Expression optimization of a human papillomavirus type 16 therapeutic vaccine candidate in *Nicotiana benthamiana* leaves

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~ Challenges are good. There is always a lesson to learn. ~

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Declaration

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Abstract

High risk human papillomaviruses (HPVs) are the causative agents of cervical cancer. The three approved prophylactic vaccines do not benefit already infected individuals; therefore, there is still an urgent need for therapeutic vaccines. The HPV oncoproteins E6 and E7 are ideal targets for the development of such vaccines, as they are expressed throughout the viral life cycle and in tumours. They could be used to elicit strong cytotoxic lymphocyte (CTL) responses which would aid in viral clearance, and could also be effective against tumours. Granadillo et al. (2011) developed an *Escherichia coli*-produced therapeutic vaccine candidate, consisting of the HPV-16 E7 protein and a cell membrane-penetrating and immunomodulatory peptide (LALF), whose fusion to HPV-16 E7 aided in the immunogenicity and antigen presentation of the oncoprotein. However, such vaccines need not only to be effective, but also to have a low cost. Plant expression systems represent an attractive alternative to conventional expression systems based on bacterial, yeast, mammalian and other cell cultures, and are potentially far more cost-effective.

The aim of the present project was to produce LALF-E7 in *Nicotiana benthamiana* leaves via *Agrobacterium*-mediated transient expression, and to optimize its expression, extraction and purification. This was done by expressing LALF-E7 using different expression vectors, testing different subcellular localizations, and testing the effect of gene silencing suppressors. By using our group's replicating expression vector and targeting LALF-E7 to the chloroplasts, the expression of the candidate vaccine in *N. benthamiana* leaves was increased 26.8 fold compared to non-replicating vectors or cytoplasmic localization. Furthermore, silencing suppressors did not significantly increase the expression of LALF-E7 when expressed by the replicating vector and targeted to the chloroplasts.

I showed, by fluorescence microscopy, that LALE-E7 was indeed being targeted to the plants' chloroplasts and that it possibly forms proteins bodies (PBs) that are closely associated to the chloroplast envelope. I further hypothesized a mechanism by which the PBs-like structures form.

Once the expression of LALF-E7 was optimized in plant leaves, a purification strategy was developed by testing different extraction methods and using metal ion affinity chromatography. The extraction protocol developed pre-purified LALF-E7 by removing the majority of soluble proteins from the final extract. However, LALF-E7 was not fully purified by affinity chromatography, suggesting that other purification strategies should be used.

Finally, I tested the partially purified plant-produced LALF-E7 candidate, and compared it to the *E. coli*-produced counterpart, in tumour regression experiments using mice as animal models. Due to low antigen doses and a large number tumourigenic cells used to inoculate the mice animal models, the effect of the plant-produced LALF-E7 as a therapeutic vaccine was inconclusive. However, it was suggested that it could potentially be comparable to the *E. coli*-produced counterpart.

In summary, I report for the first time the entire chain of research involving the expression of LALF-E7 in plants, its extraction, purification and the testing of its immunogenicity in a mouse model. This research also suggests new avenues for the use of the LALF peptide as a PB-inducer which could be useful in increasing the expression of other recombinant proteins.

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Chapter 1: Literature review

"Therapeutic vaccines for high-risk HPVs"

1.1. Introduction

It is now acknowledged that a number of infectious agents contribute to the development of malignancies in humans: in 2008 approximately 16.1% of all cancers were estimated to be caused by infectious agents (De Martel *et al.*, 2012). For example, hepatitis B and C viruses, high-risk human papillomaviruses (hr-HPVs) and *Helicobacter pylori* cause liver, cervical and gastric cancers, respectively. The fact that these cancers are caused by non-human agents allows for design of new therapeutic or preventative vaccines targeting non-human antigens, preventing auto-immune reactions, and offering a vast number of possible new strategies for the development of anti-cancer therapeutics (Zur Hausen, 2009; De Martel *et al.*, 2012; Morrow, Yan and Sardesai, 2013).

Cancer is a global leading cause of death, and HPV-related cancers represent 30% of infectious agent-born cases (Schottenfeld and Beebe-Dimmer, 2015). Of these, the most common cancer is that of the cervix, which is in turn the fourth most common cancer in women worldwide (Centers for disease control and prevention, 2012). There are at least 170 HPV genotypes described, which are categorised into two groups: low-risk types, including HPV-6, -11, -40, -42, -43, -44, -54, -61, and -72 which cause genital warts, and high-risk types including HPV-16, -18, -31, -35, -39, -45, -51, -52, -56, -58, -66 and -68, which are responsible for 99.7% of cervical cancer cases (Zur Hausen, 2002; Parkin and Bray, 2006; de Villiers, 2013). There are approximately 530,000 new cases of cervical cancer and 270,000 deaths per year (World Health Organization 2016). HPV-16 and -18 are the most prevalent types worldwide, causing more than 70% of cervical cancer cases. Developing countries carry the greatest burden of HPV infections and malignancies due to their lack of resources to implement efficient vaccination and screening programmes. Besides cervical cancer, hr-HPVs also cause vaginal, vulvar, penile, anal and oropharyngeal cancers (Zur Hausen, 2002; Parkin and Bray, 2006).

Human papillomavirus particles are non-enveloped and have an icosahedral morphology. As shown in Figure 1.1., their 8 kb double-stranded circular DNA genome encodes for six

early regulatory proteins - E1, E2, E4, E5, E6 and E7 - and the two late structural proteins L1 and L2. The virus infects basal epithelial cells through anatomically accessible points such as microlesions in the skin, genital organs and oropharyngeal areas. Capsid proteins L1 and L2 attach to epithelial cell receptors and a long process of entry commences, resulting in cytoplasmic uncoating of the virus and entry of its genome into the nucleus of the infected cell, where it is transcribed and then replicated. Early proteins are expressed first and regulate the host cell life cycle and genome replication. They also regulate the expression of late proteins in a cell differentiation-dependent manner: L1 and L2 are only expressed in mature squamous cells. Maturation of virions occurs after terminal differentiation of epithelial cells, and their release coincides with natural shedding of senescent cells at the end of epithelial cell life cycle (Zur Hausen, 2002).

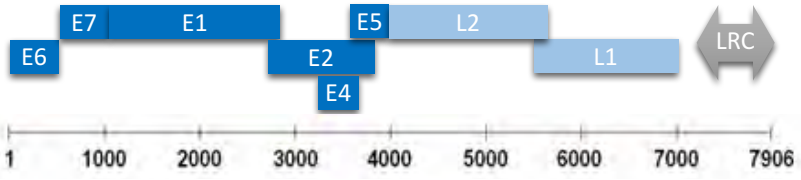

Figure 1.1. Linear genome organization of a typical HPV. E1-7, early regulatory proteins open reading frames. L1-2, late structural proteins open reading frames. LCR, long control region. Ruler, number of base-pairs (bp). Adapted from Stanley, (2006).

Most HPV infections are cleared by the host's immune system. A small portion of cases progress into persistent infections that, in the case of hr-HPVs, cause vaginal and cervical intraepithelial neoplasia (VIN and CIN, respectively), also referred to as VIL and CIL for lesions (Zur Hausen, 2002). These are graded according to the severity of the lesion as 1, 2 or 3, where CIN-1 is the least severe and CIN-3 is the most severe. Lesion progression varies between individuals and they can regress either spontaneously or with treatment. Women that have CIN-3 have a greater chance of developing cervical cancer. Similarly, women infected by HPV-16 and -18 have the highest chance of developing CIN-3 and eventually cancer (Wheeler, 2008).

In a typical hr-HPV carcinogenesis (Figure 1.2.), the genome of the virus is integrated into the host's chromosomal DNA and the *E2* sequence is disrupted during the linearization of the genome. The E2 protein is the transcriptional repressor of *E6* and *E7*; therefore, expression from these genes becomes constitutive once *E2* is disrupted. The E6 protein

subsequently promotes the degradation of the host apoptosis regulator protein p53, and activates telomerase which results in extended cell life. The E7 protein targets the tumour suppressor retinoblastoma protein (pRb) for degradation and leads to the transition of the cell life cycle to the S-phase and subsequent host cell genome replication. Overall E6 and E7 disrupt the cell cycle regulation and promote prolonged host cell life, leading to genomic instability and eventually cancer (Zur Hausen, 2002).

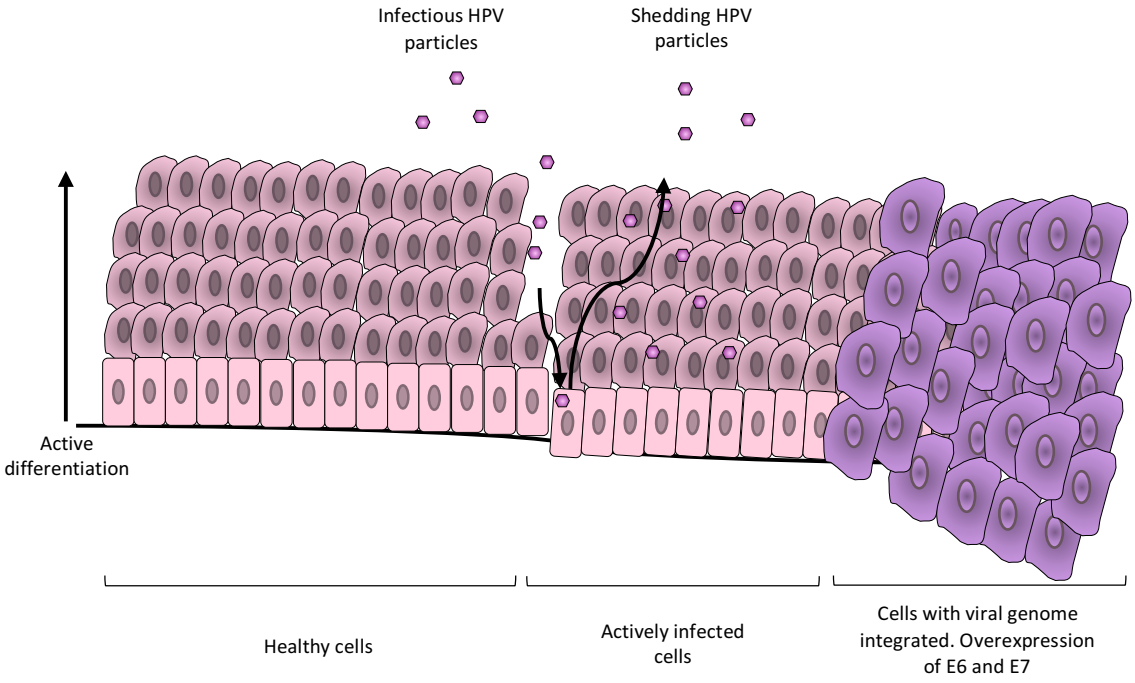

Figure 1.2. The life-cycle of a typical hr-HPV. Infection occurs at basal epithelial cells through anatomically accessible points such as microlesions. The genomes of HPVs stay as episomes in the host's cell nuclei. Cells proliferate and differentiate. The expression of structural proteins, L1 and L2, viral assembly and release only occur at late stages of the cell life cycle. Integration of the viral genome into the host's genome leads to overexpression of E6 and E7 which disrupt the cell life cycle regulation and promotes prolonged cell life leading to genomic instability and cancer. At this stage no viral structural proteins are expressed. Adapted from Moody and Laimins, (2010)

Currently, there are three commercially available HPV vaccines. All exploit the fact that HPV L1 protein can form virus-like particles (VLPs) when expressed alone in a variety of cell types, that are morphologically and antigenically highly similar to native virions (Kirnbauer *et al.*, 1992). The first, Cervarix® (GlaxoSmithKline Inc.), is a bivalent vaccine produced in insect cells using a baculovirus expression system, that targets HPV-16 and -18. The second, Gardasil® (Merck & Co., Inc.), is a quadrivalent vaccine produced in yeast cells that targets HPV-16 and -18, and the genital wart-associated HPV-6 and -11. The third vaccine, Gardasil® 9, is a nonavalent vaccine that targets five extra hr-HPV types. These

are the HPV-31, -33, -45, -52 and -58, with the potential to prevent up to 90% of cervical cancer, 20% more than its quadrivalent counterpart. This vaccine has recently been approved by the Food and Drug Administration Department (FDA) (Merck, 2014). These three vaccines effectively prevent HPV infections caused by the targeted types by eliciting the production of neutralizing antibodies that bind to the viral particles and block their entrance into host cells (Harper *et al.*, 2004; Villa *et al.*, 2005; Joura *et al.*, 2015). However, these vaccines are not effective at eliminating pre-existing infections, since the target antigens, L1 capsid proteins, are not expressed in infected basal epithelial cells (Hildesheim *et al.*, 2007, 2016; Hung *et al.*, 2008). Therefore, the large number of individuals already infected with HPV do not benefit from the current vaccines.

The lack of appropriate Pap-smear screening leads to many women only detecting infections when they have already progressed past CIN-1 or when cancer has already developed. Additionally, many women, especially in developing countries, do not have access to follow-up treatment of detected malignancies. Even if efficient vaccination programmes were implemented worldwide, their impact will only be seen in several years since these vaccines were approved only recently. In addition, the current commercially available vaccines are expensive and therefore, except when sponsored by the state or other organisations, are not accessible to low-income populations. New types of vaccines are thus needed that can eliminate established infections and that are also more accessible to poorer communities (Hildesheim *et al.*, 2007; Giorgi, Franconi and Rybicki, 2010).

Cell-mediated rather than humoral immune responses are important for the clearance of established infections. It has been observed that spontaneous clearance and slow progression of HPV infections are associated with a strong cell-mediated immune response involving mainly T-helper type 1 cells and cytotoxic T-cells derived from CD4+ and CD8+ T-cells respectively (van der Burg and Melief, 2011).

The HPV E6 and E7 oncoproteins are essential for the onset and maintenance of malignancy thus they are unlikely to escape immune responses by mutation. They are also expressed constitutively and at high levels and therefore, represent ideal targets for the development of therapeutic vaccines against established HPV infections and lesions (Zur Hausen, 2002; Lin, Doolan, *et al.*, 2010; Morrow, Yan and Sardesai, 2013). Other proteins

useful for targeting of early viral infections are E1 (viral helicase) and E2 which are expressed at higher levels than E6 and E7 at very early stages before viral genome integration (Yang *et al.*, 1993; Šmídková *et al.*, 2012). Overall, an ideal therapeutic vaccine would target these proteins to induce strong tumour-specific T-cell type 1 and cytotoxic lymphocyte (CTL) responses able to kill infected and malignant cells (van der Burg and Melief, 2011).

There are currently no HPV therapeutic vaccines approved for use in humans. Nevertheless, there have been numerous and extensive studies that have generated promising candidates tested in animal models and in clinical trials. Diverse strategies have been explored, including live vector, nucleic acid, protein, whole cell and combinatorial vaccines. These strategies will be reviewed giving the advantages, disadvantages and some important examples of each.

1.2. Types of HPV therapeutic vaccine strategies

1.2.1. Live vector vaccines

Live vectors include bacteria such as *Listeria monocytogenes, Lactoccocus lactis, and Salmonella spp.* and viruses such as adenovirus and vaccinia virus, and can be used to present the antigen of interest on their surfaces. Live vectors can replicate inside the host cells, facilitating the spread of antigens. They can drive antigen presentation through both major histocompatibility complex (MHC) class I and class II pathways, therefore stimulating CD8+ cytotoxic T-cells and CD4+ helper T-cells, respectively, thus providing high immunogenicity (Lin, Doolan, *et al.*, 2010).

Bermúdez-humarán *et al.* (2005) were the first to demonstrate the therapeutic effect of a live recombinant bacterial vector against cancer. The vaccine, LL-E7, consisted of *L. lactis* strains presenting the HPV-16 E7 antigen on the cell wall surface and secreting a form of interleukin (IL)-12. Mice were immunized intranasally and showed full protection against tumour development induced by high doses of the HPV-16 E6 and E7-expressing cell line TC-1. When tumour-bearing mice were vaccinated with LL-E7 they developed an E7-specific cell-mediated response capable of containing tumour growth. There was also detection of some E7-specific antibody production in vaccinated mice. The researchers

showed that a live vector could be used for the development of a low-cost and non-invasive HPV vaccine with therapeutic as well as prophylactic activity.

Listeria monocytogenes (Lm) is of particular interest for vaccine development since it is able to act as a natural adjuvant. It infects macrophages without being captured by phagocytosis and is able to direct antigen processing via MHCI and MHCII pathways (Goossens *et al.*, 1995; Gunn *et al.*, 2001). The latter used two live-attenuated Lm-based vaccines that secreted the HPV-16 E7 only (Lm-E7) or E7 fused to a non-haemolytic portion of listeriolysin O (Lm-LLO-E7). Both vaccines elicited specific CTL responses but only the fusion protein elicited tumour regression in mice. Lm-LLO-E7 was tested in a Phase I clinical trial on 15 patients with advanced and metastatic cervical cancer. According to the authors this trial showed that the vaccine was relatively safe to use in humans: one patient showed partial regression of lesions while seven showed stable lesions, but six had progressed by the end of the trial (Maciag, Radulovic and Rothman, 2009). This was the first Lm-based vaccine tested in clinical trials and further studies are in progress in Phase I and II clinical trials (Advaxis, 2014).

Like bacterial live vectors, live viral vectors have been used in numerous studies. For example, in a Phase II clinical trial a recombinant vaccinia virus expressing HPV-16 and -18 E6 and E7 proteins (TA-HPV) was used. The trial showed antigen-specific CTL immunity in patients with high grade anogenital intraepithelial neoplasia. Of the 12 patients, five showed reduction of at least 50% of lesion diameter and one showed complete regression of lesions. Some patients showed increased cell-mediated responses while all patients showed humoral responses to the vaccinia virus. Overall, the vaccine showed positive results but needs to be further developed to increase its therapeutic efficacy (Baldwin *et al.*, 2003).

A highly attenuated strain of the vaccinia virus, the modified vaccinia virus Ankara (MVA), was used to develop an E2-based vaccine that was able to reduce overexpression of the *E6* and *E7* oncogenes and thus decrease HeLa cell-derived tumour growth in mice (Graham *et al.*, 2000). This vaccine has been tested in a Phase II clinical trial in women with CIN-1 and -2 lesions. The MVA-E2 vaccination eliminated lesions in 59% of the 54 patients and 41% showed up to 60% reduction in lesion size (García-Hernández *et al.*, 2006). Together, these studies show that vaccinia-based vaccines are safe and effective.

Gomes-Gutierres *et al.* (2007) used calreticulin (CRT), which enhances MHC-I and MHC-II antigen presentation (Basu and Srivastava, 1999), fused to HPV-16 E7 protein expressed by a replication-deficient adenovirus. This vaccine was referred to as Ad-CRT/E7. The study was conducted in mice and showed that high E7-specific T-cell activation and proliferation, interferon-γ (INF-γ) release and CTL responses could be achieved leading to both therapeutic and prophylactic activity of the vaccine.

Although live vectors are highly immunogenic, they have drawbacks since often a greater immune response is elicited against the vector than against the target proteins. Target-specific responses are mostly humoral rather than cell-mediated, while the opposite is preferable. There is a risk of toxicity, since the vectors actively replicate inside the host. Furthermore, there is also the possibility of pre-existing immunity due to previous exposure to the vector. These factors limit the use of live vectors for the presentation of potential HPV therapeutic vaccines (Lin, Doolan, *et al.*, 2010).

1.2.2. Subunit vaccines

Subunit vaccines are antigens delivered in the form of peptides or whole proteins. They are regarded as safer than live vector vaccines for they are present in the host cells transiently, decreasing the chances of toxicity.

Peptide vaccines are simple to produce, but because of their small sizes they have limited immunogenicity and need to be administered with strong adjuvants. They also need to match the receiver's human leukocyte antigen (HLA) type, which makes this strategy unpractical to implement in mass vaccinations (Kast *et al.*, 1994; Hung *et al.*, 2008; Moscicki, 2008).

To overcome the HLA limitation, synthetic long overlapping peptides (SLPs) that contain all or most possible epitopes of the target protein can be used. In a Phase II clinical trial, Kenter *et al.* (2009) demonstrated that vaccination of women positive for HPV-16 CIN-3 with a mixture of HPV-16 E6 and E7 SLPs, induced the activation of CD4+ T-cells and CTL responses. At the end of the trial, 79% of the 19 women vaccinated showed positive clinical responses and 45% had complete regression of the lesions 12 months after vaccination.

In another Phase II clinical trial, patients that had advanced or recurrent HPV-16 associated cervical cancer were treated with a mixture of 13 overlapping SLPs covering the entire sequences of HPV-16 E6 and E7. Of 16 patients tested, 56% showed vaccine-induced, HPV-specific T-cell proliferation, and 85% of the 13 patient tested showed vaccine-induced immune responses. Furthermore, those with a longer survival time showed a stronger response than those that lived relatively shorter after vaccination, as seen by increased lymphocyte stimulation and anti-tumour agents such as INF-γ and α, and IL production (van Poelgeest *et al.*, 2013). This suggests that this vaccine was able to elicit effective CTL responses that prolonged the survival of women tested.

These studies suggest that SLP-based vaccines are safe to use, since no severe adverse events were observed, and that they can be used for the treatment of HPV-related malignancies.

Unlike peptide-based vaccines, whole protein-based vaccines contain all possible CTL epitopes required to be recognised by any individual. However, some proteins are also poorly immunogenic and efficient responses require adjuvants or fusion to immunogenic carrier molecules (Kast *et al.*, 1994; Hung *et al.*, 2008; Moscicki, 2008). Furthermore, extracellular antigens are generally processed through the MHC-II pathway, which leads to the activation of CD4+ T-cells (Banchereau and Steinman, 1998). Although this is useful for therapeutic vaccines, CD8+ T-cell activation is optimal. For this, antigens can be fused to cell penetrating peptides or to molecules that stimulate antigen presenting cells, especially dendritic cells (DCs). This facilitates antigen processing through the MHC-I pathway that leads to the activation of CD8+ T-cells (Banchereau and Steinman, 1998; Moscicki, 2008; Lin, Doolan, *et al.*, 2010; Granadillo *et al.*, 2011).

Granadillo *et al.* (2011) developed a fusion-protein vaccine consisting of the HPV-16 E7 protein and a peptide derived from the *Limulus polyphemus* anti-lipopolysaccharide factor ($LALF_{31-52}$). This is a small and hydrophobic peptide that can penetrate cell membranes and that has immunomodulatory properties (Vallespi *et al.*, 2000). The fusion to HPV-16 E7 aided in the immunogenicity and antigen presentation of E7. In a prophylactic experiment, vaccinated mice were protected against E7-expressing TC-1 cell tumour challenges. In a therapeutic experiment, tumour-bearing mice showed tumour-specific immune responses and tumour regression. The tumour protection and tumour

regression were significantly higher when compared to control mice vaccinated with E7 or LALF alone. Furthermore, the anti-tumour effect of LALF-E7 was comparable in the presence or absence of an adjuvant. These results confirmed the potentiating effect of the cell-penetrating peptide, and showed that LALF-E7 is promising as a HPV therapeutic vaccine.

Tumours generally have immunosuppressive microenvironments which may inhibit antigen-specific CTL responses. This immunosuppression is mainly due to regulatory T-cells (Tregs), tumour-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) (Sica and Bronte, 2007). Toll-like receptor (TLR) agonists are immunopotentiators which have been shown to increase activation of cell-mediated responses and viral clearance (Shen *et al.*, 2015). Wick & Webb (2011) designed a broad-spectrum HPV therapeutic vaccine, Pentarix, consisting of the E7 sequences from the major hr-HPV types 16, 18, 31, 45 and 52 that account for more than 80% of cervical cancers. They previously showed that proteins can elicit a strong cell-mediated response extracellularly when accompanied by a TLR3 agonist, poly(I:C) (Wick *et al.*, 2011). Here they also tested the TLR9 agonist, CpG DNA. Mice vaccinated with Pentarix and either one of the adjuvants showed regression of TC-1-induced tumours after one week of vaccination. They had a complete tumour regression by week three post treatment and remained tumour-free for at least three months. Furthermore, they showed that cluster immunization of mice with Pentarix + Poly(I:C) increased the cell-mediated responses in mice as well as the rate of tumour regression compared to mice vaccinated only once. Their findings suggested that the vaccine was able to elicit a strong enough cell-mediated response capable of overcoming the immunosuppressive tumour microenvironment. Pentarix could be used as a broad spectrum treatment for a variety of HPV-related pre- and cancerous conditions (Wick and Webb, 2011). A patent for Pentarix was filed in 2012 (*Pentarix. Novel HPV Therapeutic Cancer Vaccine*, 2012) however, no information on future clinical trials was available.

To further enhance the potential of subunit vaccines, self-adjuvating lipoimmunogens have been linked to therapeutic vaccine candidates, such the case of rlipo-E7m, which consists of a mutant HPV-16 E7 protein fused to a lipid moiety that has TLR2 agonist properties. The vaccine was administered to mice together with the TLR9 agonist, CpG

DNA. This study showed that rlipo-E7m in combination with the TLR9 agonist were able to eradicate large, established solid TC-1 tumours suggesting an enhanced CTL response and decrease in the immunosuppressive conditions in the tumours (Chang *et al.*, 2014).

Overall, protein-based vaccines have shown promise for future HPV therapeutic vaccine development. They have the advantage over peptide-based vaccines of containing all possible epitopes for the induction of an effective immune response, and in combination with immunopotentiators they look very promising for future vaccine designs. However, they have not progressed into Phase II clinical trials, reflecting that development lags compared to peptide-based vaccines and to other strategies.

1.2.3. Nucleic acid vaccines

Another approach to produce HPV therapeutic vaccines is the use of nucleic acids - DNA or RNA, encoding for the target antigen. These vaccines are delivered intradermally by injection followed by electroporation (EP), by gene gun or laser therapy. The target antigen is then expressed in the transfected cells. This is advantageous, as antigens are produced intracellularly and are therefore processed by the MHC-I pathway. Unlike live vector vaccines, nucleic acid vaccines do not contain or produce structural proteins which can skew the immune response towards a humoral instead of a cell-mediated response. Therefore, they can be more effective and can be administered repeatedly.

1.2.3.1. DNA vaccines

Part of the attractiveness of DNA vaccines is their relatively low cost of production. The nature of DNA makes these vaccines less heat labile and thus easier to be stored and transported. They are also more stable and provide a more continuous expression of the target antigen than RNA vaccines or subunit vaccines. Therefore, DNA vaccines could overcome some limitations of other vaccine strategies (Hung *et al.*, 2008). DNA vaccines for HPV have been extensively studied and described, with a number of clinical trials carried out. Overall they have proven to be safe, and cause no severe side effects. There are also many strategies to improve DNA vaccine efficacy, immunogenicity and potency (Lin, Roosinovich, *et al.*, 2010).

To increase their immunogenicity, Massa *et al.* (2008) genetically fused the plant potato virus X coat protein (PVX-CP) to a HPV-16 E7 mutant protein. The mutant E7, contained

three point mutations at the pRb binding site where native residues were replaced by Glycine residues, hence the name E7GGG. This abolished the transformative properties of the protein (Smahel *et al.*, 2001). The PVX-CP was chosen as a safe carrier particle for the presentation of the E7GGG antigen. They showed that mammalian cells transfected with the E7GGG-CP fusion DNA could drive protein expression and that proteins were degraded through the proteasome pathway, which suggested that the MHC-I antigen processing pathway was preserved. Mice vaccinated with the E7GGG-CP fusion DNA showed E7-specific humoral as well as cell-mediated immunity, decreased tumour growth rates and greater survival rates than mice vaccinated with the E7GGG DNA or than the placebo groups. This study showed that CPs can be used to enhance the immunogenicity of E7-based vaccines and that a plant virus CP could be a safer option compared to other strategies (Massa *et al.*, 2008).

Garcia *et al.* (2004) synthesized ZYC-101a, a DNA-based vaccine encoding the HPV-16 and -18 E6 and E7 antigens fused to a CTL antigen. The ZYC-101a vaccine was used in a Phase III trial, in women with CIN-2 and CIN-3. Complete or partial regression of the lesions was observed in 41% of the vaccinated women. However, further safety assessment of the vaccine is necessary. Nevertheless, the fact that the vaccine progressed to a Phase III clinical trial, and the results obtained, show the potential of DNA vaccines.

In order to enhance antigen processing and presentation by DCs, Kim *et al.* (2014) designed a DNA vaccine that co-expressed the HPV-16 and -18 antigens E6 and E7, and the Fms-like tyrosine kinase-3 ligand (Flt3L). The latter is a known DC activator and is commonly used in anti-cancer vaccine designs (Lynch *et al.*, 1997; Barnard *et al.*, 2012; Riediger *et al.*, 2013). The vaccine, GX-188, was tested in a Phase I clinical trial, where women with HPV-16 and -18 related CIN-3 were enrolled. Patients were vaccinated intramuscularly, followed by EP to enhance uptake of the vaccine. GX-188 elicited HPV-16 and -18 E6 and E7 specific cell-mediated responses including IFN-γ-secreting CD8+ and CD4+ T-cells, as well as polyfunctional and proliferative CD8+ T-cells. This strong response led to complete regression of lesions and viral clearance in 67% of patients after 12 weeks of treatment, and 78% of patients after 36 weeks. This study showed that GX-188 is safe and effective, and could be used as a non-invasive treatment for HPV-associated severe lesions and cancer. Furthermore, it showed the immunopotentiator effect of Flt3L.

Sun *et al.* (2015) tested the effects of different routes of vaccination and the effect of EP on DNA vaccines in a pre-clinical study. Mice were vaccinated intraperitoneally (IP) or intravaginally (Ivag), with a HPV-16 E7 sequence fused to CRT, followed by EP. Overall, EP increased the CTL responses induced by both routes of vaccination compared to the vaccination without EP. However, the Ivag + EP vaccination was able to elicit a strong E7-specific local and systemic CTL response, shown by the higher number of IFN-γ secreting CD8+ T-cells in cervicovaginal cells and splenocytes; while a strong CTL response of IP + EP vaccinated mice was only shown in splenocytes and not in cervicovaginal cells. In the same way, 60% of TC-1 tumour-bearing mice vaccinated Ivag + EP survived for at least 80 days post challenge, while mice in the control and IP + EP vaccination groups died before 40 days post challenge. This study showed that EP can enhance the immunogenicity of DNA-based vaccines and that the location of vaccination also plays a role in the vaccine efficacy.

So far, the most successful HPV therapeutic vaccine was a synthetic DNA encoding for HPV-16 and -18 E6 and E7 proteins. The vaccine, VGX-3100, was tested in a Phase IIb clinical trial (Trimble *et al.*, 2015), where CIN-2/3-positive women were vaccinated intramuscularly followed by EP. In the VGX-3100 group, 49.5% of the 105 patients had lesion regression, compared to 30.6% of the 36 patients in the placebo group. More importantly, 40.2% of the patients had lesion regression accompanied by viral clearance in the VGX-3100 group, compared to 14.3% in the placebo group. The study also showed that the vaccine elicited an E6-specific cell-mediated response that was reflected by lesion regression, and E7-specific responses which, however, did not translate clinically. Furthermore, patients also showed anti-E6 and anti-E7 humoral responses. Overall, VGX-3100 is the most advanced HPV therapeutic vaccine in terms of clinical trials, and could possibly be the first approved vaccine for the treatment of HPV-related malignancies. It is expected to enter phase III clinical trials in 2016 (Inovio Inc., 2016).

1.2.3.2. RNA vaccines

DNA vaccines are stable and easily produced but they cannot proliferate and move from cell to cell, which may decrease their immunogenicity. Additionally, they may impose a risk of integrating in the host genome which could possibly cause cell transformation. Although RNA vaccines have similar limitations as DNA vaccines and are less stable, RNA

replicon-based vaccines have advantages over DNA-based ones. These can replicate in various cell types, improving the propagation of the vaccine antigen. If derived from RNA viruses replicating via RNA intermediates, they also lack the ability to integrate into the host's genome, decreasing the risk of cellular transformation. In order to counteract the instability of RNA vaccines, a suicidal DNA vaccine encoding an RNA replicon can be used. This DNA would be transcribed into the RNA replicon which would then amplify the expression of the target antigen. The fact that the DNA is suicidal, i.e. it eventually induces cell apoptosis, decreases the chances of it becoming integrated into the host's genome (Hung *et al.*, 2008; Lin, Doolan, *et al.*, 2010; Lin, Roosinovich, *et al.*, 2010). These approaches have shown specific T-cell responses and antitumour effects (Berglund *et al.*, 1998; Herd *et al.*, 2004; Hung *et al.*, 2008). Nevertheless, RNA vaccines are more complicated to produce and more expensive. Additionally, they have not progressed into clinical trials. For these reasons they will not be further discussed here.

1.2.4. Cell-based vaccines

Cell-based vaccines involve the introduction of the major antigen-presenting cells, DCs or tumour cells, loaded with important antigens or epitopes that are needed to induce a strong tumour-specific CTL response.

The use of DCs is attractive because these cells are the major antigen presenting cells that can activate CD4+ and CD8+ T-cells (Banchereau and Steinman, 1998), bypassing the need to enhance antigen visibility that is faced by protein or DNA vaccines. Ferrara *et al.* (2003) showed the safety of this approach in a clinical trial where cervical cancer patients were vaccinated with DCs loaded with HPV-16 or HPV-18 E7 proteins. Four of the 11 patients showed E7-specific CTL responses. However, these results were not reflected clinically, maybe because of the advanced stages of the malignancies in these patients (Ferrara *et al.*, 2003). Handling human ex-vivo cells requires high technical competence and DC-based vaccine production is labour-intense, time consuming and costly, making this strategy impractical to implement in large scale vaccinations (Lin, Roosinovich, *et al.*, 2010; Morrow, Yan and Sardesai, 2013).

Using cancer cells allows the determination of useful antigens that could elicit a strong cell-mediated immunity. However, in the case of HPVs, these antigens have already been

identified; therefore, the cancer cell strategy presents limited advantages. Furthermore, it has similar limitations as the DC-based strategy with the addition that it needs to be a personalized vaccine, since tumour cells vary from patient to patient. Furthermore, there are safety concerns regarding the idea of inoculating patients with cancer cells (Lin, Roosinovich, *et al.*, 2010; Morrow, Yan and Sardesai, 2013).

Overall, cell-based vaccines do not seem to be practical for the development of a cost-effective and wide-spread treatment of HPV-related malignancies.

1.2.5. Combining different therapeutic strategies

The diversity of current therapeutic vaccine candidates for HPV-related malignancies offers an opportunity to combine some of the strategies and to better exploit their potentials.

One such strategy is the prime-boost regime, in which the immune system is primed with, for example, a DNA-based vaccine and later boosted with a protein-based vaccine. Bissa *et al.* (2015), tested three prime-boost strategies in a mouse tumour model: there were DNA/DNA, DNA/viral-vector and viral-vector/viral-vector regimens, all expressing a modified version of the HPV-16 E6. In a therapeutic setting, 80% of the mice in the DNA/DNA group remained tumour-free while surprisingly only 20% of mice remained tumour-free in the other two groups. In a preventative setting, mice showed a delayed development of TC-1 cell-induced tumours. Although the humoral response in these mice was very low, there were specific cell-mediated responses which could have accounted for the delayed tumour growth, highlighting the importance of this type of immunity in terms of cancer treatment. This study also demonstrated the potential of DNA-based vaccines to prime the immune system and allow for an enhanced cell-mediated immune response upon boosting with DNA, viral-vector or protein-based vaccines.

Another study that showed the impact of a DNA vaccine priming followed by a boost was that done by Radaelli *et al.* (2012). They immunized mice with an E7GGG DNA followed by three boosts with an E7GGG-expressing recombinant fowlpox virus live vector. This regimen resulted in E7-specific cell-mediated immune responses that were greater than that elicited by a live vector/live vector prime-boost regimen, while the humoral immune responses were comparable and low in both groups. The increased cell-mediated immune

response in the DNA/live vector group was accompanied by delayed tumour development compared to the live vector/live vector and the placebo groups.

To optimize vaccination impact, chimaeric vaccines that have both prophylactic and therapeutic effects have been studied. Ideally they would be able to induce both humoral and cell-mediated responses. De Jong *et al.* (2002) used a fusion protein consisting of HPV-16 L2, E6 and E7 (TA-CIN). Vaccinated mice showed production of E6- and E7-specific antibodies as well as T-cell immune responses. In a Phase II trial, Fiander *et al.* (2006) vaccinated 29 women with VIN-2 and VIN-3 with TA-CIN followed by a recombinant vaccinia virus encoding HPV-16 and -18 E6 and E7. Overall this prime-boost method resulted in antibody production as well as partial or complete regression of lesions. This further confirmed that prime-boost regimes can be useful in amplifying immune responses, and that combined to chimaeric vaccines, a prophylactic as well as a therapeutic effect can be achieved. This is relevant, as only one vaccine would be needed for preventing and treating HPV-related malignancies.

Pseudovirions (PsVs) consisting of a HPV VLP containing a plasmid DNA encoding for the oncogenic proteins E6 and E7 could be used as a way of delivering DNA vaccines. HPV PsVs would have the advantage of specifically targeting HPV-prone tissues and efficiently delivering the vaccine intracellularly, where MHC-I pathways would be favoured, increasing the likelihood of a strong CTL response. HPV PsVs have been used to deliver gene therapies for different types of diseases, including ovarian cancer (Hung *et al.*, 2012) and respiratory syncytial virus (Graham *et al.*, 2010; Çuburu *et al.*, 2012; Kines *et al.*, 2015), and have been shown to generate higher antigen-specific CD8+ T cell immunity than naked DNA (Peng *et al.*, 2011). HPV PsV-based vaccines could be used to also elicit protective immunity, making it a chimaeric vaccine. And, if different HPV types were used for the structural component of the PsVs and for the DNA component, a broad HPV vaccine could be generated.

Another strategy is the combination of already established therapeutic treatments such as chemo- and radiotherapy with candidate vaccines to enhance the effect of one another. For example, in a Phase II clinical trial, Daayana *et al.* (2010) tested the chimaeric vaccine TA-CIN in combination with imiquimod, an immunomodulatory topical agent. Patients were treated with imiquimod for 8 weeks prior to three immunizations with TA-

CIN. Although the regimen did not alter the natural humoral response of the patients, by week 52 after the last immunization, 63% of patients had lesion clearance on histology, 36% showed HPV-16 clearance and 79% were symptom-free. Importantly, patients whose lesion cleared had increased local numbers of CD4+ and CD8+ T-cells compared to non-responders.

Chuang *et al.* (2009) tested the combination of apigenin, a chemotherapeutic agent that enhances tumour cell apoptosis with a DNA-based vaccine encoding for the HPV-16 E7 protein fused to the *Mycobacterium tuberculosis* HSP70. They showed that the combinatorial vaccine elicited higher E7-specific CTL responses than when apigenin or E7-HSP70 were administered alone. Mice vaccinated with the combinatorial vaccine showed tumour growth rates much lower than the other groups. In the same way, 100% of mice in the combinatorial vaccine group survived 63 days after challenge with TC-1 cells, while only 20% survived in the E7-HSP70 DNA-based vaccine group and none in the apigenin group. At the end of the experiment, 60% of mice in the combinatorial group survived while none survived in the other groups. This study showed the positive impact that combining different therapeutic strategies can have in anti-tumour treatments (Chuang *et al.*, 2009).

Finally, another possible strategy would be to reduce the immunosuppressive conditions present in the tumour microenvironment. As mentioned before, this environment is influenced by Tregs, MDSCs, TAMs between other factors. By blocking the effect of these cells and factors, the potential of current therapeutic vaccine candidates could be enhanced (Pittet, 2009; Lin, Roosinovich, *et al.*, 2010; Morrow, Yan and Sardesai, 2013). A summary of the mechanism of action of an ideal HPV therapeutic vaccine is shown in Figure 1.3.

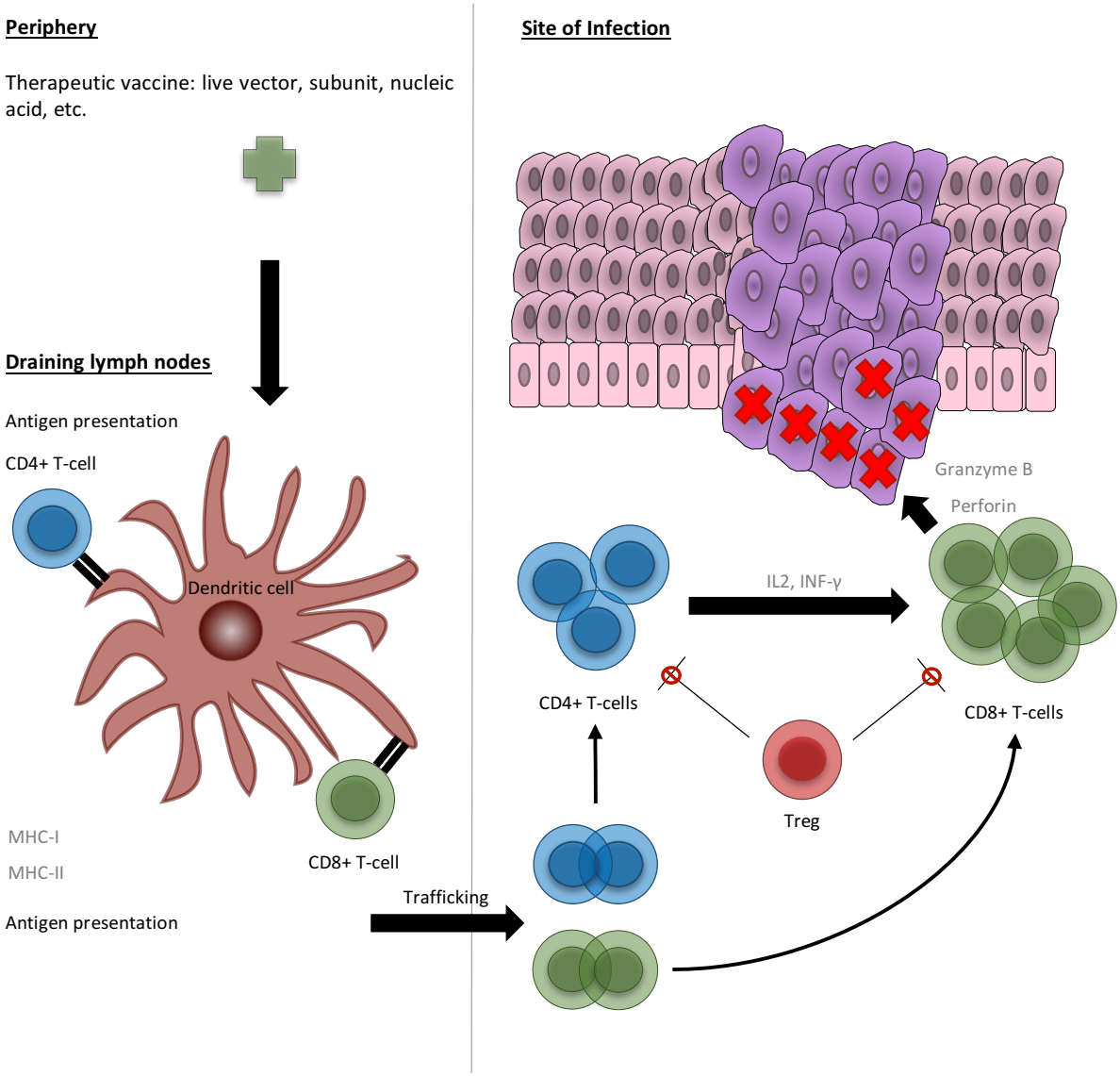

Figure 1.3. Mechanism of an ideal therapeutic vaccine against HPV-related malignancies. An ideal HPV therapeutic vaccine would elicit a strong cell-mediated immune response where CD4+ T-cells would provide support to CD8+ T-cells by secreting cytokines such as IFN-γ and IL2 labelling infected and malignant cells. Cytotoxic CD8+ T-cells would eliminate infected cells by secreting high amounts of granzyme B and perforin which lead to cell apoptosis. The response would be effective even in the presence of immunosuppressive cells such as Tregs. Adapted from Morrow *et al.* (2013).

1.3. HPV therapeutic vaccines produced in plants

Developing countries carry the greatest burden of HPV infections and malignancies due to their lack of resources to implement efficient vaccination and screening programmes. In these areas, infections are generally detected at a progressed stage, and treatment and follow-ups are difficult to obtain. Despite the current success of several HPV therapeutic vaccine candidates, including numerous clinical trials, there remains the concern that

conventional expression methods might result in expensive products (Giorgi, Franconi and Rybicki, 2010; Rybicki, 2014).

Plants represent an attractive alternative to conventional expression systems based on bacterial, yeast, mammalian and other cell cultures. Plants are not vulnerable to animal pathogen contamination like mammalian or insect cells, they do not produce endotoxins like bacterial cultures, and they are able to post-translationally modify proteins in a similar way to mammalian cells, making them efficient, safer and potentially far more cost-effective than conventional expression systems. Furthermore, they are highly scalable as they can be grown to agricultural scale in fields, greenhouses or in vertical farming units (Yusibov *et al.*, 2006; Rybicki, 2009; Merlin *et al.*, 2014).

Plants can be modified to express heterologous proteins in a transgenic or transient manner. In transient expression, unlike in transgenic expression, the gene of interest does not become integrated into the plant's genome. In general, transient expression in plants has proven to be a more efficient and generally higher yielding albeit less cost-effective method than transgenic expression. Additionally, transient expression facilitates biocontainment since genetically modified plants are not generated and no genetic material such as pollen can be transferred to adjacent plants. Plants can be transiently modified by *Agrobacterium*-mediated gene delivery of *Agrobacterium*-based expression vectors or viral expression vectors, using vacuum or syringe infiltration. This method is highly efficient and expression can be obtained as soon as 3 days post infiltration (Gleba, Klimyuk and Marillonnet, 2007; Rybicki, 2009; Hefferon, 2014).

Our group was one of the first to start developing HPV vaccines in plants, in the 1990s (Rybicki, 2009; Hefferon, 2013). Besides Biemelt *et al.* (2003), we provided one of the first proofs that plant-produced and L1-based papillomavirus vaccines could succeed by expressing the HPV-16 L1 protein in transgenic *Nicotiana tabacum* and *Solanum tuberosum* (Varsani *et al.*, 2003; Warzecha *et al.*, 2003), or the L1 protein of cottontail rabbit papillomavirus (CRPV) transiently in *Nicotiana benthamiana* via the use of a Tobacco mosaic virus vector (Kohl *et al.*, 2006). In the latter, vaccinated rabbits did not detectably produce neutralizing antibodies, but were completely protected against CRPV (Kohl *et al.*, 2006). This work was followed by numerous successful studies including optimization of HPV-16 L1 antigen expression in terms of codon usage and cell

compartment targeting (Maclean *et al.*, 2007), as well as the development of a novel geminivirus-derived replicating DNA viral vector that further enhanced expression levels (Regnard *et al.*, 2010).

Although most of the HPV vaccine development in plants has focused on second generation prophylactic vaccines, therapeutic vaccines have also been investigated more recently. One of the first groups to attempt this, used a potato virus X (PVX)-derived vector to transiently express HPV-16 E7 in *N. benthamiana* (Franconi *et al.*, 2002). They reported E7 expression levels of 3-4 mg/kg biomass. Leaf crude extracts were used to immunize mice, which produced E7-specific antibodies as well as E7-specific CTL responses. Mice showed both Th-1 and Th-2 immune responses with the Th-1 response being higher. Forty per cent of vaccinated mice survived tumour challenges with minimal tumour growth over the 52 days of the experiment. The same group was able to enhance the expression levels of E7 by 5 times by targeting it to plant secretory pathways, and the increased levels of E7 in leaf extracts enhanced the tumour protection in mice. Total tumour protection was observed in 80% of mice after 50 days of vaccination. They showed that a therapeutic HPV vaccine candidate could be expressed in plants in a biologically relevant way, although expression levels needed to be optimized. Furthermore, they suggested that plant material could behave as a natural adjuvant since stronger immune responses were seen for mice vaccinated with leaf extracts than in the positive control group vaccinated with an *E. coli*-derived E7 and the Quil A adjuvant (Franconi *et al.*, 2006). The immunomodulatory properties of *N. benthamiana* leaf extracts were later demonstrated by Di Bonito *et al.* (2009), who tested the effect of leaf extracts containing E7 on human monocyte derived DCs. The leaf extracts were able to prime these cells and induce E7-specific cytotoxic activity.

In work reported by Massa *et al.* (2007), the HPV-16 E7-based mutant E7GGG was linked to the *Clostridium thermocellum* beta-1,3-1,4-glucanase (LicKM) and expressed from the PVX-based vector in *N. benthamiana*. The LicKM-E7GGG vaccine had decreased immunogenicity, but when administered with the Quil A adjuvant it elicited strong humoral and cell mediated immunity that could prevent tumour development in 100% of vaccinated mice. They were able to purify LicKM-E7GGG to 99% purity at a yield of 100 mg/kg biomass, representing a 50% recovery rate, using immobilized metal-ion affinity

chromatography and gel filtration. They also scaled up the expression from 50 g to 1 kg of biomass with very little variation on the purification results. This showed that plant-derived LicKM-E7GGG and in general, plant-derived E7-based HPV therapeutic vaccines, can be successfully expressed, purified and scaled-up to commercially viable quantities (Buyel *et al.*, 2012).

Recently, the E7GGG candidate was expressed in chloroplasts of the well characterized unicellular alga *Chlamydomonas reinhardtii* (Demurtas *et al.*, 2013). Interestingly, they report the expression of a soluble variant of the E7 protein. This enhances its downstream processing and increases its potential to be a cost-effective product compared to other E7 derived proteins that are normally very insoluble (Giorgi, Franconi and Rybicki, 2010). Sixty per cent of immunized mice remained tumour-free at the end of the experiment and the mice that developed tumours showed delayed tumour appearance compared to control mice vaccinated with an *E. coli*-derived E7GGG, showing that this vaccine candidate expressed in an algal system retained its therapeutic relevance (Demurtas *et al.*, 2013).

Recently, Whitehead *et al.* (2014) expressed another HPV-16 E7 mutant protein in *N. benthamiana*, that had its pRb-binding ability eliminated by shuffling the protein sequence while retaining all possible CTL epitopes. This vaccine candidate, 16E7SH, was previously tested as a DNA-based vaccine with successful protective and therapeutic results in mouse models (Öhlschläger *et al.*, 2006). Here, 16E7SH was linked to the Zera® signal peptide (ERA Biotech, Spain), derived from the maize γ-zein protein, which can naturally form large membrane-bound protein bodies. The Zera®-16E7SH fusion protein was expressed at very high levels, 1.1 g/kg biomass, while when expressed without the Zera® peptide, 16E7SH was not detectable. Furthermore, Zera®-E7SH protein bodies were easily purified to a yield of 50 mg/kg biomass. Vaccinated mice showed strong cellular immunity similar to that elicited by the DNA-based counterpart, as determined by Granzyme B levels and IFN-γ levels. This was independent of the presence of incomplete Freud's adjuvant, showing a potential immunostimulatory role of the Zera® peptide. Mice were protected against tumours in prophylactic experiments, and showed tumour regression in therapeutic experiments in a similar way as mice immunized with the DNA-

based counterpart. These results were very promising and have the potential to be further enhanced by a DNA/fusion protein prime-boost regimen (Whitehead *et al.*, 2014).

Finally, our group has recently expressed HPV-16 PsVs in *N. benthamiana,* capable of infecting mammalian cells and efficiently delivering a functional reporter-expressing plasmid (Lamprecht *et al.*, 2016). This opens the possibility of producing plant-derived HPV PsVs encoding for therapeutic DNA vaccines, which would be considerably more cost-effective than conventional PsVs that are currently produced in mammalian cells. Therefore, it is evident that plant expression systems can be used to produce biologically relevant products with potential important pharmaceutical applications.

1.4. Conclusions

The future of HPV therapeutic vaccine development will probably be more focused on DNA and protein-based strategies, since these have been most successful and safer than use of live vaccine vectors. Protein and DNA-based vaccines are also simpler to produce, therefore less costly compared to the other strategies. They will probably be designed to incorporate molecules that improve CD8+ T-cell responses by facilitating uptake by professional antigen presenting cells by fusion to cell penetrating peptides, TLR-3 and -9 agonists, and engineered to overcome the immunosuppressive conditions in the tumour microenvironment. Furthermore, they will probably be more extensively studied in combination with other cancer treatments.

Until now there has been no therapeutic vaccine commercially released for the treatment of HPV infections and related malignancies. Nevertheless, there are numerous successful pre-clinical results and ever-increasing clinical results for a variety of candidate vaccines. The most promising, in terms of clinical trial progression, is the Inovio VGX-3100. With the increased accumulation of knowledge due to the detailed study of cancer pathways and characterisation of immune responses (especially cell-mediated), the design of therapeutic vaccines can only improve. More clinical studies will be done, and hopefully a HPV therapeutic vaccine is not too far in the future, even if it is one that only improves the life of those affected by the virus.

1.5. Project aims and objectives

The first aim of this study was to optimize the expression of the candidate HPV therapeutic vaccine, LALF-E7 (Granadillo *et al.*, 2011), in *Nicotiana benthamiana* leaves. This fusion protein was previously expressed in plants, but was difficult to detect and could not be purified (M. Granadillo and R. Lamprecht, unpublished data). In order to increase the expression of LALF-E7 I used our in-house self-replicating expression vector pRIC3.0 (Regnard *et al.*, 2010), which was shown to increase expression levels by increasing gene copy numbers. To further increase LALF-E7 accumulation in *N. benthamiana* leaves, I targeted it to the chloroplasts. We hypothesised that targeting proteins to cell compartments could increase their yields by increasing their stability and decreasing proteolytic degradation. Furthermore, I investigated the effect of silencing suppressors on the expression of LALF-E7.

The second aim was to determine the subcellular localization of LALF-E7 in *N. benthamiana* leaves by fluorescence microscopy. For this I fused it to the enhanced green fluorescent protein (EGFP). I wanted to see whether LALF-E7 was indeed targeted to the chloroplasts and if it remained associated to these organelles. Since LALF is a cell membrane-penetrating peptide, it could be possible that LALF-E7 would be associated with the plants' cell membranes or that it could in fact exit the plant cells.

The third aim was to scale-up the production of LALF-E7 and develop an extraction and purification strategy for it. Different crude extraction strategies were tested to pre-purify the plant-derived LALF-E7. This was followed by an automated affinity chromatography step using the ÄKTA system.

Finally, I aimed at comparing the plant-derived LALF-E7 to the *E. coli*-derived counterpart for its ability to elicit tumour regression in C57BL/6 mice. I hypothesized that the plant-derived LALF-E7 could drive tumour regression in a comparable manner as the *E. coli*-derived LALF-E7.

Chapter 2: Expression optimization of LALF-E7 in *Nicotiana benthamiana* leaves

2.1. Introduction

Recombinant proteins have a wide range of applications in the industrial, biomedical and biological research fields. There is a high demand for recombinant proteins, which in turn requires efficient and scalable production systems. Current methods to produce recombinant proteins are based on mammalian, insect or microbial cell cultures, and rely on the use of bioreactors and expensive growth media. As mentioned in Chapter 1, plants provide an alternative platform for the expression of recombinant proteins which is potentially more cost-effective than conventional methods, and highly scalable (Yusibov *et al.*, 2006; Sainsbury *et al.*, 2008; Rybicki, 2009; Pogue and Holzberg, 2012).

Plants can be modified by *Agrobacterium tumefaciens*-mediated gene delivery, where bacterial cultures transformed with an appropriate expression vector are vacuum- or syringe-infiltrated into the plant leaves, a process termed agroinfiltration. *A. tumefaciens* is a plant pathogen that is able to deliver genetic information into the plant cells nuclei via its Ti-plasmid. This plasmid contains a region flanked by 25 bp direct repeats, called the T-DNA. Any DNA sequence contained between these borders is transferred into the plant cells nuclei by a bacterially-mediated transport process involving linear single-stranded T-DNA, after which it can be integrated into the host's genomic DNA. By cloning an expression cassette into the T-DNA, transgenic plants can be generated that express a protein of interest (Kapila *et al.*, 1997; Zupan *et al.*, 2000; Gelvin, 2003).

However, not all T-DNAs become integrated: probably more often, T-DNA remains as a linear or circularised dsDNA episome. These episomal T-DNAs can drive gene expression in a transient way. This has been preferentially exploited as a more rapid and simple way of expressing recombinant proteins in plants. Transient expression is not influenced by insertion position and does not require the challenging techniques faced when regenerating transgenic plants (Kapila *et al.*, 1997; Zupan *et al.*, 2000; Gelvin, 2003). Transient expression also facilitates biocontainment since genetically modified plants are

not generated and no genetic material can be transferred to other plants via, for example, pollen (Gleba *et al.,* 2007; Hefferon, 2014; Rybicki, 2009; Yusibov *et al.,* 2006).

The choice of expression vectors plays an important role in the expression of recombinant proteins in plants. The use of plant viral vectors has often been favoured in transient expression over other methods, as they offer various advantages. Plant viruses have small genomes which allow them to be easily manipulated, especially in the case of the circular single stranded (ss) DNA geminiviruses. Plant infection with these viral vectors is simple and inexpensive, and a wide range of hosts can potentially be used (Cañizares, Nicholson and Lomonossoff, 2005; Rybicki and Martin, 2011). *Agrobacterium*-mediated delivery of the viral vectors encoded within the *Agrobacterium* T-DNA benefits from the advantages of three important biological systems: the *Agrobacterium* provides transfection efficiency and systemic delivery of the viral construct; the viral vector provides speed to the process since it contains elements that can enhance the expression of target genes encoded in its backbone; and the plant provides the correct processing of the proteins of interest as well as low production costs (Gleba *et al.,* 2007; Hefferon, 2012; Hefferon, 2014).

In this study I used pRIC3.0, a proprietary replicating plant expression vector. This vector is based on the mild strain of the bean yellow dwarf virus (BeYDV), a ssDNA geminivirus belonging to the genus *Mastrevirus*. It consists of the BeYDV genome between two viral large intergenic regions (LIRs) within which two copies of the cauliflower mosaic virus (CaMV) 35S constitutive promoter and the gene of interest replace the viral capsid protein and movement protein genes. This expression cassette is placed between the left and right borders of the *A. tumefaciens* T-DNA and includes the viral replication-associated genetic elements (Rep and RepA) expressed in *cis* from its backbone. It also contains the *bla* gene for antibiotic selection, as well as origins of replication for *E. coli* and *Agrobacterium.* Upon infiltration the T-DNA is released and re-circularizes forming a replicon. Using the host's replication machinery and specially the Rep and RepA proteins, the replicon is continuously amplified by rolling circle replication. In this way, a high copy number of the gene of interest is obtained, which leads to an increase in expression levels. Regnard *et al.* (2010) and Rybicki and Martin (2011) give a full description of this vector and discuss the use of geminiviruses and other ssDNA plant viruses as plant expression vectors.

pRIC was originally compared to the non-replicative Ti plasmid-based vector pTRAc (Regnard *et al.*, 2010). The pTRAc vector is similar to the pRIC vector, except it does not contain the BeYDV replicative elements. Instead, the gene of interest and the two copies of the CaMV 35S promoter are in between the left and right borders of the T-DNA, resembling a Ti-plasmid (Maclean *et al.*, 2007), as seen in Figure 2.1.

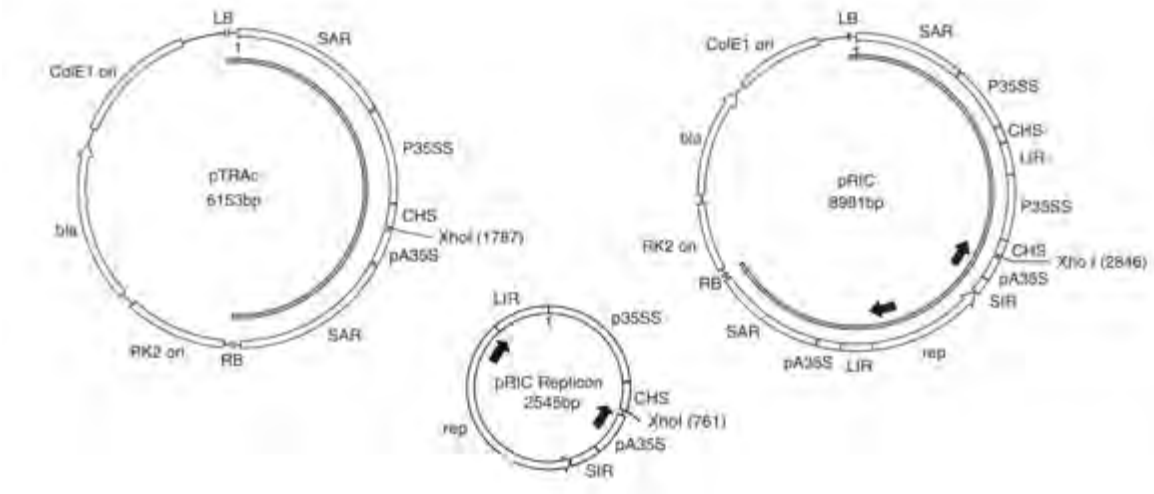

Figure 2.1. Comparison between the non-replicative pTRAc and the replicative pRIC expression vectors. The circularized pRIC replicon released from T-DNA is shown on the bottom. ColE1 ori, origin of replication for *E. coli*; RK2 ori, origin of replication for *A. tumefaciens*; *bla*, ampicillin/carbenicillin resistance gene; LB and RB, left and right borders for T-DNA integration; SAR, scaffold attachment region of the tobacco Rb7 gene; P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; CHS, chalcone synthase 5'-untranslated region; pA35S, CaMV 35S polyadenylation signal; LIR, BeYDV long intergenic region; SIR, BeYDV short intergenic region; rep, BeYDV rep gene. The T-DNA transferred into the plant cells is shown by the grey bar inside the plasmid maps. Kindly provided by Regnard *et al.* (2010).

Regnard *et al.,* (2010) obtained higher cytoplasmic expression levels of HPV-16 L1 and two other non-related proteins than previously described (Maclean *et al.,* 2007 and Meyers *et al.,* 2008). HPV-16 L1 yields were 1.6 fold higher when using pRIC (550 mg/kg biomass) than when using the pTRAc (337 mg/Kg biomass). However, the increase in expression levels was far smaller than the increase in gene copy number, which was 100 fold for HPV-16 L1.

Subcellular localization can have a dramatic effect on expression levels of recombinant proteins. It has been shown in numerous cases that targeting proteins not only to the chloroplasts, but also to the endoplasmic reticulum (ER) or the apoplast, can increase their accumulation (Streatfield *et al.*, 2003; Maclean *et al.*, 2007; Meyers *et al.*, 2008; Karg and Kallio, 2009). Significantly high levels of HPV-16 L1, up to 11% of total soluble proteins

(TSP) using the pTRAc vector, were obtained when targeting proteins to the chloroplasts compared to cytoplasmic localization. This was done using the chloroplast transit peptide (cTP) derived from the potato *rbcS1* gene of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) small subunit. It is known that targeting proteins to cell compartments increases protein accumulation possibly because proteins may be less prone to degradation than in the cytoplasm (Maclean *et al.,* 2007). It was therefore hypothesized that expression by the pRIC vector could be further enhanced by targeting proteins to the chloroplasts of the plant cells, as these might tolerate greater levels of accumulation.

As mentioned in Chapter 1, the expression of the HPV-16 therapeutic vaccine candidate LALF-E7 (Granadillo *et al.*, 2011) in *N. benthamiana* was previously attempted using pTRAc and the cowpea mosaic virus-derived pEAQ-*HT* expression vectors, but with low success (M. Granadillo and R. Lamprecht, unpublished data). In this project I aimed at increasing the expression levels of LALF-E7 by using the self-replicating vector pRIC3.0 and by targeting it to the chloroplasts using the *rbcs1*-cTP.

It is also well known that posttranscriptional gene silencing (PTGS) negatively affects the expression of recombinant proteins in plants. PTGS is a mechanism of defence against foreign RNAs in eukaryotes. In plants, PTGS is an anti-viral defence mechanism. Foreign RNA forms such as double-stranded and replication intermediate RNAs are recognized by the plant host as aberrant RNAs. The plant responds by producing homologous small interfering RNAs (siRNAs). This leads to a decrease in protein expression. Several plant viruses have developed silencing suppressor proteins to counteract the host's PTGS by binding to and blocking the effect of siRNAs (reviewed by Qu and Morris, 2005). Two well-known silencing suppressors are the tomato spotted wilt virus NSs protein (Takeda *et al.*, 2002) and the tomato bushy stunt virus P19 protein (Voinnet *et al.*, 2003). The expression of recombinant proteins was dramatically increased when plants were co-infiltrated with such silencing suppressors. Therefore, I also investigated the effect of co-expression of silencing suppressors on the accumulation levels of LALF-E7 in *N. benthamiana.*

2.2. Materials and Methods

2.2.1. Bacterial strains and culture conditions

2.2.1.1. *Escherichia coli*. All constructs were maintained in DH5-α chemically competent *E. coli* cells (*E. cloni™,* Lucigen). Cells containing pRIC3.0-LALF-E7 and pRIC3.0-cTP-BFDV were kindly provided by Renate Lamprecht and Guy Regnard respectively, and were obtained from the culture collection of the Biopharming Research Unit at the Department of Molecular and Cell Biology (MCB), University of Cape Town (UCT). These were streaked onto Luria-Bertani (LB) agar [1.0 % tryptone, 0.5 % yeast extract, 1.0 % NaCl, 1.5 % agar, pH 7.0] plates. Liquid cultures were grown in LB broth with agitation at 120 revolutions per minute (rpm), at 37 °C, for 16 to 18 h. Antibiotic selection was done using 100 µg/ml ampicillin.

2.2.1.2. *Agrobacterium tumefaciens*. For pTRAc, pRIC3.0 and pRIC3.0-cTP constructs, *A. tumefaciens* strain GV3101 containing the helper plasmid pMP90RK, was used. For pEAQ-*HT* and pBIN constructs, *A. tumefaciens* strain LBA4404 was used. Cells were grown in LB broth, with agitation at 120 rpm, at 27 °C for 2-3 days. Antibiotic selection was done using 50 µg/ml carbenicillin (only used for recombinant GV3101::pMP90RK cells), 50 µg/ml rifampicin and 30 µg/ml kanamycin. To prevent clumping of LBA4404 cultures, liquid media were supplemented with $MgSO_4$ to a final concentration of 2 mM.

2.2.2. Construct generation

2.2.2.1. pRIC3.0-LALF-E7. The sequence encoding LALF-E7 was kindly provided by our Cuban collaborator, M. Granadillo (Centre for Genetic Engineering and Biotechnology, Havana, Cuba). The E7 sequence was previously modified to contain a base substitution (T/G) in the codon encoding for the first cysteine to abolish the carcinogenic effect of the protein. The LALF sequence encodes a small linear peptide containing residues 32 to 51 from the original LALF protein (Granadillo *et al.,* 2011; Vallespi *et al.,* 2000). The LALF-E7 sequence was codon-optimized for expression in *N. benthamiana*, synthesized by GenScript (USA) and provided in the cloning vector pUC57. The LALF-E7 sequence was excised from pUC57 using the restriction enzymes (REs) Nco*I*/Xho*I.* The sequence was inserted into the expression vector pRIC3.0 by R. Lamprecht (Biopharming Research Unit, MCB, UCT) using the RE sites Afl*III*/Xho*I*.

2.2.2.2. Subcloning of LALF-E7 into pRIC3.0-cTP. The vector pRIC3.0-cTP-BFDV, was kindly provided by G. Regnard (Biopharming Research Unit, MCB, UCT) with the beak and feather disease virus capsid protein (BFDV) inserted between the *MluI* and *XhoI* restriction sites. To confirm insertions, pRIC3.0-cTP-BFDV and pRIC3.0-LALF-E7 were digested with Eco*RV*. To subclone the LALF-E7 sequence from pRIC3.0 into pRIC3.0-cTP, a Mlu*I* restriction site was added at the 5'-end of the sequence by PCR using the LALF-E7 primers (Table 2.1). The PCR was carried out using the *Pfu* polymerase, a final concentration of 4 mM $MgSO_4$, determined by $MgSO_4$ titration, and 1 µM of each primer. The PCR profile was initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s and extension at 72 °C for 1 min; and final extension for 7 min. LALF-E7 amplicons were gel-extracted using the QIAQuick® Gel extraction kit (QIAGEN®) according to manufacturer instructions, and ligated into pJET1.2/blunt cloning vector overnight, at 4 °C, using the T4 ligase with a 1:4 vector to insert ratio. *E. cloni* cells were transformed with pJET-LALF-E7, as per manufacturer's protocol. Clones were confirmed by *MluI* RE digestion. Five µg of pJET-LALF-E7 DNA were RE-digested with *XhoI* and *MluI* in order to excise LALF-E7. To linearize pRIC3.0-cTP while excising the BFDV-CP sequence from it, 2 µg of pRIC3.0-cTP-BFDV were RE-digested with the same enzymes. The DNA fragments of interest were gel-extracted and ligated. *E. cloni* cells were transformed with pRIC3.0-cTP-LALF-E7 and putative recombinants were then confirmed by RE digestion using Eco*RV*. The constructs pRIC3.0-LALF-E7 and pRIC3.0-cTP-LALF-E7 were further confirmed by sequence analysis (Macrogen Inc.) using the pTRAkc primers (Table 2.1). All REs, the DNA polymerase and ligase as well as pJET1.2/blunt used here were provided by Thermo Scientific.

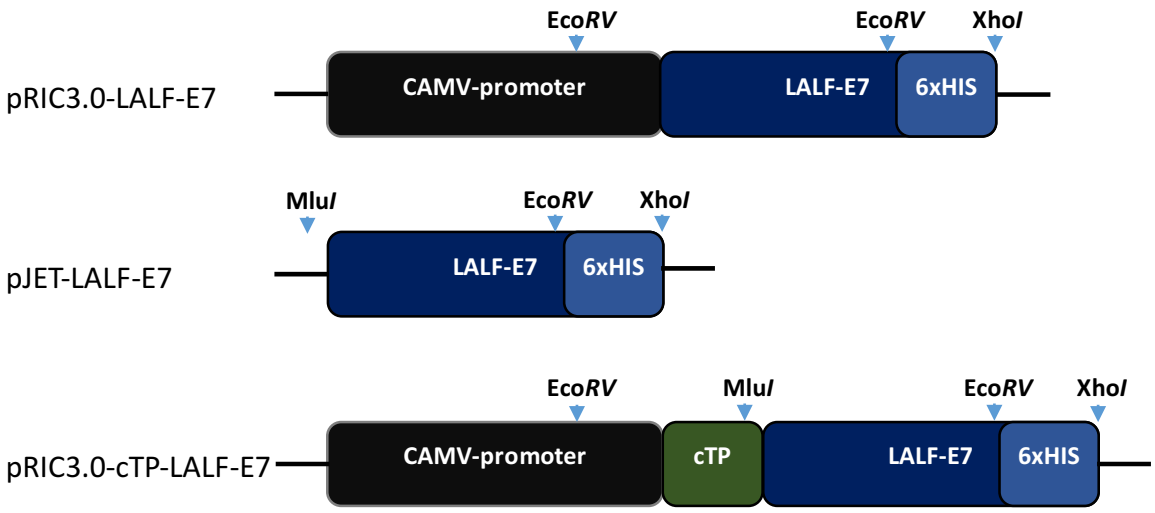

Figure 2.2. Construct generation for the expression of LALF-E7 in *N. benthamiana*. The LALF-E7 sequence (dark-blue boxes) was plant codon-optimized and synthesised by GenScript. To generate pRIC3.0-cTP-LALF-E7, a 5'-end *MluI* restriction site was added to the LALF-E7 sequence by PCR amplification from pRIC3.0-LALF-E7. The LALF-E7 amplicon was cloned into pJET1.2/blunt to give the intermediary construct, pJET-LALF-E7. pRIC3.0-cTP-BFDV and pJET-LALF-E7 were digested with *MluI/XhoI* REs and the LALF-E7 fragment and pRIC3.0-cTP vector were ligated to create pRIC3.0-cTP-LALF-E7. Light-blue arrows, RE sites. Black boxes, the cauliflower mosaic virus 35S constitutive promoter. Light-blue boxes, LALF-E7 hexa-histidine tag. Green box, the *rbcs1*-cTP. Not drawn to scale.

2.2.3. Electroporation of *A. tumefaciens*

Electro-competent *A. tumefaciens* cultures were prepared as described by Wen-jun and Forde, (1989) and electroporated as described by Maclean *et al*., (2007). GV3101::pMP90RK cells were electroporated with pRIC3.0-LALF-E7 and pRIC3.0-cTP-LALF-E7 in this study. The construct pRIC3.0 empty vector in GV3101::pMP90RK cells was kindly provided by J. Els and the constructs pTRAc-LALF-E7 and pRIC3.0-LALF-E7 in GV3101::pMP90RK cells as well as pEAQ-*HT*-LALF-E7 in LBA4404 cells were kindly provided by R. Lamprecht (both in the Biopharming Research Unit, MCB, UCT). The constructs pEAQ-*HT* which contains the p19 expression cassette, and pBIN-NSs, both in LBA4404 cells were obtained from the Biopharming Research Unit culture collection. Recombinant *A. tumefaciens* pRIC3.0-LALF-E7 and pRIC3.0-cTP-LALF-E7 were confirmed by colony PCR using the pTRAkc primers (provided by R. Lamprecht; Table 2.1) and GoTaq® Flexi DNA Polymerase (Promega). The I PCR conditions were an initial denaturation cycle at 95 °C for 5 minutes, subsequent 30 cycles of denaturation for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension cycle for 7 min.

Table 2.1. Primers used in this work

Name	Sequence	Feature	Application
LALF-E7-Fw	5' AAA CGC GTT AGG TGC ATG GCC GAG TTT CAT TAC AG 3'	Contains the Mlu*I* site.	PCR amplification and addition of the Mlu*I* restriction site.
LALF-E7-Rv	5' AAT CGA GGG TCT CAT TAG TGG TGG TGG TGA TGG TGA G 3'	N.A.	PCR amplification.
pTRAkc-Fw	5' CAT TTC ATT TGG AGA GGA CAC G 3'	Complementary to pRIC3.0 and pRIC3.0-cTP backbone.	Sequencing insertions and confirmation of recombinant *A. tumefaciens* GV3101::pMP90RK.
pTRAkc-Rv	5' GAA CTA CTC ACA CAT TAT TCT GG 3'	Complementary to pRIC3.0 and pRIC3.0-cTP backbone.	Sequencing insertions and confirmation of recombinant *A. tumefaciens* GV3101::pMP90RK.

LALF-E7 primers were designed using the CLC-Main Workbench 6 (QIAGEN® Bioinformatics) and synthesised by the DNA Synthesis Unit (MCB, UCT). N.A., not applicable.

2.2.4. Agroinfiltration and transient expression of LALF-E7 in *N. benthamiana leaves*

2.2.4.1. Determination of the optimal conditions for the expression of LALF-E7 in *N. benthamiana* leaves. Recombinant *A. tumefaciens* cultures were prepared for small scale agroinfiltration as described by Maclean *et al.,* (2007). Two plants per construct were syringe-infiltrated using *A. tumefaciens* cultures at different optical densities of 0.25, 0.5, 1.0 and 1.5 at 600 nm (OD_{600}), to determine the optimal OD_{600} for the expression of LALF-E7. A time trial was performed to determine the accumulation profile of LALF-E7. This was done by harvesting 3 x leaf clippings (obtained using the lid of a 1.5 ml microcentrifuge tube) on 3, 5 and 7 days post infiltration (dpi). The control, pEAQ-*HT*-LALF-E7, was infiltrated at an OD_{600} of 0.75 as previously determined by M. Granadillo (2013; unpublished data). The negative control, pRIC3.0-empty, was infiltrated at an OD_{600} of 1.0.

Thereafter, the time trial was repeated three times independently, using the optimal OD_{600}, three plants per construct and vacuum infiltration. The construct pTRAc-LALF-E7 was used as a more appropriate positive control, as its backbone is similar to that of pRIC3.0. For vacuum infiltration, *Agrobacterium* cultures were grown as described by Maclean *et al.,* (2007) in LBB enriched medium [0.25 % tryptone, 1.25 % yeast extract,

0.50 % NaCl, 10 mM 2-(N-Morpholino) ethanesulfonic acid (MES), pH 5.6], induced overnight with 20 μM acetosyringone and diluted to the desired final OD_{600} in resuspension solution [5 mM MES, 20 mM $MgCl_2$, 0.2 mM acetosyringone].

2.2.4.2. Investigating the effect of silencing suppressors on the expression of LALF-E7. Plants were vacuum infiltrated with pRIC3.0-LALF-E7, pRIC3.0-cTP-LALF-E7 and pRIC3.0-empty, with or without a silencing suppressor-containing constructs: LBA4404-pEAQ-*HT*-P19 and LBA4404-pBIN-NSs. All *A. tumefaciens* cultures were set to an OD_{600} of 1.0. Three plants per construct were vacuum infiltrated. One leaf per plant was harvested on 3, 5 and 7 dpi, giving a total of 3 leaves per construct. This set of experiments was repeated twice, independently.

2.2.5. Preparation of plant crude extracts

2.2.5.1. Small scale expression studies. Samples of three or six leaf discs were harvested by clipping the leaves with a 1.5 ml microcentrifuge tube lid. Samples were flash-frozen in liquid nitrogen, ground with a pestle and suspended in 200 μl or 400 μl 8 M urea extraction buffer [8 M urea in 1 mM carbonate-bicarbonate buffer, pH 10.6], respectively. Extracts were clarified by centrifugation at 13,000 rpm for 10 min. The supernatant was prepared for SDS-PAGE or stored at -20 °C.

2.2.5.2. Medium scale expression studies. For vacuum-infiltrated plants, leaves were weighed immediately after being harvested. Leaves were flash-frozen in liquid nitrogen and ground up using a mortar and pestle. Crude extracts were prepared by homogenizing leaves in 2 v/w of extraction buffer using a T25 digital ULTRA-TURRAX® homogenizer (IKA). Extracts were clarified by centrifugation at 13,000 rpm (BeckmanT Coulter Avanti® J25TI centrifuge) for 10 min.

2.2.6. Total soluble protein (TSP) and % TSP determination

Total soluble protein concentrations of crude extracts were determined using the *DC* Protein Assay (Bio-Rad). A serial dilution of 1 mg/ml to 0.06 mg/ml bovine serum albumin (BSA; Sigma-Aldrich) was used as standard. Crude extracts were diluted to fall within the range of the standard. The A_{750} of the samples was determined using the microplate spectrophotometer PowerWave XS™ (BioTek®). The output data were analysed by Microsoft® Excel 2015. For %TSP determination, western blot densitometry was used. The

plant-derived LALF-E7 was quantified using the 6xHis Protein Ladder (QIAGEN®) as a protein standard. The anti-Histidine and goat anti-mouse IgG whole molecules conjugated to alkaline phosphatase (AP; Sigma-Aldrich) were used at a dilution of 1:2,000. The Syngene Gene Genius imaging system (Artisan Technology Group) and GeneTools software (Syngene) were used. The %TSP fold change was calculated using the equation bellow, where values above 1 indicated increase, a value of 1 indicated no change and a value below 1 indicated decrease.

$$Fold\ change\ = \frac{\%\ TSP\ pRIC3.0 - cTP - LALF - E7}{\%\ TSP\ pRIC3.0 - LALF - E7}$$

2.2.7. SDS-PAGE and western blotting

Crude extracts were mixed with 6x SDS sample application buffer [25 % (v/v) glycerol, 0.5 M DDT, 5 % (w/v) bromothymol blue] to a final concentration of 1x and boiled at 90 °C for 10 min. Fifteen per cent SDS-PAGE-gels were loaded and electrophoresed in a Bio-Rad Tetra Cell system at 120 volts. Gels were stained with Aqua Stain™ (Vacutec) for 1 h, at 37 °C. Protein sizes were estimated using PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific) or with the Colour Prestained Protein Standard, Broad Range (11–245 kDa) (NEB).

For western blots, proteins were transferred from SDS-PAGE-gels to nitrocellulose membranes using a Bio-Rad Trans-Blot® Semi-dry transfer cell at 15 volts, for 1 h. The primary and secondary antibodies used were the polyclonal anti-HPV-16-E7 rabbit serum (I. Hitzeroth, Biopharming Research Unit, MCB, UCT) and the goat anti-Rabbit IgG whole molecules-AP (Sigma-Aldrich). Blots were visualized with NBT/BCIP substrate (Sigma-Aldrich).

2.2.8. Bacterial expression of LALF-E7

To be used as positive control for plant-derived LALF-E7 expression, the LALF-E7 sequence was cloned into the bacterial expression vector pPROEX-HTb (Addgene). LALF-E7 was expressed in *E. coli* according to the pPROEX-HTb Prokaryotic Expression System protocol. LALF-E7 was partially purified using the BugBuster® Protein Extraction Reagent (Novagen) protocol for inclusion body purification with additional 3 x washes with 0.1§% Triton X

100 (Sigma-Aldrich) in phosphate buffered saline [PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM PO_4^{3-} (0.144 % Na_2HPO_4 and 0.024 % KH_2PO_4), pH 7.4].

2.3. Results

2.3.1. Construct generation

Initial constructs were confirmed by RE digestion with Eco*RV*. The DNA band patterns obtained were as expected, 1 kb and 5.84 kb for pRIC3.0-cTP-BFDV, and 0.45 kb and 5.85 kb for pRIC3.0-LALF-E7 (not shown). For subcloning into the chloroplast-targeting expression vector, pRIC3.0-cTP, the restriction site for *MluI* was added at the 5'-end of LALF-E7 by PCR amplification. Gel-extracted amplicons were first ligated into the pJET1.2/blunt cloning vector to produce pJET-LALF-E7, in order to confirm that the amplicons were positive for the *MluI* restriction site. Plasmid DNA from 11 clones were digested with *MluI* and 9 were determined *MluI*-positive by obtaining the expected 3.4 kb band (Figure 2.3A). One clone was selected to proceed with subcloning. The constructs pJET-LALF-E7 and pRIC3.0-cTP-BFDV were digested with Xho*I* and Mlu*I*. Gel-extracted fragments of interest were ligated, resulting in pRIC3.0-cTP-LALF-E7. All recombinant pRIC3.0-cTP-LALF-E7 clones selected were successfully confirmed by RE digestion using Eco*RV* that resulted in a band pattern of 0.6 Kb and 5.9 Kb (Figure 2.3B). The constructs of interest, pRIC3.0-LALF-E7 and pRIC3.0-cTP-LALF-E7, were sequenced and the results agreed with predicted sequences on CLC Main Workbench 6. *A. tumefaciens* cells were successfully electroporated with these constructs as shown by positive colony PCRs using the pTRAkc primers. These primers amplified products of 0.6 kb for pRIC3.0-LALF-E7 and 0.75 kB for pRIC3.0-cTP-LALF-E7 which were as expected (Figure 2.3C).

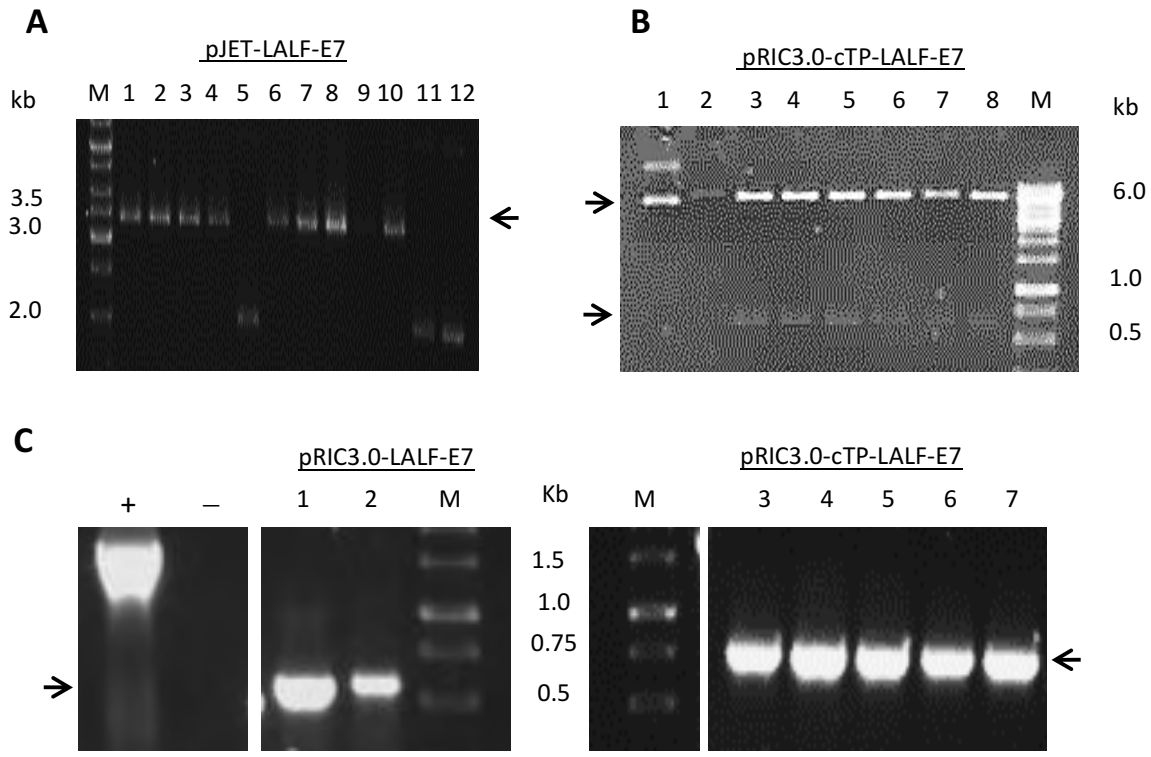

Figure 2.3. pRIC3.0-cTP-LALF-E7 construct generation and *A. tumefaciens* transformation. (A) pJET-LALF-E7 screening by *MluI* RE digestion of DNA isolated from 11 single colonies (Lanes 1-11). Lane 12, no RE added. Band pattern expected: 3.4 kb linearized plasmids. **(B)** pRIC3.0-cTP-LALF-E7 screening by *EcoRV* RE digestion. Lane 1, no RE added. Lanes 2-8, digested DNA isolated from 7 single colonies. Band pattern expected: 0.6 kB + 5.9 kb. **(C)** Confirming recombinant *A. tumefaciens* by colony PCR. (+) positive control, 1.5 kb. (–) negative control, PCR mixture with no DNA added. Lanes 1 and 2: pRIC3.0-LALF-E7, 0.6 kb. Lanes 3-7, pRIC3.0-cTP-LALF-E7, 0.75 kB. For all images: (M), 1kb DNA molecular weight marker. (kb), sizes shown in kilobases. Arrows point at relevant bands.

2.3.2. Optimization of LALF-E7 transient expression in *N. benthamiana*

To determine the optical density (OD_{600}) of recombinant *A. tumefaciens* that resulted in highest expression levels, plants were syringe-infiltrated using recombinant GV3101::pMP90RK-pRIC3.0-LALF-E7 and –pRIC3.0-cTP-LALF-E7 cultures at different OD_{600}s. Two plants were syringe-infiltrated per OD_{600}, per construct and the plants were monitored over 7 days. pRIC3.0-empty vector and pEAQ-*HT*-LALF-E7 were used as negative and positive controls, respectively. Leaf clippings were collected on 3, 5 and 7 dpi. On 3dpi, all plants infiltrated with pRIC3.0 constructs presented signs of infection. By 5 dpi these plants showed signs of damage which became severe by 7 dpi, including brittle leaves and chlorosis (data not shown for syringe-infiltrated plants). In the plants infiltrated with pEAQ-*HT*-LALF-E7 the severe symptoms were delayed, only appearing by the end of the time trial (data not shown).

Anti-E7 polyclonal antibody western blots showed that the expression of LALF-E7 (≈22 kDa) using vectors pRIC3.0 and pRIC3.0-cTP was successful (Figure 2.4). LALF-E7 was absent or poorly detected for some pRIC3.0 samples (Figure 2.4, upper panel) but it was detected in all pRIC3.0-cTP samples (Figure 2.4, bottom panel). The expression of LALF-E7 in pRIC3.0 appeared strongest at an OD_{600} between 1.0 and 1.5, on 3 dpi. The expression in pRIC3.0-cTP appeared strongest at an OD_{600} between 0.5 and 1.0, also on 3 dpi. The expression of LALF-E7 when using the positive control, pEAQ-*HT* was also detected, however, it appeared to be lower than that when using pRIC3.0 and pRIC3.0-cTP. No LALF-E7 was detected in the negative control samples of pRIC3.0-empty vector.

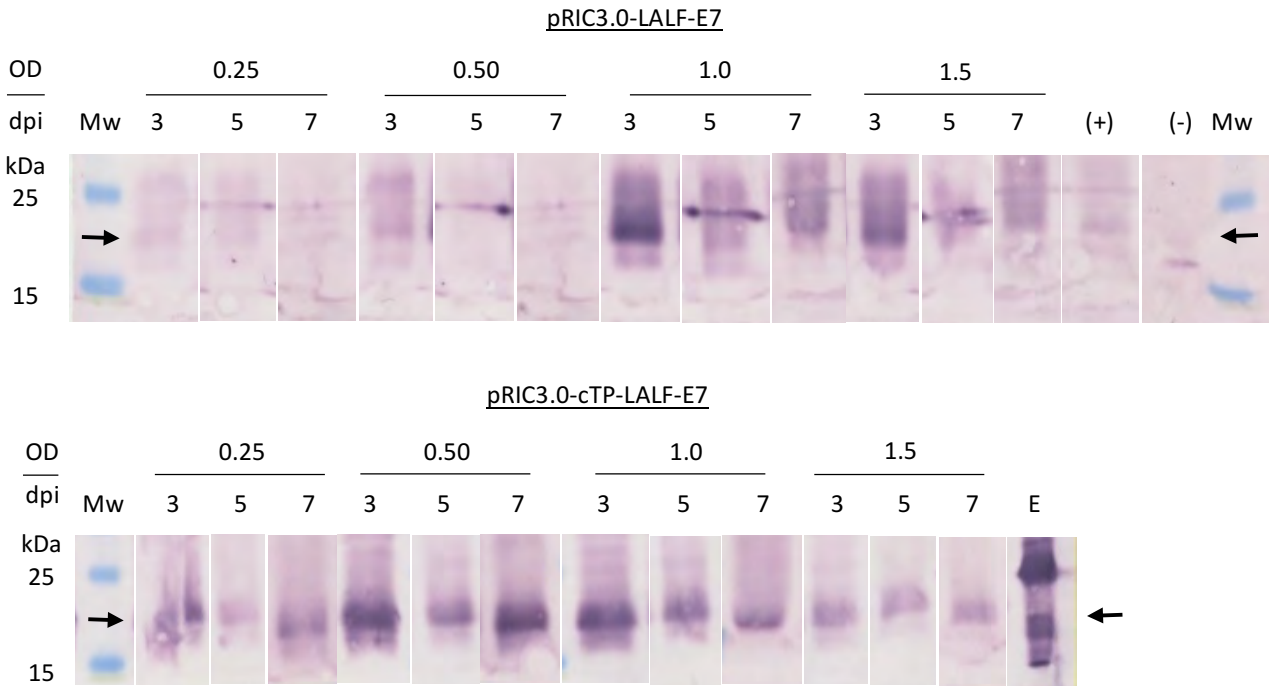

Figure 2.4. Small-scale detection of LALF-E7 expression in *N. benthamiana* leaves. Western blots (1:1,000) of equal volumes of crude extracts from *N. benthamiana* leaves syringe-infiltrated with pRIC3.0-LALF-E7 (top) and pRIC3.0-cTP-LALF-E7 (bottom). Different recombinant *Agrobacterium* OD_{600}s were used. Leaf clippings were harvested on 3, 5 and 7 dpi. Mw, Page Ruler™ molecular weight marker. Band sizes (kDa) are indicated on the left-hand-side. (-), pRIC3.0-empty vector, harvested on 5 dpi. (+), pEAQ-*HT*-LALF-E7, harvested on 5 dpi. E, purified *E. coli*-derived HPV-16 E7 provided by M. Granadillo. Arrows indicate the expected position of LALF-E7, ≈22 kDa.

2.3.3. Comparing the accumulation of LALF-E7 in the cytoplasm versus the chloroplasts

In order to better compare the accumulation of LALF-E7 in the cytoplasm versus the chloroplasts, the set of experiments described in section 3.2. were repeated three times, independently and at a larger scale. Three plants were vacuum-infiltrated with pRIC3.0-LALF-E7 and pRIC3.0-CTP LALF-E7, using the overlapping optimum OD_{600} of 1.0. pRIC3.0-empty vector and pTRAc-LALF-E7 were used as controls. pTRAc-LALF-E7 was chosen as a more appropriate control than pEAQ-*HT*-LALF-E7 as its backbone is similar to that of pRIC3.0. Plants were monitored for 7 days and leaves were harvested on 3, 5 and 7 dpi. All plants showed similar but less severe symptoms as described in section 2.3.2. (Figure 2.5A). Plants infiltrated with pTRAc-LALF-E7 showed delayed and less severe symptoms than plants infiltrated with pRIC3.0 constructs (data not shown).

Equal volume western blots showed how LALF-E7 was almost undetectable when expressed from pTRAc, even when using concentrated antibodies (1:1,000; Figure 2.5B, left hand side). However, large molecular weight aggregates were detected in these samples at approximately 46 and 58 kDa. The total soluble protein (TSP) content of pRIC3.0-LALF-E7 and pRIC3.0-cTP-LALF-E7 crude extracts was determined by Bradford's assay. In order to normalize for any variation in protein extraction efficiency, equal amounts of TSP were used to analyse the accumulation of LALF-E7 in the cytoplasm versus the chloroplasts (Figure 2.5B, right hand side). Unlike in the small-scale expression experiments, when expressed in pRIC3.0, LALF-E7 peaked on 5 dpi and was undetectable on 3 and 7 dpi. No LALF-E7 was detected in pRIC3.0-empty vector extracts. As determined by western blot densitometry, it accounted for up to 0.017 % TSP in the extracts analysed (Figure 2.5C). On the other hand, LALF-E7 accumulation when expressed in pRIC3.0-cTP fluctuated less than in pRIC3.0, peaking on 3 and 5 dpi. This accounted for up to 0.56% TSP, however on average this was 0.44% TSP. The accumulation of LALF-E7 in the chloroplasts was 26.8 fold higher than the accumulation in the cytoplasm when taking into account the average %TSP on 3 and 5 dpi.

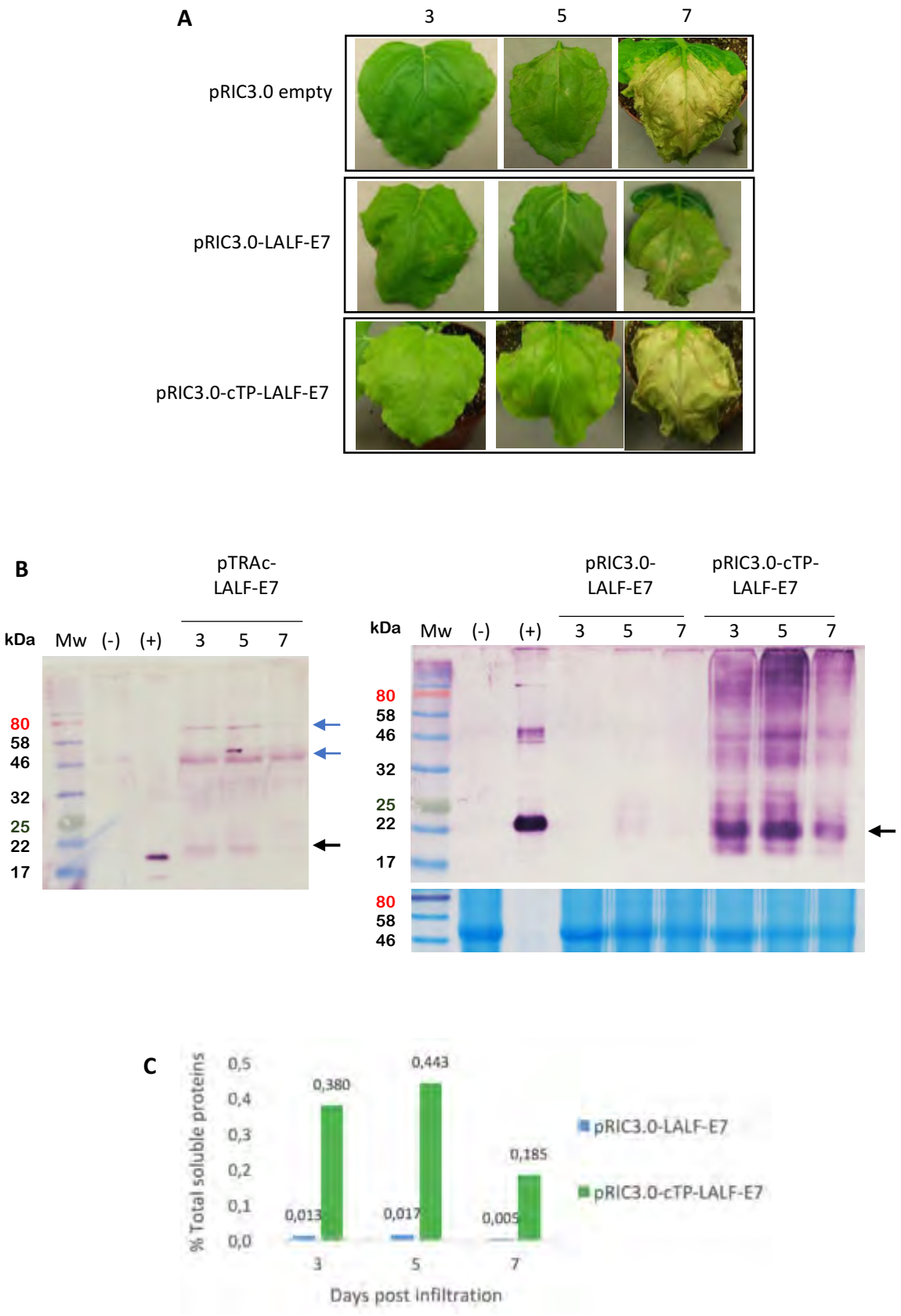

Figure 2.5. Comparing the effects of expression vector and subcellular localization on the expression of LALF-E7. **(A),** Leaf physiology monitored over time after vacuum infiltration with pRIC3.0-LFLF-E7 and pRIC3.0-cTP-LFLF-E7. All cultures used were set to an OD_{600} of 1.0. Shown are representative leaves on 3, 5 and 7 dpi. pRIC3.0-empty vector was used as a negative control. Shown here are representative images of

three independent repeats. **(B)** The effect of expression vector on LALF-E7 expression. (Left hand side), equal volume western blots (1:1,000) of LALF-E7 expression from pTRAc. (+), purified *E. coli*-derived LALF-E7, provided by M. Granadillo. (-), pRIC3.0-empty vector crude extract. Mw, weight marker. Black arrow indicates the expected position of LALF-E7, ≈22 kDa. Blue arrows show higher molecular weight aggregates. (Right hand side), analysis of 50 μg of TSP of pRIC3.0-LALF-E7 and pRIC3.0-cTP-LALF-E7 crude extracts. Top panel: western blot (1:5,000). Bottom panel: corresponding AquaStained SDS-PAGE gels showing a native protein of approximately 50 kDa as an internal control for equal TSP loading. Mw, molecular weight marker. (-) pRIC3.0-empty vector crude extract. (+), *E. coli*-derived LALF-E7 inclusion bodies. Arrow indicates the position of LALF-E7, ≈22 kDa. Shown here are representative images of three independent repeats. **(C)** The effect of subcellular localization on LALF-E7 expression determined by the %TSP.

2.3.4. Investigating the effect of silencing suppressors on the expression of LALF-E7

To determine the effect of silencing suppressors on the expression of LALF-E7 in *N. benthamiana,* leaves were co-infiltrated with pRIC3.0-LALF-E7 or pRIC3.0-cTP-LALF-E7 and P19 or NSs. pRIC3.0-empty vector was used as a negative control. This set of experiments was done twice independently, by vacuum infiltration. The plants were monitored over a period of 7 days. Symptoms were as described in section 2.3.2. However, signs of necrosis were exacerbated by the co-infiltration with P19, and seemed to be attenuated by co-infiltration with NSs (Figure 2.6A).

Western blot analysis of leaf extracts from each construct and silencing suppressor combination showed that LALF-E7 in pRIC3.0 peaked on 5 dpi, with or without silencing suppressor, which was consistent with previous experiments. The best day for pRIC3.0-cTP was 3 dpi, except when in the presence of P19, in which case it was 5 dpi. No LALF-E7 was detected from pRIC3.0-empty vector leaf extracts (data not shown).

Based on the above results, TSP content of the leaf extracts from the best expression day of each combination was determined. Fifty μg TSP per sample were used to compare the effect of silencing suppressors on the expression of LALF-E7 (Figure 2.6B). The expression of LALF-E7 in pRIC3.0 did not seem to change in the presence of either silencing suppressor. It was not possible to accurately quantify these samples due to their poor detection. The expression of LALF-E7 in pRIC3.0-cTP only increased in the presence of P19, on average by 1.3 fold, while it seemed to decrease by on average 0.92 fold in the presence of NSs.

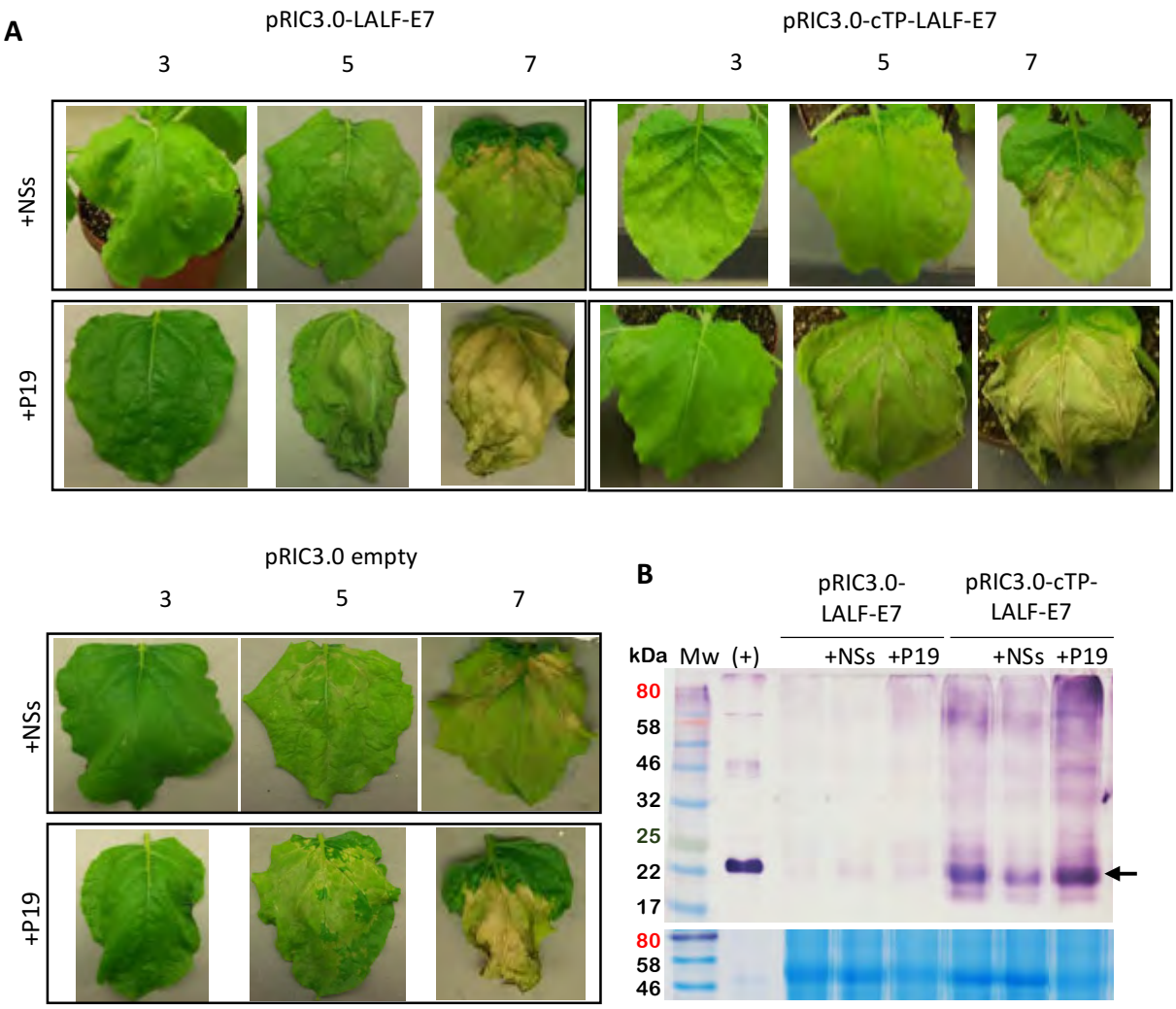

Figure 2.6. **The effects of silencing suppressors on the expression of LALF-E7. (A)** Leaf physiology monitored over time after vacuum co-infiltration with pRIC3.0-LFLF-E7 and pRIC3.0-cTP-LFLF-E7 with silencing suppressors. All cultures used were set to an OD_{600} of 1.0. Shown are representative leaves on 3, 5 and 7 dpi. pRIC3.0-empty vector was used as a negative control. **(B)** Analysis of 50 µg of TSP crude extracts of leaves vacuum-infiltrated with or without a silencing suppressor on the best expression dpi. Top panel: western blot (1:5,000). Bottom panel: corresponding AquaStained SDS-PAGE gel showing a native protein of approximately 50 kDa as an internal control for equal TSP loading. Mw, molecular weight marker. (-) pRIC3.0-empty vector crude extract. (+), *E. coli*-derived LALF-E7 inclusion bodies. Arrow indicates the position of LALF-E7, ≈22 kDa. Shown here are representative images of two independent repeats.

2.4. Discussion

The fusion protein LALF-E7 is a potential therapeutic vaccine candidate for HPV-16. It was previously expressed in *E. coli* and was successful in eliciting tumour regression in trials in animal model (Granadillo *et al.*, 2011, 2013). However, it would be potentially more economical to use plant expression systems to produce this candidate vaccine, given that the expression is sufficiently high (Fischer *et al.*, 2004; Rybicki, 2010). LALF-E7 has been expressed in *N. benthamiana* leaves before, using the expression vectors pTRAc and pEAQ-*HT* (Granadillo and Lamprecht, unpublished data). However, the expression level was very low.

In order to determine whether the plant-derived LALF-E7 has similar immunogenic effects in animal models as its bacterial-derived counterpart, I aimed at increasing and optimising the expression of LALF-E7 in *N. benthamiana.* This was done by expressing LALF-E7 in the self-replicating viral-based expression vector pRIC3.0 (Regnard *et al.*, 2010). We also compared the accumulation of LALF-E7 when targeted to the chloroplast by the potato *rbcs1* chloroplast targeting peptide (cTP), to that when localized in the cytoplasm. And finally, I tested whether silencing suppressors would affect the accumulation of LALF-E7 in the cytoplasm and in the chloroplasts.

The expression of LALF-E7 as detected by western blots was successful using both pRIC3.0 and pRIC3.0-cTP vectors. The expression of LALF-E7 in pRIC3.0 and pRIC3.0-cTP was notably higher compared to pEAQ-*HT* and pTRAc. This agreed with the results of Regnard *et al*. (2010), in that increasing gene copy number can lead to increase in expression of recombinant proteins. Several other studies have also shown that using replicating vectors, especially those derived from geminiviruses, can lead to high expression levels (Kim *et al.*, 2007; Huang *et al.*, 2009, 2010; Dugdale *et al.*, 2013).

It was noted that LALF-E7 appeared at ≈ 22 kDa in western blots, while its predicted size is ≈ 15 kDa. This was consistent with observations by Granadillo *et al.,* (2011). The net negative charge of E7 is known to influence the migration of this protein in SDS-PAGE gels, making it appear larger (Armstrong and Roman, 1993). This has also been observed with other E7-based fusion proteins, like 16E7SH (Whitehead *et al.*, 2014).

It was observed that using the self-replicating vector induced strong infection-like symptoms in the plants, even when no insert was present. Other studies have reported that the Rep proteins of other geminiviruses can induce hypersensitive responses in plants (Van Wezel *et al.*, 2002): it could be that the presence of the BeYDV Rep and RepA proteins induces the symptoms observed. Alternatively, the high level of replication of the pRIC3.0 vector could induce a hypersensitive response in the plant cells, especially in later stages of infection. However, the protein expression levels were not negatively affected by these strong symptoms, even on 7 dpi, compared to the expression obtained from non-replicative vectors. The strong symptoms could also be a result of the high OD_{600} used.

The choice of subcellular localization for the expression of recombinant proteins is an important factor to be considered (Fischer *et al.*, 2004; Benchabane *et al.*, 2008; Daniell *et al.*, 2009). This has been demonstrated in previous studies from our lab. The highest expression levels of HPV-16 L1 variants where obtained when these were targeted to the chloroplasts, compared to cytoplasmic localization. On the other hand, the same proteins were hardly detectable when targeted to the endoplasmic reticulum (Maclean *et al.*, 2007). Meyers *et al.* (2008) obtained similar results for a human immunodeficiency virus type 1 (HIV-1) Gag-derived antigen by targeting it to the same cell compartments. However, a 5-fold increase in expression levels of HPV-16 E7 in *N. benthamiana* leaves was reported when E7 was targeted to the plant secretory pathways (Franconi *et al.*, 2002, 2006), suggesting that the effect of cell compartment targeting varies from protein to protein.

To properly compare the accumulation of LALF-E7 in the cytoplasm to that in chloroplasts, the preliminary set of experiments was repeated three times. The total soluble protein (TSP) content was determined for each sample and accounted for when analysing the expression of LALF-E7. The accumulation of LALF-E7 in the cytoplasm peaked on 5 dpi and it was undetectable on 3 and 7 dpi. On the other hand, the accumulation detected in the chloroplasts did not fluctuate and represented the highest accumulation levels of LALF-E7. The increase in % TSP of 26.8-fold agrees with the hypothesis that proteins in cell compartments are less prone to degradation. It is thought that protease concentrations are lower in these than in the cytoplasm (Fischer *et al.*, 2004; Maclean *et al.*, 2007; Meyers

et al., 2008). The higher accumulation of LALF-E7 in the chloroplasts seen here could also be due to the cTP signal increasing the stability of the LALF-E7 transcripts in the cytoplasm, making it less prone to PTGS (Maclean *et al.,* 2007). To confirm this, quantitative reverse transcription PCR (qRT-PCR) could be done on pRIC3.0-LALF-E7 and pRIC3.0-cTP-LALF-E7 samples from different time points post infiltration.

The fact that targeting LALF-E7 to the chloroplasts dramatically increased its expression compared to localizing it in the cytoplasm, suggest that subcellular localization played a greater role in the accumulation of LALF-E7 than the increase in gene copy number provided by the self-replicating vector. This agrees with the findings of Regnard et al. (2010), in that the increase in gene copy number was not proportional to the fold increase in protein expression. Therefore, it would not be surprising if comparable results were obtained if expressing LALF-E7 from pTRAc versus pTRA-cTP; although higher *A. tumefaciens* OD_{600} would probably be needed.

Previous work with ZERA-16E7SH expressed using pTRAc (Whitehead *et al.,* 2014) showed that the accumulation of this protein was enhanced in the presence of the NSs protein. This suggested that PTGS could play a role in the accumulation of E7-based recombinant proteins. To determine whether PTGS does play a role in LALF-E7 accumulation, *N. benthamiana* plants were co-infiltrated with pRIC3.0-LALF-E7 or pRIC3.0-cTP-LALF-E7 and NSs or P19 silencing suppressors. It was shown that co-infiltration with silencing suppressors did not have a big impact on the expression of LALF-E7, as the increase was only 1.3-fold: this can be considered insignificant when compared to the 26.8-fold increase seen when targeting to the chloroplast alone versus cytoplasmic localization. These results were consistent with those of Maclean et al. (2007), who reported that targeting hL1 to the chloroplasts allowed for its detection in western blots, while it was undetectable when localized in the cytoplasm, even in the presence of NSs.

The only instances where silencing suppressors did seem to have an effect on the expression of LALF-E7 were when looking at 7 dpi samples (data not shown). This is not surprising as PTGS takes place in later stages of infection. However, the expression levels at 7 dpi in the presence of silencing suppressors were still lower than on days 3 and 5 with or without silencing suppressors. The replication of geminivirus-based vectors is very rapid and high expression levels are often obtained before the onset of PTGS (Chen *et al.*,

2011). Furthermore, the poor expression of LALF-E7 in pEAQ-*HT,* which encodes for the P19 silencing suppressor, further suggests that amplification of gene copy number by the replicative vectors can have a greater impact on expression levels than that of silencing suppressors.

In conclusion, I was able to enhance the expression of LALF-E7 in *N. benthamiana* leaves over previous studies. I found that subcellular localization of LALF-E7 played the most important role in increasing its accumulation levels. Furthermore, this allowed me to proceed to expressing LALF-E7 in large scales to test it in animal models.

Chapter 3: Subcellular localization of LALF-E7 in *Nicotiana benthamiana leaves*

3.1. Introduction

The green fluorescent protein (GFP) and its derivatives are flexible proteins that have been used for numerous applications in cell biology, including plant imaging. They have facilitated the study of subcellular localization and trafficking visualization of diverse proteins, and have helped elucidate different cellular pathways. Therefore, fluorescent proteins have gained great importance as cell biology tools (Tsien, 1998; Hanson and Köhler, 2001).

In our group, the use of the *rbcS1* gene-derived chloroplast transit peptide (cTP), from the RuBisCO small subunit of *Solanum tuberosum*, has proven useful (Maclean *et al*. 2007; Meyers *et al*. 2008). In Chapter 2 it was seen that the highest accumulation level of LALF-E7 was obtained when targeted to the chloroplasts by the same cTP. This was 26.8-fold higher than when LALF-E7 was not targeted to any cell compartment. It is not likely that this difference is due to a difference in gene copy numbers, since the same replicative vector was used for cytoplasmic and chloroplast-targeted LALF-E7 expression. In order to show whether my protein of interest was in fact being targeted to the chloroplasts, I fused LALF-E7 to the enhanced GFP (EGFP). In this way, I aimed to determine the subcellular localization of LALF-E7 by indirectly imaging it using confocal laser scanning microscopy (CLSM).

N. benthamiana leaves expressing fluorescent proteins can easily be visualized by CLSM *in vivo* without the need for fixation or staining procedures (Fang and Spector, 2010). Furthermore, the autofluorescence of chlorophylls can be used as a means of detecting the plant chloroplasts. The advances in confocal microscopy, the autofluorescence of chlorophylls and the properties of GFP make the latter an appropriate tag candidate to determine the subcellular localization of LALF-E7.

3.2. Materials and Methods

3.2.1. Bacterial strains and growth conditions

Refer to section 2.2.1.

3.2.2. Construct generation

3.2.2.1. Modification of LALF-E7. In order to fuse LALF-E7 to EGFP, the DNA sequence encoding LALF-E7 (section 2.2.2.1) was modified to remove the hexa-histidine (His)-tag and the stop codon, to add appropriate restriction enzyme (RE) sites to be used in a stepwise cloning strategy, and to insert a linker between the two peptides. The RE sites Bam*HI*, Mlu*I* and Nco*I* were added at the 5'-end of the LALF-E7 open reading frame (ORF), while a rigid linker, Proline(P)-Alanine(A)-P-A-P, and the restriction site Bsp*HI* were added to the 3'-end (Figure 3.1). The modified LALF-E7 was plant codon-optimized and synthesized by GenScript (USA), and provided in a pUC57 plasmid.

3.2.2.2 Subcloning the modified LALF-E7 into plant expression vectors. The pUC57 vector containing the modified LALF-E7 and the pEGFP plasmid were digested with Bam*HI*/Bsp*HI.* The relevant fragments, 428 bp and 3,340 bp, respectively, were gel-extracted and ligated as described in section 2.2.2.2. The plasmid generated, pLALF-E7-EGFP (pLG), was RE-digested with Bam*HI/*Not*I* to excise the fusion sequence LALF-E7-EGFP (LG; 1,100 bp). This fusion fragment was ligated into pPROEX-HTb previously linearized using the same REs. The pPROEX-HTb-LALF-E7-EGFP (pX-LG) plasmid was digested with Nco*I/*Xho*I* to insert LG into pRIC3.0 using the restriction sites Nco*I/*Afl*III*, generating pRIC3.0-LALF-E7-EGFP (pR-LG). To insert LG into pRIC3.0-cTP, the plasmids pX-LG and pRIC3.0-cTP-BFDV were digested with Mlu*I/*Xho*I*, thereby excising the beak and feather disease virus (BFDV) capsid protein sequence and inserting the LG sequence into pRIC3.0-cTP, generating pRIC3.0-cTP-LALF-E7-EGFP (pT-LG; Figure 3.1 and table 3.1).

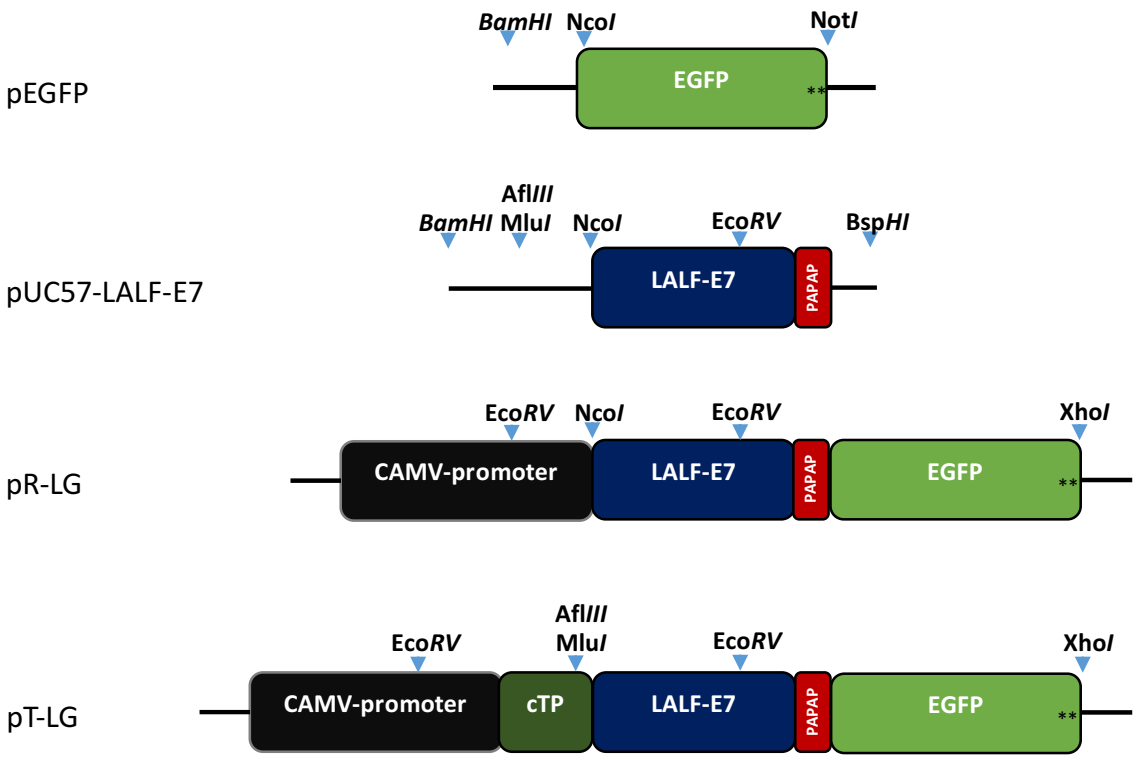

Figure 3.1. Representation of the relevant constructs used in this work. pEGFP, contains the target EGFP sequence (light-green box) to be fused to LALF-E7, and contains a stop codon (black stars). pUC57-LALF-E7 contains the plant codon-optimized LALF-E7 sequence (dark-blue box), appropriate restriction sites for subcloning (shown by the light-blue arrows), a rigid linker (PAPAP, red box) and no His-tag or stop codons. The final constructs pR-LG and pT-LG were generated in a stepwise manner, having the p-LG and pX-LG as intermediates (not shown). Also shown are the cauliflower mosaic virus (CAMV) 35 S constitutive promoter (black box), and the chloroplast targeting peptide (cTP, dark-green box). Not drawn to scale.

Table 3.1. Summary of the construct generation for the expression of LG in plants.

Step #	Initial construct	Target vector	REs used	Construct generated			
				Name	Short name	Confirmed by REs	Expected fragments (bp)
1	pUC57-LALF-E7	pEGFP	BamHI/BspHI into BamHI/NcoI	pLALF-E7-EGFP	p-LG	NotI/EcoRV	2,934 822
2	pLALF-E7-EGFP	pPROEX-HTb	BamHI/NotI	pPROEX-HTb-LALF-E7-EGFP	pX-LG	EcoRV	3,778 1,114 626 366
3	pPROEX-HTb-LALF-E7-EGFP	pRIC3.0	NcoI/XhoI into AflIII/XhoI	pRIC3.0-LALF-E7-EGFP	pR-LG	EcoRV	6,597 479
3	pPROEX-HTb-LALF-E7-EGFP	pRIC3.0-cTP	MluI/XhoI	pRIC3.0-cTP-LALF-E7-EGFP	pT-LG	EcoRV	6,597 626

For all steps, *E. cloni* cells were transformed as described in section 2.2.2.2. Colonies were screened by PCR using the LALF-E7-GenScript primers (Table 3.2). The KapaTaq HiFi HotStart ReadyMix PCR kit (Kapa Biosystems) was used according to the manufacturer's instructions. The PCR profile used was one cycle of initial denaturation at 95 °C for 3 min; 25 cycles of denaturation at 98 °C for 20 s, annealing at 65 °C for 15 s, and extension at 72 °C for 15 s; and one cycle of final extension at 72 °C for 7 min. The expected size of the PCR product was 403 bp. Putative positive colonies were then confirmed by RE digestion. All REs used were provided by Thermo Scientific, except for Afl*III* (New England Biolabs). One μg of plasmid DNA was used for sub-cloning steps and 300-500 ng of DNA were used for confirmation of putative recombinant colonies. The final constructs pR-LG and pT-LG were further confirmed by sequence analysis (Macrogen Inc.) using the pTRAkc primers (Table 3.2).

3.2.3. Electroporation of *A. tumefaciens*

Electrocompetent *A. tumefaciens* GV3101::pMP90RK cultures were prepared and electroporated as described in section 2.2.3. Recombinant *A. tumefaciens* pR-LG and pT-LG were confirmed by colony PCR using the KapaTaq ReadyMix PCR kit (Kapa Biosystems) and the pTRAkc primers (Table 3.2). The PCR profile used was as in section 2.2.3. with the annealing temperature being at 54 °C and extension period for 1 min. The pRIC3.0 empty vector DNA was used as positive control and a no template reaction was used as negative control. The expected PCR products were 1,347 bp and 1,494 bp for pR-LG and pT-LG, respectively.

Table 3.2. Primers used in this work

Name	Sequence	Feature	Application
Fw-LALF-E7-GenScript	5' aaT CCA TGG CCG AGT TCC ATT 3'	Complementary to the modified LALF-E7 sequence.	*E. cloni* colony PCR screening.
Rv-LALF-E7-GenScript	5' ttc tcg agt tat taA GCA CGG CTA 3'	Complementary to the modified LALF-E7 sequence.	*E. cloni* colony PCR screening.
pTRAkc-Fw	5' CAT TTC ATT TGG AGA GGA CAC G 3'	Complementary to pRIC3.0 and pRIC3.0-cTP backbone.	Sequencing insertions and confirmation of recombinant *A. tumefaciens.*
pTRAkc-Rv	5' GAA CTA CTC ACA CAT TAT TCT GG 3'	Complementary to pRIC3.0 and pRIC3.0-cTP backbone.	Sequencing insertions and confirmation of recombinant *A. tumefaciens.*

LALF-E7-GenScript primers were designed using the CLC-Main Workbench 6 (QIAGEN Bioinformatics) and synthesised by the DNA Synthesis Unit (MCB, UCT).

3.2.4. Agroinfiltration of *N. benthamiana* leaves

Recombinant *A. tumefaciens* cultures were prepared for agroinfiltration as described in section 2.2.4.1. For small scale expression studies, *A. tumefaciens* cultures were diluted in infiltration medium to final OD_{600}s of 0.25, 0.50 and 1.00 to determine the optimal OD for the expression of LG. Two plants were used per construct:OD_{600} combination. A time trial was done to determine the expression profile of pR-LG and pT-LG, as well as to monitor leaf symptoms. This was done by visualizing infiltrated leaves under white light and ultraviolet light (Spectroline Long Life ™ Filter, set at 365 nm) on 3, 5 and 7 days post infiltration (dpi), and harvesting plant tissue on 3 and 5 dpi. The construct pRIC3.0-EGFP was used as a positive control at an OD_{600} of 0.25 as determined by Regnard *et al*. (2010). As negative control, the pRIC3.0 empty vector was used at an OD_{600} of 0.5. One plant was used per control.

For fluorescence CLSM, young *N. benthamiana* plants, 3-4 weeks old, were vacuum-infiltrated with *A. tumefaciens* cultures at an OD_{600} of 0.25 as described in section 2.2.4.1. The pR-LG construct was also vacuum-infiltrated at an OD_{600} of 0.5.

3.2.5. Fluorescence confocal laser scanning microscopy (CLSM)

3.2.5.1. Sample preparation. Vacuum-infiltrated leaves were manually sectioned with a razor blade into very fine strips and mounted in distilled water on a glass slide. A cover slip was placed on top of the sections for visualization.

3.2.5.2. Data collection. Thin leaf sections were imaged using a Zeiss LSM 510 Meta NLO multiphoton confocal microscope and the ZEN 2009 software (Zeiss). The lenses used included a 20x air lens and a 40× water immersion lens (numerical aperture of 1.1). The EGFP was excited by an argon laser at 488 nm and the emission was detected at 500-550 nm. The chloroplasts were indirectly detected by exciting the chlorophyll by a DPSs laser at 561 nm and the emission was detected at 650-710 nm. Imaging parameters were fixed for all data acquisition for both rounds of microscopy done.

For colocalization studies, images obtained during sample visualization were analysed using the colocalization set-up on the ZEN software. To accurately set the crosshairs, single label control samples must be prepared. For the red label sample (chloroplasts), the negative control was used. Due to the nature of the samples, I did not have a green single label. Therefore, the positive control was used. These controls were imaged with the same microscope settings as the experimental sample. All imaging settings, including the crosshair positions, were kept fixed throughout the colocalization analysis.

3.2.6. Preparation of plant crude extracts and western blot detection of LG

For the small scale expression optimization, crude extracts were prepared as described in section 2.2.5.1. For plants used for CLSM, vacuum-infiltrated leaves were harvested and crude extracts were prepared as described in section 2.2.5.2. after the visualization was done.

Samples were prepared for SDS-PAGE electrophoresis as described in section 2.2.7. Twenty μl of crude extracts were used per sample. Crude extracts from plants infiltrated with pRIC3.0-EGFP and pRIC3.0 empty vector were used as positive and negative controls, respectively. The primary and secondary antibodies used were mouse monoclonal anti-GFP (Sigma-Aldrich) and goat anti-Mouse IgG whole molecules conjugated to alkaline phosphatase (AP; Sigma-Aldrich), respectively. Both were used at a dilution of 1:5,000.

3.3. Results

3.3.1. LALF-E7-EGFP fusion construct generation

The original sequence of LALF-E7 was modified to generate the fusion protein LALF-E7-EGFP (LG). The His-tag and stop codons were removed, while adding appropriate RE sites and a rigid linker sequence to separate the two peptides (Figure 3.1). The rigid linker, PAPAP (Zhao *et al.*, 2008; Chen, Zaro and Shen, 2013), was chosen to prevent interactions between LALF-E7 and EGFP, and therefore prevent possible misfolding of the EGFP, since LALF-E7 is highly hydrophobic and could associate with hydrophobic patches on the EGFP. The modified LALF-E7 sequence was successfully cloned into the pEGFP plasmid (Figure 3.2A). The LG fusion was subcloned into pPROEX-HTb in order to place a Xho*I* RE site at the 3'-end of the sequence which was used in subsequent cloning steps (see section 3.2.2. and Figure 3.2B). From pPROEX-HTb, LG was subcloned into the final vectors pRIC3.0 and pRIC3.0-cTP, generating pR-LG and pT-LG respectively (Figure 3.2C). Furthermore, the sequencing results of the final constructs coincided with predicted sequences on CLC Main Workbench 6. These constructs were subsequently electroporated into *A. tumefaciens* cells and recombinant colonies were confirmed by PCR (Figure 3.2D).

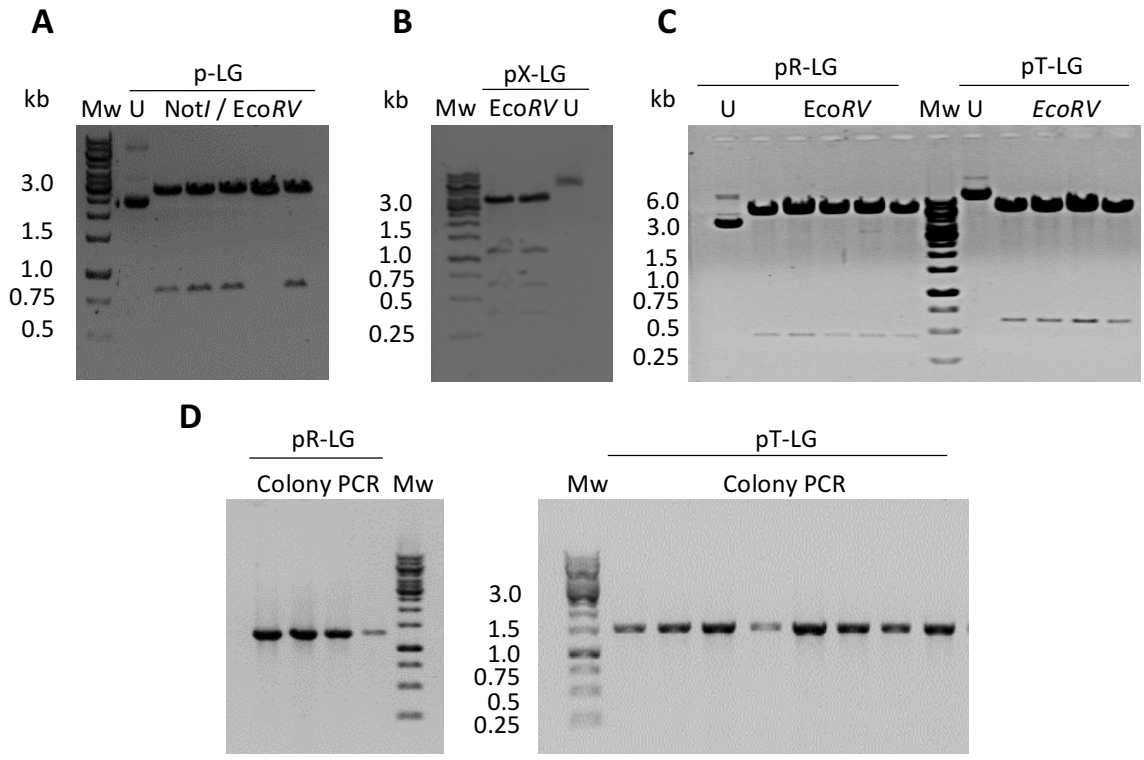

Figure 3.2. Confirming recombinant *E. cloni* and *A. tumefaciens* containing LALF-E7-EGFP fusion constructs. (A-C), Screening putative positive *E. cloni* colonies by RE digestion. REs used are shown above each image. U, undigested plasmid. Mw, 1 kb DNA molecular weight marker. Relevant molecular weight marker band sizes are shown. **(D)**, Screening putative positive *A. tumefaciens* GV3101::pMP19RK-pR-LG and -pT-LG by colony PCR. Shown are representatives of 10 and 30 colonies selected, respectively.

3.3.2. Optimization of LG expression in *N. benthamiana* leaves

In order to determine the best OD_{600} and dpi for the expression of the EGFP-tagged LALF-E7, plants were syringe-infiltrated with pR-LG and pT-LG at different OD_{600}s. The plants were visualized under UV-light on 3, 5 and 7 dpi. The positive control fluoresced green throughout the experiment as expected (Figure 3.3); however, due to tissue necrosis by 7 dpi, the fluorescence decreased at this time point (not shown). The negative control fluoresced red, except in areas of leaf damage, such as at the points of infiltration and areas affected by necrosis. Therefore, a white-blue fluorescence was seen on 5 and 7 dpi. Experimental plants, pR-LG and pT-LG, expressed LG on 3 and 5 dpi regardless of the OD_{600} used. However, higher OD_{600} and longer incubation periods were associated with more necrotic symptoms and lower green fluorescence. For fluorescence CLSM, healthy, undamaged leaf tissue was desirable, since these are easier to handle during sample preparation and unspecific fluorescence is prevented. Therefore, OD_{600} of 0.25-0.5, and 3 dpi were considered to be the optimal conditions for LG expression from both vectors.

This was further confirmed by western blot analysis of crude extracts from 3 and 5 dpi (not shown).

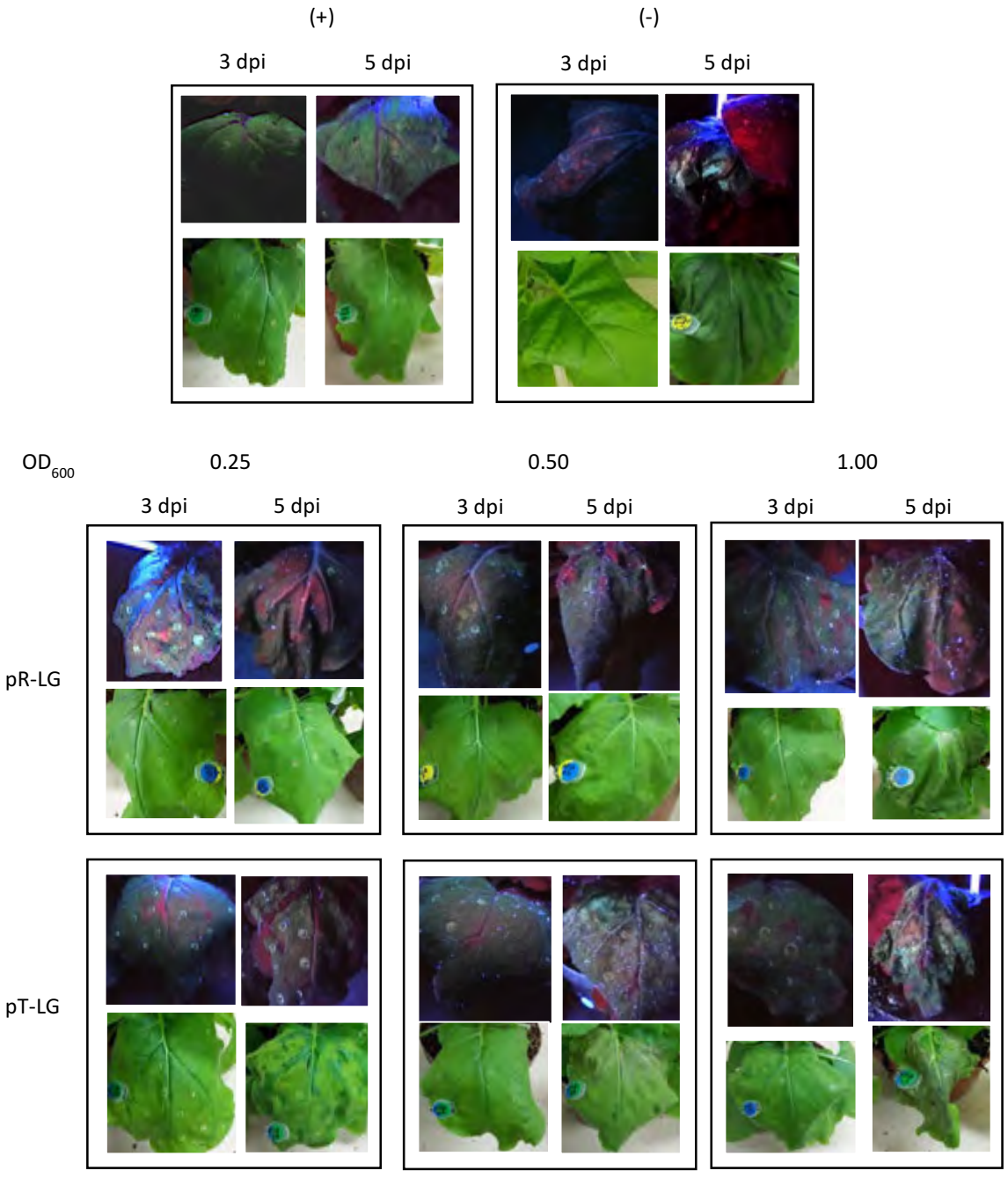

Figure 3.3. Optimization of LALF-E7-EGFP expression in syringe-infiltrated *N. benthamiana* leaves. Plants infiltrated with pRIC3.0-EGFP and pRIC3.0 empty vector were used as positive (+) and negative (-) controls, respectively. Experimental plants were infiltrated with pR-LG and pT-LG at different OD_{600}. Shown here is a representative picture of each vector:OD_{600} combination under UV-light (top) and white light (bottom).

3.3.3. Subcellular localization of LG by fluorescence CLSM

Once the optimal conditions were determined for the expression of LG, plants were vacuum-infiltrated and leaves were visualized under a confocal laser scanning microscope. Young plants were used as their leaves are thinner than that of older plants. This allowed for appropriate leaf sectioning and better visualization.

In cells expressing the positive control, the green fluorescence could be seen evenly distributed in the cytoplasm. No green fluorescence was detected inside the chloroplasts as they were seen as black or empty spheres in the green channel and no yellow was detected when the green and red channels were merged. This represented a typical cytoplasm-localized protein (Figure 3.4A). In samples of the negative controls, pRIC3.0 empty vector and uninfiltrated leaves, no green fluorescence was observed, as expected, and only chloroplasts were detected (Figure 3.4B).

The fluorescence pattern in the pR-LG samples resembled that of the positive control, however it was less intense (Figure 3.4C), as also seen during the optimisation experiments. Besides the evenly spread green fluorescence within the plant cells, large and bright globular structures could also be seen that resembled protein bodies (PBs; Figure 3.4D, yellow arrows).

Interestingly, cells expressing pT-LG presented a very different fluorescence pattern. The LG in these samples were seen as numerous bright small dots also resembling PBs, but smaller and much more abundant than those seen in some pR-LG samples (Figure 3.4E-F). The pattern formed by these PB-like structures followed the pattern formed by the chloroplasts and appeared yellow when seen at a lower magnification (Figure 3.4F, white arrows). On the other hand, the LG PB-like structures did not appear to overlap or to be within the chloroplasts when seen at a higher magnification. They appeared rather associated to the surface of the chloroplasts (Figure 3.4E, blue arrow). However, no LG was seen separated from chloroplasts, even though chloroplasts that did not have a LG PB-like structures attached to them could be seen.

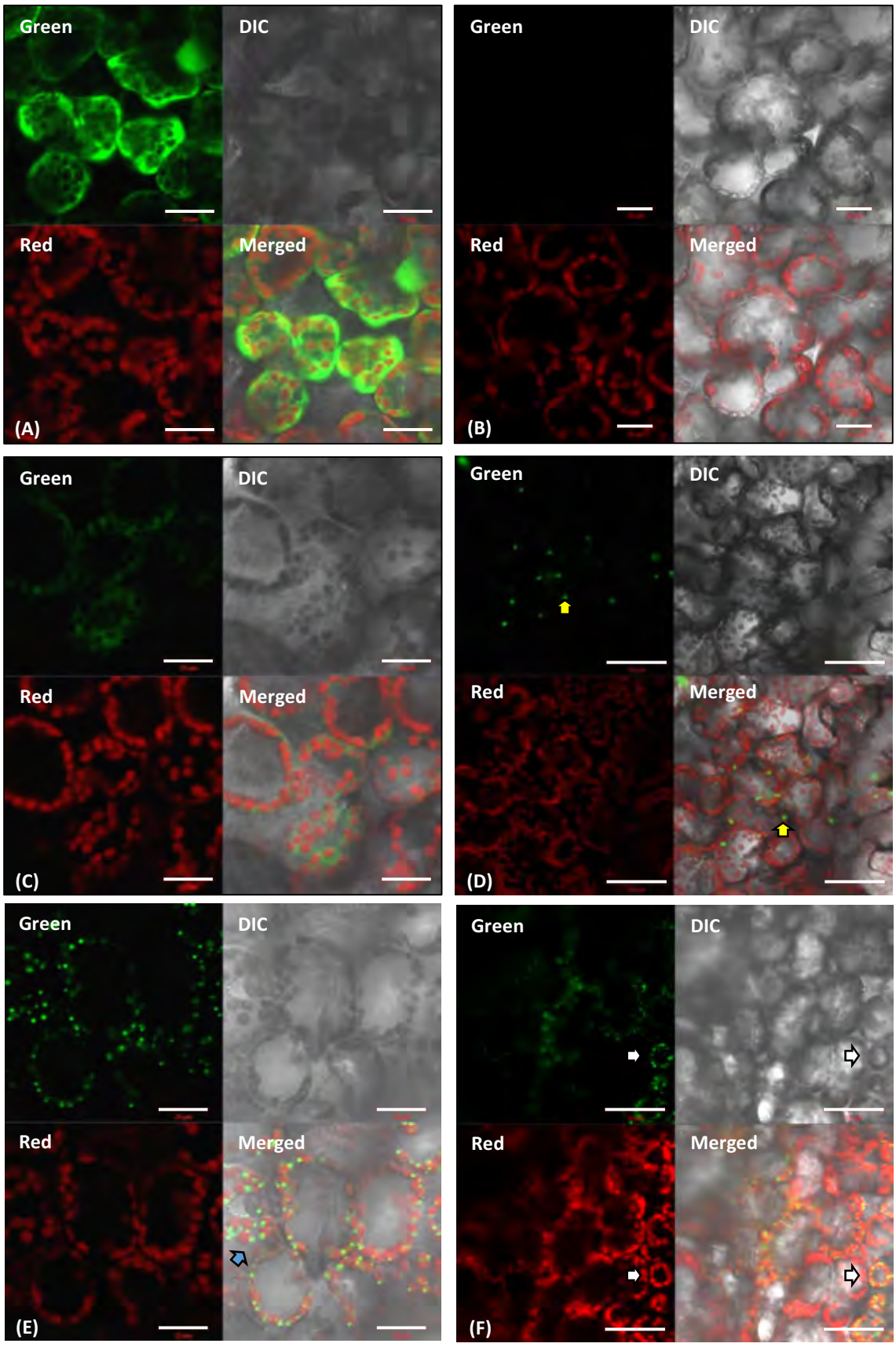

Figure 3.4. Fluorescence CLSM images of the subcellular localization of LG. *N. benthamiana* leaves were vacuum-infiltrated with the relevant constructs. On 3 dpi live leaf sections were prepared for visualization. **(A),** positive control, pRIC3.0-EGFP. **(B),** negative control, pRIC3.0 empty vector. **(C-D),** cytoplasmic pR-LG. **(E-F),** chloroplast-targeted pT-LG. Green channel, EGFP fluorescence. Red channel, chlorophyl

autofluorescence. DIC, differential interference contrast. Merged, an overlap of the Green, Red and DIC channel images. For A-C and E: scale bar = 20 µm, magnification = 40x. For D and F: scale bar = 50 µm, magnification = 20x. Shown here are representative images of several images taken over two independent sets of experiments.

Images taken during the fluorescence microscopy were analysed using the colocalization settings of the Zeiss' Zen software. Shown in Figure 3.5 are representative images of the colocalization profiles obtained. Overall, the pattern seen for the positive control was that as the green fluorescence curve increased, the red fluorescence curve decreased, and vice-versa (Figure 3.5A). Overlapping of the red and green fluorescence curves was not common, as expected, indicating that the EGFP was not localized inside the chloroplast. The negative control contained only a red fluorescence curve (Figure 3.5B). The curve pattern for the cytoplasmic pR-LG was similar to that of the positive control, except that the green fluorescence was lower (Figure 3.5C). On the other hand, the green and red fluorescence curves for chloroplast-targeted pT-LG followed the same pattern, as one increased so did the other. This indicated that pT-LG was associated with the chloroplasts (Figure 3.5D). Here, the green fluorescence curve was at times very high, suggesting that the PB-like structures seen for pT-LG were very concentrated.

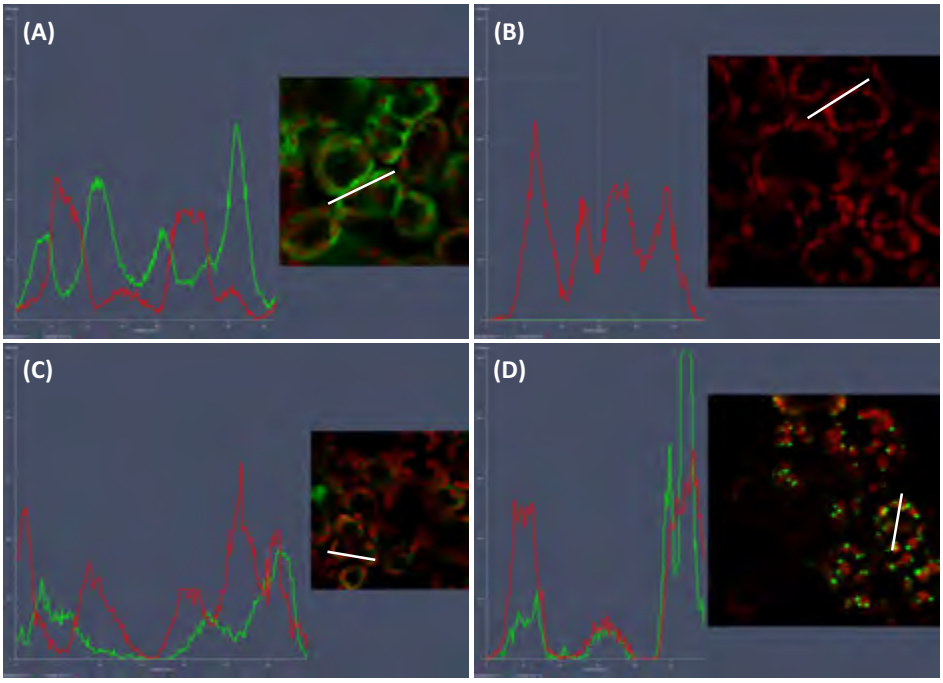

Figure 3.5. Colocalization profiles of LG and chloroplasts. Colocalization profiles were generated using the colocalization feature of the Zeiss Zen software. **(A)**, positive control, pRIC3.0-EGFP. **(B)**, negative control, pRIC3.0 empty vector. **(C)**, cytoplasmic pR-LG. **(D)**, chloroplast-targeted pT-LG. Green and red curves correspond to EGFP fluorescence and the chlorophyl autofluorescence, respectively. White bars represent the area used to generate the colocalization profiles. Magnification of 40X. Shown here are representatives of several images taken over two independent sets of experiments.

After fluorescence confocal microscopy, the remaining leaves were harvested from the vacuum-infiltrated plants. The western blots of the resulting crude extracts confirmed that LALF-EGFP was expressed in these plants. Cytoplasmic and chloroplast-targeted LG formed the same banding patterns, showing high molecular weight aggregates that were not disrupted by boiling with SDS and beta-mercaptoethanol and that were larger than 190 kDa. Furthermore, the most distinct band for both samples appeared at the same molecular weight, of approximately 50 kDa (Figure 3.6).

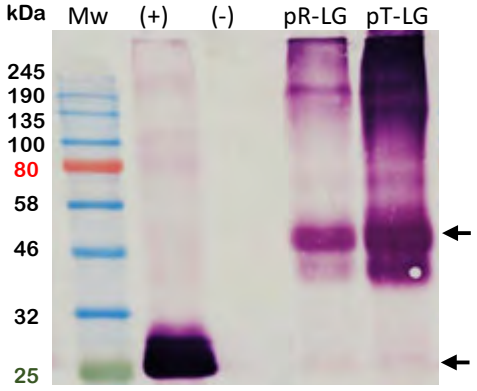

Figure 3.6. Western blot of leaf crude extracts of samples viewed under the fluorescence CLSM. Crude extracts were prepared after visualizing vacuum-infiltrated *N. benthamiana* leaves. **(+)**, positive control, pRIC3.0-EGFP. **(-)**, negative control, pRIC3.0 empty vector. **pR-LG**, cytoplasmic LG. **pT-LG**, chloroplast-targeted LG. Mw, NEB protein molecular weight marker. Sizes are shown on the left hand side in kilodaltons (kDa). Arrows point at the position of LG ≈ 50 kDa and EGFP ≈ 25 kDa. Primary and secondary antibodies: 1:5,000 monoclonal anti-GFP and anti-mouse, respectively.

3.4. Discussion

LALF-E7, accumulated to the highest levels when targeted to the chloroplasts of *N. benthamiana* leaves than when not targeted to any cell compartment. In order to prove whether this protein was indeed being targeted to the plant chloroplasts, I fused it to EGFP and determined its subcellular localization by fluorescence CLSM. LALF is a cell membrane-penetrating peptide (Vallespi *et al.*, 2000), therefore I also wanted to determine whether LALF-E7 interacted with the plant cell membranes.

The predicted molecular weight of the EGFP-tagged LALF-E7 (LG), was 42 kDa, however, it appeared in western blots at 50 kDa. This observation is therefore consistent with previous expression of LALF-E7 and other HPV-16 E7-based proteins which run higher on

acrylamide gels than predicted by their molecular weight (Armstrong and Roman, 1993; Granadillo *et al.*, 2011; Whitehead *et al.*, 2014).

The optimal OD_{600} for the expression of LG was lower than that for the untagged LALF-E7. This was because, here it was desirable to detect LG via fluorescence while maintaining minimum leaf symptoms, unlike in Chapter 2 where the optimization was focused on reaching high expression levels. The leaf symptom development after agroinfiltration with the pRIC3.0 constructs was consistent with that seen in previous expression experiments in Chapter 2, this suggested that they are mainly due to the expression vector, rather than the target protein. However, a toxic effect of the target protein cannot be ruled out.

From the images taken during fluorescence CLSM, it could be seen that LG expressed from pRIC3.0 behaved similarly to the positive control, a typical cytoplasmic protein. It could often also be seen that the cytoplasmic LG formed large PB-like aggregates, however, these were less common and more heterogeneous than those seen in chloroplast-targeted LG samples. It has been shown that LALF-E7 forms inclusion bodies in *E. coli* cells and that it forms large molecular weight aggregates of varying sizes, even after its purification (Granadillo *et al.*, 2011, 2013). However, these PB-like aggregates were not associated with the chloroplasts. This sample also showed a less intense fluorescence than the chloroplast-targeted LG, agreeing with previous accumulation levels obtained for the untagged cytoplasmic LALF-E7.

The fluorescence patterns seen for the chloroplast-targeted LG was distinctively different from that of the cytoplasmic LG. The small spherical structures seen for pT-LG samples clearly formed the same pattern as the chloroplasts, confirming they were associated. These spherical structures were comparable in shape and size to the PBs formed by the chloroplast-targeted γ-Zein-DsRed fusion protein seen by Hofbauer et al. (2014). This group demonstrated that PBs can form in different subcellular localizations, not only in the ER. This suggested that LALF-E7 could form PBs, not only when targeted to the chloroplasts. However, the chloroplast-targeted LG PB-like structures appeared more concentrated and abundant. Unlike in the cytoplasm, no other form of LG was seen when targeted to the chloroplasts. A similar punctuated pattern was also seen by immunofluorescence microscopy of mammalian cells incubated with *E. coli*-produced LALF-E7 (Granadillo *et al.*, 2011).

Scatter plots of the colocalization of EGFP or LALF-E7-EGFP and chloroplasts were generated. These showed colocalization for all samples, except for the negative control, which was not surprising, since the cytoplasmic EGFP and LG were within the cells, surrounding the chloroplasts and other organelles. Furthermore, the chloroplast-targeted LG was not localized within the chloroplasts, at least not within the stroma, making the colocalization scatter plots unreliable for this particular study. Fluorescence profiles proved to be more useful. These further illustrate that, as expected, the LG expressed from pRIC3.0-cTP was associated with chloroplasts, while no association was seen for the same protein expressed from pRIC3.0.

Taken together the microscopy results obtained, I showed that the cTP signal indeed targeted LALF-E7 to the chloroplasts. This agreed with immunogold labelling studies previously conducted in our laboratory [Maclean *et al*. (2007), unpublished data]. Furthermore, I am confident that LALF-E7 entered the chloroplasts, as in western blots it was detected at the same molecular weight as the cytoplasmic LALF-E7, showing that the cTP signal peptide was cleaved during the membrane translocation into the chloroplasts. The same was observed for cytoplasmic and chloroplast-targeted LG.

Chlorophyll pigments are located within the thylakoid membranes in the stromal compartment of chloroplasts. At the resolution power at which the fluorescent CLSM images were taken here, it is not possible to differentiate between the chloroplast compartments. However, if LG was located within the stroma, where it is targeted to, most of the higher magnification images would show yellow PB-like structures. As this was not the case, I hypothesize that LG is located on the surface or in the intermembrane space of the chloroplast envelope. It was not possible to determine whether the PB-like structures were membrane-bound. For that, transmission electron microscopy or higher resolution CLSM imaging would be necessary, as done by Hofbauer *et al*. (2014).

I further hypothesize a mechanism that leads to the formation of the protein PB-like structures seen here (Figure 3.7). The LALF-E7 genetically fused to the cTP is expressed in the plant cell cytoplasm. It is targeted to the chloroplasts by the cTP signal. It enters the chloroplasts stroma through the translocon at the outer envelope membrane of chloroplasts/translocon at the inner envelope membrane of chloroplasts (TOC/TIC) pathway (Li and Chiu, 2010). In the stroma, the cTP signal is cleaved by stromal processing

peptidases. Thereafter, the LALF portion of LALF-E7 mediates the exit from the chloroplast stroma. Alternatively, LALF-E7 exits the chloroplasts by an unknown mechanism. PBs or protein aggregates are subsequently formed either in the envelope intermembrane space or on the surface of the outer envelope membrane. Furthermore, it is possible that the PB-like structures seen are membrane-bound.

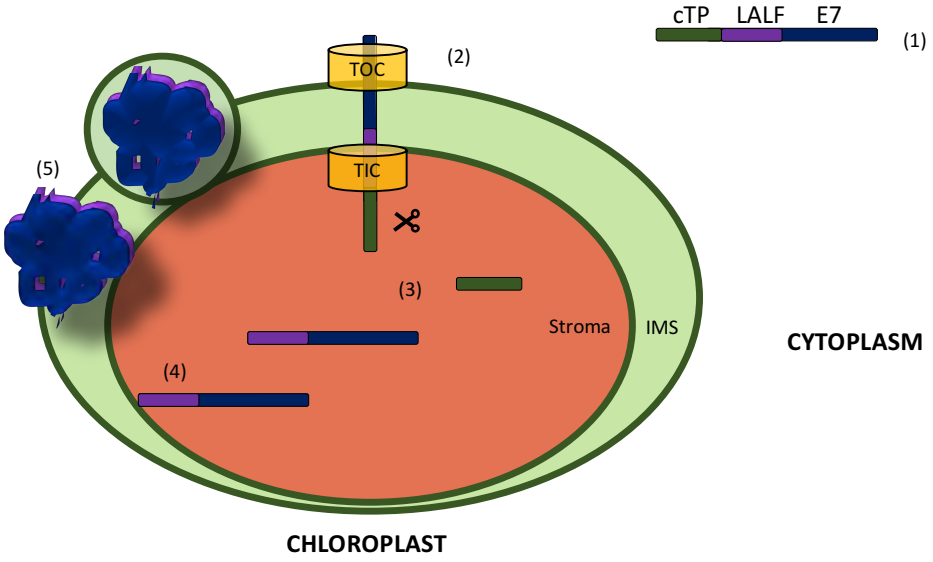

Figure 3.7. Hypothesized mechanism by which LALF-E7 forms protein body-like structures when targeted to the chloroplsts. (1) cTP-LALF-E7 is expressed in the cytoplasm. **(2)** The cTP signal targets LALF-E7 to the chloroplasts where it interacts with TOC complex. cTP-LALF-E7 enters the chloroplast stroma via the TOC/TIC pathway. **(3)** In the stroma, the cTP signal is proteolitically processed by stromal processing peptidases. **(4)** LALF-E7 interacts with the chloroplast envelope membranes mediated by the LALF peptide. **(5)** LALF-E7 exits the stroma and forms protein bodies that could be membrane bound. These are located in IMS of the chloroplast envelope, or on its surface (but remaining in close association). cTP, chloroplast transit peptide. TOC, translocon at the outer envelope membrane of chloroplasts. TIC, translocon at the inner envelope membrane of chloroplasts. IMS, intermembrane space.

Overall, I have shown how LALF-E7 probably forms PBs when targeted to the chloroplasts, which are more concentrated and abundant than for the cytoplasmic LALF-E7. Besides other reasons stated in Chapter 2, these results could explain why LALF-E7 accumulated to much higher levels when targeted to the chloroplasts than when left in the cytoplasm. This further confirms the importance of cellular localization, and highlights that chloroplasts are a useful compartment to target proteins to.

Chapter 4: Large-scale production of LALF-E7 and Immunogenicity studies

4.1. Introduction

4.1.1. Scaling-up plant production and purification of plant-produced recombinant proteins

Plant expression systems offer an advantage over other expression systems in terms of lower upstream production costs (Fischer *et al.*, 2004; Rybicki, 2010; Sack *et al.*, 2015). Plant produced antigens have been shown to elicit immune responses, showing that plants can be used to produce biologically functional pharmaceuticals (Di Bonito *et al.*, 2009; BioTherapeutics, 2010; Whitehead *et al.*, 2014; Beiss *et al.*, 2015). However, the downstream processing of plant-produced proteins has similar costs to those of other expression systems, these accounting for the bulk of production costs. Therefore it is important to optimize their large scale expression, extraction and purification (Rybicki, 2009; Fischer, 2013; Sabalza, Christou and Capell, 2014).

In the case of plant-produced proteins, scaling up expression does not represent the challenge it does in other systems. A large number of plants can be used, depending on the requirements, without changing the expression conditions, since the expression unit continues to be the plant leaf. This is in contrast to cell culture expression systems, in which the conditions change as the volumes increase, which can therefore be more difficult to scale up (Rybicki, 2009).

In terms of protein extraction from plant leaves, the optimization depends on the nature of the recombinant protein and its localization (Fischer, 2013). Proteins targeted to the apoplast can be easily extracted without the disruption of the plant cells. This is useful, as it minimizes the presence of host contaminant proteins in the final crude sample. On the other hand, proteins localized within the plant cells require the breakage of these cells (Łojewska *et al.*, 2016).

Protein solubility is also an important factor in purification. If the protein of interest is insoluble, there is the possibility of removing soluble host proteins before solubilizing the target protein. This is advantageous, since most host proteins are soluble, including the

well-known and common plant contaminant, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), that is known to associate with numerous recombinant proteins expressed in plants and adds to the difficulty of purifying the protein of interest (Buyel, Twyman and Fischer, 2015).

There are different strategies to purify plant-produced proteins, ranging from density gradient ultracentrifugation techniques to precipitation ammonium sulphate (A.S.) treatment, to the more sophisticated metal ion affinity chromatography. The first mentioned method is the most widely used laboratory-scale technique for the purification of virus-like particles (VLPs). Ammonium sulphate can be used to salt out subunit proteins according to their solubility at different A.S. percentages, and is commonly useful for the removal of host proteins in pre-purification steps. Lastly, affinity chromatography can be used to specifically purify the protein of interest, resulting in good recovery rates and purity levels (Buyel *et al.*, 2012; Atkinson *et al.*, 2016; Łojewska *et al.*, 2016).

The hexa-histidine (His)-tag binds to immobilized nickel ions (Ni^{2+}) and is a widely used affinity tag for the purification of recombinant proteins. Nickel column chromatography is a highly specific method that can lead to low protein loss, and often leads to significant enrichment of the protein of interest. Ni^{2+} resins can withstand flexible conditions and numerous regeneration cycles, making them practical and relatively inexpensive to use (Schmitt, Hess and Stunnenberg, 1993; Waugh, 2005). They can also support denaturing conditions, which is advantageous for the purification of highly hydrophobic proteins, which is the case for LALF-E7.

When expressed in *E. coli* cells, LALF-E7 could be purified using a single step of Ni^{2+} affinity chromatography (Granadillo *et al.*, 2011, 2013). In the previous chapters of this dissertation, it was shown how the expression of LALF-E7 in *N. benthamiana* leaves was optimized. Subsequently, it was desirable to scale-up its production and develop a purification strategy to obtain enough of the candidate vaccine to test in animal experiments. For this, I optimized the extraction of LALF-E7 from leaf material and used Ni^{2+} affinity chromatography to purify it.

4.1.2. Evaluation of HPV therapeutic vaccine candidates

The propagation of HPVs in mammalian cells has been difficult, which complicates the characterization of HPV vaccines *in vitro.* HPV vaccine development has benefited from the development and use of tumourigenic mouse cell lines, such as C3 (Feltkamp *et al.*, 1993) and TC-1 (Lin *et al.*, 1996) cells, that express the HPV-16 oncogenes E6 and E7. The C3 line is one of the most commonly used tumour cell lines and contains the entire HPV-16 genome, whereas the TC-1 cell line only contains the HPV-16 E6 and E7 oncogenes. Another important difference between the two cell lines is that in C3 cells, the HPV genes are under the control of their natural promoters, whereas in TC-1, the E6 and E7 genes are under the control of a heterologous retroviral promoter.

The most well studied animal models used for HPV-induced tumours are based on challenging immunised female C57BL/6 mice with these tumour cells, or looking for regression of established tumours after therapeutic immunisation. These are suitable pre-clinical tumour models for the evaluation of HPV therapeutic vaccines targeting E6 and E7 oncogenic proteins, since the above tumourigenic cell lines were derived from these mice.

The candidate HPV-16 therapeutic vaccine LALF-E7 produced in *E. coli* cells was originally evaluated in pre-clinical studies using the TC-1 cell line (Granadillo *et al.*, 2011), and clearly elicited strong specific cell-mediated immune responses capable of inducing both tumour regression and tumour protection. Similar immune responses were obtained when the same vaccine was tested in C3 cell line-induced tumours by the detection of interferon-γ-producing cytotoxic cells identified by Enzyme-Linked ImmunoSpot (ELISPOT) assays (M. Granadillo and R. Lamprecht, 2015, unpublished data).

It was hypothesized that the plant-produced LALF-E7 would have comparable anti-tumour properties as the *E. coli*-produced counterpart. Therefore, the second aim of the work reported in this chapter was to test the plant-produced LALF-E7 in a tumour regression study using female C57BL/6 mice as animal model and C3 cells as HPV-induced tumour models. This was a proof of concept study to show that plant produced LALF-E7 can result in tumour regression, which would then lead to a follow-up study with a tumour challenge experiment.

4.2. Methods

4.2.1. Large scale agroinfiltration of *N. benthamiana* leaves

For large scale expression and purification of LALF-E7, 20-40 plants were vacuum-infiltrated with *A. tumefaciens* GV3101::pMP90RK – pRIC3.0-cTP-LALF-E7 cultures diluted to an OD_{600} of 1.0 as described in section 2.2.4.1. Leaves were harvested on 3 days post infiltration (dpi) and stored at -80 °C until needed.

4.2.2. Preparation of LALF-E7 crude extracts

4.2.2.1. Protocol 1. Leaf material was homogenized with 8 M urea extraction buffer [8 M urea in 1 mM carbonate-bicarbonate buffer, pH 10.6] as in section 2.2.5.2. Homogenates were passed through a double-layer of Miracloth (EDM Chemicals). Extracts were clarified twice by centrifugation. The final crude extracts were stored at -20 °C.

4.2.2.2. Protocol 2. Vacuum-infiltrated leaves were flash-frozen in liquid nitrogen and ground up using a mortar and pestle. Ground leaf material was washed 3 times with PBS or 5-6 times with T-PBS [0.1%Triton X-100 (Sigma-Aldrich) in PBS buffer] by homogenizing in 4 v/w using a T25 digital ULTRATTURRAX® homogenizer (IKA) and subsequently centrifuging at 13,000 rpm (Beckman T Coulter Avanti® J25TI centrifuge) for 10 min at 4 °C. LALF-E7 was extracted from the washed and pelleted plant material with 2-5 v/w of 8 M urea extraction buffer at 4 °C with shaking for 0-4 h and overnight. Extracts were passed through a double-layer of Miracloth and were clarified twice by centrifugation at 13,000 rpm for 20 min at room temperature (RT). The final crude extracts were stored at -20 °C.

4.2.2.3. Ammonium sulphate precipitation to enrich crude extracts. The amount of ammonium sulphate (A.S.) to add to crude extracts for a final concentration of 20% (w/v) was determined via an online ammonium sulphate calculator (EnCore Biotechnology, 2014). The crude extracts were mixed with A.S. for 1 h, at 4 °C with shaking. Precipitated proteins were pelleted by centrifugation at 13,000 rpm for 20 min at 4 °C. The pellet was resuspended in extraction buffer. The supernatant was subsequently treated with 40%, 60% and 80% ammonium sulphate.

4.2.3. Purification of LALF-E7 by nickel ion (Ni^{2+}) affinity chromatography.

LALF-E7 crude extracts were prepared from 50 g FLW using protocol 2. Imidazole (Merck Millipore) and NaCl were added to a final concentration of 20 mM and 500 mM respectively. A 1 ml or 5 ml nickel affinity column (HisTrap™ HP, GE Healthcare) was equilibrated with 5 column volumes (CV) of equilibration buffer [500 mM NaCl, 8 M urea, 20 mM imidazole in 50 mM Na-PO_4 pH 8.0]. The crude extract was loaded onto the column using a 150 ml SuperLoop. Thereafter the column was washed with 10 CV of equilibration buffer or wash buffer [equilibration buffer at pH 6.5]. LALF-E7 was eluted over 20 CV of a linear gradient (from 0% to 100%) of elution buffer [equilibration buffer with 500 mM imidazole] and an additional 5 CV step of 100% elution buffer. The ÄKTA Explorer™ system and the UNICORN 4.11 software (GE Healthcare) were used. All fractions were collected in 1 or 5 ml aliquots, depending on the column used, and stored at -20 °C. Relevant fractions were analysed by western blots and SDS-PAGE gels.

Relevant elution fractions were pooled and extensively dialysed against 130 v/v renaturing buffer [10 mM Tris, pH 8.0]. The dialysis was carried over 4 h, followed by an overnight round and a final round of 4 h. Thereafter, dialysed samples were filter-sterilized through a 0.2 µm Corning® syringe filters (Sigma-Aldrich) and stored at 4 °C.

4.2.4. Detection and quantification of LALF-E7

4.2.4.1. SDS-PAGE and western blots. Refer to section 2.2.7. Gels were stained with Coomassie Brilliant Blue stain (Bio-Rad) for 2 h at 37 °C and destained with destaining solution [30% (v/v) methanol and 10% (v/v) glacial acetic acid in distilled water]; or they were stained with Aqua Stain™ (Vacutec) for 1 h at 37 °C and rinsed in distilled water. For protein detection on western blots the primary antibodies used were the polyclonal anti-HPV-16 E7 rabbit serum (I. Hitzeroth, Biopharming Research Unit, MCB, UCT), monoclonal mouse anti-HPV-16-E7 antibodies [716-218] (Abcam) and anti-6xHis (Sigma-Aldrich). The secondary antibodies used were the goat anti-rabbit and goat anti-mouse IgG whole molecules conjugated to alkaline phosphatase (Sigma-Aldrich). Polyclonal and anti-rabbit antibodies were used at a dilution of 1:5,000. The remaining antibodies were used at a dilution of 1:2,000.

4.2.4.2. Dot blots. A piece of nitrocellulose membrane was divided into squares of approximately 1.5 cm^2, 3 μl of sample were dotted per square and allowed to air-dry. The nitrocellulose membrane was then treated as for normal western blots.

4.2.4.3. Western blot densitometry. The plant-produced LALF-E7 was quantified by western blot densitometry using a serial dilution of *E. coli*-produced LALF-E7 inclusion bodies as a protein standard and anti-His antibodies as a detection method. The Syngene Gene Genius imaging system (Artisan Technology Group) and GeneTools software (Syngene) were used.

4.2.5. Liquid chromatography mass spectrometry (LCMS)

Selected Ni^{2+} affinity chromatography elution fractions containing LALF-E7 were electrophoresed in a 15% SDS-PAGE gel and putative LALF-E7-containing bands were sequenced by LCMS at the Centre for Proteomic and Genomic Research (CPGR; Cape Town).

4.2.6. Tumour regression experiments

4.2.6.1. Animals. Female C57/BL6, SPF-1 and SPF-2 mice from the Research Animal Facility (Health Sciences Faculty, UCT) were maintained under biosafety level 2 (BSL-2) at the Animal Unit (Health Sciences Faculty, UCT). Permission for this study was granted by the Research Animal Ethics Committee, UCT (AEC 013/024).

4.2.6.2. Tumour cells. The C3 and C3-3 cells used in this study were grown and maintained in RPMI media supplemented with 10% foetal calf serum, 1%pen-strep, 0.1 mg/ml Kanamycin and 0.8 mg/ml G418 (Sigma-Aldrich). The C3-3 tumour cell line was generated by R. Lamprecht (BRU, UCT) as follows: C3 tumour cells were used to inoculate 8-9 weeks old female C57/BL6 mice. Thereafter, two tumours from these mice were excised, cut into small fragments and trypsinized. Released cells were washed and expanded in RPMI media. To test the tumorigenicity of these cells, female C57/BL6 were inoculated and had 90% tumorigenicity. The resulting cell line was named C3-3 and was stored at -80 °C until needed. The C3-3 cells were kindly prepared and scaled up to 10 ml or 2.5 x 10^6 cells for animal inoculations by R. Lamprecht and M. Hendrikse (BRU).

4.2.6.3. Tumour inoculation and mice immunizations. Thirty-nine 8-9 weeks old mice were inoculated with 5 x 10^5 C3-3 cells in a final volume of 200 µl subcutaneously (s.c.), into the right inguinal site, after intraperitoneal (i.p.) anaesthesia. Tumour development was monitored daily. When tumours were palpable (approximately 2-3 mm in size), 10 mice received the placebo vaccine [200 µl of 10 mM Tris, pH 8.0], 10 mice received the positive control vaccine [4.6 µg *E. coli*-produced LALF-E7 in 10 mM Tris, pH 8.0 to a final volume of 200 µl], and 19 mice received the plant-produced LALF-E7 vaccine [4.6 µg 10 mM Tris, pH 8.0 to a final volume of 200 µl]. Each group was composed of equal numbers of SPF1 and SPF2 mice. The immunizations were administered by s.c. injections and were repeated weekly to a total of 4 immunizations per mice.

The plant-produced LALF-E7 vaccine was prepared in this study. All steps from extraction to purification were done using depyrogenized glass ware and endotoxin-free, sterile plastic ware. The Tris buffer and the *E. coli*-produced vaccines were prepared by M. Granadillo as reported in Granadillo *et al*. (2011). All vaccines were filter-sterilized through a 0.2 µm membrane and a sample of each was plated on a LB agar plate without antibiotics. Plates were incubated at 27 °C and 37 °C, and examined for growth after 24 and 48 h. In the absence of bacterial growth, the vaccinations were considered safe for animal immunizations.

4.2.6.5. Data collection. Tumour sizes were monitored daily after inoculation and twice a week after the first immunization using a caliper when possible. Tumour volumes were calculated as:

$$Volume\ (mm^3) = \frac{Length \times Width^2}{2}$$

In the case of multiple tumours, all tumours were included to calculate the total tumour volume. Mice were euthanized if one of the tumours reached 20 mm in length or if signs of discomfort were shown.

4.3. Results

4.3.1. Optimization of LALF-E7 crude extraction

Automated chromatography purification systems require crude extracts that have low viscosity and no particulate matter. The initial LALF-E7 crude extracts obtained using the extraction protocol 1 were viscous and could not be passed through a 0.45 µm membrane filter. In order to obtain more appropriate crude extracts to purify LALF-E7 the initial crude extract was treated by A.S. precipitation. It was seen by western blots that LALF-E7 was soluble at 20% A.S., as it was found in the supernatant fraction and was not detected in the pellet fraction (Figure 4.1). Thereafter it was found insoluble at 40% A.S. LALF-E7 was also more concentrated after these precipitation steps compared to the crude extract. From this it was concluded that at the 20% A.S. step, all the plant particulate matter could be removed from the crude extract while concentrating LALF-E7, and that it could be further concentrate at the 40% A.S. step. However, stained gels showed that the A.S. treatment did not remove contaminant host proteins (see lane 40p). Additionally, after resuspending the pellet obtained at the 40% A.S. precipitation step in 8 M urea extraction buffer, the extract was still viscous and could not be completely dissolved.

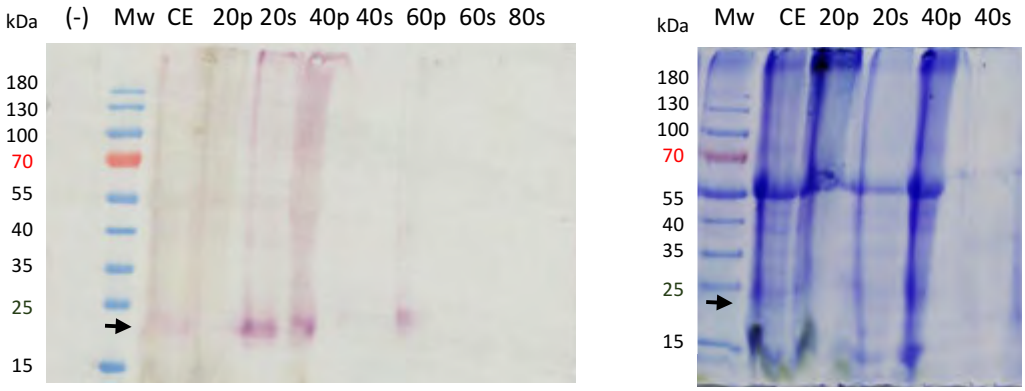

Figure 4.1. Ammonium sulphate precipitation of LALF-E7. LALF-E7 crude extract (CE) from 5 g FLW, prepared using protocol 1 was subjected to 20-80% A.S precipitation. Insoluble (20-60p) and soluble (20-80s) fractions were analysed by western blot using anti-E7 polyclonal antibodies (left-hand-side) and by Coomassie-stained SDS-PAGE gel (right-hand-side). No pellet was formed at the 80% A.S. precipitation step. (-), pRIC3.0 empty vector. Mw, Page Ruler™ molecular weight marker. Band sizes (kDa) are indicated on the left-hand-side. The arrows point to the expected size of LALF-E7 of ≈ 22 kDa. No pellet was formed at the 80% A.S. precipitation step.

Another approach for the extraction of LALF-E7 was developed which was analogous to the extraction of the *E. coli*-produced counterpart, described by Granadillo et al. (2013). Here the leaf material was washed with PBS in order to remove the majority of the plant host soluble proteins, while the highly hydrophobic LALF-E7 stayed in the insoluble fraction. Thereafter, LALF-E7 was solubilized from the plant material with urea extraction buffer. Two concentrations of urea were tested, 6 M and 8 M.

The extraction protocol worked as expected (Figure 4.2A), no LALF-E7 was lost during the PBS washes, and the final crude extract was cleaner than the extracts obtained with the previous extraction protocol (compare the lanes 6M and 8M to PE). There seemed to be slightly more LALF-E7 when extracted with 8 M urea than with 6 M, which was as expected. Furthermore, there were two contaminant proteins, which only appeared after the solubilisation of LALF-E7. One of approximately 35 kDa and the second, larger than 55 kDa. These were in excess compared to LALF-E7 which was not clearly detectable in stained gels. Furthermore, the addition of 0.1% Triton X-100 to the PBS washes and a total of 5-6 washes done in later crude extract preparations, did not remove these contaminant proteins, suggesting they were also highly hydrophobic. However, the addition of Triton X-100 seemed to increase the extraction of LALF-E7 (data not shown). It was concluded that the extraction protocol 2 could be used as a pre-purification step, as most proteins were removed from the final LALF-E7 crude extract, and that Triton X-100 could continue to be used.

An additional problem encountered during the extraction of LALF-E7, was that there were relatively large amounts of LALF-E7 remaining in the washed and pelleted leaf material after the solubilisation step with 8 M urea extraction buffer (Figure 4.2B). To maximize the amount of LALF-E7 extracted from the leaf material, 4-5 v/w extraction buffer was used, instead of 2-3 v/w. Additionally, a time trial was done in which the leaf material was incubated with extraction buffer for 0-4 h and overnight. It was found that the increase in 8 M urea extraction buffer volume did not seem to decrease the amount of LALF-E7 found in the insoluble fraction. Increasing the incubation period slightly increased the amount of LALF-E7 extracted, however it degraded during the overnight step (data not shown).

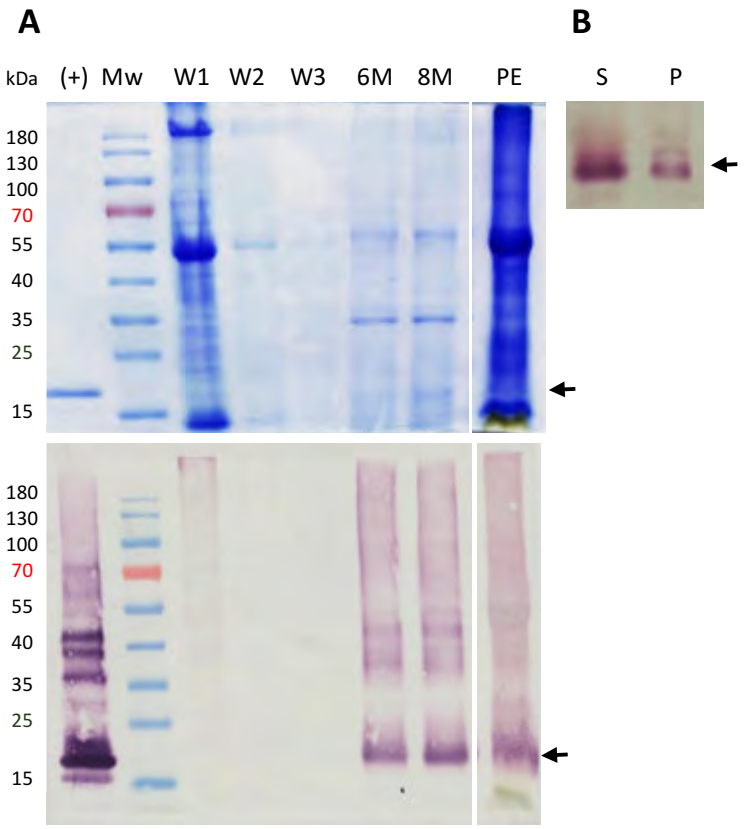

Figure 4.2. LALF-E7 crude extraction protocol 2. (A) Leaf material expressing LALF-E7 was ground up with liquid nitrogen and washed 3 times with PBS (W1-3). LALF-E7 was solubilized from the leaf material with extraction buffer containing 6 M or 8 M urea (6M and 8M, respectively). The final crude extracts are compared to a crude extract sample prepared using the previous extraction protocol (PE). (+), purified *E. coli*-produced LALF-E7. Mw, Page Ruler™ molecular weight marker. Band sizes are indicated on the side in kDa. Top panel, Coomassie-stained SDS-PAGE gel. Bottom panel, anti-E7 polyclonal western blot. **(B)** Anti-E7 polyclonal western blot comparing the amount of LALF-E7 in the soluble and insoluble fractions after the solubilisation step with 8 M urea extraction buffer. (S), 8 M urea-solubilized LALF-E7. (P), insoluble LALF-E7 found in the pelleted leaf debris. Arrows point at the expected size of LALF-E7 of ≈ 22 kDa.

The plant-produced LALF-E7 could also be characterized in terms of its stability in crude extracts and in frozen leaf material. Three temperatures were used to test the stability of LALF-E7 in crude extracts, 4 °C, -20 °C and -80 °C, as well as storage for 1, 2 and 3 weeks. LALF-E7 crude extracts degraded at 4 °C, while storing the crude extracts at -20 °C and -80 °C was comparable, and longer storage periods of time did not seem to lead to losses in LALF-E7 (Figure 4.3). Likewise, it was seen that LALF-E7 was stable in -80 °C frozen leaves for up to 17 months after harvesting. This leaf material was used throughout this study. Storing leaf material did not seem to have adverse effects on LALF-E7 extraction or purification.

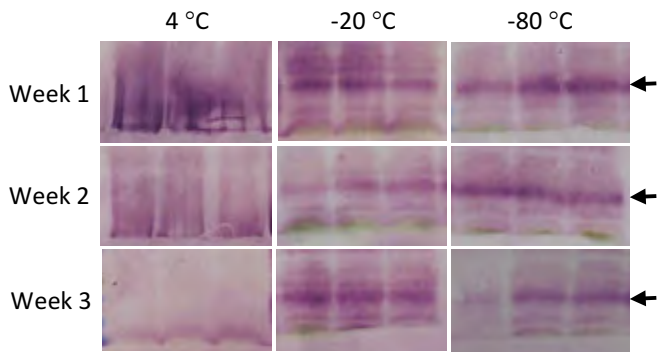

Figure 4.3. Stability of LALF-E7 in crude extracts. LALF-E7 was extracted using protocol 1. Aliquots were stored in triplicates at 4 °C, -20 °C and -80 °C for 1, 2 and 3 weeks. Arrows show expected LALF-E7 size of ≈ 22 kDa.

4.3.2. Purification of LALF-E7 by Ni^{2+} chromatography

Initially, a 1 ml HisTrap column was used to purify LALF-E7. LALF-E7 eluted within elution fractions 1 and 2 as seen by a single large peak in the chromatogram of more than 5,000 Milli absorbance units (mAU) at 280 nm (A_{280}; not shown). The eluted LALF-E7 was concentrated and partially pure. A contaminant protein of approximately 17 kDa and possibly another of approximately 30 kDa could be seen in the Coomassie-stained SDS-PAGE gel (Figure 4.4). Additionally, a precipitation was found in crude extracts before the purification which corresponded to a 25 kDa contaminant protein and degraded LALF-E7 (Figure 4.4, lane P).

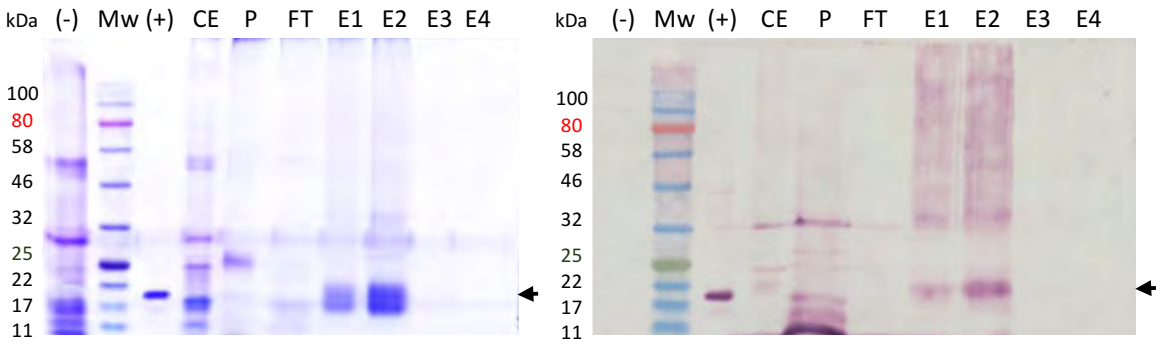

Figure 4.4. Purification of LALF-E7 using a 1 ml HisTrap Ni^{2+} chromatography column. Left panel, Coomassie-stained SDS-PAGE gel. Right panel, anti-E7 polyclonal western blot. (-), pRIC3.0 empty vector crude extract using protocol 2. Mw, NEB protein molecular weight marker. Band sizes are shown on the left hand side. (+), purified *E. coli*-LALF-E7. CE, LALF-E7 crude extract from 50 g FLW, prepared using protocol 2. P, precipitation found before purification. FT, flow through during crude extract loading onto the column. E1-E4, elution fractions 1-4. Arrows point at the expected size of LALF-E7 of ≈ 22 kDa.

In order to purify large amounts of LALF-E7 to be used in animal experiments, I scaled up to a 5 ml HisTrap column. This purification was repeated several times, testing different conditions such as crude extracts prepared using 2-5 v/w ratio of 8 M urea extraction buffer, eliminating the wash buffer step during purification, as well as using imidazole only at the elution step to prevent the loss of LALF-E7. When extracting LALF-E7 with large volumes of extraction buffer, the purified LALF-E7 concentration was very low, as indicated by A_{280} values of 1,100 to 1,300 mAU (not shown). However, when the extraction volume was between 2-3 v/w, more concentrated LALF-E7 was obtained and within less elution fractions, resulting in a A_{280} value of more than 4,000 mAU. Eliminating the wash step at low pH and eliminating the addition of imidazole in the crude extracts and equilibration buffer also prevented the loss of LALF-E7 (Figure 4.5A).

The Ni^{2+} chromatography elution fractions from the 5 ml HisTrap purifications containing LALF-E7 did not correspond to the elution peak, instead they corresponded to adjacent later fractions. This suggested that another protein or proteins eluted with LALF-E7. The AquaStained gel of these fractions indicate that there was at least one contaminant protein in these fractions between 46 and 58 kDa (Figure 4.5B). Furthermore, LALF-E7 appeared as a double-band in the AquaStained gel and in the western blot.

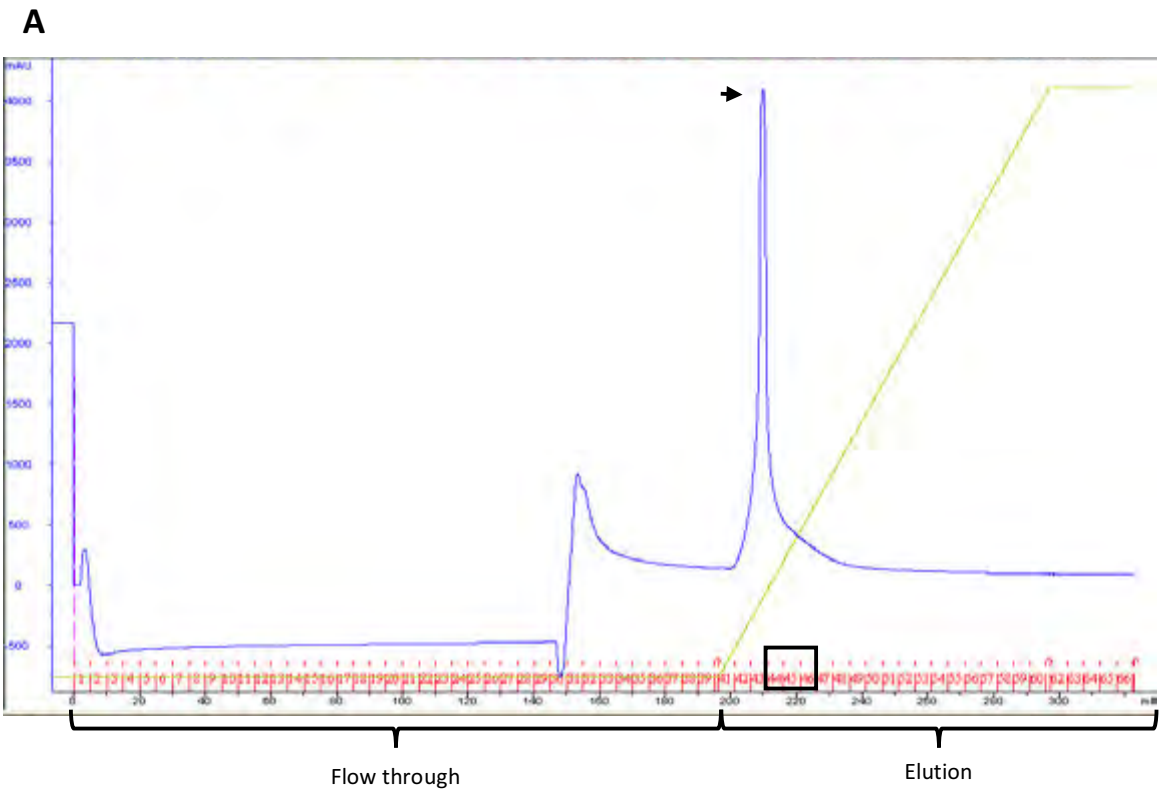

Figure 4.5. Purification of LALF-E7 using a 5 ml HisTrap Ni^{2+} chromatography column. (A), ÄKTA chromatogram. Blue curve, A_{280} representing protein levels. Light-green curve, elution buffer gradient. Arrow shows the elution peak corresponding to approximately elution fractions 1-7. Black box, elution fractions 5-7 corresponding to LALF-E7. (B), analysis of relevant fractions by AquaStained SDS-PAGE gel and anti-E7 polyclonal antibodies western blot (left and right hand side, respectively). Mw, NEB protein molecular weight marker. Band sizes are shown on the left-hand-side. (+), *E. coli*-LALF-E7 inclusion bodies. CE, LALF-E7 crude extract from 50 g FLW, prepared using protocol 2. FT, flow through during crude extract loading onto the column. E4-E13, elution fractions 4-13. Arrows point at the doublet bands representing LALF-E7 ≈ 20 and 22 kDa.

The LALF-E7 double bands were sequenced by mass spectrometry. The results indicated that LALF-E7 was indeed present in these samples by the presence of HPV-16 E7 protein hits. However, there seemed to be other contaminant proteins, such as the Histone H4 and H2b as well as the gremin-like protein of *N. benthamiana*. Therefore, after dialysis,

the quantification of LALF-E7 was done using western blot densitometry, with *E. coli*-produced LALF-E7 as a standard, which was more specific and representative of the true amount of LALF-E7 in the samples selected (Figure 4.6). The partially purified and dialyzed LALF-E7 appeared as a double band again, and so did the purified LALF-E7 provided by M. Granadillo for the animal experiments, unlike the non-purified plant-produced LALF-E7 and *E. coli*-produced LALF-E7 inclusion bodies. It was also seen that the apparent molecular weight of LALF-E7 was lower in the purified *E. coli*-produced preparation (Figure 4.6), and it was higher in the *E. coli*-produced inclusion bodies compared to the plant-produced LALF-E7 (compare the "Plant" lane to the inclusion body protein standard and "*E. coli"* lanes).

The yield of the plant-produced LALF-E7 was calculated as 15 mg/kg FLW at a concentration of 0.0231 mg/ml. The expression of LALF-E7 from pRIC3.0-cTP on 3 dpi was extrapolated to be on average 49.7 mg/Kg FLW in crude extracts prepared using protocol 1. Therefore, 30% of expressed LALF-E7 was recovered during the purification process.

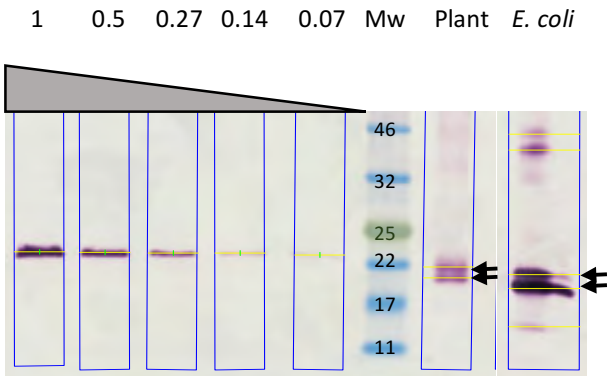

Figure 4.6. Quantification of partially purified and dialysed LALF-E7. Relevant fractions obtained during the purification of LALF-E7 by Ni^{2+} affinity chromatography were pooled and dialyzed against Tris buffer. The dialyzed LALF-E7 was then quantified by western blot densitometry using *E. coli*-produced LALF-E7 inclusion bodies as standard (grey triangle with loaded amounts shown above in μg). Mw, NEB protein molecular weight marker with sizes shown in kDa. Plant, partially purified and dialyzed plant-produced LALF-E7. *E. coli,* purified *E. coil*-produced LALF-E7. Arrows point at the LALF-E7 doublet.

4.3.3. Tumour regression experiments

In order to determine the efficacy of our plant-produced LALF-E7 and to compare it to the *E. coli*-produced counterpart, these candidate vaccines were tested in tumour regression experiments in a mouse model.

Female mice were inoculated with tumourigenic C3-3 cells. Seven days post inoculation, tumours were palpable in all mice. Mice were immunized on day 7 post inoculation and at weekly intervals thereafter for a total of 4 immunizations per mouse. Mice received a placebo vaccine, Tris buffer; 4.6 μg of the *E. coli*-produced LALF-E7 vaccine or 4.6 μg of the plant-produced LALF-E7 vaccine. Thereafter, tumours were measured twice a week.

Most mice showed rapid tumour growth reaching more than 500 mm^3 and were euthanized if a tumour reached 20 mm in length. Most mice presented multiple tumours on day 24 post inoculation. However, mice in the *E. coli*-LALF-E7 group showed less multiple tumour formation than the other groups (Table 4.1). The tumours in the plant-LALF-E7 and the placebo group grew very rapidly in the last three days of the experiment, reaching more than 1,000 mm^3. In the *E. coli*-LALF-E7 group, mice showed a mean tumour volume of less than 800 mm^3 (not shown).

Tumour regression was observed in one mouse in the *E. coli*-LALF-E7 group and two mice in the plant-LALF-E7 group. Of these, the mouse in the *E. coli*-LALF-E7 group and one mouse of the plant-LALF-E7 group had complete tumour regression from 4.0 and 13.5 mm^3, respectively, to undetectable tumours. Furthermore, although, not showing tumour regression, some mice showed relative slow. Mice were considered slow progressors if their total tumour volume, accounting for multiple tumours as well, did not reach more than 599 mm^3 by the end of the experiment, since most mice presented total tumour volumes above this value on the day they were euthanized.

Overall, the plant-produced LALF-E7 was comparable to the *E. coli*-LALF-E7 in terms of tumour regression and slow progression. However, it seemed to not prevent multiple tumour formation and tumours that did not progress slower, seemed to progress to higher volumes than the *E. coli*-LALF-E7 group, comparable to the Tris control, except for four outliers that showed extreme tumour growth towards the end of the experiment.

These observations did not seem to be influenced by the SPF type or age of the mice used in this study.

Table 4.1. Summary of the tumour regression experiment

Treatment	Tris buffer	*E. coli*-produced LALF-E7	Plant-produced LALF-E7
Multiple tumour formation	8/10 (80%)	3/10 (30%)	14/19 (74%)
Slow progressors	2/10 (20%)	3/10 (30%)	5/19 (26%)
Tumour regression	0/10 (0.0%)	1/10 (10%)	2/19 (10.5%)
Mice remaining tumour-free	0/10 (0.0%)	1/10 (10%)	1/19 (5.0%)

The survival rate of mice vaccinated with the plant-LALF-E7, representing the percentage of mice not euthanized at each time-point, was higher than that for the placebo mice, but lower than that for the *E. coli*-LALF-E7 mice by the end of the experiment (Figure 4.7).

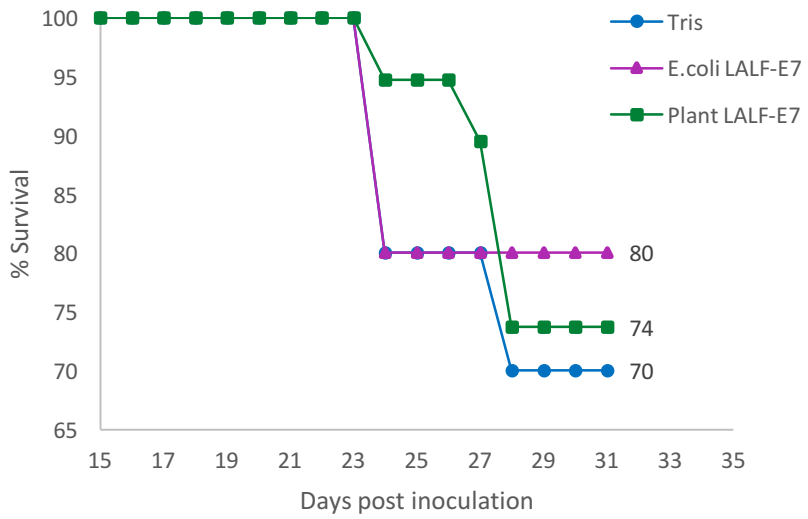

Figure 4.7. Mouse survival rate. Percentage of mice alive at each time point was calculated for each group. Blue spheres, Tris buffer group. Purple triangles, *E. coli*-produced LALF-E7 group. Green squares, plant-produced LALF-E7 group. Days 0-14 post inoculation are omitted for illustration purposes.

On the other hand, the rate of tumour volumes reaching 500 mm^3 was comparable between the vaccine groups. These were relatively different only at the end of the

experiment, where mice in the plant-LALF-E7 group seemed to have fewer tumours over 500 mm^3 than in the *E. coli*-LALF-E7 and the placebo groups (Figure 4.8).

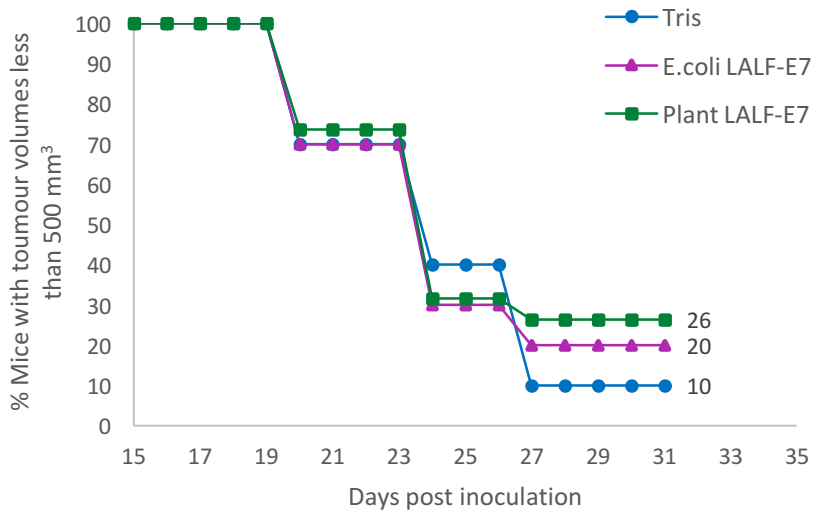

Figure 4.8. Mice showing tumour volumes of less than 500 mm^3. Percentage of mice with a total tumour volume of less than 500 mm^3 at each time point was calculated for each group. Blue spheres, Tris buffer group. Purple triangles, *E. coli*-produced LALF-E7 group. Green squares, plant-produced LALF-E7 group. Days 0-14 post inoculation are omitted for illustration purposes.

4.4. Discussion

4.4.1. Optimization of LALF-E7 crude extraction

The fact that higher accumulation of LALF-E7 was obtained using pRIC3.0 and chloroplast targeting than when expression vectors pEAQ-*HT* and pTRAc were used or when no targeting was used, suggested that pRIC3.0-cTP-LALF-E7 could possibly allow the production of enough quantities of LALF-E7 for its purification and subsequent animal testing. Scaling-up of LALF-E7 expression, extraction and purification strategies were thus explored.

For consistency, I used Agrobacterium cultures at an OD_{600} of 1.0 during the large-scale production of LALF-E7. However, this could be reduced to 0.5 as it was as effective as an OD_{600} of 1.0 (see Chapter 2). Since the addition of a silencing suppressor did not have a major impact on the expression of LALF-E7, I opted to express LALF-E7 targeted to the chloroplasts alone. Using fewer bacterial strains would also simplify the scaling-up process and would be more economical in a possible future industrial set-up.

In order to obtain appropriate crude extracts to purify LALF-E7 through the automated ÄKTA chromatography system, I pre-purified LALF-E7 crude extracts prepared using the protocol 1 by A.S. precipitation (Englard and Seifter, 1990). However, this treatment did not remove host proteins and it did not result in a less viscous final crude extract, which is required for automated affinity chromatography systems. In fact, it appeared that the A.S. treatment concentrated every protein in the crude extract, and therefore did not have the desirable effect that was expected. This could have been a result of all proteins being denatured by 8 M urea which could have eliminated the selective precipitation of proteins according to their intrinsic properties, resulting in all proteins precipitating at one step. Therefore, A.S. precipitation was of limited usefulness.

I subsequently focused on developing an extraction protocol that would result in a "cleaner" crude extract that would also minimize the manipulation of LALF-E7-containing material. The new protocol was analogous to the crude extract preparation used for *E. coli*-produced LALF-E7 (Granadillo et al., 2013). Most host soluble proteins were successfully removed by washing the plant material with PBS before solubilising LALF-E7 with urea. This protocol represented a more efficient way of extracting LALF-E7 from plant material and could be used as a pre-purification step.

Two urea concentrations were tested, 6 M which was the concentration used by Granadillo *et al.* (2013), and 8 M which was the concentration used before in this study. No significant difference was seen, and therefore, for consistency 8 M urea continued to be used throughout this study.

It was seen that even with 8 M urea extraction buffer, and a 4-hour incubation period, the extraction of LALF-E7 from plant material was not complete. Increasing the volume of extraction buffer, did not seem to have an effect on the extraction efficiency of LALF-E7. This suggested a close association of LALF-E7 and leaf material. As seen in Chapter 3, the chloroplast-targeted LALF-E7 forms protein body (PB)-like structures that possibly remain associated to the chloroplast envelope membranes. To circumvent this, differential ultracentrifugation of plant material could be done to purify the plant chloroplasts before solubilizing LALF-E7 (Elias and Givan, 1978; Ferro *et al.*, 2002). This could benefit from more extraction buffer being available to solubilize less plant material, which could increase the extraction of LALF-E7. Alternatively, other stronger denaturing conditions

could be tested to determine if more LALF-E7 can be recovered without leading to its degradation or break down as seen for the overnight incubation of plant material with 8 M urea extraction buffer.

It was seen that LALF-E7 was stable in crude extract preparations only for a short period of time when stored at 4 °C. This could be due to urea-induced carbamylation that can lead to protein degradation (Sun *et al.*, 2014). On the other hand, LALF-E7 was highly stable in frozen plant material at -80 °C, being detected after 17 months post infiltration and harvest. This suggests that the plant material acts as a protective matrix where the protein can be kept for long periods of time. This can also be due to the PB-like structures formed by LALF-E7 in the chloroplasts, that might concentrate it and stabilize it (Torrent *et al*., 2009; Hofbauer and Stoger, 2013). This is useful, as plant material does not need to be processed immediately after harvesting and can be used as a natural long-term storage method.

4.4.2. Purification of LALF-E7

Once the extraction of LALF-E7 was optimized, its expression was scaled-up to further develop a purification strategy based on Ni^{2+} affinity chromatography. A drawback of the large-scale extraction of LALF-E7 using protocol 2 was that more host contaminant proteins became more detectable. This was not unexpected and is often seen that when scaling up starting material for extraction, more proteins can be extracted.

I first tested a HisTrap column volume of 1 ml as a pilot experiment. This showed that LALF-E7 could be concentrated as seen by the high A_{280} obtained during the chromatography and the fact that LALF-E7 was clearly visible in stained gels, despite a possible 17 kDa contaminant being present. However, LALF-E7 eluted at a very low elution buffer percentage, suggesting that it was loosely bound to the Ni^{2+} resin. This further suggested that LALF-E7's His-tag might not have been fully exposed to the resin, possibly influenced by the conditions used.

Using a larger column volume, removing the imidazole present in the equilibration buffer and eliminating the wash step at low pH proved to enhance the purification conditions. However, complete removal of contaminant proteins was not achieved. Nevertheless, LALF-E7 seemed to be in excess to contaminant proteins in later elution fractions.

The fact that a small portion of LALF-E7 co-eluted with most contaminant proteins in early elution fractions, while it was more strongly detected in later elution fractions, suggested that a portion of LALF-E7 molecules is associated with these host proteins. The contaminant proteins were not found in the flow-through. In fact, no proteins seemed to be detected in the flow-through as seen in the chromatogram and stained gel in Figure 4.5. The apparent interaction of LALF-E7 and host proteins was not completely eliminated by the denaturing conditions used in the process, nor by the addition of the detergent Triton X-100, suggesting this association was strong. This could account for the incomplete exposure of the LALF-E7's His-tag leading to loose binding and for the co-elution of these proteins with LALF-E7. This could be seen in both column volumes used, but was more prominent when the 5 ml HisTrap columns were used, probably due to the larger volume of resin available that could have led to a better binding of LALF-E7 and LALF-E7-host protein complexes. This may be confirmed by the fact that LALF-E7 eluted in later fractions when a larger HisTrap column was used.

Overall, the use of Ni^{2+} affinity chromatography seemed to have only concentrated LALF-E7, but not purified it. It appeared that LALF-E7 was mostly purified by the extraction protocol 2 which removed the bulk of the host contaminant proteins.

It was noted that after the affinity chromatography, the plant-produced LALF-E7 appeared as a double band. This was also seen for the *E. coli*-produced LALF-E7. Similar doublet formation was observed by Demurtas *et al.* (2013), who proposed these were phosphorylated forms of the protein. The HPV-16 E7 protein is known to be phosphorylated at serine residues (Munger and Halpern, 1997).

The final concentration of LALF-E7 after the affinity chromatography was 0.0231 mg/ml, equivalent to a yield of 15 mg/kg FLW which represented a recovery rate of 30%. This low recovery rate can be attributed to the incomplete extraction of LALF-E7 from leaf material and the manipulation of crude extracts during affinity chromatography. Manipulation of protein extracts can lead to protein degradation and protein loss. There is a negative relationship between protein recovery and number of purification steps.

The downstream processing of most recombinant proteins can account for 80% of the production cost, which in fact currently represents a bottleneck in the development of low-cost biopharmaceuticals (Rybicki, 2009; Fischer *et al.*, 2012; Sabalza, Christou and

Capell, 2014). Therefore, it is important that an economic and efficient purification method is applied to increase the recovery of LALF-E7, if it is to have a future as a therapeutic. However, the low yield of LALF-E7 could be a reflection of its low expression levels in plant leaves. Even though the best conditions yet achieved for plant-produced LALF-E7 were used, namely, a replicative expression vector and chloroplast targeting which increased the expression of LALF-E7 by 26.8-fold, LALF-E7 only expressed at levels up to 0.5% total soluble protein (TSP) or 50 mg/kg. This yield was lower than those reported for other E7-based plant-expressed proteins. For example, Buyel et al. (2012) report the expression of a HPV-16 E7 fusion protein of 233 mg/kg FLW in *N. benthamiana.*

Whitehead *et al.,* (2014) report expression levels of a Zera®-fused HPV-16 E7 protein of 150 mg/kg, while expression levels of 1,100 mg/kg were obtained when the E7 protein sequence was shuffled. This suggests that this protein might be detrimental to the plant cells. Since E7 can interact with other proteins besides the retinoblastoma protein (Moody and Laimins, 2010; Roman and Munger, 2013), it is possible that it interferes with plant cell pathways, which is not seen for E7 expression in prokaryotic expression systems (Granadillo *et al.*, 2013). The effects of E7 together with symptoms induced by the replicative vector seen in Chapter 2 might have compromised the expression of LALF-E7, compared to other plant-produced proteins. Furthermore, Demurtas et al. (2013) reported a His-tag being detrimental to the expression of transplastomic HPV-16 E7-based protein (E7GGG) in algae cells. When a different affinity tag was used, the protein expression level was increased.

Overall, it could be worth to test the expression of LALF-E7 in a different expression vector, such as pTRA-cTP, while removing the His-tag and developing non-chromatographic purification strategies. For example, chloroplasts could be purified by differential ultra-centrifugation techniques and thereafter, LALF-E7 could be extracted from the chloroplast envelopes (Elias and Givan, 1978; Ferro *et al.*, 2002). This would provide a more practical means of purifying LALF-E7. Alternatively, targeting LALF-E7 to secretory pathways fusing it to carrier molecules and could increase its expression levels, as demonstrated by Franconi *et al.* (2006) for HPV-16 E7, and Massa *et al.* (2007) and Buyel *et al.* (2012) for E7GGG.

4.4.3. Tumour regression experiments

The *E. coli*-produced LALF-E7 was previously shown to induce tumour-specific cell-mediated responses and tumour regression in mice (Granadillo *et al.,* 2011). In this study, I tested the plant-produced LALF-E7 to determine whether it would have the same successful effects.

Numerous HPV E7-based plant-produced therapeutic vaccine candidates have been tested in tumour protection as well as tumour regression experiments (Franconi *et al.*, 2002, 2006; Massa *et al.*, 2007; Venuti *et al.*, 2009; Demurtas *et al.*, 2013). However, since it is important that the therapeutic vaccine be efficient at eliminating already existing tumours or at least at minimizing their growth (Ma *et al.*, 2010; Morrow, Yan and Sardesai, 2013), in the current pilot experiment, I prioritized the determination of whether the plant-produced LALF-E7 could induce tumour regression in mice animal models.

It was previously observed that mice inoculated with our group's tumour-inducing C3 cell line showed spontaneous tumour regression even in placebo groups (M. Granadillo and R. Lamprecht, unpublished data). Therefore, a more tumorigenic cell line (C3-3) was developed in order to distinguish between spontaneous and vaccine-induced tumour regression. These rapidly formed tumours in 90% of inoculated mice in previous experiments (R. Lamprecht, unpublished data). The high tumorigenicity was also observed in the present study that led to tumour development in all mice by 7 days post inoculation.

The vaccine dosage used in the present study was 4.6 μg of protein per immunization. This was lower than generally used for tumour regression studies based on HPV E7-derived vaccines. For example, Granadillo *et al.* (2011) used 30-120 μg of LALF-E7 and Wick and Webb, (2011) used 100 μg of Pentarix. However due to technical limitations, this represented the maximum possible dose, given that the maximum injection volume allowed was 200 μl per mouse.

Other groups have used low antigen doses of E7-based vaccines and demonstrate their anti-tumour effects as well as the induction of antigen-specific humoral and cell-mediated immune responses (Franconi *et al.*, 2002, 2006; Massa *et al.*, 2007; Venuti *et al.*, 2009; Demurtas *et al.*, 2013). However, they only test the prophylactic activity of the vaccine (Demurtas *et al.*, 2013), vaccinate on days 0 or 3 post tumour cell inoculations (Franconi

et al., 2002; Massa *et al.*, 2007), and/or measure the tumour progression in terms of tumour-free mice (Massa *et al.*, 2007; Venuti *et al.*, 2009; Demurtas *et al.*, 2013). In the present study, mice were only immunized after tumour development and tumour progression was measured in terms of tumour volumes. Therefore, the above mentioned studies are of limited comparison to the current study.

Due to the tumour cell strength and low vaccine dosages, it was seen that tumour development was very rapid and did not seem to reach a plateau before mice had to be euthanized. This was seen in the plant-LALF-E7 group as well as in the *E. coli*-LALF-E7 and the placebo groups, suggesting it was not due to an adverse effect of the antigens.

Whitehead et al. (2014) used antigen dosages of 5 μg Zera®-16E7SH fusion protein in tumour regression experiments. The fact that they could observe differences in tumour progression of mice in the vaccinated group compared to the placebo group, suggests that, indeed, the high tumorigenicity of the C3-3 cells might have obscured the potential of the plant and *E. coli*-produced LALF-E7 vaccinations. Furthermore, the Zera® peptide acted as an adjuvant in these experiments, and so did LALF in the studies performed by Granadillo et al. (2011). Therefore, the differences seen here compared to Whitehead et al. (2014) are unlikely to be entirely due to the Zera® peptide.

In the present study, differences between the vaccination groups could be better seen in terms of survival rates and the relative ability to delay tumour growth. It could be seen that by the end of the experiment the plant- and *E. coli*-LALF-E7 groups had better survival rates than that of the placebo group, however, the plant-LALF-E7 group survival rate was lower than that of the *E. coli*-LALF-E7. It was also noticed that the *E. coli*-LALF-E7 group showed less multiple tumour formation. On the other hand, the plant-produced LALF-E7 vaccine seemed to limit tumour growth to 500 mm^3, to a better extent than the *E. coli*-produced vaccine. The differences seen between the vaccine groups were very small and not statistically relevant. However, they suggested that the two vaccines are somewhat comparable and did have an effect on the immune response of the mice tested, since these groups performed slightly better that the placebo group. The fact that 10% of mice showed tumour regression in the vaccine groups versus no regression in the placebo group could further confirm that this is due to the immunizations. However, spontaneous regression cannot be ruled out.

The *E. coli*-produced LALF-E7 was previously tested against TC-1 and C3 cell-tumours by Granadillo et al. (2011) and by our group (unpublished data), using high vaccination doses of 60 μg. LALF-E7 was able to induce strong tumour-specific cell mediated responses as seen by ELISPOT assays. Therefore, in future studies, higher vaccine doses, lower number of C3-3 cells in inoculums as well as larger group sizes could allow for a more conclusive characterization of the effects of plant-produced LALF-E7 in tumour regression experiments.

Chapter 5: Conclusions

Therapeutic vaccines are greatly needed for human papillomavirus infections due to the large number of individuals that are already infected by the viruses who will not benefit from the prophylactic vaccines, and for those that have developed HPV-associated malignancies. Since more than 85% of HPV-associated cervical cancer cases and deaths occur in developing countries, it is important that a future therapeutic vaccine is not only efficient, but also cost-effective (Giorgi, Franconi and Rybicki, 2010).

The current study explored the possibility of producing a cost-effective therapeutic vaccine candidate for HPV-16 which is based on a mutated viral E7 oncoprotein fused to a cell membrane-penetrating and immunomodulatory peptide, LALF - creating LALF-E7 (Granadillo *et al.*, 2011).

The aims of this study were (1), to optimize the expression of LALF-E7 in *Nicotiana benthamiana* leaves; (2), to determine the subcellular localization of LALF-E7 in plant cells; (3), to develop a large-scale extraction and purification strategy, and finally (4), to test the plant-produced LALF-E7 in a mouse tumour model to determine whether it was comparable to the *E. coli*-produced LALF-E7 in terms of tumour regression.

The highest expression levels of LALF-E7 obtained were achieved by using the replicating expression vector pRIC3.0, and targeting LALF-E7 to the plant chloroplasts via the rbcS1 chloroplast transit peptide (cTP). Compared to cytoplasmic localization, chloroplast targeting increased the expression of LALF-E7 by 26.8-fold. Silencing suppressors did not have a significant impact on the expression of LALF-E7 as they only increased it by 1.3 fold. I concluded that the effect of increased gene copy number early post infiltration of the plant leaves by the replicating expression vector, and the subcellular localization of LALF-E7 had a major impact on its accumulation levels, circumventing the effects of gene silencing.

I proved, by fluorescent confocal microscopy, that LALF-E7 is indeed targeted to the plant chloroplasts. I further described how LALF-E7 potentially forms protein bodies (PBs) when targeted to these compartments, and hypothesized a mechanism for this. The potential of the LALF peptide to induce PB formation has important implications. It could possibly be fused to other proteins to increase their yields, since PB formation increases stability

and concentration of target proteins as well as facilitates their purification (Torrent *et al.*, 2009; Hofbauer and Stoger, 2013). Furthermore, it would be interesting to determine if targeting LALF-E7 to the endoplasmic reticulum (ER), where PBs are naturally derived from, would increase the accumulation of this protein. Another advantage of targeting LALF-E7 to the ER would be that the PBs would possibly be easier to purify, by for example, differential ultracentrifugation, as done by Whitehead *et al.* (2014), unlike the current PB-like structures seen for cTP-LALF-E7 that were closely associated with the chloroplast envelopes.

It was seen that the cTP-LALF-E7 expressed to ≈ 50 mg/kg fresh leaves [0.5% total soluble proteins (TSP)]. Even though this was the highest accumulation level obtained for LALF-E7 produced in plants and that this was obtained using the best conditions described so far, this expression level was low compared to that of other plant-produced proteins. This could be a consequence of strong symptoms induced by the replicating vector used, or possible cell toxicity induced by the target protein. To circumvent this, the use of the non-replicating vector, pTRAc and targeting to the chloroplasts, ER or apoplast could be tested. Alternatively, the HPV-16 E7 sequence used here could be replaced with the shuffled sequence used by Whitehead *et al.* (2014), creating LALF-16E7SH which could possibly be less toxic. Since viable expression levels for plant expression systems are considered to be > 1% TSP (Fischer *et al.*, 2004), further increase in the accumulation of LALF-E7 in *N. benthamiana* leaves is probably needed.

Scaling up the production of LALF-E7 was not a challenge, and it was possible to develop an extraction strategy that removed the bulk of host contaminant proteins, acting as a partial purification step. In fact, this protocol was more successful at removing contaminant proteins than the use of affinity chromatography. This is hypothesized to be due to the close interaction of LALF-E7 with other insoluble host proteins that led to their co-elution during affinity chromatography. It suggested that LALF-E7 was closely associated to these proteins since this interaction did not seem to have been prevented by the denaturing conditions used throughout the extraction and purification process.

It would be useful to develop an alternative non-affinity chromatography purification strategy for LALF-E7, that would also lead to a further more concentrated final preparation. For example, chloroplasts could be purified by differential ultra-

centrifugation techniques and thereafter, LALF-E7 could be extracted from the chloroplast envelopes (Elias and Givan, 1978; Ferro *et al.*, 2002). This could facilitate the purification process and would provide a means of enrichment, since the final extracts could have smaller volumes than the current LALF-E7 extracts.

It was shown by Beiss *et al.* (2015) that a range of high temperature treatments can be applied to plant crude extracts that promote host contaminant protein removal. Such treatments could be tested at the last step of soluble protein removal during LALF-E7 crude extract preparations. It is known that LALF-E7, and HPVs' E7 proteins in general, are heat-resistant since they contain cysteine residues that form disulphide bonds resistant to reducing conditions and boiling for long periods of time (Imai *et al.*, 1991; Demurtas *et al.*, 2013; Roman and Munger, 2013). Therefore, high temperature treatments of the plant material containing LALF-E7 could be used to remove the few proteins that remain possibly associated to LALF-E7. High temperatures could as well increase the extraction efficiency of LALF-E7 from the plant material.

Furthermore, the His-tag could be removed from the LALF-E7 sequence. As suggested by Demurtas *et al.* (2013), this affinity tag could be detrimental for the expression of certain proteins. If this is true for LALF-E7, removing the His-tag could lead to higher protein accumulation levels.

Finally, I tested the plant-produced LALF-E7 in tumour regression experiments using female C57/BL6 mice as animal models and an enhanced C3 cell line (C3-3) as a HPV-specific tumour model. It was not possible to evaluate the anti-tumour effects of the plant-produced and the *E. coli*-produced LALF-E7 in the present study due to low antigen doses and the high tumorigenicity of the C3-3 cell line. However, differences in survival rate and percentage of mice having very large tumour volumes compared to that of the placebo group may suggest that the plant-produced LALF-E7 had an influence in the immune response of these mice. This needs to be confirmed using higher doses of antigen, lower number of tumorigenic cells and larger vaccination groups. Another alternative could be the use of a homologous prime-boost vaccination strategy, as done by Wick and Webb (2011). Nevertheless, it is known that LALF-E7 is able to elicit tumour-specific immune responses and tumour regression in mice, if a higher antigen dose than

that used in this study is given (Granadillo *et al.*, 2011). It is unlikely that the plant-derived LALF-E7 is unable to elicit similar responses.

A possible alternative to the vaccine design implemented in this study is the use of pseudovirions (PsVs) to deliver the therapeutic vaccine in the form of encapsidated DNA. Our group has recently succeeded in producing plant-made HPV-16 PsVs capable of encapsidating a reporter gene-encoding plasmid that were able to infect mammalian cells and express the reporter gene in a similar way to a natural viral infection mechanism (Lamprecht *et al.*, 2016). The reporter gene in the encapsidated plasmid could therefore be replaced by the sequence encoding for LALF-E7 to create such a vaccine. This could further act as a chimeric vaccine, eliciting anti-L1 and anti-L2 humoral as well as anti-E7 cell-mediated immune responses.

In summary, I report here for the first time the entire chain of research involving the expression of LALF-E7 in plants, and the testing of its immunogenicity and possible therapeutic activity in a mouse model. There have been numerous improvements to its accumulation in *N. benthamiana* leaves, but there is further possible optimizations and different strategies that can be implemented. This study was also important as a proof of concept that protein localization plays a great role in protein accumulation in plant expression systems. It was also a proof of concept that our group's cTP efficiently targets proteins to the chloroplasts as shown by fluorescent microscopy. This study further indicated new avenues for the possible applications of the LALF peptide as a potential PB-inducer that could enhance the expression of other recombinant proteins.

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