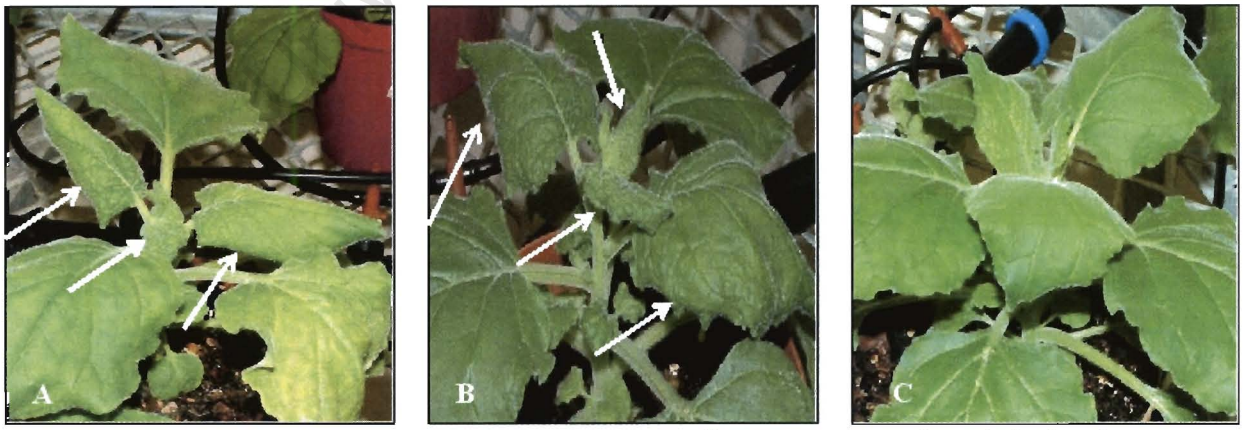


Figure 4.5: *N. benthamiana* plants showing symptoms directly resulting from infection by recombinant TMV. Signs of systemic infection include the curling of leaves (as indicated by arrows) and the typical mosaic pattern. **A** and **B** show plants infected with recombinant TMV expressing the HPV-11 L1 NLS$^-$ and CRPV L1 proteins respectively, whereas C represents an uninfected *N. benthamiana* plant.

4.3.2. Detection of expression of the HPV-11 *L1 NLS*⁻ and CRPV *L1* genes

On the day prior to harvesting the infected *N. benthamiana* plants (Day 13), leaf disc samples were taken from each leaf including the inoculated through to the last leaf on the apex and total RNA was extracted. Since the replication of TMV does not include a DNA intermediate step, the extracted total RNA did not have to be treated with DNase to prevent contamination prior to RT-PCR analysis. RT-PCR analysis was performed to confirm the presence of the respective HPV-11 *L1 NLS*⁻ and CRPV *L1* messenger RNAs. In parallel, total RNA samples were also analysed for the TMV coat protein mRNA (Figures 4.6 **A** and **B**). Amplification of the respective mRNAs was achieved using the internal primer pairs 9, 12 and 13 as listed in table 4.1.

The expression of the HPV-11 *L1 NLS*⁻ gene showed a delayed pattern: the 303 bp transcription product could only be detected from the 4th through the 10th leaf (Figure 4.6 **A**). In contrast, lower levels of expression of the CRPV *L1* gene could be detected in the inoculated through to the 5th leaf, whereafter it was not possible to detect the 421 bp amplicon (Figure 4.6 **B**). Concurrent RT-PCR analysis detected the 474 bp TMV coat protein mRNA in all leaves of infected plants, confirming the systemic spread of TMV from the inoculated through to the apical leaf.

RNA analysis was not done by northern blot as already mentioned in chapters 2 and 3, so it was not possible to show whether the *L1* messenger RNA was degraded. As presented, these results confirm that regions of the *L1* mRNA (HPV-11 L1 nucleotides 593-895 and CRPV nucleotides 433-854) were detectable by RT-PCR.

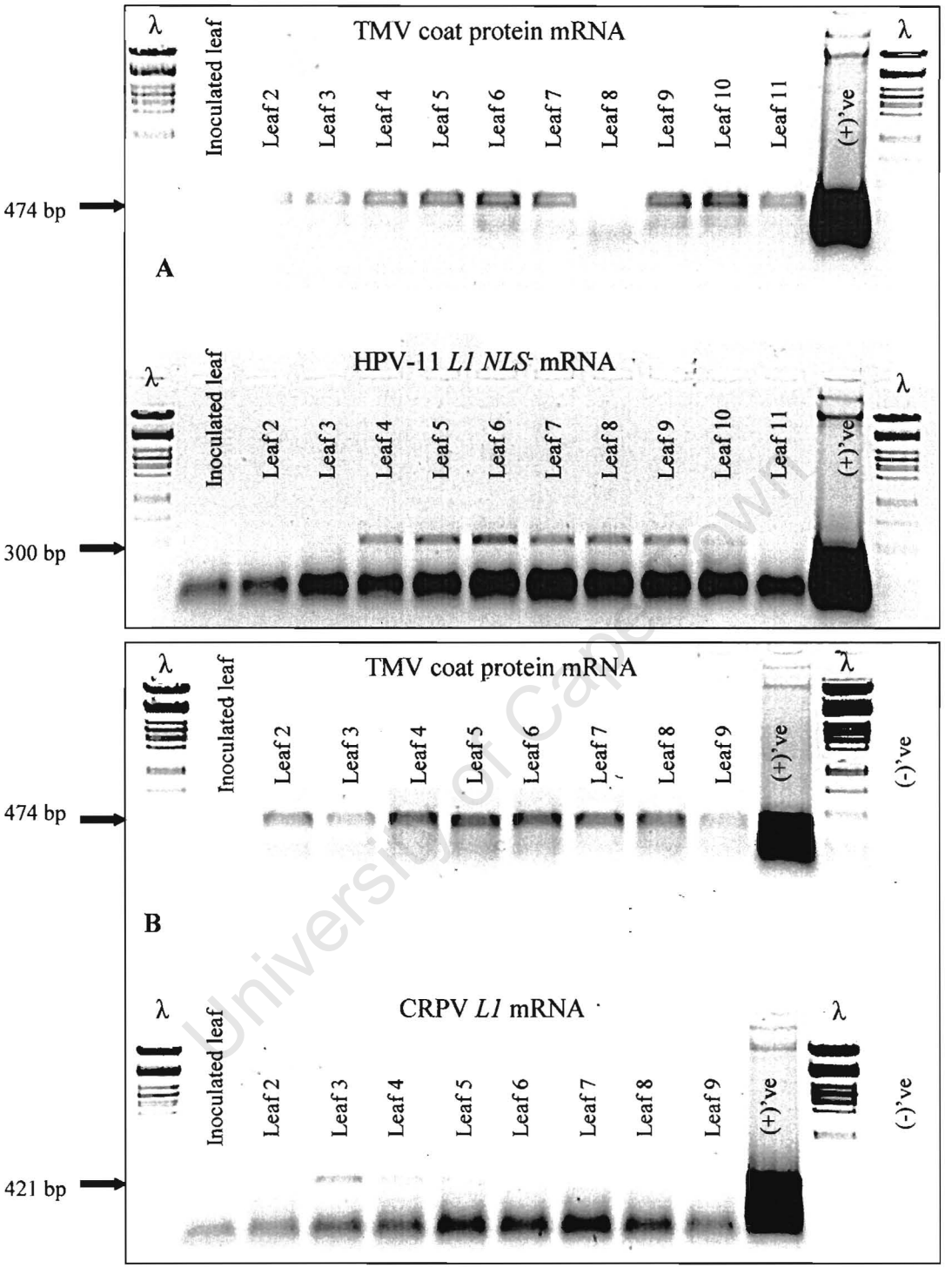


Figure 4.6: RT-PCR RNA analysis of *N. benthamiana* plants 13 days post inoculation (dpi) with TMV-HPV-11 *L1 NLS*- (**A**) and CRPV *L1* (**B**) synthesized transcripts. Delayed expression of the HPV-11 *L1 NLS*- gene (leaves 4 through 10) is indicated by a 303 bp product in **A**, whereas the expression the 421 bp CRPV *L1* cDNA product appears to be terminated after the 6th leaf stage. Total RNA extracted from a non-infected plant represents the negative control, whereas the positive controls are represented by synthesized transcripts prepared form pBSG-HPV-11 *L1 NLS*- and pBSG-CRPV *L1* plasmid DNA.

4.3.3 HPV-11 L1 NLS⁻ and CRPV L1 protein product analysis

Upon confirmation of expression of the HPV-11 *L1 NLS⁻* and CRPV *L1* genes, complete plants were harvested on day 14 and processed according to figure 4.4. The putatively 25 times concentrated protein extract (Number ⑧, Figure 4.4) was analyzed using a panel of MAbs to HPV-6, HPV-11 and CRPV L1s (Figure 4.7 **A** and **B**). Analysis of the TMV-derived HPV-11 L1 NLS⁻ protein extract revealed that the two conformation-specific and HPV-11 neutralising MAbs H11:B2 and H11:H3 bound the plant-derived protein extract with great affinity, displaying an even higher $O.D._{405nm}$ reading than the insect cell-derived HPV-11 VLP spiked positive control (Figure 4.7 **A**). HPV-6 MAbs H6:C6 and H6:I2, recognizing surface-linear epitopes and cross-reacting with HPV-11, also bound the HPV-11 L1 NLS⁻ protein extract, with MAb H6:I2 displaying the greatest binding affinity resulting in an $O.D._{405nm}$ reading higher than the positive control. The inability of the HPV-6 surface linear MAb, H6:E51, to recognize HPV-11 L1 NLS⁻ protein could suggest that the region of this surface linear epitope possibly differs in VLPs derived from plants and insect cells.

Detection of TMV-derived CRPV L1 protein using MAbs CRPV:5A and CRPV:10B was weak in comparison to the detection of the insect cell-derived CRPV VLP spiked positive control (Figure 4.7 **B**). The weak detection of the plant-derived protein could be the direct result of failure of CRPV *L1* gene expression in most of the infected plant (Figure 4.6 **B**) and will be addressed in the discussion.

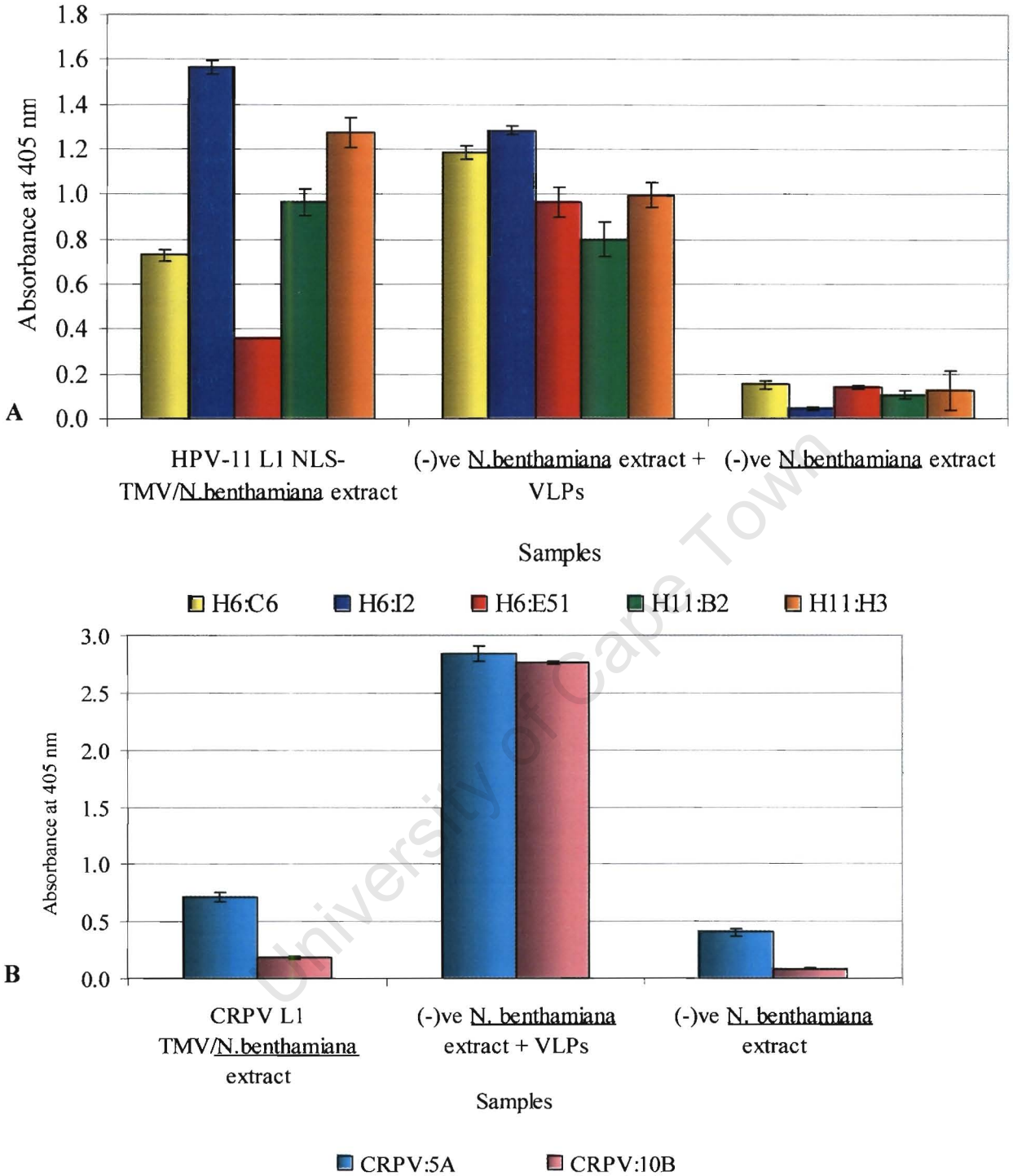


Figure 4.7: Characterization of the TMV transiently expressed HPV-11 L1 NLS$^-$ protein using a panel of MAbs to HPV-6 and HPV-11 (**A**). Two MAbs against CRPV L1 were used to characterize the transiently expressed CRPV L1 protein (**B**). Non-infected *N. benthamiana* protein extract was used as negative control; whereas the positive control consisted of non-infected *N. benthamiana* protein extract spiked with 4µg/ml (0.4µg/100µl) and 1.2µg/ml (0.12µg/100µl) insect cell-derived HPV-11 L1 NLS$^+$ and CRPV L1 VLPs respectively. Error bars represent the standard deviation calculated from triplicate analysis of samples.

4.3.4 Quantification of the transiently expressed HPV-11 L1 NLS⁻ and CRPV L1 proteins

The amounts of transiently expressed HPV-11 L1 NLS⁻ and CRPV L1 protein in plant extracts were measured by comparison to a standard curve of the $O.D._{405nm}$ values plotted against known concentrations of insect cell-derived HPV-11 L1 NLS⁺ and CRPV L1 proteins by ELISA. Non-infected *N. benthamiana* plant protein extract was spiked with insect cell-derived HPV-11 L1 NLS⁺ and CRPV L1 VLPs resulting in known concentrations of 0.4µg and 0.12µg per well (100µl) respectively. These spiked samples represented the respective positive controls and $O.D._{405nm}$ comparisons allowed for the calculation of the total amounts of HPV-11 L1 NLS⁻ and CRPV L1 proteins from respective infected *N. benthamiana* plant material batches of which the total weight and homogenization buffer volume was known (Table 4.2).

Table 4.2: Calculated yields of HPV-11 L1 NLS⁻ and CRPV L1 protein derived from 1 kg of recombinant TMV-infected leaf material

Samples	**Concentration of HPV-11 L1 NLS⁻ and CRPV L1 protein per kilogram of fresh leaf material (mg/kg)**				
	H6:C6	**H6:E51**	**H6:I2**	**H11:B2**	**H11:H3**
N. benthamiana HPV-11 L1 NLS⁻	4.923±0.191	9.747±0.176	**2.969**±0.020	9.639±0.593	**10.190**±0.509
N. benthamiana CRPV L1	**CRPV:5A**		**CRPV:10B**		
	0.603±0.036		**0.159**±0.008		

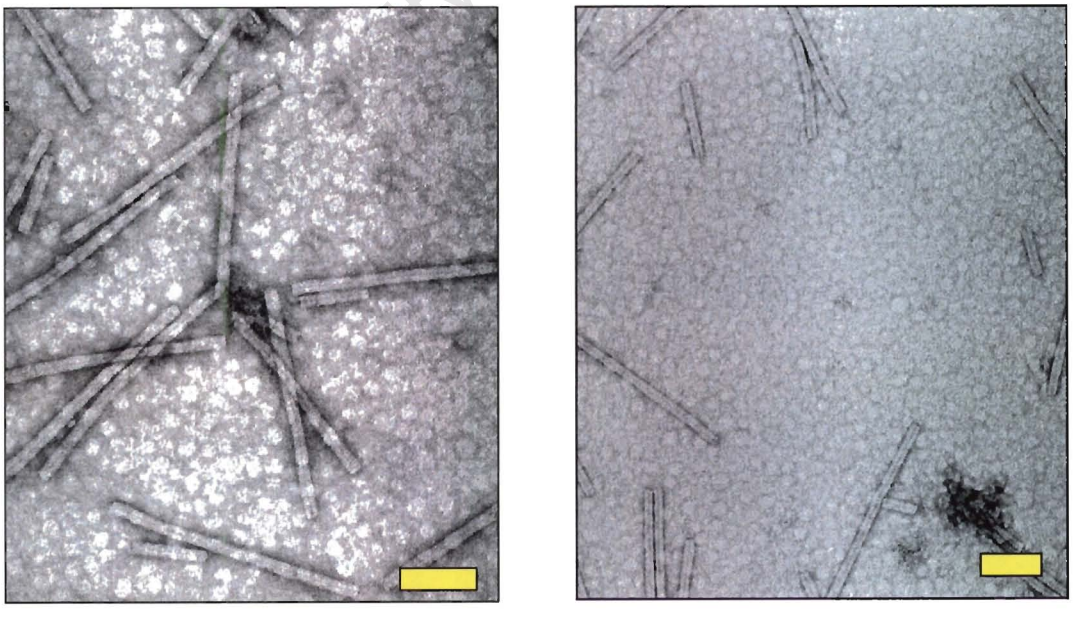
Standard error calculated from triplicate analysis of samples.

The yield of transiently expressed of HPV-11 L1 NLS⁻ protein was calculated to range from between 2.9 to 10.6 mg/kg of fresh leaf material (shown in bold face in Table 4.2), whereas the yield per kilogram of fresh leaf material established for expression of the CRPV L1 protein was only between 0.15 and 0.6mg.

4.3.5 TMV-derived protein extracts: analysis by electron microscopy

TMV-derived protein extracts were immunotrapped using the following MAbs (1:1000 dilution): H6:I2 for trapping HPV-11 L1 NLS$^-$ protein and CRPV:5A for trapping CRPV L1 protein. For immunogold-labelling purposes, grids were coated with respective rabbit polyclonal antiserum (HPV-11 L1 and CRPV L1 pAb at 1:50 dilution) before immunotrapping the respective protein extracts. Immunotrapped HPV-11 L1 NLS$^-$ and CRPV L1 protein was decorated with MAbs H6:I2 (HPV-11 L1 NLS$^-$) and CRPV:5A (CRPV L1) (1:1000 dilution) respectively, before being detected with an anti-mouse-gold-conjugated secondary antibody (1:100 dilution). Two additional grids were prepared by absorbing samples from before (supernatant 1, number ②) and after (supernatant 2, number ④) the 4% polyethylene glycol (PEG) cut (Figure 4.4.), aimed at removing most of the TMV particles in the purification procedure.

Although it was impossible to completely remove all TMV particles from the plant protein extract by precipitation with 4% PEG, markedly fewer TMV particles were observed in supernatant 2 (Figure 4.8 **A** and **B**).



A **B**

Figure 4.8: Electron micrographs showing the difference in recombinant TMV particles before (**A**) and after (**B**) the 4% PEG precipitation step (**Scale bar = 100 nm).**

Examination of the immunotrapped HPV-11 L1 NLS⁻ TMV-derived protein extract revealed a number of uniform particles, displaying an approximately diameter of 50 to 60 nm. However; these particles do not display the typical "doughnut" shape characteristic of insect cell-derived HPV-11 VLPs (Figure 4.9 **A**). Like their transgenic plant-derived counterparts, this suggests that they package foreign nucleic acid to some degree (Figure 2.13, Chapter 2). Immunogold-labeling of the identical protein extracts resulted in the detection of 30 nm gold particles as presented in figure 4.9 B.

In comparison to the above, analysis of the TMV-derived CRPV L1 protein extract by electron microscopy revealed a completely different result. Although figure 4.9 **C** shows the presence of 30nm gold particles, no distinct pentameric aggregates or higher order structures (VLPs) could be identified within the TMV-derived CRPV L1 protein extract upon examination using the CRPV:5A MAb. Parallel analyses of proteins extracted from a non-infected *N. benthamiana* plant revealed no presence of PV-specific structures made from L1 proteins (Figure 4.9 D).

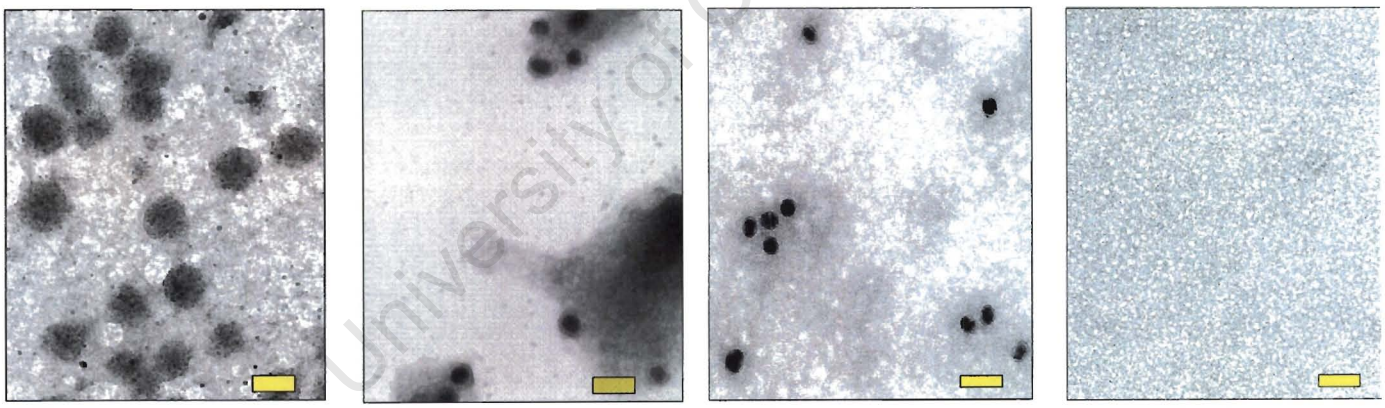
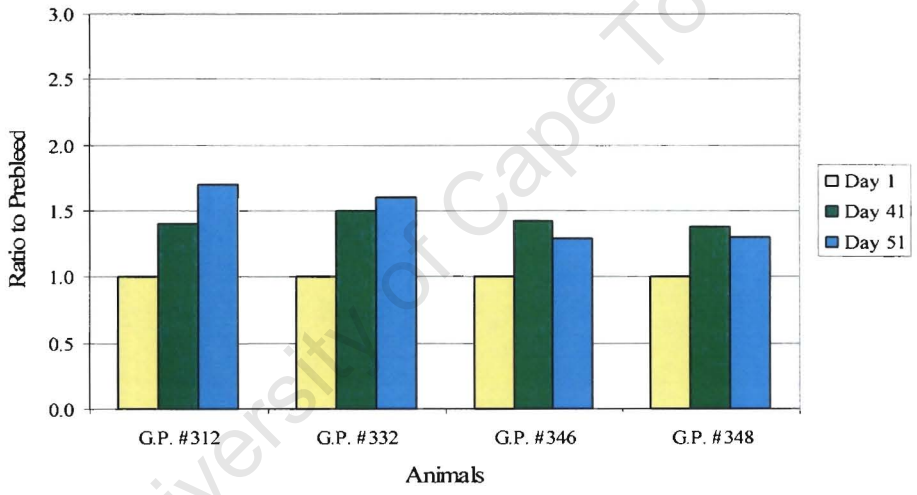


Figure 4.9: (**A**) TMV-produced HPV-11 L1 NLS⁻ VLPs, immunotrapped with monoclonal antibody H6:I2. Particles are on average approximately 60 nm in diameter and appear not to encapsidate nucleic acid, neither do they display the typical "doughnut-shaped" appearance. (**B**) Immunogold-labelling of the TMV-derived protein extract showing the presence of 30 nm gold particles. (**C**) No distinct higher order structures (VLPs) were observed upon examination of the TMV-derived CRPV L1 protein extract, however immunogold-labelling of the protein extract with CRPV MAb 5A shows the presence 30 nm gold particles. (**D**) Immunogold-labeling of non-infected plant protein extract does not show any presence of gold particles. Scale bar = **50 nm** in **A** and **B**, and **100 nm** in **C** and **D**.

4.3.6 Analysis of the immune response of animals immunized with TMV-derived HPV-11 L1 NLS⁻ and CRPV L1 protein extract

In order to determine the antigenicity of the TMV-derived HPV-11 L1 NLS⁻ plant protein extract, a dose calculated to contain between 3.6µg and 13.3µg per 100µl of concentrated HPV-11 L1 NLS⁻ protein was administered intramuscularly to a group of four guinea pigs. Two additional booster inoculations were given on days 23 and 41. Serum samples were collected on days 1, 41 and 51 and evaluated by direct ELISA against insect cell-derived HPV-11 L1 and L1 NLS⁻ VLPs. These results are shown in figure 4.10 **A** and **B** respectively).

A



B

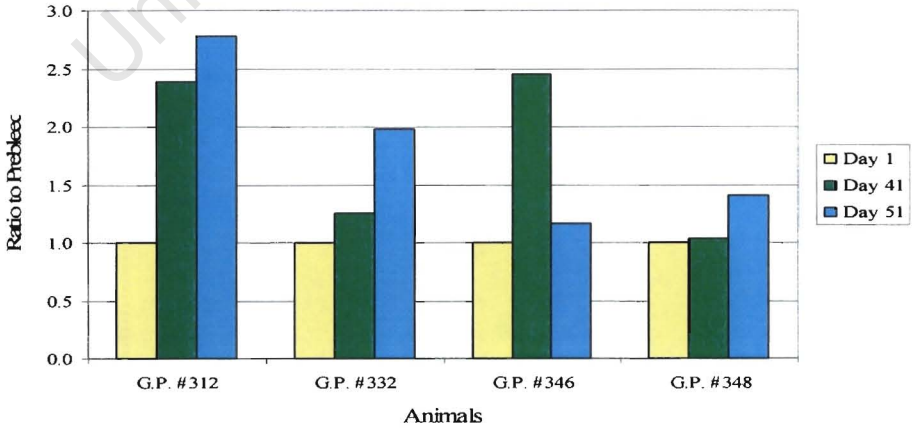


Figure 4.10: Analysis of antisera from TMV-produced HPV-11 L1 NLS⁻ immunized guinea pigs. Antiserum collected on days 1, 41 and 51 was tested by direct ELISA against 0.4µg/well insect cell-derived HPV-11 L1 (**A**) and L1 NLS⁻ (**B**) and is presented in the form of ratio to prebleed.

Results in figure 4.10 **B** indicate that the induced antibody response is primarily targeted against HPV-11 L1 NLS$^-$ VLPs. Guinea pigs #312 and #346 show the strongest reaction against HPV-11 L1 NLS$^-$ VLPs, followed by that of guinea pig #332. Although an upward trend in immune response is projected, none of the antisera collected from all the animals were observed to strongly react against HPV-11 L1 VLPs (Figure 4.10 **A**). Antiserum from guinea pig #348 did not react against HPV-11 L1 NLS$^-$ (**B**) or native VLPs (**A**) The variability of animals in terms of response to a particular antigen could definitely account for this result. The above mentioned results were confirmed when the guinea pig serum was tested by western blotting against insect cell-derived HPV-11 L1 NLS$^-$ and L1 VLPs. Chemiluminescent detection of the anti-guinea pig-horse radish-peroxidase-conjugated secondary antibody with pConmaric Acid and 5-Amino-2,3 Dihydro-1,4-Phtahalazinedione showed the detection of HPV-11 L1 NLS$^-$ protein, but not the native protein.

Results obtained from the animal experiments in this chapter are very similar to those in Chapter 2. Although TMV-derived HPV-11 L1 NLS$^-$ VLPs appear more uniform and not as pleomorphic as transgenic plant-derived particles when examined by electron microscopy, they induce a similar pattern in antibody response once administered to animals. This response is again primarily targeted at HPV-11 L1 NLS$^-$ VLPs, as was also observed for rabbits immunized with transgenic plant-derived HPV-11 L1 NLS$^-$ protein. Hoping to obtain some clarification as to why this distinct antibody-antigen recognition pattern exists, I immunized two groups of four guinea pigs with insect cell-derived HPV-11 L1 NLS$^-$ and native VLPs. Each animal in a group was immunized with 20µg of VLPs, and was given two further booster inoculations of 20µg before all serum was collected on day 42. The answer to the question whether this antibody response pattern would repeat itself upon administration of the insect cell-derived VLPs was of utmost interest. All sera obtained from guinea pigs were analyzed against non-denatured and denatured HPV-11 L1 NLS$^-$/native VLPs by direct ELISA. The results are shown in figures 4.11 **a** and **b**.

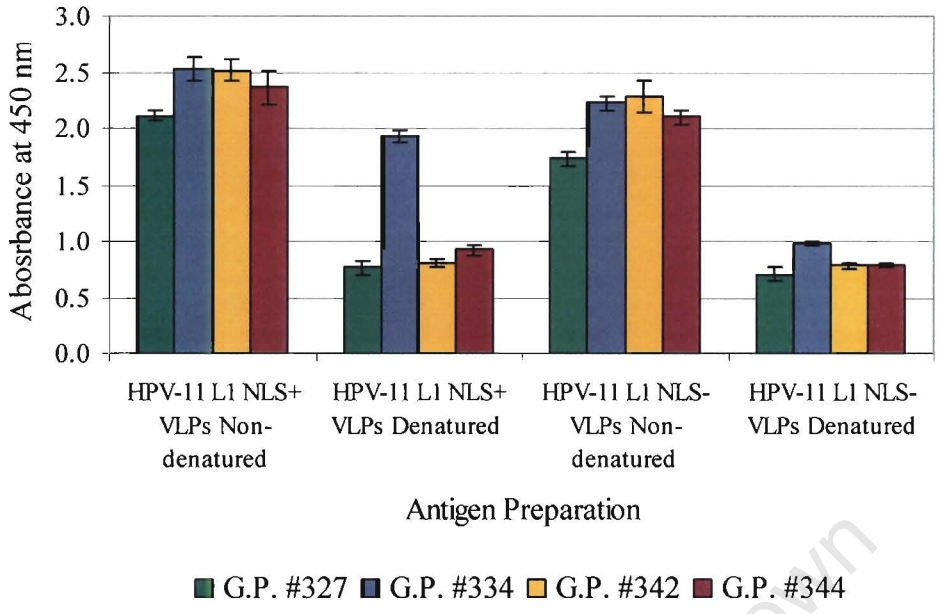


Figure 4.11a: Analysis of sera from insect cell-derived **HPV-11 L1 NLS$^+$ VLP** immunized guinea pigs. Serum was tested against non-denatured and denatured insect cell-derived HPV-11 L1 NLS$^-$ and native VLPs at a concentration of 0.4µg/100µl. Error bars represent the standard deviation calculated from triplicate analysis of samples.

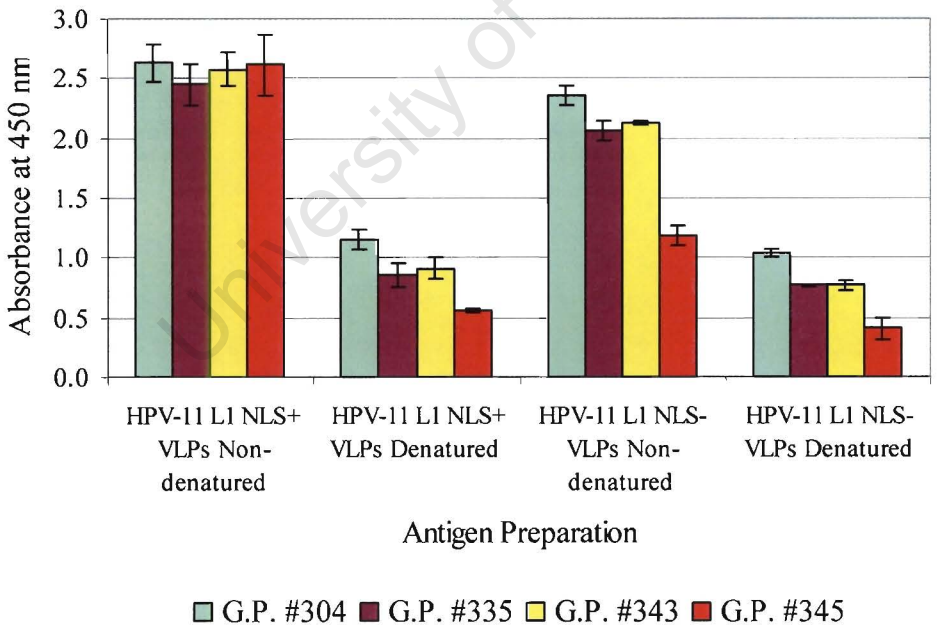


Figure 4.11b: Analysis of sera from insect cell-derived **HPV-11 L1 NLS$^+$ VLP** immunized guinea pigs. Serum was tested against non-denatured and denatured insect cell-derived HPV-11 L1 NLS$^-$ and NLS$^+$ VLPs at a concentration of 0.4µg/100µl. Error bars represent the standard deviation calculated from triplicate analysis of samples.

The results indicate that sera collected from both groups of guinea pigs do not preferentially react with either HPV-11 L1 NLS$^-$ or native VLPs. Unlike the observed pattern of serum reactivity from animals injected with either transgenic- or TMV-derived HPV-11 L1 NLS$^-$ antigen (Figures 2.15 and 2.16 **a** and **b**, Chapter 2; Figure 4.10 **A** and **B**), no apparent differences in reactivity were observed in the detection pattern of serum collected from the guinea pigs (Figures 4.11 **a** and **b**). As expected, sera reacted more strongly with non-denatured insect cell-derived HPV-11 VLPs than with denatured protein. This suggests that both plant-produced HPV-11 L1 proteins are subject to modifications within their respective hosts, changes that bring about the noticeable difference in antibody response. As to what protein modifications are possibly involved, this shall be addressed in the discussion.

The production of CRPV L1 protein in plants using the recombinant TMV vector was complicated by several issues, the first being the final vector stability. RT-PCR analysis confirmed that the CRPV *L1* mRNA could no longer be detected in the sixth leaf of infected plants. This could be attributed to reversion of the vector to its wild type state. Without the presence of the *L1* gene throughout the entire plant, one cannot expect a high protein yield. The formation of CRPV L1 VLPs was not confirmed and is probably directly linked to the low concentration of the L1 protein in the cell (see Chapter 2). Nevertheless, transiently expressed CRPV L1 protein extract in the most concentrated form was injected into a group of three New Zealand white rabbits. Serum was collected on days 1, 23, 41 and 51 and analyzed by direct ELISA against CRPV L1 insect cell-derived VLPs at a concentration of 0.12µg/well. Results are shown in figure 4.12.

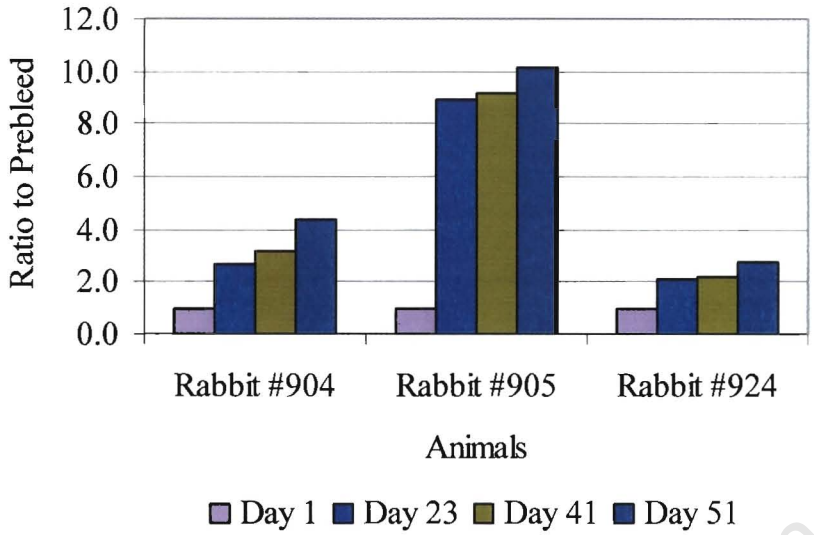


Figure 4.12: Analysis of sera collected from rabbits inoculated with transiently-derived CRPV L1 protein extract. Serum was collected on days 1, 23, 41 and 51 and tested by direct ELISA against insect cell-derived CRPV L1 VLPs at a concentration of 0.12µg/well.

Analytical results for the serum collected from rabbits inoculated with TMV-derived CRPV L1 protein were not as expected. The initial and follow-up dosages of concentrated CRPV L1 protein injected into the rabbits were respectively 9 to 18 times lower than the HPV-11 L1 NLS$^-$ dosages (calculated per 500µl of inoculum) given to the guinea pigs, yet figure 4.12 shows the induction of distinct antibody responses. Although rabbit antiserum was pre-absorbed as described in section 4.2.9, rabbit #905 showed a last bleed antiserum (day 51) reaction of 10 times higher compared to the reaction of the prebleed (day 1) serum. Although not to the same order of magnitude, a similar trend in increasing reaction levels was observed for the serum collected from rabbits #904 and #924 respectively. The induction of an immune response is dosage dependent and since the inoculum consisted of low amounts of transiently-derived CRPV L1 protein, it remains unclear as to why such highly elevated response levels were detected.

I evaluated the same pre-absorbed sera (1/20 dilution) by western blot. Figure 4.13 indicates that denatured insect cell-derived CRPV L1 protein could be detected by the last bleed (Day 51) in all cases whereas no detection is achieved by the prebleed (Day 1) serum as anticipated.

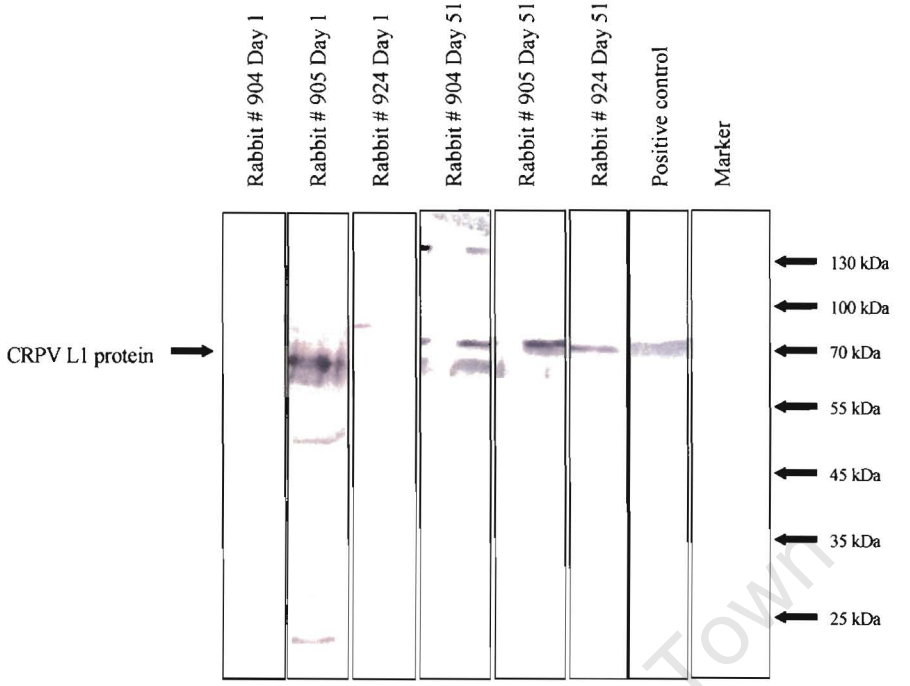


Figure 4.13: Analysis of serum collected from New Zealand white rabbits immunized with TMV-derived CRPV L1 protein extract. Serum was tested against denatured insect cell-derived CRPV L1 protein at a final concentration of 2μg per lane. Day 1 represents the prebleed serum, whereas Day 51 represents the last bleed serum. The presence of a band in the last three lanes indicates the detection of CRPV L1 protein by the day 51 serum collected from individual rabbits. The presence of two bands of identical size in 1 lane is due to the incorrect cutting of the membrane after transfer. Although all sera were pre-absorbed, insect cell debris and residual baculovirus proteins are still detected by some of the prebleed sera (rabbit #905 and #924). Detection of denatured insect cell-derived CRPV L1 protein using MAb CRPV:10B is represented as the positive control.

Given the results of chapters 2 and 3, in which I found that the antibodies induced after vaccination with transgenic plant-derived HPV-11 L1 NLS$^-$ protein do not induce virus neutralising antibodies (Chapter 2) and that the CRPV pseudovirus neutralisation capability is possibly induced through application of the actual virus during the challenge experiment (Chapter 3), I proceeded to have all sera collected from animals in this study evaluated in the *in vitro* neutralisation assay. Thanks to the help of my colleague Debbie Stewart, these results are presented in the next section.

4.3.7 *In vitro* HPV-11 L1 neutralization assay.

Results obtained form the evaluation of the pre-inoculation serum of guinea pigs were as expected: sera collected from naïve guinea pigs did not neutralise the HPV-11 pseudovirus. These results are presented in figure 4.14 A, while neutralisation results for post-inoculation immune sera are shown in figure 4.14 B.

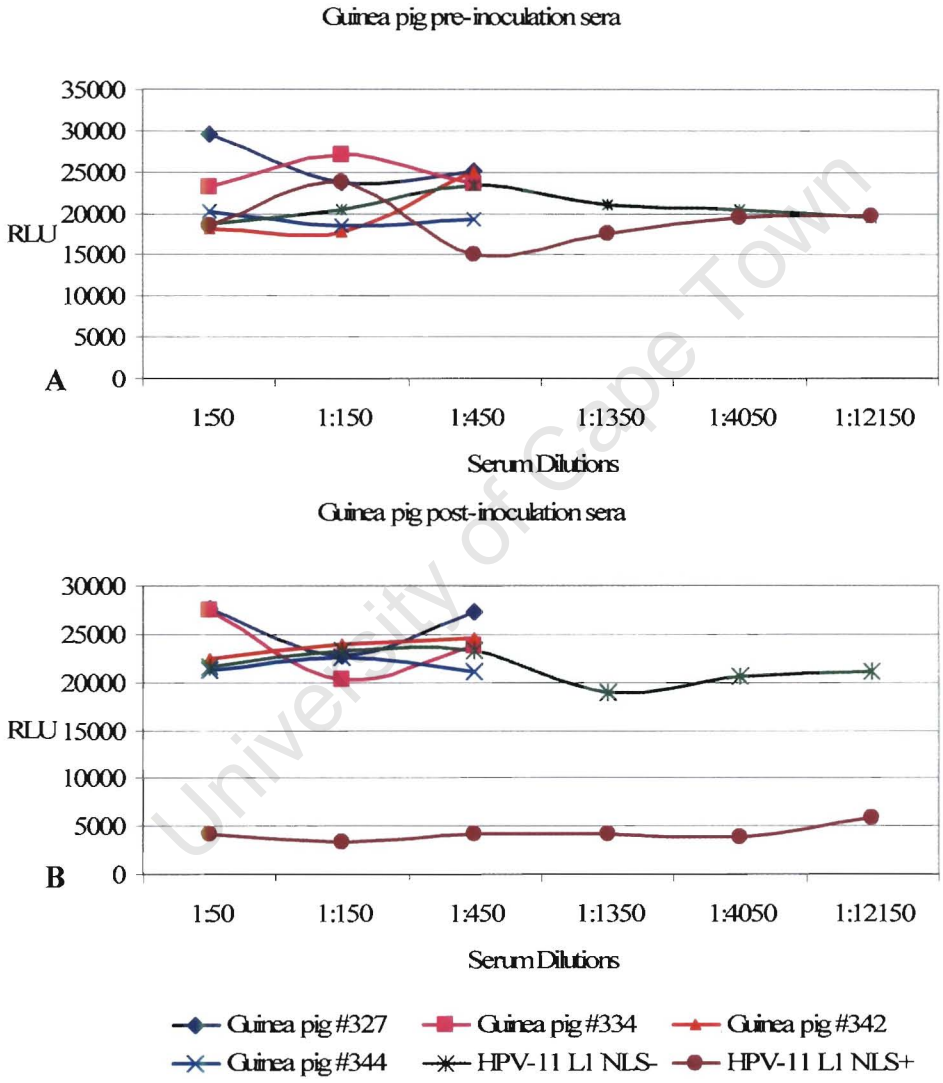


Figure 4.14: HPV-11 pseudovirus neutralising assay using the pre-inoculation serum (Day 1) collected from naïve guinea pigs before immunization with the transiently expressed plant-derived HPV-11 L1 NLS^- protein extract (**A**). The evaluation of the post-inoculation sera (Day 51) from all animals is shown in **B**. Included in the data set is the serum collected from one of the guinea pigs that was vaccinated with insect cell-derived HPV-11 L1 NLS^- VLPs (NLS^-) as well as the serum from one of the guinea pigs inoculated with insect cell-derived HPV-11 L1 VLPs (NLS^+). The data is presented as relative light units (RLU) observed at various dilutions of the tested serum.

The results presented in figure 4.14 B are disappointing. As was also shown in the neutralisation experiments conducted in chapter 2, the antisera collected from guinea pigs on day 51 of this study all failed to neutralise the HPV-11 pseudovirus – with native L1 protein – *in vitro*. Knowing that the antibody response is again predominantly targeted against the L1 NLS$^-$ version of the protein (Figure 4.10), the question still remains as to what exactly causes that.

Results of greater concern are also reflected in figure 4.14. Included in these HPV-11 pseudovirus neutralisation assays were sera collected from guinea pigs that were immunized with either insect cell-derived HPV-11 L1 NLS$^-$ or native VLPs. As expected, analysis of the pre-inoculation serum from both animals does not indicate any ability to neutralise the pseudovirus (Figure 4.14 **A**). However, figure 4.14 **B** also indicates something completely unexpected. The serum collected on the last day from a guinea pig vaccinated with HPV-11 L1 VLPs is capable of neutralising the HPV-11 pseudovirus *in vitro*, however, the serum collected from an animal that was vaccinated with HPV-11 L1 NLS$^-$ VLPs, is not. These neutralisation experiments have since been repeated and have resulted in identical findings.

4.3.8 Animal challenge and the CRPV pseudovirus neutralization assay

Similar to the experiments described in chapter 3, three transiently expressed TMV-derived CRPV L1 protein inoculated rabbits were challenged with infectious virus.

Immunized rabbits were challenged with two dosages of infectious virus as described in section 3.2.11 of chapter 3. Papillomas were observed and measured 14 dpi over a period of 63 days. Results obtained from all animals were combined and are presented in figure 4.15.

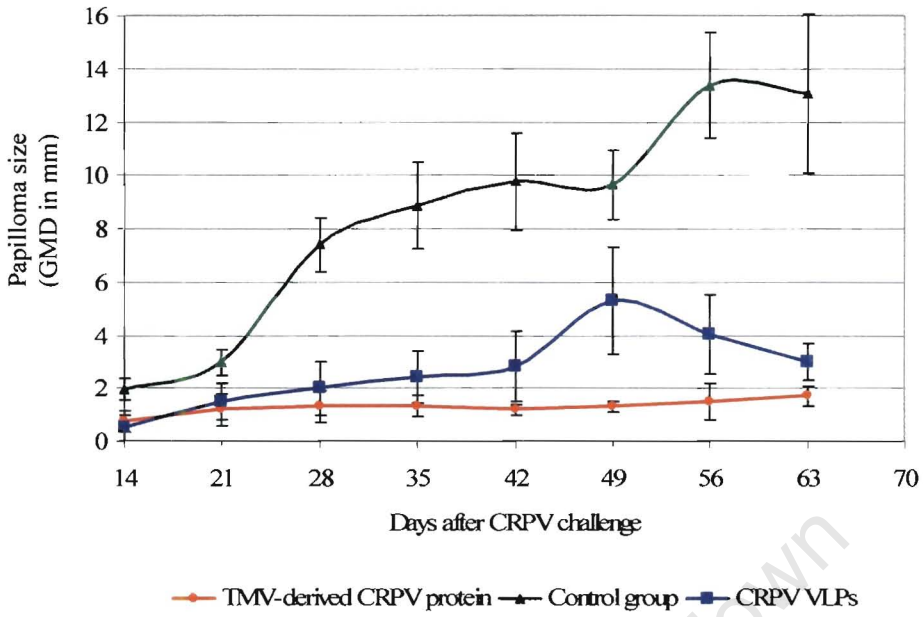


Figure 4.15: Papilloma growth on the backs of TMV-derived CRPV L1 immunized rabbits following challenge with infectious CRPV. Rabbits were challenged with 10-fold dilutions of infectious CRPV (two sites per dilution). Papilloma sizes were measured weekly beginning on day 14 and the geometric mean diameter (GMDs) calculated. The GMDs and standard error of measurement (SEM) of papillomas were plotted against time for the sites challenged with 10^{-2} dilution of infectious CRPV.

The data presented in figure 4.15 clearly reflects a similar pattern to that observed after challenge of the transgenic plant-derived immunized rabbits (Figure 3.9, Chapter 3). Again it indicates that vaccination of rabbits with transiently plant-expressed CRPV L1 protein appears to protect from challenge with infectious CRPV, and that at the higher dosage (10^{-2} dilution) of virus stock. Although these results indicate that over the period of 63 days the geometric mean diameter of papillomas in the CRPV L1 protein immunized animals is just below 2mm, these results are a combination of all measurements taken from all rabbits. On an individual level, rabbit #905 only developed papillomas on day 35 of this experiment. Measurements taken on day 35 showed papillomas of 0.5 mm in diameter. On the final day (Day 63), this diameter had increased to approximately 1 mm. In contrast the remaining two challenged rabbits (rabbit #904 and rabbit #924) had already developed papillomas on day 14. Measurements showed that the diameter of papillomas on the backs of rabbits #904 and #924 was around 0.8

mm and 1.45 mm respectively. Over the period of 63 days these papillomas grew in size, resulting in measurements of 2 mm and 2.1 mm for rabbits #904 and #924 respectively.

In comparison to the control in which papillomas were measured to be an average of 13 mm in diameter on day 63, these results are quite impressive. In order to determine whether generation of virus neutralising antibodies resulted from vaccination with TMV-derived CRPV L1 protein, we evaluated the serum antibodies collected from all TMV-derived CRPV L1 vaccinated animals in the *in vitro* CRPV pseudovirus neutralisation assay.

Serum collected from all animals was tested for the ability to neutralise CRPV pseudoviruses *in vitro*. These results are presented in figure 4.16. Figure 4.16 **A** specifically evaluates the serum collected from naïve rabbit before vaccination. As expected, the absence of type-specific antibodies in the serum results in the inability of the serum to neutralise the CRPV pseudovirus. However, no neutralisation of the pseudovirus was observed in the post-inoculation serum (Day 51) harvested from all animals either (Figure 4.16 B).

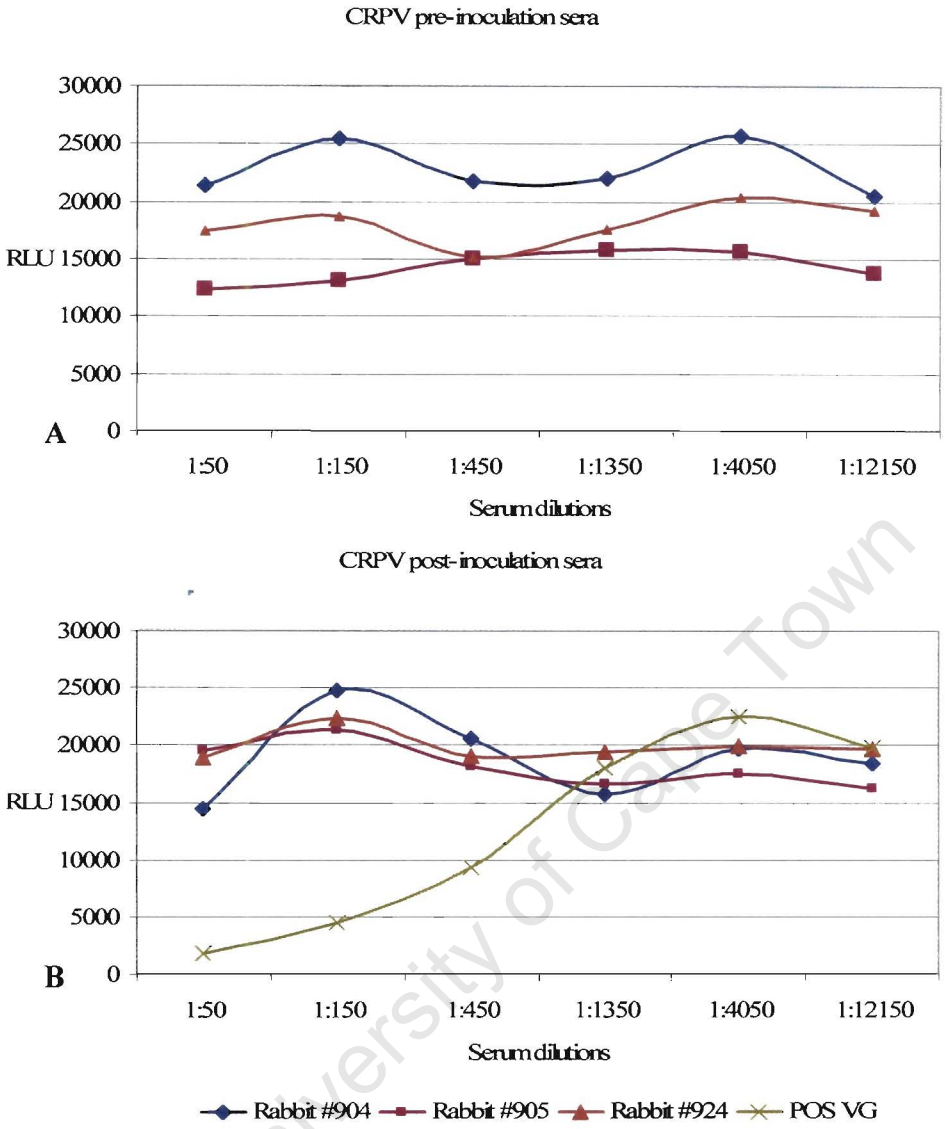


Figure 4.16: CRPV pseudovirus neutralising assay using the pre-inoculation serum (Day 1) collected from 3 rabbits immunized with the transiently expressed TMV-derived CRPV L1 protein (**A**). The evaluation of the post-inoculation sera (Day 51) from all animals is shown in **B**. Included in the data set is the anti CRPV serum provided by Dr Vandana Govan (pos GV). The data is presented as relative light units (RLU) observed at various dilutions of the tested serum. Neutralisation assay results obtained from incubation of CRPV pseudoviruses with known conformation-specific and neutralising MAb CRPV:5A is shown in **C**. Data is presented as a collection from six individual experiments

As already described in Chapter 3, the post-inoculation serum collected from rabbits before challenge with infectious virus is not capable of neutralising the CRPV pseudovirus; whereas antiserum against insect cell-derived CRPV L1 VLPs was neutralising.

4.4 DISCUSSION

This chapter investigated the use of *N. benthamiana* plants for the transient expression of the HPV-11 L1 NLS$^-$ and CRPV L1 proteins using the TMV-based plant virus vector pBSG1057. Various virus-based transient expression system allow for the synthesis of large amounts of antigen within a short period of time. Given the not so satisfactory yields obtained from the expression of the above mentioned genes in transgenic plants (Chapters 2 and 3), the overall aim of this study was to produce more quantity of protein without loss of quality. However, this virus-based expression system does have a disadvantage: The expression of a foreign gene inserted into a virus-based vector remains the most crucial aspect. In order to obtain the highest yield possible, the gene needs to be stably expressed; this however is not always guaranteed.

In a series of consecutive infections of *Nicotiana benthamiana* plants with a TMV-based vector expressing the *30B-GFP3C* mutant gene, Rabindran & Dawson (2001) have shown the appearance of a diffuse pattern of fluorescence after the second passage, indicating that sequences were being deleted from the recombinant virus thus resulting in the loss of GFP expression. RNA analysis of these individual plants revealed an additional RNA species, smaller than the *30B-GFP* mRNA. Donson *et al.* (1991) implicated the size of the inserted gene as being the direct cause of vector instability.

Given that the size difference between the *30B-GFP* and the HPV-11 *L1 NLS*$^-$ and CRPV *L1* genes is approximately 800 bp, a similar loss of gene expression appears to emerge in attempts to express the native, non-codon-optimized CRPV *L1* gene in *N. benthamiana* plants using the TMV-based vector (Figure 4.3). However, since both genes are of similar size, why does the expression of the HPV-11 *L1 NLS*$^-$ gene remain unaffected? Upon analysis of gene transcription by RT-PCR, I have not shown the presence of an additional RNA species as reported by Rabindran & Dawson (2001), but rather revealed that the CRPV *L1* mRNA is absent from leaves 6 through 9. Although expressed in the first five leaves, concurrent RT-PCR analysis detected the TMV coat protein mRNA in leaves 1 (inoculated) through 9, thus suggesting that the CRPV *L1* gene must have been wholly or

partially excised and that the recombinant virus had essentially reverted back to wild-type. This phenomenon had previously been observed in attempts to express the HPV-16 *L1* gene using the TMV-based expression vector (personal communication with Dr Arvind Varsani). The causes of the CRPV *L1* gene instability have not been investigated. Such an investigation could have potentially identified certain regions of the gene that were repeatedly deleted or appeared to be particularly unstable. Investigations such as these could have furthered our understanding and help in resolving the gene instability issue, and remain a potential project for others.

I observed a repeated pattern of loss of CRPV L1 expression in a number of individual experiments attempting to express the CRPV *L1* gene. Besides the absence of the CRPV *L1* mRNA, a distinct difference in the development of infectious symptoms was observed throughout the growth of infected plants. Recombinant plant viruses are said to induce milder and more delayed symptoms (Rabindran *et al.*, 2001), symptoms including the curling of leaves and the familiar mosaic pattern. Nemichov *et al.* (2000) has described these symptoms to occur 14 to 21 days post inoculation (dpi) with recombinant TMV expressing the hypervariable region 1 (HVR1), a potentially neutralising Hepatitis C virus (HCV) epitope. These classical symptoms can be observed 14 dpi of *N. benthamiana* plants with the TMV-based vector expressing the HPV-11 *L1 NLS*$^{-}$ or CRPV *L1* genes (Figure 4.5 **A** and **B**). However, reversion to the wild-type virus classically displays all of the above mentioned symptoms at greater severity within a shorter time period.

The probable deletion of the CRPV *L1* gene (Figure 4.6 **B**) from the TMV-based vector stands in contrast to the stable expression of the gene reported in Chapter 3. Expressed in transgenic plants, this native non-codon-optimized CRPV *L1* gene is not subjected to deletion.

In contrast to the CRPV results, expression of the HPV-11 *L1 NLS*$^{-}$ gene in *N. benthamiana* showed a delayed pattern in gene expression (not expressed in the inoculated leaf through to 3rd leaf) thus only detecting stable expression in leaves 4

through 10 (Figure 4.6 **A**), and no apparent loss of expression. The ability of the recombinant TMV to replicate and spread systemically has been confirmed by detection of the TMV coat protein mRNA in leaves 1 through 11. The question as to why a delayed pattern in HPV-11 *L1 NLS*$^{-}$ gene expression is observed remains unanswered, as does the difference in stability of expression compared to CRPV.

The substantial difference in protein yield is directly attributable to the gene expression or lack thereof. The direct ELISA analysis of the CRPV L1 plant protein extract (Figure 4.7 **B**) reflects the effects of gene deletion. Expression of the CRPV *L1* gene in *N. benthamiana* yields approximately 159 to 603µg of CRPV L1 protein per kilogram of fresh leaf material. Not only is this substantially less than harvested from expression of the same gene in transgenic plants, but it is also not comparable to yields of various other antigens expressed in plants via different plant virus vectors. Although thus far plant virus vectors – including derivatives of Alfalfa -, Cowpea -, and Tobacco mosaic virus; Plum pox virus, Potato virus X and Tomato bushy stunt virus (see Table 1.5. Chapter 1) – have mainly been used for the expression of viral epitopes, some reports focus on the expression of complete proteins.

Fernández-Fernández *et al.* (2001) have reported on the stable expression of the structural protein VP60 from Rabbit haemorrhagic disease virus in Nicotiana clevelandii using a Plum pox virus (PPV)-based vector. A Potato virus X (PVX)-based vector has been used to express the rotavirus VP6 protein in *N. benthamiana*; rotavirus being the causative agent of gastroenteritis in children (O'Brien *et al.* 2000). Expression of the free VP6 protein led to the assembly of para-crystalline sheets, whereas a PVX coat protein fusion not only resulted in flexuous rods, but also in icosahedral VLPs. Overall total yields of 50 mg of VP6 protein per kilogram of fresh leaf material were harvested using this PVX-based vector. Yet another report describes the expression of a cholera toxin/Hepatitis C virus fusion protein (CTB/HVR1) vaccine using a recombinant TMV vector (Nemchinov *et al.*, 2000b). Although the calculated yield is highly variable, explained by the authors to be a result of different protein accumulation in randomly sampled leaves, it has been determined to amount to between 6 and 80 mg per kilogram of fresh leaf material.

Unlike the reported assembly of the VP6 protein, but similar to what happened with transgenic plants, the TMV-derived CRPV L1 protein does not assemble into VLPs. This is most probably due, as was speculated in the first case, to the protein expression levels being too low to drive the equilibrium towards the assembly of VLPs. The lower expression level than seen for the transgenics was almost certainly due to loss of the CRPV *L1* gene from the vector. The susceptibility of foreign genes to eventually be deleted from a variety of different vectors has been described by various research groups (Choi *et al.* 2000; German-Retana *et al.* 2000; Guo *et al.* 1998). It is possible that the time allowed for replication and spread of the recombinant TMV virus – fourteen days – was too long. Even at initial low expression levels, the translated protein should have accumulated and assembled into pentameric or possibly higher order structures (VLPs), but as was found with the expression of HPV-11 L1 NLS$^-$ protein in transgenic tobacco, this might also have been subject to rapid degradation by plant trypsin-like proteases. There was no evidence of this, however, in western blot analysis in western blot analysis of protein produced via recombinant TMV. It is most likely that the assembly of CRPV L1 pentamers and/or VLPs might initially require a much higher concentration of L1 monomers than is the case for HPV-11.

The failure to increase expression of the CRPV *L1* gene using the TMV plant virus vector does not negate the results achieved by expression of the native HPV-11 *L1 NLS*$^-$ gene. This particular gene has not been codon-optimized, yet its insertion into the virus vector appears to be stable. Gene transcription throughout all leaves of infected plants resulted in assembly of uniform particles, displaying an average size between 50 and 60 nm in diameter (Figure 4.9**A**). This was unlike their pleomorphic transgenic *N. tabacum* or *A. thaliana* plant expressed counterparts, which ranged in size from 20 to 60 nm in diameter. This could reflect a difference between expression and/or assembly between *N. benthamiana* and *N. tabacum* in particular, where L1 protein was extensively degraded.

Through use of the TMV-based vector, a total yield ranging from 3 to 10 mg of HPV-11 L1 NLS$^-$ protein was harvested from one kilogram of fresh plant material. Although this yield falls within the bottom range of the reported highly variable 6 to 80 mg per

kilogram of TMV-derived CTB/HVR1 fusion protein (Nemchinov *et al.*, 2000), it is approximately 4 to 14 times more than the yield achieved by expression of the same protein in transgenic *N. tabacum* plants. Thus, the overall aim of synthesizing more HPV-11 L1 NLS$^-$ protein had been fulfilled; however the quality of the protein remained to be determined.

Direct ELISA results showed that a panel of MAbs against HPV-6, 11 and 16 were able to detect the TMV-derived HPV-11 L1 NLS$^-$ protein (Figure 4.7 A). HPV-11 conformation-specific and neutralising MAbs H11:H3 and H11:B2 were able to bind the plant-derived protein with great affinity, more so in fact than in the insect cell-derived HPV-11 L1 NLS$^-$ VLP spiked positive control. This pattern of HPV-11 L1 NLS$^-$ protein recognition is different to the one observed in figure 2.8 of chapter 2, in which the MAb H6:I2 rather than the two conformation-specific MAbs predominantly detected the transgenic *N. tabacum*-produced HPV-11 L1 NLS$^-$ protein. This was a significant observation suggesting that TMV-derived particles detected by these conformation-specific and neutralising MAbs might also be able to induce a virus neutralising immune response once administered to animals. However, cautioned by the information gathered from the evaluation of the *A. thaliana*-derived HPV-11 L1 NLS$^-$ protein extract which initially displayed similar antigenic properties, but failed to induce virus neutralising antibodies in rabbits, I proceeded to test the TMV-produced HPV-11 L1 NLS$^-$ protein in guinea pigs.

Respective immunization of guinea pigs and New Zealand white rabbits with the TMV-derived HPV-11 L1 NLS$^-$ and CRPV L1 proteins resulted in interesting findings. The injection of guinea pigs with 3.6 to 13.25μg of HPV-11 L1 NLS$^-$ protein resulted in an antibody response in three out of four guinea pigs (Figure 4.10**B**). As was seen in Chapter 2, type-specific antibody reactivity was only observed when sera were tested against insect cell-derived HPV-11 L1 NLS$^-$ and not native VLPs. Although dosages calculated to contain more HPV-11 L1 NLS$^-$ protein were administered to determine the properties of the transgenic plant-derived antigen (up to 47μg per inoculum), it is a common finding that the inoculation of animals with HPV-11 L1 NLS$^-$ protein extract, whether produced

by means of transgenic or transient expression, results in an antibody response that primarily reacts with insect cell-derived HPV-11 L1 NLS$^-$ and not native HPV-11 L1 VLPs.

In experiments aimed at determining the antigenic properties of the TMV-derived CRPV L1 protein, I have observed the following. A high immune response was observed in rabbits after immunization with only 2 to 8µg of TMV-derived CRPV L1 protein. Additional booster inoculations containing only half the amount of CRPV L1 protein were administered, yet rabbit #905 showed the highest antibody response of all animals evaluated in this particular study (Figure 4.12). Displaying a ratio of prebleed to last bleed serum reactivity of almost 10 times, it surpasses even the 4 and almost 3 times higher reactivity levels of the remaining two rabbits #904 and #924 injected with the identical antigen. It is an interesting question as to how so little CRPV L1 protein, compared to the much higher HPV-11 L1 NLS$^-$ antigen concentration, could induce an immune response of this magnitude. It is possible that the strong response to CRPV L1 in rabbits was the result of boosting a weak pre-existing immunity, to CRPV or a related virus – however, no evidence has been found for this.

With the help of my colleagues, we determined whether the induced antibody response in rabbits resulting from inoculation with TMV-derived CRPV L1 protein is capable of neutralising the virus upon challenge. Results showed that animals were effectively protected, meaning that the average size of papillomas formed after challenge was approximately 6 times smaller than in the control group (Figure 4.15). The same observation had been made after challenge of transgenic plant-derived CRPV L1 protein vaccinated rabbits in chapter 3.

Independent researchers have evaluated various other plant virus vector-derived antigens. For example, Fernández-Fernández *et al.* (2001) have injected rabbits with 1ml of PVX-derived VP6 protein. Although they did not mention the dosage, they showed that rabbits were completely protected from lethal challenge with Rabbit haemorrhagic disease virus after having been vaccinated twice.

Langeveld *et al.* (2001) administered an UV-inactivated recombinant Cowpea mosaic virus (CPMV) displaying the canine parvovirus VP2 capsid protein epitope (17 amino acids) fused to the coat protein and displayed on the surface of the virus particle to disease-free beagles. Approximately 150μg of displayed VP2 epitope was calculated to be contained in 7.5 mg of recombinant CPMV. Dogs were injected subcutaneously with this formulation on two occasions (days 0 and 28) before being challenged with canine parvovirus on day 42. Vaccinated dogs developed high titre epitope-specific antibodies, capable of detecting the complete parvovirus VP2 protein. Complete protection from lethal challenge associated with no appearance of clinical symptoms, allowed the conclusion that recombinant CPMV was as an effective carrier of foreign viral epitopes.

Analysis of serum collected from TMV-derived CRPV L1 injected rabbits showed that neither serum collected from naïve rabbits (day 1) nor serum collected on day 51 were capable of neutralising the virus *in vitro* (Figure 4.16). This repeated the discoveries described in chapter 3: the overall conclusion to be drawn from this is that protection from virus challenge is probably due to a cell-mediated response, contrary to popular doctrine. Although the presence of virus neutralising antibodies cannot be entirely ruled out, the CRPV animal model might not be appropriate because the infection is non-productive in domesticated rabbits and lesions do not produce much (if any) infectious virus.

We also analyzed all sera collected from animals vaccinated with the TMV-derived HPV-11 L1 NLS^- protein. Although assembled VLPs resulting from TMV expression of the HPV-11 *L1* NLS^- gene were uniform in size and were detected by all the HPV-11 conformation-specific and neutralising MAbs, they failed to induce neutralising antibody responses in animals (Figure 4.14). While this repeats the results of Chapter 2, I note that in contrast to the failure of conformation-specific and neutralising MAbs in that work to recognize the transgenic tobacco-derived protein, VLPs produced in *N. benthamiana* via TMV were recognised, and were obviously less degraded and more similar to protein made in transgenic *A. thaliana*. Furthermore, the differences in VLPs are distinct: transgenic plant-derived VLPs are pleomorphic whereas TMV-produced VLPs are more

uniform in size, possibly suggesting that the original conformation of the virus capsid is preserved in the TMV-derived VLPs. Not only is the assembly of VLPs concentration-dependent, but although not shown in this chapter, the HPV-11 L1 monomer is severely degraded after synthesis in *N. tabacum* plants. Overall one question remains to be answered. What causes the changes in the plant-synthesized HPV-11 L1 NLS$^-$ VLPs that bring about the changes seen here? The inability to induce a virus neutralising antibody response brings any potential vaccine developmental efforts to a halt. While I have achieved the overall goal of this particular study, that is to produce more quantity of antigen that could potentially be used for HPV-11 vaccination purposes, what good is more of something if the quality is not preserved and it does not serve its purpose?

Another fundamental discovery made throughout the HPV-11 VLP evaluation process was that serum collected from guinea pigs inoculated with either insect cell-derived HPV-11 L1 NLS$^-$ or native VLPs did not cross-react with the non-cognate antigen in the direct ELISA (Figure 4.11 a and b), unlike what was seen for the analysis of serum collected from animals inoculated with plant-derived HPV-11 L1 NLS$^-$ protein. Results from figure 4.14 conclusively indicate that the post- inoculation serum collected from the animal vaccinated with insect cell-derived HPV-11 L1 VLPs was capable of neutralising the HPV-11 pseudovirus *in vitro*, whereas the serum collected from the insect cell-derived HPV-11 L1 NLS$^-$ inoculated animal is not. This raises important questions for all the research described in this thesis, but it more importantly raises the question as to how this happens. The biopsy-derived HPV-11 *L1 NLS*$^-$ gene was sequenced after each amplification and cloning step and does not differ from the internationally accepted HPV-11 *L1* gene nucleotide sequence (Accession number M14119, see Figure 2.3 Chapter 2) with the exception of one silent mutation at *L1* nucleotide position 257 (C to T), and the amino acid level it is identical to the sequence published by Warzecha *et al* (2003). I note those workers did not report any neutralisation experiments; thus, their antigen may have been exactly the same as mine in terms of lack of induction of neutralising antibodies.

The research presented in this chapter not only confirms the results described in chapters 2 and 3, but also raises further questions. In summary, the aim of improving the overall

yield of the plant-derived HPV-11 L1 NLS$^-$ and CRPV L1 proteins was partially fulfilled. Although the final yield per kilogram of fresh leaf material of TMV-derived HPV-11 L1 NLS$^-$ protein was equivalent to that of *A. thaliana* transgenic expression system, it was higher than that harvested from transgenic *N. tabacum* plants; moreover, the protein was antigenically 'intact' compared to the latter. Particles isolated from *A. thaliana* and *N. benthamiana* plants display similar antigenic properties (Figure 4.7**A**), yet *N. benthamiana*-produced HPV-11 L1 NLS$^-$ VLPs are more uniform in size (Figure 4.9**A**). As for the unforeseeable instability of the CRPV *L1* gene directly contributing to the low yield of CRPV L1 protein, the evaluation of both TMV-derived proteins *in vivo* and *in vitro* has questioned the suitability of these plant-produced antigens for use as potential vaccines. Remaining aspects that justify further investigations are the following: Why does the plant-derived HPV-11 L1 NLS$^-$ protein, whether produced by means of transgenic or transient expression, induce an antibody response that predominantly recognizes the HPV-11 L1 NLS$^-$ particle, when antiserum from guinea pigs injected with either insect cell-derived L1 NLS$^-$ or native VLPs is not capable of distinguishing between them (Figure 4.11**a** and **b**)? Would transient expression of the HPV-11 L1 gene in plants, as described by Warzecha *et al.* (2003), provide conclusive answers to the differences in antibody responses generated upon inoculation of animals?

In conclusion, one of the most important lessons to be learnt from the results obtained throughout the research compiling this chapter is that one cannot rely on being able to amplify different antigens by TMV expression in *N. benthamiana* just because expression of the identical antigen works similarly in transgenic plant expression systems. As is the difference between the North and South Pole, so too is there a difference between transgenic and transient plant expression system, further warranting that each individual step in the production of a potential vaccine candidate be thoroughly investigated.

Chapter 5

Conclusion

Worldwide, efforts have been made to develop efficacious vaccines to combat the detrimental impact of cancer- and morbidity-causing human papillomaviruses. HPV-11 does not cause cancer, yet it remains one of the most prevalent sexually transmitted HPVs. A high morbidity and reduction in quality of life results from the development of unpleasant genital warts.

Development efforts on candidate prophylactic HPV vaccines have primarily focused on the HPV L1 subunit and have culminated in successful phase I, II and III trials (Brown *et al.* 2001; Emeny *et al.* 2002; Fife *et al.* 2004; Harper *et al.* 2004; Koutsky *et al.* 2002), which have shown that a variety of HPV L1 subunit vaccines are efficacious in stimulating a long-lasting antibody-mediated immune response in humans. Indeed, Merck & Co. Inc. have in late 2005 presented data indicating that their GARDASIL™ combination HPV-6, -11, -16 and -18 L1 VLP-based vaccine prevented 100% of HSIL and CIN 2/3 associated with HPV-16 and -18 in a major Phase III clinical trial involving over 25000 people in 33 countries worldwide (taken from http://merck.com/newsroom/press_releases/research_and_development/2005). While these achievements are impressive, success comes at a price. Modelled on an already existing HBV vaccination program, it has been estimated that the implementation of an equivalent HPV vaccination strategy might involve costs of up to around US$ 300 per vaccinated individual (Sanders *et al.* 2003). This dampens the enthusiasm of having achieved something that might prevent the continuous spread of the virus-associated disease, because it is impossible to realize such a program in most of the developing world; ironically, a world burdened by the highest prevalence rate of HPV-associated disease.

Cheaper alternatives for the production of equivalent candidate HPV vaccine have to be tailored and developed in order to implement their final cost-effective use in

disadvantaged regions of the globe, including countries in which the infrastructure for traditional vaccine production, storage, proper distribution and administration simply does not exist.

Efforts described in this thesis revolved around the development of alternative candidate HPV subunit vaccine production possibilities. I explored the possible expression of the HPV-11 major capsid protein (L1) in non-nutritious plants, the associated purification thereof and the evaluation of such in stimulating the much desired antibody-mediated immune response in animals.

Chapters 2 and 4 explore and compare two different plant-based protein expression systems for the potential production of a candidate HPV-11 L1 NLS$^-$ subunit vaccine. Chapter 2 exclusively describes the determinants of transgenic expression of the HPV-11 *L1 NLS*$^-$ gene in both *A. thaliana* and *N. tabacum.* Stable integration and gene inheritance over many generations have been recorded. Protein expression resulted in the assembly of HPV-11 L1 VLPs; these however display a pleomorphic phenotype and do not resemble insect cell-derived VLPs. It was found that the HPV-11 protein produced in *N. tabacum* is subject to degradation by plant proteases, much more so than in *A. thaliana*, predominantly resulting in a 36 kDa species of the protein. Nonetheless, conformation-specific and surface linear neutralising MAbs to HPV-6 and HPV-11 did bind the HPV-11 VLPs, thus providing evidence that transgenic plant-produced antigens possess the potential ability to induce virus-neutralising antibodies. The preliminary evaluation of the protein in animals has shown that the transgenic plant-derived HPV-11 L1 NLS$^-$ VLPs are indeed capable of inducing an antibody-mediated type-specific immune response, a prerequisite for any successfully applied efficacious vaccine.

The transgenic expression system was the first to be explored. After having obtained the necessary data, we looked at exploring the options of extending the limits of HPV-11 L1 NLS$^-$ protein production and attempted to express the L1 protein via a plant virus-based transient expression system. This transient expression system was the commercially available genetically modified TMV-based vector (Chapter 4), and yielded mixed results.

Expression of the HPV-11 *L1 NLS*$^-$ gene via recombinant TMV successfully produced VLPs that are not only antigenically similar to those of insect cell and transgenic plant origin, but also display a more uniform appearance. The drawback however, was presented in the form of total yield. I had hoped for an increase in protein production, yet after having calculated the final protein yield per kilogram of fresh leaf material, it was obvious that a protein production was not much increased: the final yield in *N. benthamiana* was equivalent to that obtained in transgenic *A. thaliana.* However, it presents a 4 to 14 fold increase in HPV-11 L1 NLS$^-$ protein production in a tobacco species, a fact that cannot be ignored. As was also done with the transgenic plant-derived particles, the TMV-derived HPV-11 L1 NLS$^-$ particles were evaluated in terms of their efficacy in inducing an immune response. Immunization results have shown that they are indeed capable of inducing reactive antibodies, but as was found with transgenic-derived VLPs, these primarily recognized the truncated (NLS$^-$) version of the protein.

I and others subsequently tested the sera collected from all animals that were either vaccinated with the transgenic plant-derived or transiently expressed HPV-11 L1 NLS$^-$ protein. Our neutralisation results indicate that none of the sera were capable of neutralising the HPV-11 pseudovirus *in vitro*. The implications of this are far reaching. Insect cell-derived HPV-11 L1 NLS$^+$ VLPs have elicited neutralising antibodies (Rose *et al.* 1999). My plant-derived HPV-11 L1 NLS$^-$ protein expressed transgenically and via TMV did assemble into pleomorphic and more uniform VLPs respectively, which we thought would mean they would similarly elicit neutralising antibodies; thus we did not expect this result at all. The result from tobacco-produced L1 was at least understandable, given the significant proteolysis of the L1: is it possible that VLP formation is initiated, but that all appropriate conformational determinants may well have been lost in particles assembled from severely degraded L1 protein? However, material produced in *A. thaliana* and in *N. benthamiana* was not so degraded, and the *N. benthamiana* material moreover resembled insect cell-produced L1 VLPs more closely than any other, yet still did not elicit neutralising antibodies. Expression of the HPV-11 L1 gene by recombinant baculovirus has been the preferred production method in two publications by Christensen *et al.* (1994) and Rose *et al.* (1994). The inability of insect cell-derived HPV-11 L1 NLS$^-$

VLPs to adequately elicit neutralising antibodies in guinea pigs is worrying. Warzecha *et al.* (2003) showed that potato-derived HPV-11 L1 NLS^- VLPs induce an antibody response, but no further record of whether the antibody response elicited upon vaccination with these particles is capable of virus neutralisation has been published.

The developmental process of a potential vaccine against HPV-11 has not been without difficulties. In addition to the results published by Neeper *et al.* (1996), the Merck Research Laboratories published their findings on the expression of the synthetic HPV-11 *L1* gene in *Saccharomyces cerevisiae* (Cook *et al.* 1999). Included in their report are findings similar to the ones described in this thesis. Expression of the HPV-11 *L1* gene in *S. cerevisiae* results in the production of the HPV-11 L1 protein. However, the authors noted the persistent appearance of a 45 kDa protein species and attributed this to proteolysis of L1. Furthermore, the assembly of *S. cerevisiae*-derived VLPs resulted in the formation of pleomorphic VLPs, described by the authors to range from 32 to 97 nm. These findings are similar to these described in chapter 2, although the HPV-11 *L1* gene expressed in *N. tabacum* and *A. thaliana* did not have a nuclear localization signal nor was it codon-optimized for the use in plants. When this project started some 6 years ago, not enough funds were available to justify such codon-optimization. It would however be very interesting to evaluate and compare expression levels of native and either plant or human codon-optimized HPV-11 *L1* NLS^- genes in all described plant expression systems.

An independent study was initiated with the aim to validate the use of plants for the production of a variety of different antigens that could potentially be used as future vaccines: this is presented in chapters 3 and 4 of this thesis. I explored the possibilities of expressing the CRPV *L1* gene in both the transgenic plant and transient expression systems. Expression of this particular *L1* gene was successful in transgenic plants in comparison to expressing using the engineered TMV-based vector. Insertion of the full-length CRPV L1 gene in the TMV genome appeared to be highly unstable, resulting in the eventual loss of expression. Nevertheless, the overall CRPV L1 protein harvested from both expression systems was capable of binding conformation-specific and surface

linear epitope recognizing MAbs. Although the protein derived from both systems did not appear to assemble beyond the pentameric stage, its capacity to stimulate an immune response in domestic rabbits was surprising. This data forms part of a proof-of-concept study aimed at providing evidence that plant-derived antigens are capable of inducing an antibody response, but more importantly whether this response would protect the natural host from infection. We successfully obtained the answer to this very important question. All rabbits that were initially vaccinated with either the transgenic plant-derived or the transiently expressed CRPV L1 protein were subsequently challenged with infectious CRPV. Our results have confirmed that almost complete protection was obtained in vaccinated rabbits, with these papillomas that were up to 6 times smaller than in the control group. Given the positive outcome of the challenge experiment, we evaluated the serum collected from all animals before the actual challenge in the *in vitro* CRPV pseudovirus neutralisation assay. The results were surprising: none of the sera were capable of virus neutralisation, suggesting that the protective effect seen in the challenge experiment could be attributed to a cellular response, or perhaps the live virus inoculum acting as a booster.

With certainty it can be concluded that the expression of this HPV-11 *L1 NLS*$^{-}$ gene in plants results in VLPs that are not able to elicit a virus neutralising antibody response, and thus do not meet the requirements of a potential efficacious subunit vaccine against HPV-11. Such a product cannot under any circumstances be considered for vaccinations of humans. I note that in the work reported on by Warzecha *et al.* (2003), in which the low antibody titre elicited by feeding mice with transgenic HPV-11 L1 NLS^{-} potato was boosted by administering sub-immunogenic dosages of insect cell-derived VLPs, there was no report of neutralising antibodies having been elicited: however, they speculated that plant-derived HPV proteins could potentially be used as boosters throughout an HPV vaccination program. This however requires a subunit protein that has proven to meet the requirements of a vaccine, which ours – and potentially theirs – has not.

High level production of HPV L1 proteins has successfully been performed using insect-yeast- or mammalian expression systems, yet the expression of these L1 proteins in

transgenic plants generally remains low. Possible reasons for this are the associated modifications that could potentially occur at the post-transcriptional or post-translational level. However, if such modifications were not to occur, the accumulating L1 protein resulting from translation of the introduced gene might still be too low, thus not allowing for the nucleation of molecular L1 aggregates. Furthermore, L1 protein expression in the plant cytosol might not provide the ideal environment necessary to promote the formation of inter-subunit disulphide bonds known to be required for the formation of HPV L1 VLPs (Li *et al.* 1998). To date several modifications to the original plant binary vectors have proven to be extremely useful. Currently it is possible to target HPV L1 proteins to various organelles within the plant cell including the chloroplasts and the endoplasmic reticulum (ER). Targeting the protein to specific destinations within the cells promotes the local accumulation of L1 protein inside these organelles thus obtaining higher protein concentration levels required for the assembly of VLPs. In addition, the targeting of the HPV L1 protein to the ER ensures an environment which is not only oxidizing in nature, but also provides the protein disulphide isomerase enzyme necessary for disulphide bond formation.

Technological advances have not only been developed for the expression of foreign proteins in transgenic plants, but are constantly evolving to overcome barriers of all plant expression systems. For example, the *in vitro* infection of susceptible plant species with synthesized plant virus-based vector RNA transcripts is a straight forward process, yet to be considered are the implicated costs of such inoculations especially if large numbers of expression constructs are to be tested on large amounts of plants (Scholthof, 1999). To overcome this constraint, Scholthof (1999) has explored the possibilities of adapting the Tomato bushy stunt virus (TBSV) vector cDNA to do without the required in vitro synthesis of RNA. Constructs containing the full-length TBSV cDNA inserted downstream from the CaMV35S promoter and upstream from the nopaline synthase poly(A) signal have proven to efficiently express foreign genes upon simple rub-inoculation of miniprep DNA.

As presented, the research conducted throughout this PhD has opened Pandora's Box; it has raised more questions to those already answered. Yes, it is possible to express the HPV-11 *L1* gene in the transgenic plant expression system; however, only the truncated version (*NLS*$^-$) and not the full-length (*NLS*$^+$) gene appears to be stable and non-toxic to the host. This result was obtained prior to the publication of Warzecha *et al.* (2003), but confirms their experience. Besides the apparent quantitative differences in terms of protein expression, the results vary between the two expression systems. For one, why do the resulting transgenic-derived HPV-11 L1 particles display a pleomorphic phenotype, whereas the TMV-produced particles display a more uniform assembly? Is this purely a L1 protein concentration dependency? Whether expressed in *A. thaliana* or *N. tabacum* or *N. benthamiana*, all HPV-11 L1 NLS$^-$ particles appear to encapsidate foreign nucleic acid to varying degrees. Only one publication reporting on the possible nucleic acid encapsidation by plant-derived PV particles has thus far been recorded in the literature. Does the expression and consequent post-translational modification of the protein in plants lead to the possible exposure of a DNA binding domain or is it just random packaging of nucleic acid? The efforts described in this developmental process of a potential HPV-11 subunit vaccine have questioned why all antisera produced from immunization of animals with plant-derived HPV-11 L1 NLS$^-$ particles predominantly recognize insect cell-derived NLS$^-$ and not NLS$^+$ VLPs. It could be argued that apparent differences between antiserum reactions with insect cell-derived HPV-11 L1 NLS$^-$ and native VLPs are due to differences in the coating antigen concentrations; however, antisera derived from injection of insect cell-derived NLS$^-$ and NLS$^+$ VLPs do not show this difference, indicating that the observed differences seen for antisera against plant-derived antigens are real. The display of a putative immunodominant epitope(s) on the surface of plant-produced NLS$^-$ particles might account for these results, although Biemelt *et al.* (2003) and Varsani *et al.* (2003) have reported on no apparent protein modifications when expressing the HPV- 16 *L1* gene in transgenic tobacco and potato plants. This aspect was not explored by Warzecha *et al.* (2003) in their paper on HPV-11 *L1 NLS*$^-$ expression in potato plants; it is vitally important to consider this aspect since the efficacy of antibodies raised to such an altered antigen have proven not to neutralize the HPV-11 pseudovirus in our experiments.

The proof-of-concept experiment exploring the expression of the CRPV *L1* gene in plants has raised a serious question. Is vaccination with plant-derived L1 protein really inducing a virus neutralising antibody response or is this response only seen upon challenge and contact with the actual virus? This aspect of chapter 3 demands further investigation and clarification.

In summary, it can be said that this thesis has not explored all the different possibilities of expressing either proteins in plants. Although the results of several years of research is described, various new technological developments such as targeting the HPV-11 L1 protein to various organelles within the plant cell by means of protein expression after leaf infiltration with recombinant *Agrobacterium* could have provided further insight into a more stable and effective production mechanism. Several aspects remain unaccounted for and include questions such as whether the HPV-11 L1 nuclear localization signal could be substituted by the nuclear localization signals of other PV L1 genes, including the CRPV and HPV-16 L1 genes, in order to alleviate the toxicity aspect upon expression of the gene in transgenic plants. As reported throughout all chapters, low expression levels and/or modifications of the CRPV L1 and HPV-11 L1 NLS^- proteins in either plant system account for the formation of either pentameric aggregates or pleomorphic VLPs. The PV L2 protein enhances the formation of a tighter assembled PV capsid, but is not required for the assembly of VLPs. Would the co-expression of the HPV-11 L1 and L2 proteins in plants enhance the formation of more homogeneous VLPs? The answer is probably not, because the formation of homogeneous VLPs does not depend on how tightly the individual components of the capsid are bound, but rather on the availability of individual components to form the capsid.

In comparison to various other publications on the expression of HPV L1 proteins in plants and noting the fact that the expression of HPV-11 L1 has proven problematic in yeast, this work has shown that HPV-11 L1 protein expression can be observed in plants, but the resulting proteins are unfortunately not suitable for the potential use as plant-derived subunit vaccines for the prevention of HPV-11 infection as they do not induce a protective neutralising antibody response. Altogether, the work presented in this thesis

has shown a substantial increase in HPV-11 L1 protein expression levels in plants, and forms part of the first proof-of-concept study in which the protective viability of a plant-produced CRPV L1 protein has been evaluated.

Appendix A

Papillomavirus types

Contents

Table A.1: Characterized *Human papillomavirus* types

PV type	Abbreviation	Accession number	Site of infection/source
Human papillomavirus type 1a	HPV-1	V01116	Plantar warts
Human papillomavirus type 2a	HPV-2	X55964	Common warts
Human papillomavirus type 3	HPV-3	X74462	Flat warts
Human papillomavirus type 4	HPV-4	X70827	Common warts
Human papillomavirus type 5	HPV-5	M17463	Benign warts, laryngeal papillomatosis
Human papillomavirus type 6b	HPV-6	X00203	Genital warts, laryngeal papillomatosis
Human papillomavirus type 7	HPV-7	X74463	'Butchers' warts, oral papillomas of HIV patients
Human papillomavirus type 8	HPV-8	M12737	Benign and malignant Epidermodysplasia verruciformis (EV) lesions
Human papillomavirus type 9	HPV-9	X74464	EV lesions
Human papillomavirus type 10	HPV-10	X74465	Flat warts
Human papillomavirus type 11	HPV-11	M14119	Laryngeal papillomas, genital warts
Human papillomavirus type 12	HPV-12	X74466	EV lesions
Human papillomavirus type 13	HPV-13	X62843	Oral focal epithelial hyperplasia
Human papillomavirus type 14	HPV-14	X74467	EV lesions
Human papillomavirus type 15	HPV-15	X74468	EV lesions
Human papillomavirus type 16	HPV-16	K02718	Anogenital intraepithelial neoplasia and cancers
Human papillomavirus type 17	HPV-17	X74469	EV lesions
Human papillomavirus type 18	HPV-18	X05015	Anogenital intraepithelial neoplasia and cancers
Human papillomavirus type 19	HPV-19	X74470	EV lesions
Human papillomavirus type 20	HPV-20	U31778	EV lesions
Human papillomavirus type 21	HPV-21	U31779	EV lesions
Human papillomavirus type 22	HPV-22	U31780	EV lesions
Human papillomavirus type 23	HPV-23	U31781	EV lesions
Human papillomavirus type 24	HPV-24	U31782	EV lesions
Human papillomavirus type 25	HPV-25	U74471	EV lesions
Human papillomavirus type 26	HPV-26	X74472	Common warts under immunosuppression
Human papillomavirus type 27	HPV-27	X73373	Common warts
Human papillomavirus type 28	HPV-28	U31783	Flat warts
Human papillomavirus type 29	HPV-29	U31784	Common warts
Human papillomavirus type 30	HPV-30	X74474	Laryngeal carcinomas
Human papillomavirus type 31	HPV-31	J04353	Anogenital intraepithelial neoplasia and cancers
Human papillomavirus type 32	HPV-32	X74475	Oral focal epithelial hyperplasia, oral papillomas
Human papillomavirus type 33	HPV-33	M12732	Anogenital intraepithelial neoplasia and cancers
Human papillomavirus type 34	HPV-34	X74476	Anogenital intraepithelial neoplasia
Human papillomavirus type 35	HPV-35	X74476	Anogenital neoplasia and cancers
Human papillomavirus type 36	HPV-36	U31785	Actinic keratosis, EV lesions
Human papillomavirus type 37	HPV-37	U31786	Keraroacanthoma
Human papillomavirus type 38	HPV-38	U31787	Melanoma
Human papillomavirus type 39	HPV-39	M62849	Anogenital intraepithelial neoplasia and cancers
Human papillomavirus type 40	HPV-40	X74478	Anogenital intraepithelial neoplasia
Human papillomavirus type 41	HPV-41	X56147	Cutaneous squamous cell carcinomas
Human papillomavirus type 42	HPV-42	M73236	Anogenital intraepithelial neoplasia
Human papillomavirus type 43	HPV-43	AJ620205	Anogenital intraepithelial neoplasia
Human papillomavirus type 44	HPV-44	U31788	Anogenital intraepithelial neoplasia
Human papillomavirus type 45	HPV-45	X74479	Anogenital intraepithelial neoplasia and cancers

Appendix A: Papillomavirus types

PV type	Abbreviation	Accession number	Site of infection/source
Human papillomavirus type 46	HPV-46		
Human papillomavirus type 47	HPV-47	M32305	EV lesion
Human papillomavirus type 48	HPV-48	U31790	Cutaneous squamous cell carcinoma
Human papillomavirus type 49	HPV-49	X74480	Flat wart under immunosuppression
Human papillomavirus type 50	HPV-50	U31790	EV lesion
Human papillomavirus type 51	HPV-51	M62877	Anogenital intraepithelial neoplasia and cancers
Human papillomavirus type 52	HPV-52	X74481	Anogenital intraepithelial neoplasia and cancers
Human papillomavirus type 53	HPV-53	X74482	Anogenital intraepithelial neoplasia
Human papillomavirus type 54	HPV-54	U37488	Anogenital intraepithelial neoplasia
Human papillomavirus type 55	HPV-55	U31790	Anogenital intraepithelial neoplasia
Human papillomavirus type 56	HPV-56	X74483	Anogenital intraepithelial neoplasia and cancers
Human papillomavirus type 57	HPV-57	X55965	Oral papillomas and inverted maximmary sinus papillomas
Human papillomavirus type 58	HPV-58	D90400	Anogenital intraepithelial neoplasia and cancers
Human papillomavirus type 59	HPV-59	X77858	Anogenital intraepithelial neoplasia
Human papillomavirus type 60	HPV-60	U31792	Epidermoid cysts
Human papillomavirus type 61	HPV-61	U31793	Anogenital intraepithelial neoplasia
Human papillomavirus type 62	HPV-62	U12499	Anogenital intraepithelial neoplasia
Human papillomavirus type 63	HPV-63	X70828	Myrmecia wart
Human papillomavirus type 64	HPV-64	U12495	Anogenital intraepithelial neoplasia
Human papillomavirus type 65	HPV-65	X70829	Pigmented wart
Human papillomavirus type 66	HPV-66	U31794	Cervical carcinoma
Human papillomavirus type 67	HPV-67	D21208	Anogenital intraepithelial neoplasia
Human papillomavirus type 68	HPV-68	X67161	Anogenital intraepithelial neoplasia
Human papillomavirus type 69	HPV-69	AB027020	Anogenital intraepithelial neoplasia and cancers
Human papillomavirus type 70	HPV-70	U21941	Vulvar papilloma
Human papillomavirus type 71	HPV-71	AB040456	Anogenital intraepithelial neoplasia
Human papillomavirus type 72	HPV-72	X94164	Oral papilloma (HIV patients)
Human papillomavirus type 73	HPV-73	X94165	Oral papilloma (HIV patients)
Human papillomavirus type 74	HPV-74	U40822	Anogenital intraepithelial neoplasia
Human papillomavirus type 75	HPV-75	Y15173	Common warts in organ allograft recipient
Human papillomavirus type 76	HPV-76	Y15174	Common warts in organ allograft recipient
Human papillomavirus type 77	HPV-77	Y15175	Common warts in organ allograft recipient
Human papillomavirus type 78	HPV-78		
Human papillomavirus type 79	HPV-79		
Human papillomavirus type 80	HPV-80	Y15176	
Human papillomavirus type 81	HPV-81	AJ620209	
Human papillomavirus type 82	HPV-82	AB027021	Anogenital intraepithelial neoplasia
Human papillomavirus type 83	HPV-83	AF151983	Anogenital intraepithelial neoplasia
Human papillomavirus type 84	HPV-84	AF293960	Anogenital intraepithelial neoplasia
Human papillomavirus type 85	HPV-85	AF131950	
Human papillomavirus type 86	HPV-86	AF349909	Anogenital intraepithelial neoplasia
Human papillomavirus type 87	HPV-87	AJ400628	Anogenital intraepithelial neoplasia
Human papillomavirus type 88d	HPV-88d		
Human papillomavirus type 89	HPV-89	AF436128	Anogenital intraepithelial neoplasia
Human papillomavirus type 90	HPV-90		Anogenital intraepithelial neoplasia
Human papillomavirus type 91	HPV-91	AF131950	Anogenital intraepithelial neoplasia
Human papillomavirus type 92	HPV-92	AF531420	

PV type	Abbreviation	Accession number	Site of infection/source
Human papillomavirus type 93b	HPV-93b	AY382778	
Human papillomavirus type 94a	HPV-94a	AJ620211	
Human papillomavirus type 95c	HPV-95c	AJ620210	
Human papillomavirus type 96b	HPV-96b	AY382779	

Table A.2: Characterized animal papillomavirus types

PV Type	Abbreviation	Accession number	Host
Bovine papillomavirus type 1	BPV-1	X02346	Domestic cattle (*Bos taurus*)
Bovine papillomavirus type 2	BPV-2	M20219	
Bovine papillomavirus type 3	BPV-3	X59062	
Bovine papillomavirus type 4	BPV-4	X05817	
Bovine papillomavirus type 5	BPV-5	U43367	
Bovine papillomavirus type 6	BPV-6	X59064	
Canine oral papillomavirus	COPV	L22695	Domestic dogs (*Canis familiaris*)
Colobus monkey papillomvirus	CCPV		
Cottontail rabbit papillomavirus	CRPV	K02708	Cottontail rabbit (*Sylvilagus floridanus*)
Deer papillomavirus	DPV	M11910	Deer (*Odocoileus virginianus*)
European elk papillomavirus	EEPV	M15953	European elk (*Alces alces*)
Equus caballus papillomavirus	EcPV	AF498323	Horse (*Equus caballus*)
Felis domesticus papillomavirus	FdPV	AF377865	Domestic cats (*Felis domesticus*)
Fringilla coelebs papillomavirus	FcPV	AY957109	Chaffinch (*Fringilla coelebs*)
Mastomys natalensis papillomavirus	MnPV	U01834	Multimammate rat (*Mastomys natalensis*)
Micromys minutus papillomavirus	MmPV	X65200	Old World harvest mouse
Ovine papillomavirus type 1	OvPV-1	U83594	Domestic sheep (*Ovis domesticus*)
Ovine papillomavirus type 2	OvPV-2	U83595	
Psittacus erithacus timneh papillomavirus	PePV	AF420235	Grey parrots (*Psittacus erithacus*)
Rabbit oral papillomavirus	ROPV	AF227240	Rabbit (*Sylvilagus*)
Reindeer papillomavirus	RPV	AF443292	Reindeer (*Rangifer tarandus*)
Rhesus monkey papillomavirus	RhPV	NC_001678	Rhesus monkey

Appendix B

Maps of non-commercial vectors

Contents

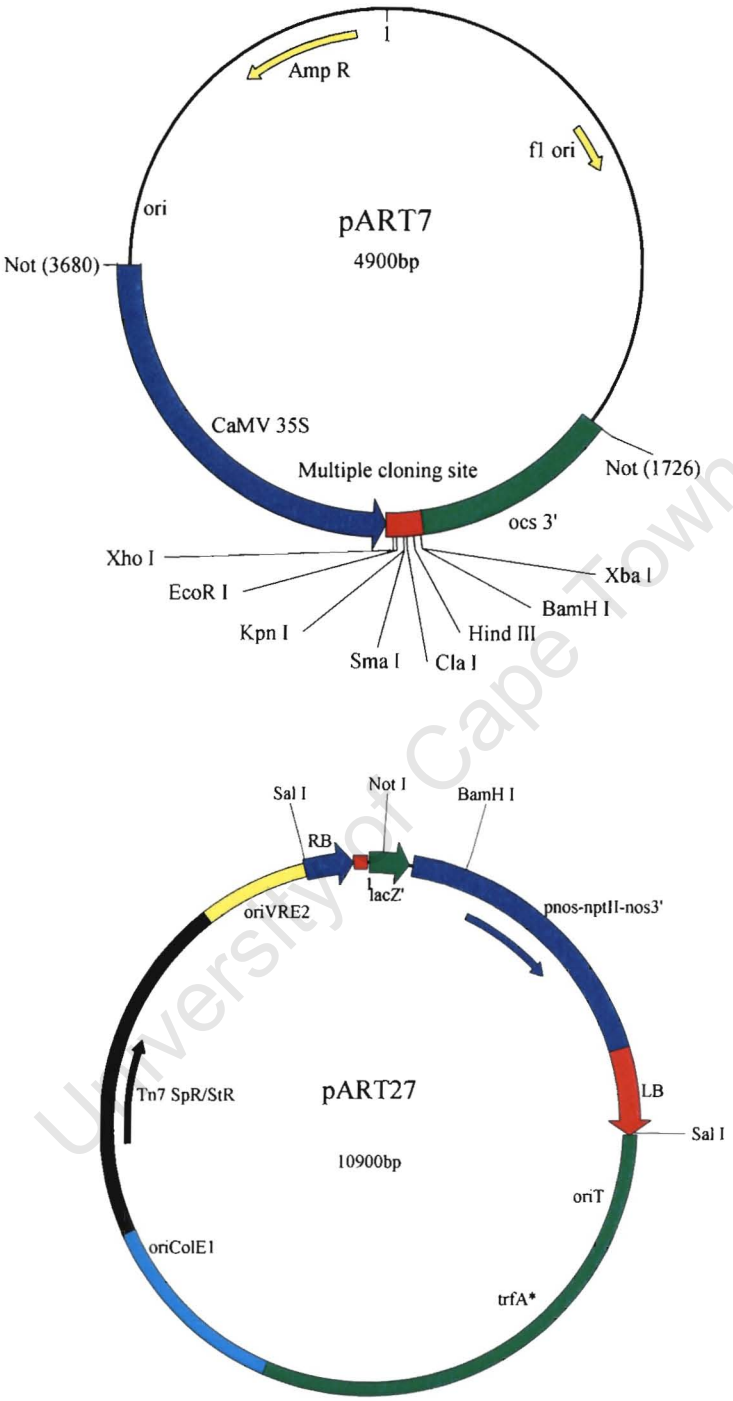


Figure B.1: Map of pART7 and binary vector pART27 (Gleave, 1992).

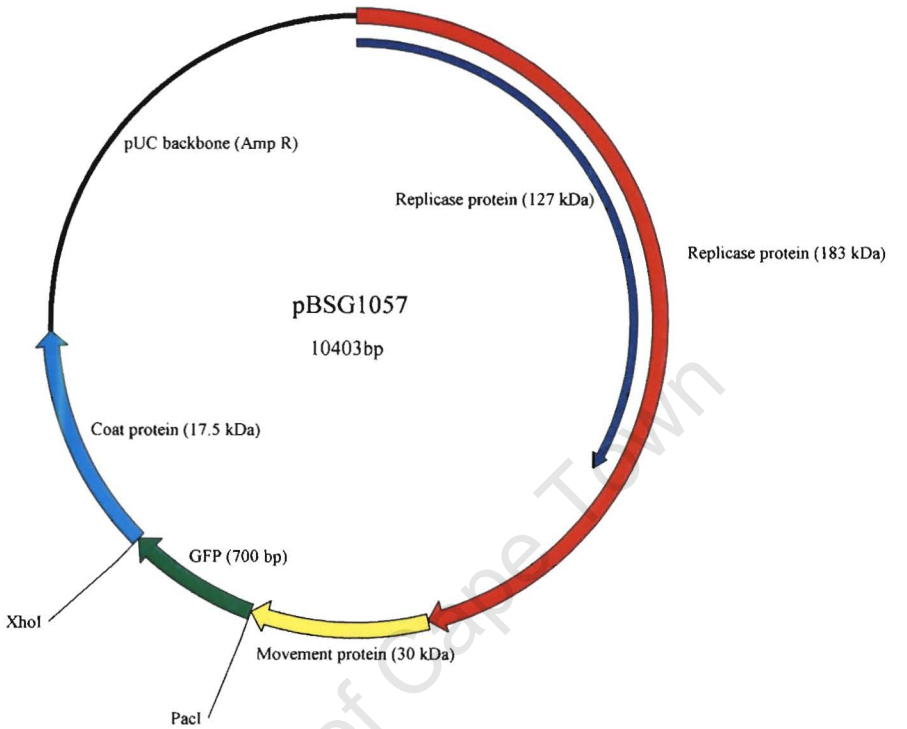


Figure B.2: Map of TMV-based vector pBSG1057 (provided by Dr Kenneth Palmer, Large Scale Biology Corporation, Vacaville, CA, USA)

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