Development of Rift Valley fever virus candidate vaccines and reagents produced in *Nicotiana benthamiana*

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Declaration

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Abstract

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Rift Valley fever (RVF) is a haemorrhagic fever agent caused by an infection with an enveloped negative-stranded RNA Rift Valley fever virus (RVFV). It belongs to the genus *Phlebovirus* in the family *Bunyaviridae*. The virus is spread by infected mosquitoes and affects ruminants and humans, causing high numbers of neonatal fatalities in animals and occasional fatalities in humans. It is endemic to parts of Africa and the Arabian Peninsula, but is described as an emerging virus due to the wide range of mosquitoes that could spread the disease into non-endemic areas, posing serious health and agricultural problems. The disease can be prevented by vaccination, but there is currently no Food and Drug Administration-approved RVFV vaccine that can be used outside endemic areas, while there are two live attenuated vaccines available for use in endemic areas. These vaccines have the potential for reversion, and are therefore not recommended for use in countries where RVFV is not endemic. This indicates the need for more RVFV vaccine research and development. This work focused on the development of a RVFV vaccine candidate that would allow for differentiation between infected and vaccinated animals as well as humans.

A readily available wild type Egyptian ZH548 RVFV *GnGc* polyprotein-encoding gene sequence was initially used for developing the candidate vaccines. The *GnGc* genes were cloned into various plant expression vectors with and without a histidine tag. Initial strategies and efforts made to express GnGc protein resulted in a lack of recombinant protein detection. Thus, a different *GnGc* DNA sequence representing a South African isolate M35/74 was selected to make putative vaccine candidates; this gene was *Nicotiana* sp.- and human-codon optimised and both were synthesised. Both genes were modified for various cloning strategies into different plant expression vectors. Gene expression was conducted by transient expression of recombinant constructs in *Nicotiana benthamiana* via *Agrobacterium tumefaciens*-mediated gene transfer. Protein expression analysis was verified by western blotting of crude leaf extracts separated using SDS-PAGE. Protein expression could not be detected from the full length *GnGc* glycoprotein gene.

The lack of recombinant protein expression from the full length *GnGc* lead to the RVFV *Gn* gene being used for further studies. *Gn* was modified by truncation of its transmembrane domain and cytoplasmic tail (TMD-CT) and substituting its native signal peptide with two heterologous signal peptides, namely protein disulphide isomerase (PDI) and murine mAB24 heavy chain (LPH), to make a soluble recombinant protein vaccine candidates (PDI-ΔtGn and LPH-ΔtGn). Virus-like particles are known to be more immunogenic than recombinant proteins alone because they display authentic conformation of the virus, thus displaying their immunogenic epitopes. In addition to the recombinant protein vaccine, a second type of vaccine candidate was designed to form chimaeric VLPs by fusing the LPH-ΔtGn to the influenza hemagglutinin TMD-CT (LPH-ΔtGnHA).

Gene expression was conducted by transient expression of recombinant constructs in *N. benthamiana* via *A. tumefaciens*-mediated gene transfer. Protein expression analysis was verified by western blotting of crude leaf extracts separated using SDS-PAGE. Unfortunately, no recombinant protein expression was detected using the PDI-ΔtGn vaccine candidate. Expression was successfully detected using the modified RVFV LPH-ΔtGn and LPH-ΔtGnHA vaccine candidate crude leaf extracts. Production

of these recombinant vaccine candidates was scaled up and protocols for their purification were developed. The soluble LPH-ΔtGn was initially partially purified by ammonium sulphate precipitation and then by affinity chromatography. The protein yields were calculated to be ~ 56.81 mg/kg fresh weight. The chimaeric particulate LPH-ΔtGnHA candidate was purified by differential centrifugation. The protein yields were calculated to be ~ 59 mg/kg fresh weight. Protein was characterised by transmission electron microscopy (TEM). TEM showed chimaeric Gn particles of ~49 – 60 nm. Preliminary immunogenicity studies of both modified RVFV Gn candidate vaccines was carried out in BALB/c mice. The vaccine candidates were found to be immunogenic in mice.

The second focus of this research was on the development of a diagnostic reagent. Diagnosis of RVFV is carried out by various methods including ELISA, using the nucleocapsid protein (N-protein) as a diagnostic agent. N-protein is the most abundant non-structural protein in the virion. It has been used as a diagnostic reagent for other viruses in the *Bunyaviridae* family. N-protein is currently produced from live virus preparations which involve high levels of biosafety, high production costs and have restricted use outside of RVF endemic areas due to potential escape of the virus in non-endemic areas. This work describes the production of a cost-effective and safer diagnostic N-protein antigen using plants as an expression system.

The South African isolate M35/74 RVF N-protein was human-codon optimised and synthesised. Recombinant protein expression was successfully detected in plants when fused with a histidine tag. Purification by affinity chromatography yielded high amounts of protein ranging from 500 - 558 mg/kg fresh weight. TEM of purified preparations showed that the protein forms ring shaped structures of ~10 nm. Preliminary data carried out using purified protein showed that the N-protein is stable after at least 8 months when stored at both 4 and - 80 ˚C. Preliminary ELISAs carried out revealed that plant-produced N-protein was functional as it could successfully differentiate between serum isolated from infected and non-infected animals, thus indicating it has potential use as a diagnostic reagent.

In conclusion, this research demonstrates the potential of LPH-ΔtGn and LPH-ΔtGnHA vaccine candidates. They both elicited anti-Gn immune responses in vaccinated mice without the use of adjuvant. These result show that these vaccine candidates have potential as RVFV candidate vaccines and their development should be further investigated. The plant-produced N-protein detected IgG antibodies against RVFV-infected sheep serum.

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While my name maybe alone on the front cover of this thesis. I am by no means its sole contributor. There are several people behind this piece of work, who deserve to be both acknowledged and thanked here:

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PREFACE

This thesis is presented as a compilation of nine chapters. Each chapter is introduced separately and is written to the style of the **Frontiers-Science Journal**

CHAPTER 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

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CHAPTER 4 **RESEARCH RESULTS**

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List of abbreviations

Abbreviations

5FU	5-fluorouracil
6xhis	Histamine
aa	Amino acids
ACT2	Arabidopsis actin 2
ARC-OVI	Agricultural Research Council – Onderstepoort Veterinary Institute
BeYDV	Bean yellow dwarf virus
bp	Base pair
BRU	Biopharming Research Unit
BSL	Biosafety level
BTI	*Bacillus thuringiensis israeliensis*
CaMV	Cauliflower mosaic virus
Carb	Carbenicillin
CDC	Centre for Diseases Control
CPMV	Cowpea mosaic virus
CTL	Cytotoxic T lymphocyte
DIVA	Differentiate Infected and Vaccinated Animals
dNTP	deoxy-ribosenucleoside triphosphates (dATP, dCTP, tTTP and dGTP)
dpi	Days post infiltration
DTT	DL-dithiothreitol

EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
Env	Envelope protein
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
FMD	Food and Mouth Disease
FMDV	Foot and mouth disease virus
FRhL-2	Entebbe strain in diploid cells
FW	Fresh weight
Gc	Carboxy-terminal glycoproteins
GFP	Green fluorescent protein
Gn	Amino-terminal glycoproteins
HA	Hemagglutination assay
HI	Hemagglutination inhibition
HT	Hyptertranslatable
IFA	Immunofluorescence assay
IPTG	Isoprophylthio-β-D-galactoside
Kan	Kanamycin
L	Large

LA	Luria agar
LAMP	Loop mediated isothermal amplification
LB	Left border/ Luria-broth
LC-MS	Liquid chromatography – mass spectrometry
LIR	Long intergenic region
LPH	Murine mAb24 heavy chain
LSDV	Lumpy skin disease virus
M	Medium/molar
M1	Matrix protein
MCS	Multiple cloning site
MES	2-morpholineethanesulforic acid
MP	Movement protein
MRC5	Human diploid lung cells
N	Nucleocapsid protein
nAbs	Neutralising antibodies
NBT/BCIP	Nitro blue tetrazolium chloride/5-bromo-4chloro-3-indolyl phosphate
NP	Nucleoprotein
NSR	Nonspreading RVFV
O/N	Overnight
OIE	Office for International des Epizooties
Ori	Origin of replication

PAGE	Polyacrylamide gel electrophoresis
PB(s)	Protein bodies
PDI	Protein disulphide isomerase
PTGS	Post-translational gene silencing
R/Rev	Reverse
RB	Right border
Rif	Rifampicin
RNP	Ribonucleoprotein
RT	Room temperature
RVF	Rift valley fever
RVFV	Rift Valley Fever virus
SANS	South African National Accreditation System
SIR	Short intergenic region
Sp.	Species
TBSV	Tomato bushy stunt virus
T-DNA	Transfer-DNA
TE	Transient expression
Ti	Tumour-inducing
TMV	Tobacco mosaic virus
Tris	Tris(hydroxymethyl)aminomethane
TSP	Total soluble protein
TSWV	Tomato spotted wilt virus
U	Unit

USDA	U.S Department of Agriculture
UTR	Untranslated region
VEEV	Venezuelan equine encephalitis virus
VLP	Virus-like particles
VN	Virus neutralization
wt	weight
WT	Wild type
x-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Contents

Chapter 1: General Introduction and project aims

1.1 General Introduction

Rift Valley fever (RVF) is a zoonotic infectious disease which primarily affects ruminants, and is caused by the enveloped Rift Valley fever virus (RVFV) with a three-component single-stranded RNA genome, in the genus *Phlebovirus* of the family *Bunyaviridae*. It is predominantly spread by mosquitoes (Moutailler et al., 2008). Infections in animals occur predominantly as a result of bites from mosquitoes carrying the virus but can also be spread by direct contact with infected animal tissues and fluids, which is the main route by which humans are infected (Morrill et al., 1997b; Moutailler et al., 2008). Symptoms of infection in ruminants vary between different animal species, but include the occurrence of near simultaneous abortions in pregnant animals, high neonatal mortality rates, hepatic damage and deformed young. Case fatality rates vary between 20 – 70% (Chevalier et al., 2010; Gerdes, 2004). Infection of humans is often asymptomatic, but after an incubation period of 2 to 6 days symptoms can manifest as headaches, muscular pain, photophobia, weakness or onset of fever (McIntosh et al., 1980a). Most patients recover from the disease completely after several weeks, but those more susceptible can develop retinal macular change which causes defective vision for a while. Less than 1% of those infected develop complications which can lead to haemorrhagic fever or encephalitis, with a case fatality rate of about 45% (LaBeaud et al., 2008; McIntosh et al., 1980b).

RVFV poses a great economic threat to the Agricultural and Health sectors, and is thus of interest in the One Health paradigm. The World Organization for Animal Health (Office for International des Epizooties (OIE)) has classified this virus as a high-consequence pathogen with the potential for international spread (OIE - List A) (Bird et al., 2009). The lack of efficient prophylactic and therapeutic measures make infection with this virus a serious public concern not only in endemic, developing countries, but also in many non-endemic, developed countries. The spread of RVFV can be prevented by vaccination of animals and humans, but there are currently no

licensed RVFV vaccines for use in animals and humans (Ikegami and Makino, 2009; Ly et al., 2016).

Currently available animal vaccines are available as live attenuated RVF virus which is effective, but is also expensive due to the requirement for high biosafety levels during production (Muller et al., 1995; Smithburn, 1949). An inactivated RVFV vaccine has also been used on animals: however, this is not as effective, requires booster inoculations and also has high biosafety level requirements for production (Randall et al., 1962). For humans, there is no commercially available vaccine. However, a killed vaccine which is made under stringent safety restrictions is used to immunise individuals who work in regions with high risk of infections (army workers and laboratory workers). This vaccine requires several booster administrations to maintain complete immunity (Eddy and Peters, 1980; Niklasson, 1982).

The envelope glycoproteins of RVFV (Gc and Gn), found on the outermost surface of the virions, are the most exposed parts of the virus. They show a high degree of sequence conservation, and there is effectively only one serotype of the virus - therefore, a single vaccine should be able to provide protective immunity against currently circulating RVFV strains (Ikegami, 2012). Recombinant Gn and Gc glycoproteins have been produced in bacterial, mammalian and insect cell expression systems and have been shown to elicit virus neutralising antibodies (de Boer et al., 2010; El Salam, 2014; Habjan et al., 2009; Schmaljohn et al., 1989). These neutralising antibodies (nAbs) have been shown to protect against the disease. Further, some research has shown that sufficient neutralising antibodies are induced by Gn protein alone to protect mice and sheep against RVFV challenge. However, all the above production systems are costly, not easy to scale up, they do not produce glycosylated proteins, contaminating endotoxins are difficult to remove and recombinant proteins often form inclusion bodies. As an alternative to the above-mentioned systems, is the use of the plant-production system. an attempt was recently made to develop a RVFV candidate vaccine using transgenic *Arabidopsis thaliana* (Kalbina et al., 2016). The vaccine candidate was orally immunised and was shown to be immunogenic.

Detection of the virus using traditional culture methods is time-consuming, and requires biosafety level 3 or 4 facilities. This has led to the development of enzyme-linked immunosorbent assays (ELISA) as a highly sensitive and rapid diagnostic tool (Paweska et al., 1995). This is based on use of an inactivated antigen which binds to RVFV antibodies, production of which also requires biosafety containment facilities, and which binds poorly to the ELISA plates (Jansen van Vuren et al., 2007). In addition, such antigen does not allow for distinguishing between infected and vaccinated animal or human serum.

There is therefore a need for a cheap, safe and reliable vaccine, and for antigens for diagnostic use which allow for detecting RVFV antibodies in animal and human sera. Plants offer a viable alternative to the above-mentioned conventional expression systems. Production can be rapidly scaled up, and yields may be high, resulting in potential for significantly reducing costs of raw material (Lomonossoff and D'Aoust, 2016; Marsian and Lomonossoff, 2016; Rybicki, 2009; Rybicki, 2010). The plant production system is more economical than the convensional expression sytems, as plants do not need expensive materials for growth and maintenance, and their use reduces concerns over human pathogen contamination of vaccine and reagent preparations.

1.2 Project Aims

The main objective of my study was to produce a Rift Valley fever candidate vaccine in *Nicotiana benthamiana,* as well as a diagnostic reagent for differentiating between infected and vaccinated animals. In order to achieve this, the specific aims of the study were four-fold.

1. **To identify Rift Valley fever genes suitable for development of plant-made candidate vaccines**

   Objectives:

   a. To clone RVFV *GnGc* glycoprotein genes into plant expression vectors

b. To assess recombinant GnGc levels in plants after infiltration on a small scale, and to scale up production if successful
c. To purify and characterise the selected candidate vaccine protein(s) by chromatography and transmission electron microscopy

2. **To use the results obtained from Aim 1 to produce one or more candidate RVFV vaccines by fusing Gn with an Influenza virus HA transmembrane domain and co-expression with influenza M1 and RVFV nucleocapsid protein (N-protein)**

Objectives:

a. To clone constructs derived from the relevant genes into plant expression vectors and do protein expression analysis to pick the best-expressing constructs for further analysis
b. To purify proteins and putative virus-like particles and characterize extracts by ultracentrifugation and electron microscopy

3. **To test the immunogenicity of candidate plant-produced vaccines in mice**

Objectives:

a. To analyse the immunogenicity of the plant-produced candidate vaccines in BALB/c mice

4. **To develop a diagnostic reagent for detection of RVFV in animal serum using the N-protein**

Objectives:

a. To clone the *N* gene into plant expression vectors and do protein expression analysis to select the best expressing plasmid for further analysis
b. To purify the N-protein and characterise by chromatography and electron microscopy
c. To test the functionality of the purified N-protein in ELISA tests using sera from animals previously shown to be infected or not infected with RVFV and compare it to conventional reagents

Chapter 2: Literature review

2.1 Rift Valley Fever

Rift Valley fever (RVF) is a mosquito-transmitted viral zoonotic disease which affects animals primarily, as well as humans. RVF was first identified in the Rift Valley in Kenya in 1931 when it caused an outbreak of disease in sheep (Daubney et al., 1931). The causative agent of RVF is the Rift Valley fever virus (RVFV), which belongs to the *Phlebovirus* genus in the *Bunyaviridae* family (Elliott, 1997). RVFV infects and is transmitted by more than 30 mosquito species which belong to seven genera, of which *Aedes. sp* and *Culex*. sp are considered the most significant. RVFV persists for a long time in the mosquito population. This virus can also be harboured by other vectors such as ticks and sand-flies.

No antigenic differences have been reported between viral isolates involved in the different outbreaks in African countries; however, there is some evidence that there may be differences in virulence of certain isolates. Infections in animals occur predominantly as a result of bites from mosquitoes carrying the virus (Moutailler et al., 2008). The main route of transmission to humans, however, is by direct contact with infected animal tissues and fluids, generally during the preparation of meat. (Morrill et al., 1997b; Wilson, 1994). They can also be infected by mosquito bites (Davies and Martin, 2006) during periods of high vector population levels, such as during rainy seasons. No human to human transmission has been reported to date.

2.2 Viral structure

RVFV is a glycoprotein-enveloped virus with a tri-segmented single-stranded RNA genome, with genes mostly translated in the negative sense. The genome is composed of a large segment (L), a medium segment (M), and a third small (S) segment which is ambisense in terms of having ORFs in both (+) and (-) senses. Each segment is enclosed in a separate helical nucleocapsid protein (N-protein) within the same virion (Garcia et al., 2001). The L segment encodes the viral RNA polymerase (~6.4 kb) which is used for replication and mRNA transcription. The M (~3.8 kb) segment encodes a polyprotein consisting of an amino terminal glycoprotein (Gn) and a carboxy-terminal glycoprotein (Gc) as well as two non-structural proteins, NSm1 (78

kDa) and NSm2 (14 kDa), which play an important role in RVFV infection and pathogenesis (Freiberg et al., 2008; Gerrard et al., 2007; Won et al., 2006) (Figure 2.1).

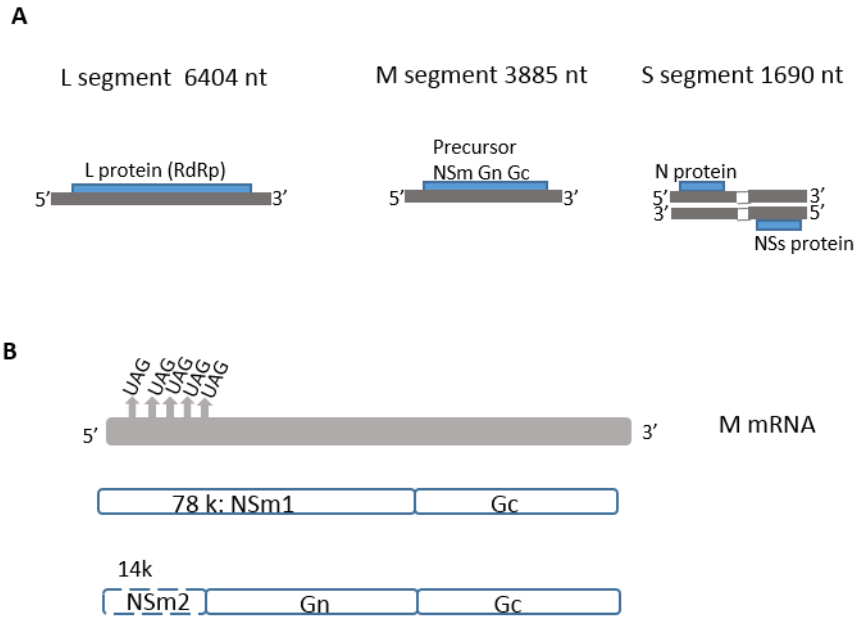

Figure 2.1: The illustration of the Rift Valley fever virus genome. **A**) The three genomic large segments (L), medium (M) and small (S) and proteins encoded by them. **B**) The mRNA transcribed from the M segment contains five in-frame start codons which give rise to the NSm1 and NSm2 proteins, as well as some minor products (Bouloy and Flick, 2009).

RVFV particles consist of an enveloped virion measuring 90 - 100 nm in diameter (Figure 2.2) (Ellis et al., 1979). The envelope is composed of a lipid bilayer containing the Gn and the Gc glycoprotein surface subunits which are 5 – 8 nm in length. These are regularly arranged on its surface forming an icosahedral shell of 122 capsomers (Huiskonen et al., 2009), similar to those reported for the related Uukuniemi Phlebovirus (Pettersson and von Bonsdorff, 1975). Within the inner envelope of the virion, ribonucleoprotein (RNP) complexes composed of the three genomic segments associated with the nucleoprotein and the RNA-dependent RNA polymerase are packed proximal to the inner envelope, suggesting a possible interaction between the glycoproteins and RNPs (Boshra et al., 2011; Bouloy and Flick, 2009; Pepin et al., 2010).

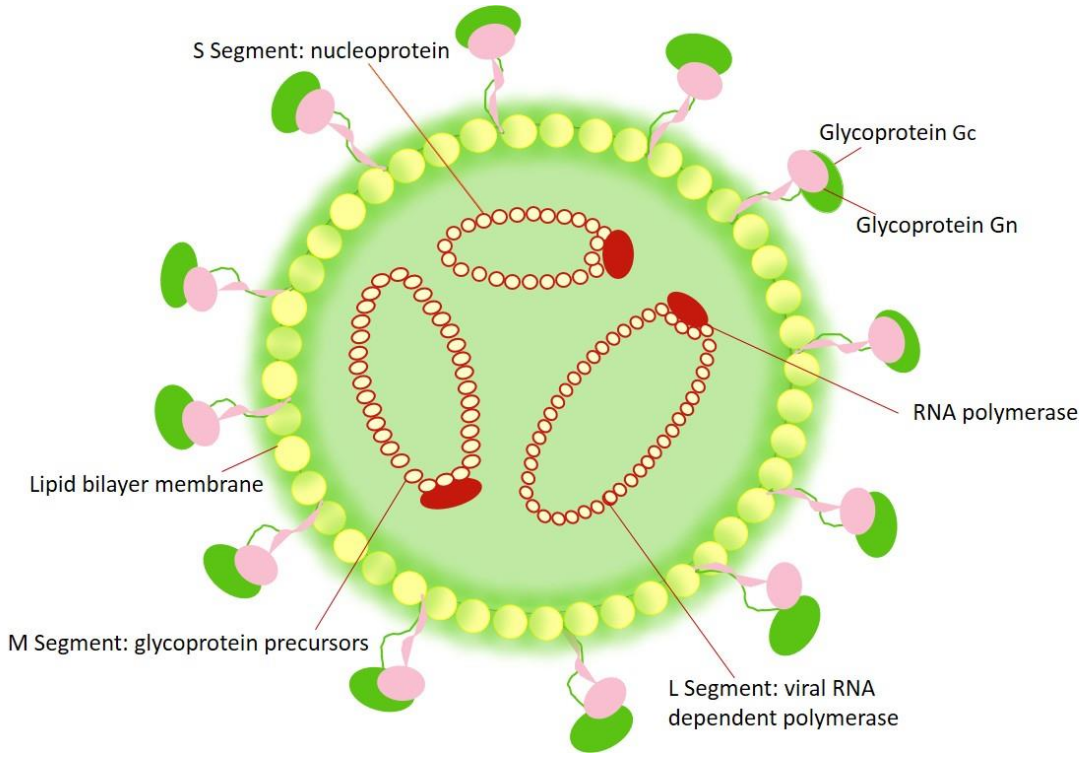

Figure 2.2: Illustration of the Rift Valley fever virus virion adapted from Pepin et al. (Pepin et al., 2010).

2.3 Viral entry and transcription

RVFV binds to unidentified cellular receptors, then enters the cells using a class II fusion mechanism which is activated by low pH following endocytosis of the virion. Viral transcription and replication take place in cytoplasm of the infected cells (de Boer et al., 2012; Filone et al., 2006). The L segment allows RNA replication and viral RNA transcription. The M (~3.8 kDa) segment contains four nested protein genes in a single open reading frame (ORF): these are the two structural glycoproteins Gn (55 kDa) and Gc (58 kDa) and two non-structural proteins, the 14 kDa NSm and the 78 kDa NSm-Gn fusion protein (Lagerqvist et al., 2009; Suzich et al., 1990). The NSm1 and NSm2 proteins are produced by alternative use of the first and second of the 5 in-frame AUG codons, respectively, which are present at the 5' end of the M mRNA upstream of the Gn. They function as virus virulence factors and determinants for host pathogenesis. NSm is dispensable for efficient RVF virus growth in both interferon-competent and interferon-deficient cell cultures (Bird et al., 2007; Gerrard et al., 2007; Le May et al., 2004; Vialat et al., 2000; Won et al., 2006).

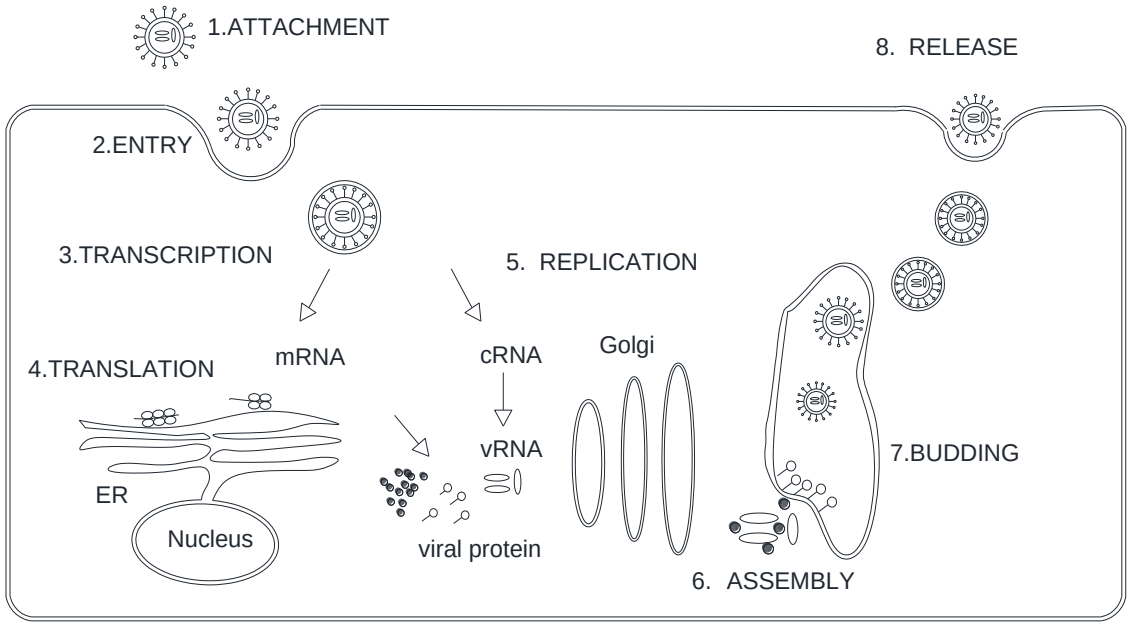

Figure 2.3: A model showing the RVFV replication cycle

The Gn and Gc polyproteins are produced when translation initiates at the second, fourth or fifth of the 5 in-frame AUG codons (Gerrard and Nichol, 2002). They are inserted in the virus lipid envelope and are responsible for cell tropism and membrane fusion. When Gn is expressed on its own, it is transported to the Golgi apparatus due to the specific Golgi localization signal at the C-terminus, whereas Gc is localized in the endoplasmic reticulum (ER) due to the presence of an ER retention signal (Gerrard and Nichol, 2002). The RVFV GnGc polyprotein localizes to the Golgi apparatus. The Gc moves to the Golgi apparatus due its physical association with Gn (Wasmoen et al., 1988); the remaining structural proteins and the genome are recruited by the glycoproteins, followed by viral budding. The mature virions are released from the cell through fusion of virion-filled Golgi elements with the plasma membrane (Elliott, 1997; Gerrard and Nichol, 2002). The endodomain of Gn interacts with the N-protein and this interaction is important for genome packaging into infectious virus particles.

The S segment (~1.6 kb) encodes the viral N-protein (27 kDa) in its antisense orientation, while the complementary orientation encodes the non-structural protein NSs (Gerrard et al., 2007; Struthers et al., 1984). After viral uncoating, viral ribonucleocapsids (RNP) composed of genomic RNA segments and N-protein are

released into the cytoplasm. The NSs is a filamentous nuclear protein that functions as a virus virulent factor, by the inhibition of the host's RNA polymerase II activity indirectly disrupting the host cell antiviral response and assembles in the cytoplasm of the infected cell, thus, plays an important role in pathogenesis (Bird et al., 2007; Vialat et al., 2000; Won et al., 2006).

2.4 RVFV outbreaks

Outbreaks of RVFV are often associated with abnormal heavy rains resulting in an accumulation of surface water in dams and depressions. These are ideal conditions for hatching mosquito eggs, which can survive in dry mud for a number of seasons (Davies et al., 1985; Linthicum et al., 1985). The virus is mainly detectable when weather conditions allow for a sufficient number of mosquitoes to breed. RVFV is transmitted transovarially in mosquitoes, which provide a reservoir of infection during epizootics. During an outbreak, mosquitoes feed on livestock such as cattle, sheep and goats. Infection with the virus results in significant losses of livestock and results in abortion storms and death of new-borns and young animals.

Table 2.1 lists the major RVFV outbreaks occurring from 1974 to 2010. The RVF epidemic that occurred in Saudi Arabia between 2000 and 2001 had a 13% human fatality rate (Control and Prevention, 2000). However, statistics from recent outbreaks that occurred in 2006 - 2007 in East Africa, Somalia and Tanzania revealed that the case fatality rate was significantly increased, to about 45% for those with complications (Bouloy and Flick, 2009; LaBeaud et al., 2008), when more than 300 patients died (2007a; 2007b). The biggest outbreak in South Africa occurred in 1974 – 1976 when more than 10 000 infections were reported. The most recent outbreak occurred from February to September in 2010, with 242 human RVF cases, of which 25 were deaths were reported (Archer et al., 2013; Métras et al., 2012).

Table 2.1: Major Rift valley fever outbreaks from 1974 - 2010

Year	Country (human infections)	Reference
1974 - 1974	South Africa (10 000 – 20 000)	(McIntosh et al., 1980b)
1977 - 1978	Egypt (200 000, 594 deaths)	(El-Akkad, 1978; Meegan, 1979; Meegan et al., 1979)
1987	Mauritania, Senegal (8900, 220 deaths)	(Digoutte et al., 1989; Jouan et al., 1989)
1997 - 1998	Kenya (89 000, 478 deaths)	(Woods et al., 2002)
2000 - 2001	Saudi Arabia, Yemen (2000, 245 deaths)	(Control and Prevention, 2000)
2006 - 2007	Somalia, Kenya, Tanzania (1 062, 315 deaths)	(2007a; 2007b)
2010	South Africa (242, 25 deaths)	(Métras et al., 2012)
2012	Mauritania (36, 18 deaths)	http://www.who.int/mediacentre/factsheets/fs207/en/
2016	Niger (348, 33 deaths)	http://www.who.int/mediacentre/factsheets/fs207/en/

The virus was restricted to sub-Saharan Africa prior to the middle 1970s, but has now become endemic to parts of North Africa and the Arabian Peninsula. Recently, large outbreaks have been reported in Kenya, South Africa, Senegal, Mauritania, Egypt and Madagascar (Control and Prevention, 2000). For the first time in 2000, RVFV cases were reported in Saudi Arabia and Yemen. These cases of the disease outside the African continent raise concerns that the virus could extend to other parts of Asia and Europe (Control and Prevention, 2000). Due to the large number of mosquitoes that can transmit or spread the virus to non-endemic areas, it is considered an important emerging disease.

2.5 Clinical Symptoms

2.5.1 RVFV in animals

Symptoms of infection in ruminants vary between different animal species but include the occurrence of near simultaneous abortions in pregnant animals in the same herd, high neonatal mortality rates, hepatic damage and deformed young (Arishi et al., 2006). Clinical manifestations vary depending on the age of the animal. In sheep, a fever of 41 - 42 °C is observed after a short incubation period. Newborn lambs usually die within 36 to 40 h after the onset of symptoms, with mortality rates reaching 95%. Older animals either die or develop mild infection. In pregnant ewes, abortions are frequent, ranging from 5 – 100%. Twenty percent of aborting ewes die. These animals may also experience fever with depression, haemorrhagic diarrhoea, blood staining muco-purulent nasal discharge and icterus. Case fatality rates vary between 20 – 30% (Gerdes, 2004). In cows, calves often develop acute illness, with fever, diarrhoea and dyspnoea and mortality rates vary from 10 – 70% (Chevalier et al., 2010).

2.5.2 RVFV in humans

Infection of humans yields no symptoms until after an incubation period of 2 to 6 days. The high-risk individuals are herders, farmers, slaughterhouse workers and veterinarians. Most people develop inapparent infections, with some having moderate to severe non-fatal flu-like symptoms characterized by fever, headache, light sensitivity, muscle and joint pain. Some people develop neck stiffness, sensitivity to light, loss of appetite and vomiting. Complete recovery usually occurs in two weeks (Ma et al., 2015; McIntosh et al., 1980a; McIntosh et al., 1980b).

In severe cases, however, people infected can develop ocular lesions (0.5 – 2%) resulting in blurred vision which may resolve with time, but detachment of the retina may occur in a few cases causing permanent blindness. Less than 1% of people infected develop severe haemorrhagic syndrome or meningoencephalitis, which may be fatal. These symptoms usually occur within four weeks of infection. Clinical features of encephalitis include intense headache, loss of memory, hallucinations, confusion, disorientation, vertigo, convulsions, lethargy and coma. Neurological

complications can appear at later stages. Haemorrhagic fever begins with the evidence of severe liver impairment, such as jaundice. This is subsequently followed by signs of haemorrhage such as vomiting blood, blood in the faeces, a purplish discolouration of the skin or subcutaneous spots of blood, bleeding from the nose or gum, menorrhagia and bleeding from venepuncture sites. Death usually occurs three to six days after the onset of symptoms (Bird et al., 2009; Chevalier et al., 2010; Flick and Bouloy, 2005; Gerdes, 2004; Le May et al., 2004; Magurano and Nicoletti, 1999; McIntosh et al., 1980b).

2.6 Bioterrorism concerns

The global spread of RVFV presents a serious bioterrorism or biosecurity concern due to movement of a wide range of mosquitoes, humans and animals into different countries: this can easily introduce the virus into non-endemic countries, resulting in serious health and agricultural problems (Chevalier et al., 2010; Gubler, 2002; House et al., 1992). More so, since RVFV was reportedly weaponised by the US offensive biological weapons program, illustrating the real threat and utility of RVFV as a bioweapon. The lack of treatment or no Food and Drug Administration (FDA) or U.S Department of Agriculture (USDA) approved RVFV vaccine for human or animal use makes infection with the virus a major concern and a need for more RVFV vaccine research and development (Bird et al., 2009; McElroy et al., 2009). Handling of RVFV requires high containment facilities, including biosafety level 3 enhanced (BSL-3E) or BSL 4 laboratories (Mandell et al., 2010a; Mandell et al., 2010b).

RVFV is classed as a RNA viral haemorrhagic fever agent: the OIE has classified RVFV as a high-consequence pathogen with the potential for international spread (List A). In the United States of America, RVFV is number 3 on the list of the 17 most dangerous animal threats, behind only highly pathogenic avian influenza virus and Foot-and-Mouth disease virus (FMDV). Therefore it is classified as a Category A agent by the Centers for Diseases Control and Prevention (CDC) and the USDA (Bird et al., 2009; Ikegami and Makino, 2009). In Europe, the constraints are the same as

for Foot-and-Mouth disease and other epizootic diseases. They were instituted in 1972 (directive 72/462/CEE, which has been modified recently to be more stringent).

2.7 Diagnosis

RVF can be diagnosed using various methods, with serological test results being combined with clinical observation and epidemiological history. The virus can be detected in blood during early phases of illness, or in post-mortem tissue using various techniques, including virus propagation, antigen detection and RT-PCR (Wilson et al., 2014). RT-PCR for detection of RVFV have been described (Drosten et al., 2002; Garcia et al., 2001; Sall et al., 2002) and shown to be valuable in mobile diagnosis (Njenga et al., 2009) as well as detection of the virus in serum samples (Chengula et al., 2014; Odendaal et al., 2014). There are three main diagnostic methods for detecting antibodies against RVFV in serum. The traditional methods for detection of antibodies to RVFV are hemagglutination inhibition (HI), virus neutralization (VN) and immunofluorescence assays (IFA). Disadvantages of these tests include the high health risk to laboratory workers that handle the live virus, and that their use is restricted outside RVF endemic areas. This has led to the development of an enzyme-linked immunosorbent assay (ELISA), which is a rapid and reliable diagnostic tool. N-protein is the most abundant viral protein, and has been successfully used for the detection of antibodies for many viruses in the family *Bunyaviridae* (Fischer et al., 2015; Schwarz et al., 1996; Swanepoel et al., 1986a). A highly sensitive and specific indirect ELISA based on the use of β-propiolactone-inactivated or gamma irradiated, sucrose-acetone extracted antigens derived from tissue culture or mouse brain tissue for the detection of IgG and IgM antibodies to RVFV in animals and humans, has been developed and validated (Paweska et al., 2003a; Paweska et al., 2005a; Paweska et al., 2005b; Paweska et al., 2003b). However, the production of this inactivated antigen requires high biosafety containment facilities. The main drawbacks associated with the ELISA test as published are that production of the antigen is a costly process, and there is poor binding of the antigen to ELISA plates as it is too crude (Jansen van Vuren et al., 2007; Moutailler et al., 2008). Therefore, there is a need for a cheap, safe and more reliable test for the presence of antibodies in animals and in humans, which can differentiate between infected and vaccinated animal (DIVA) as well as humans.

The N-protein is the major component of ribonucleoprotein complex and it plays a role in RNA transcription and replication (Schmaljohn and Hooper, 2001) as well as viral assembly (Liu et al., 2008). It has been expressed and very well characterised in various expression platforms and revealed to form complex multimers (Ferron et al., 2011; Le May et al., 2005; Liu et al., 2008; Raymond et al., 2010). It has been shown to be highly immunogenic but does not exhibit neutralising activity (Boshra et al., 2011; Faburay et al., 2014; Lagerqvist et al., 2009).

2.8 Control and prevention

During a RVF outbreak, farmers can attempt various treatments to prevent the spread of mosquitoes. Treatments are aimed at the most vulnerable animals, namely pregnant and young livestock. The preferred used insecticide is pyrethroid deltamethrin, which has a knockdown effect, reducing the number of mosquitoes (Diallo et al., 2008). Larvicidal measures at mosquito breeding sites are the most effective form of vector control, if the breeding sites can be clearly identified and are limited in size and extent. During periods of flooding, however, the number and extent of breeding sites is usually too high for application of larvicides to be feasible. Methoprene and *Bacillus thuringiensis israeliensis* preparations are hormonal larvae growth inhibitors and microbial larvicides, respectively; these are commercially available and can be successfully used to treat temporary ponds and watering places where mosquitoes proliferate (Lacey and Lacey, 1990).

2.9 RVFV Vaccines

RVFV outbreaks in animals can be prevented by a sustained programme of animal vaccination. Live attenuated and inactivated vaccines have been developed for veterinary use. Only one dose of the live attenuated vaccine is required to produce long term immunity, but the vaccine may result in spontaneous abortion if given to pregnant animals (Smithburn, 1949). On the other hand, the inactivated vaccine does not have side effects but requires multiple doses in order to provide adequate protection, which may be problematic in endemic areas (Caplen et al., 1985). Animal immunization must be implemented before an outbreak if an epizootic is to be prevented. Once an outbreak has occurred, animal vaccination should not be

implemented because there is a high risk of intensifying the outbreak. During mass animal vaccination programmes, animal health workers may, unintentionally, transmit the virus through the use of multi-dose vials and re-use of needles and syringes, if some of the animals in the herd are already infected and viraemic. Restricting or banning movement of livestock may be effective in slowing the expansion of the virus from infected to uninfected areas.

Global health and animal health agencies or departments agreed to start a “One Health” initiative, which has as a priority to develop RVFV countermeasures whether for human or animals or both. The “One Heath” initiative aims to yield a highly effective and long term protective immunity through interdisciplinary collaborations and communications in all aspects of health care for humans, animals and the environment. Novel vaccines should be cost-effective and application should be safe regardless of the physiological state of the animal. Be DIVA complient by use of appropriate discriminatory assays would also be beneficial. Conventional vaccines such as live attenuated vaccines or even some inactivated vaccines are not suitable for DIVA, since the antibody responses elicited by these vaccines are similar or the same for attenuated vaccines, and may be similar for inactivated, as those induced by wild type virus. DIVA strategies can help in the detection of specific antibodies which are induced by natural infection rather than by artificial immunisation in animals, or vice-versa, ensuring safe trade of products from immunised animals (Liu et al., 2013a). DIVA vaccines allow serological differentiation between vaccinated and infected animal in conjunction with proper diagnostic tests that detect antibodies against the protein (Mebatsion et al., 2002; van Oirschot et al., 1986; Wehrle et al., 2007). The spread of RVFV can be prevented by vaccination of animals and humans but development of safe and effective vaccines has been difficult (Bouloy and Flick, 2009; Ikegami and Makino, 2009). Numerous candidate vaccines have been developed.

2.9.1 Live attenuated vaccines

The Smithburn vaccine was developed by K C Smithburn and his fellow researchers in 1944 during a yellow fever outbreak in Uganda (Smithburn, 1949; Smithburn et al., 1949). The strain was isolated from mosquitoes, and then attenuated by sequential

intracerebral passages in suckling mice, which reduced the hepatotropism of the strain. Smithburn showed that this virus strain could protect lambs challenged with virulent RVF virus. Further analysis revealed that the vaccine protected the animals 19 months after a single vaccination. However, the vaccine may induce abortion and teratology in sheep, ewes, and cows (Botros et al., 2006; Kasari et al., 2008). The strain's affinity for foetus neural tissue causes cephalic disorder and arthrogryposis, resulting in abortion or stillborn young. From 1953 – 1958 the Smithburn vaccine was used for vaccination purposes in South Africa, and is currently produced by Onderstepoort Biological Products (Barnard, 1979; Barnard and Botha, 1977). Although the vaccine is significantly attenuated, it has the potential for reversion, and hence it is not recommended for use in countries where RVFV is not endemic (1983). This vaccine is pathogenic to humans as well, causing febrile syndrome. Despite the drawbacks it is recommended by the Food and Agriculture Organization (FAO) of the United Nations and it remains the most widely used vaccine against RVF in Africa (1983).

The MP-12 attenuated strain of RVFV originates from the Egyptian RVFV strain ZH548, which was isolated from a human patient. Attenuation was accomplished by 12 serial mutagenesis passages of the wild type RVFV ZH548 strain in human diploid lung (MRC-5) cells using the chemical mutagen 5-fluorouracil (5FU). The virus was propagated in the presence of medium containing 5FU on a monolayer of MRC-5 cells that were plaqued under agarose, and large plaque clones were harvested and amplified on monolayers of MRC-5 cells with medium lacking 5FU. The supernatant from the passage of cells was then used to initiate another mutagenesis cycle for a total of 18 cycles. A series of RVFV mutants with increasing attenuation was obtained by this approach; the product of the 12^{th} mutagenesis passage is referred to as MP-12 (Caplen et al., 1985). MP-12 was selected for further investigation on the basis of its immunogenicity and favourable attenuation in a preliminary investigation in mice (Caplen et al., 1985; Schirmbeck et al., 1996; Vialat et al., 2000). It was demonstrated that MP-12 had three mutations in the three segments (L, M and S) (Saluzzo and Smith, 1990). Further analysis of the vaccine strain revealed that it protected sheep and cattle after the first trimester (Baskerville et al., 1992; Morrill et al., 1991). The vaccine provides a low level of protection and its production is expensive; two

inoculations and frequent booster shots are required to induce protection (Hunter et al., 2002; Morrill et al., 1991; Morrill et al., 1997a). This is not feasible in countries where a large proportion of the animals are roaming around free. Furthermore, this vaccine strain also showed teratogenic effects in sheep (Hutchinson et al., 1996), same safety concern as observed with the Smithburn vaccine. Nonetheless, more efforts are being made to attenuate the MP-12 vaccine virus strain further using reverse genetics to delete the NSs gene in the pre-Gn region of the M segment (Habjan et al., 2009; Morrill et al., 2013).

Another approach is using engineered RVFV replicon particles which are highly effective in inducing a protective immune response, nonspreading RVFV (NSR) (Kortekaas et al., 2011; Oreshkova et al., 2014; Oreshkova et al., 2013; Wichgers Schreur et al., 2014a) and the four-segmented RVFV 4s that was constructed by splitting the wild type M genome segment into two segments encoding either the Gn or Gc protein. *In vivo* studies have demonstrated that RVFV-4 is unable to spread the infection and disease in mice even in the presence of the main virulent factor NSs, but induced a protective immune response against a lethal challenge (Wichgers Schreur et al., 2015; Wichgers Schreur et al., 2014b).

The Clone 13 vaccine was isolated from a mild human case of RVF in the Central African Region (Weinbren et al., 1957). The strain showed heterogeneous plaques when cultured *in vitro* and was found to have an in-frame deletion in most of the NSs, lacking 70% of the coding sequence for the interferon antagonist (Muller et al., 1995; Nakitare and Elliott, 1993). When assessed for safety and efficacy in sheep, the strain was effective without teratogenic effects (Albarino et al., 2007; Bouloy et al., 2001; Dungu et al., 2010; Muller et al., 1995), making it a suitable alternative to Smithburn and MP-12. The deletion in the sequence encoding the virulence factor makes it a good alternative due to its inability to revert. The Clone 13 based vaccine was commercialized in South Africa by Onderstepoort Biological Products in 2010. Despite the high safety profile of Clone 13 vaccine, more research efforts are being carried out to further attenuate mutations on the L and M segments of MP-12 with the S segment of Clone 13 virus. The re-assortant virus, named R566, contains mutations in each of the three genome segments (Bouloy and Flick, 2009; Kortekaas et al., 2014).

Currently in South Africa, both Smithburn and Clone 13 vaccines are commercially available for use in endemic areas.

2.9.2 Inactivated vaccines

The National Drug Biological Research (NDBR) 103 vaccine strain was initially isolated from mosquitoes in Uganda harbouring the Entebbe strain of RVFV. The vaccine virus was developed from mouse serum master seed (184th passage) and amplified in primary monkey kidney cells, before being used routinely for formalin inactivation (Randall et al., 1962). It was further shown that the vaccine prepared from chicken embryos or chick embryo cell culture was less immunogenic when it was prepared using primary rhesus or African green monkey kidney cells (Randall et al., 1962) .

The formalin-inactivated Salk Institute–Government Services Division (TSI-GSD) 200 vaccine was subsequently manufactured under more stringent safety regulations by using the new master seed prepared from two passages of the mouse serum master seed of the Entebbe strain in diploid cells (FRhL-2 cells) derived from the lungs of a foetal rhesus monkey (Eddy and Peters, 1980). TSI-GSD 200 is available in limited amount to the US for protection of military personnel and laboratory staff, but it has not been licensed. The vaccine is not commercially available, but is used by veterinarians who work in areas endemic to RVFV as well as others who are at high risk of contracting the disease including lab workers in high containment areas. As with most inactivated antiviral vaccines, this vaccine is expensive, difficult to make, and several inoculations are required to maintain immunity. In addition, this vaccine is in short supply (Kark et al., 1982; Randall et al., 1964). The NDBR 103 and TSI-GSD 200 vaccines have been used for immunization of human volunteers (Eddy and Peters, 1980; Niklasson, 1982) and no adverse side effects were reported (Kark et al., 1982; Niklasson, 1982; Rusnak et al., 2011).

2.9.3 Recombinant viral vaccines

Recombinant viral vectors can express the antigen of interest through viral infection, thus enabling the host cell to process the recombinant protein in a manner similar to that of natural infection. Advantages of using recombinant viruses are that antigens are not expressed in the context of the natural virus, therefore limiting the risks associated with attenuated vaccines and allowing DIVA.

Protective epitopes in RVFV Gn and Gc glycoproteins have been mapped (Dalrymple, 1989). These epitopes been reported to be highly immunogenic and able to protect animals (Besselaar and Blackburn, 1991; Schmaljohn et al., 1989). Different strategies have been used to assess the competence of recombinant Gn and Gc proteins for eliciting neutralising antibodies in animals. Expression vectors such as the Venezuelan equine encephalitis virus (VEE) vector and the lumpy skin disease virus (LSDV) vector (Wallace et al., 2006; Wasmoen et al., 1988) alphavirus (Gorchakov et al., 2007; Heise et al., 2009) adenoviruses (Holman et al., 2009; Hoogstraal et al., 1979; Warimwe et al., 2013), Newcastle disease virus (Kortekaas et al., 2012; Kortekaas et al., 2010a; Kortekaas et al., 2014), and other poxviruses (Collett et al., 1987; Papin et al., 2011; Soi et al., 2010) have all been used to generate RVFV glycoproteins which have elicited RVFV-specific immune responses and induced protection against RVFV challenge in animals. In South Africa, rLSDV was used for the expression of Gn and Gc to protect sheep from RVFV (Wallace et al., 2006). These studies suggest that viral vectors are useful for the expression of Gn and Gc, and may be further improved as veterinary vaccines.

2.9.4 Virus-like particles as vaccines

Virus-like particles (VLPs) are composed of one or several recombinantly expressed viral proteins that spontaneously assemble into molecular structures resembling the infectious virus. VLPs are structurally similar to infectious viruses, and they are much more highly immunogenic than recombinant proteins alone (Grgacic and Anderson, 2006). They also lack viral nucleic acids, making them non-infectious and safe to use. VLPs do not elicit antibody responses to internal or non-structural proteins encoded by viruses, therefore allowing for vaccines to be produced that can distinguish between

vaccinated and non-vaccinated animals, the so-called DIVA attribute (Capua et al., 2003). Due to the highly repetitive surface structures, VLPs are able to elicit strong B cell responses (Bachmann et al., 1997), which are strong enough to elicit T cell-independent induction of IgM antibodies (Bachmann et al., 1993; Chackerian et al., 1999; Kouskoff et al., 2000). VLPs also induce T-helper cells that trigger IgG secretion as well as affinity maturation and the generation of long-lived memory B-cells, thereby fulfilling the fundamental goal for any vaccine. Multivalent antigens, like VLPs, can activate B-cells at much lower concentrations than monomeric antigens. In addition to their ability to stimulate B-cell mediated immune responses, VLPs have been shown to be highly effective at stimulating CD4 proliferative responses and even cytotoxic T lymphocyte (CTL) responses (Meyers et al., 2008; Paliard et al., 2000; Schirmbeck et al., 1996).

VLPs can be used as an alternative to the traditional live attenuated and formalin-inactivated RVFV vaccines. By expressing RVFV N-protein and GnGc together in a dual baculovirus expression vector, Liu and co-workers (Liu et al., 2008) showed that enveloped VLPs resembling wild type RVFV virions could be produced in insect cells. VLPs were also produced by the dual baculovirus vector system with just N-protein and Gc without the Gn. These VLPs were more pleomorphic than the VLPs comprising both glycoproteins. Habjan et al (Habjan et al., 2009) produced RVFV VLPs in mammalian cells by co-expressing recombinant RVFV RNA-dependent polymerase and N-protein together with a minireplicon RNP and additional expression of the viral glycoproteins. These have been shown to protect mice from a lethal challenge of RVFV (Naslund et al., 2009). It was also shown by de Boer et al. (de Boer et al., 2010) that RVFV VLPs can be produced in a *Drosophila* insect cell system expressing the Gn and Gc proteins separately. Furthermore, these VLPs provided 100% protection for mice when challenged with wild type virus. The effect of soluble Gn in vaccinated mice was also tested and it has shown that full protection was afforded from lethal challenge with RVFV (de Boer et al., 2010). Therefore, RVFV VLPs and even soluble Gn are a possible candidate for vaccination against RVF as they are stable, they are more immunogenic than recombinant proteins alone, and they maintain their conformational epitopes which induce neutralising antibodies.

Despite their efficacy, producing VLPs in mammalian or insect cells is very expensive, thus hindering future development and commercialization of these candidate vaccines. An alternative production method such as plant-based expression for vaccine antigens may be more efficient and cost-effective compared to mammalian and insect cell production of these VLPs (; Mortimer et al., 2012).

2.10 Plant production systems

Advances in molecular biology and biotechnology have opened up new opportunities for development of new genetically engineered vaccines. One of the cheaper ways of producing vaccines is by using plants as an expression system (Daniell et al., 2001; Lomonossoff and D'Aoust, 2016; Santi et al., 2006; Streatfield and Howard, 2003). Plant-based expression systems are safe to use as they do not contain human or animal pathogens, thus reducing the risk of contamination for pharmaceutical proteins. Plants offer a number of advantages compared to other expression systems, which include flexibility in the production platform and easy scale up for manufacturing (Rybicki, 2010; Xu et al., 2012; Youssef and Donia, 2001). Plant expression systems also provide the majority of eukaryotic post-translational modification machinery required for complex proteins and simple low-cost scale up for manufacturing. N-linked glycosylation and other post-translational modifications are important for the folding of some eukaryotic proteins and glycoproteins are efficiently glycosylated when expressed in the plant expression system. These biosafety advantages positively impact their commercial aspects, since they reduce purification costs and minimize risks associated with potential production shut-downs and facility decontamination and supply chain limitations leading to unmet patient or customer demand (Xu et al., 2012).

Various plant species can be used as hosts for plant-based production; these include *in vitro*-grown cells (cell suspension cultures), and plant tissue culture as well as whole plants grown in growth chambers and in the field. *Nicotiana* species, alfalfa, rice, wheat, potatoes, carrot, tomatoes, maize, soybeans among others can be used for production of recombinant proteins (Gils et al., 2005; Gleba et al., 2005; Marillonnet et al., 2004; Rybicki, 2009; Yusibov et al., 2002). Certain plants such as the horticulturally very well understood *Nicotiana benthamiana* can be grown at high density and produce large amounts of biomass in a few weeks. Heterologous proteins

can be produced in plants by two approaches: stable transformation and transient expression. Stable transformation involves the integration of genes into the plant nuclear or chloroplast genomes (Berberich et al., 2005; Huang et al., 2005; Pogrebnyak et al., 2005). This approach has advantages in terms of reproducibility and large-scale production. However, it is very time consuming and it is unsuitable for rapid screening of a wide variety of different constructs (Kusnadi et al., 1997).

Transient expression (TE) allows for robust high levels of protein production in several days, and while it is mostly used for rapid screening of expression constructs, TE can also be reliably used for large-scale recombinant protein production. TE of heterologous proteins in plant leaves was first demonstrated by infiltration of suspensions of *Agrobacterium tumefaciens* harbouring a binary vector into plant leaves (Kapila et al., 1997), followed by the demonstrations of expression of many proteins that was easily achieved by *A. tumefaciens* mediated somatic gene transfer (Circelli et al., 2010; Collens et al., 2007; Fischer et al., 1999b; Maclean et al., 2007; Sijmons et al., 1990; Warzecha and Mason, 2003).

A. tumefaciens is a Gram-negative bacterium that contains a naturally occurring plasmid known as Ti (tumour-inducing), which is capable of directing the transfer of part of the plasmid (the transfer DNA or T-DNA) into a plant cell and integrating the T-DNA into the plant genome (Chilton et al., 1977; Eddy and Peters, 1980; Escobar and Dandekar, 2003). With transient gene expression, foreign genes are generally introduced into the leaf tissue of intact plants by direct injection via syringe, or at larger scale by vacuum infiltration of whole plants with *Agrobacterium* containing gene(s) of interest within its T-DNA. Recombinant protein production (based on the extra-chromosomal gene expression within the plant cell) is initiated in the leaves within 24 hours of infiltration and continues for several days up to as long as several weeks, depending on the vector used and protein of interest being expressed (Gleba et al., 2005). While chromosomal integration is possible, it occurs at considerably lower frequency in comparison to extra-chromosomal expression of the gene of interest (Circelli et al., 2010; Sheludko, 2008). The success of this process has been well developed for scaling up the production of recombinant proteins in plants (D'Aoust et al., 2010).

Transient expression vectors can also be based on plant viruses such as ssDNA geminiviruses, the ssRNA agents Potato virus X (PVX), Tobacco mosaic virus (TMV) and Cowpea mosaic virus (CPMV). These are ideal for suitable expression vectors due to rapid recombinant protein production, and a variety of possible regulatory components (Canizares et al., 2006; Giritch et al., 2006; Lindbo, 2007; Mor et al., 2003; Regnard et al., 2010; Sainsbury and Lomonossoff, 2008). Most recently, during the outbreak of Ebola virus disease in Western Africa, much focus was on plant-derived pharmaceuticals; this was because the experimental drug ZMApp™, a combination of three humanized monoclonal antibodies that recognised an Ebola virus surface glycoprotein, was manufactured by agroinfiltrated rTMV-mediated transient expression in *N. benthamiana* plants by Kentucky BioProcessing under licence from Mapp Biopharmaceuticals Inc. (Qiu et al., 2014).

2.11 Plant expression vectors

One of the major drawbacks of plant-produced vaccines is the potentially low yields of protein. Increasing protein yield remains a challenge for economic feasibility; key strategies used to try and improve product recovery in plant expression systems are summarized in Table 2.2 (Hood et al.; Rybicki, 2010). These strategies include the choice of expression vector, use of signal peptides/sequences and gene sequence codon optimisation.

For transient expression, an ideal expression vector should:

- Have a wide selection of multiple cloning sites.
- Have a broad host range origin of replication (ori) as well as an ori for a high copy number plasmid (ego li E1 from pBR322) to allow high yielding DNA preparations in *E. coli*.
- Be of a small size, i.e. 10 – 13 Kb.
- Have a good choice of selection markers for both bacterial and plant host.
- Have a high transformation efficiency (Hellens et al., 2000).
- Have regulatory elements including a promoter such as Cauliflower mosaic virus (CaMV) 35S promoter, which is a constitutive promoter sequence and is responsible for the generation of full-length RNA copies in CaMV infected cells (Odell et al., 1985).

- Have translational enhancer elements such as the *Tobacco mosaic virus* (TMV) 5' omega leader sequence, which enhances the expression of heterologous protein *in vitro* and *in vivo* (Gallie et al., 1987) and Tobacco etch virus (TEV) (Carrington and Freed, 1990).

The introduction of such elements into pre-existing viral backbones has resulted in the development of replicating viral vectors and non-replicating viral vectors (Regnard et al., 2010; Sainsbury et al., 2009a; Saunders and Lomonossoff, 2013).

Table 2 2: Strategies used to improve recombinant protein yields in plants

Production Stages	***Strategies***
Transformation	Attachment or fusion of SAR or LIR to DNA
Transcription	Development of strong promoters, double enhanced promoter, hybrid promoter
	Use of inducible promoters
	Engineering of better enhancers, activators or repressors
Translation	Optimization of 5'- and 3'-UTR (untranslated region)
	Codon optimisation
	Changes in initiation codon
Post-translation	Targeting to sub-cellular compartments such as ER or apoplast
	Co-expression with protease inhibitors and protein co-factor/subunit; co-expression of antibody with antigen
	Expression as a fusion to a highly expressed and stable peptide

Modified from (Desai et al., 2010; Xu et al., 2012)

2.11.1 Plant organelle targeting vectors

These vectors mainly involve the pTRAc (Figure 2.4) vector series (Maclean et al., 2007). The backbone is a derivative of pPAM (Genbank AY027531). They have an RK2 *ori*, *mob* and are *tra* deficient. They consist of a CaMV 35S promoter with a

duplicated translational enhancer, chalcone synthase 5'-unltranslated region and a CaMV 35S polyadenylation signal. The selection markers are ampicillin (amp) in *E. coli* and carbenicillin (carb) in *Agrobacterium*. The expression cassette is flanked by a scaffold attachment region (SAR) on the tobacco Rb7 gene to enhance gene expression (Halweg et al., 2005), which in turn is flanked by the left and the right borders required for T-DNA integration.

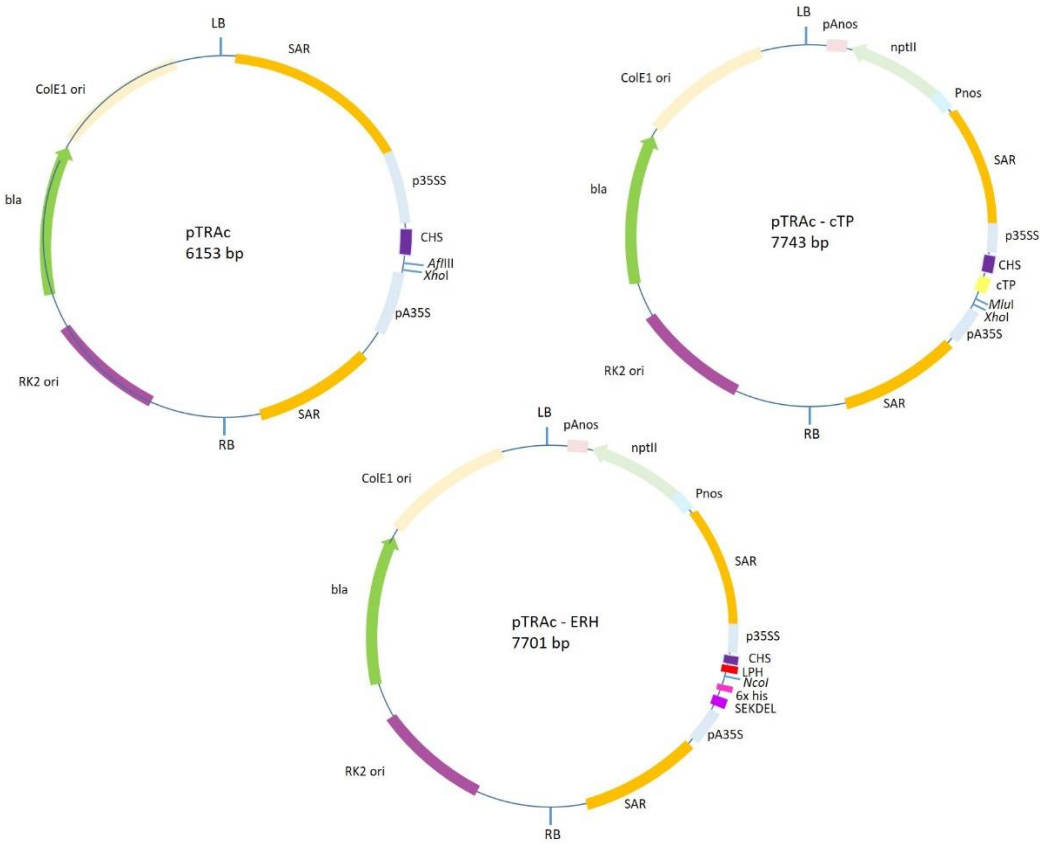

Figure 2.4: Organelle targeting *Agrobacterium* vectors pTRAc, pTRAc-cTP and pTRAc-ERH. ColE1 origin of replication for *E. coli*; RK2 ori, origin of replication for *A. tumefeciens*; bla, ampicillin/carbenicillin resistance *bla* gene, LB and RB, left and right borders for T-DNA integration; SAR, scaffold attached 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; LHP, murine mAb24 heavy chain signal peptide; 6xhis, 6 x histidine tag; SEKDEL, ER retention signal; cTP, chloroplast transit peptide of RuBisCO small sub-unit; nptII , kanamycin resistance gene; Pnos and pAnos, promoter and polyadenylation signal of the nopaline synthase gene (Maclean et al., 2007).

This vector series was used to demonstrate that codon optimization of the human papillomavirus (HPV) 16 L1 gene and targeting of the protein to different plant cell compartments can influence the accumulation of the L1 protein by up to 80-fold, resulting in yields of 108 – 375 mg/kg fresh weight (FW) (Maclean et al., 2007). The vector were also used for the production of candidate vaccines for avian influenza

H5N1, where yields of 300 – 675 mg/kg FW of HA were obtained (Mbewana et al., 2015; Mortimer et al., 2012); production of HPV 16 E7 protein bodies with yields from 400 to 1100 mg/kg FW (Whitehead et al., 2014); as well as, the production of human rotavirus candidate vaccines (Pera et al., 2015).

2.11.2 Geminivirus-based expression vector

Bean yellow dwarf virus (BeYDV) belongs to the genus *Mastrevirus*, family *Geminiviridae*. It is a twinned-capsid spherical virus with a single stranded circular DNA genome (Zhang et al., 2001). The genome contains short and long intergenic regions (SIR and LIR respectively), which allow for the initiation of second strand synthesis, and initiation of linear ssDNA synthesis by rolling circle replication by viral Rep/RepA proteins, respectively (Halley-Stott et al., 2007; Liu et al., 1997). Mor et al (Mor et al., 2003) showed that this allowed for the deployment of LSL (LIR SIR LIR) vectors, in which a linear construct based on BeYDV containing a SIR and flanked by two LIRs in an *Agrobacterium* vector can be delivered into plant cells, liberated as a linear ssDNA by Rep/RepA action, and circularise to form replicons. A self-replicating vector, pRIC (Regnard et al., 2010), has a similar backbone to the pTRA vector series (Maclean et al., 2007) except it has a Rep gene from the BeYDV inserted into it between the left and right intergenic regions and has no signal sequence. It makes use of the same selection markers as the pTRA vectors. This allows for vector replication *in planta*. The pRIC vector (Figure 2.5) multiplied to ~1000 copies per cell, and allowed overexpression of HPV-16 L1 (550 mg/kg FW) and HIV capsid protein p24 (3.23 mg/kg FW), but only by factors of up to 4-fold, so that increases in protein translation levels did not directly correlate with gene copy number.

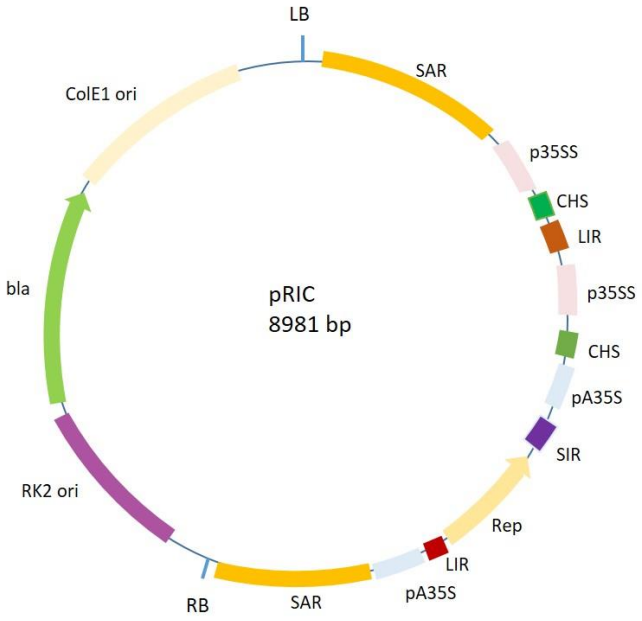

Figure 2.5: Self-replicating *Agrobacterium* vector pRIC. ColE1 origin of replication for *E. coli*; RK2 ori, origin of replication for *A. tumefeciens*; bla ampicillin/carbenicillin resistance *bla* gene, LB and RB, left and right borders for T-DNA integration; SAR, scaffold attached region of the tobacco *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 (Regnard et al., 2010).

2.11.3 Comovirus-based expression vector

The pEAQ-*HT* vector series is based on and contains a mutated version of a 5' untranslated region (UTR) from a CPMV RNA-2 gene, which enhances protein expression from recombinant protein genes in plants when they are cloned downstream of the modified UTR (Sainsbury et al., 2008; Sainsbury and Lomonossoff, 2008). Protein expression is further enhanced by placing the 3' UTR from CPMV RNA-2 after the coding sequence and co-expression of the P19 silencing suppressor from the Tomato bushy stunt virus on the same vector, to stabilize the mRNA (Sainsbury et al., 2009b). The backbone of this vector is modified from the commercially available pBIN plant expression vector. The selection is carried out using kanamycin . The pEAQ-*HT* (Figure 2.6) expression system has been used for the production of VLPs for different viruses such as Hepatitis B core particles (Peyret et al., 2015), Bluetongue virus (Thuenemann et al., 2013) as well as human (Matic et al., 2012) and bovine (Love et al., 2012) papillomavirus particles.

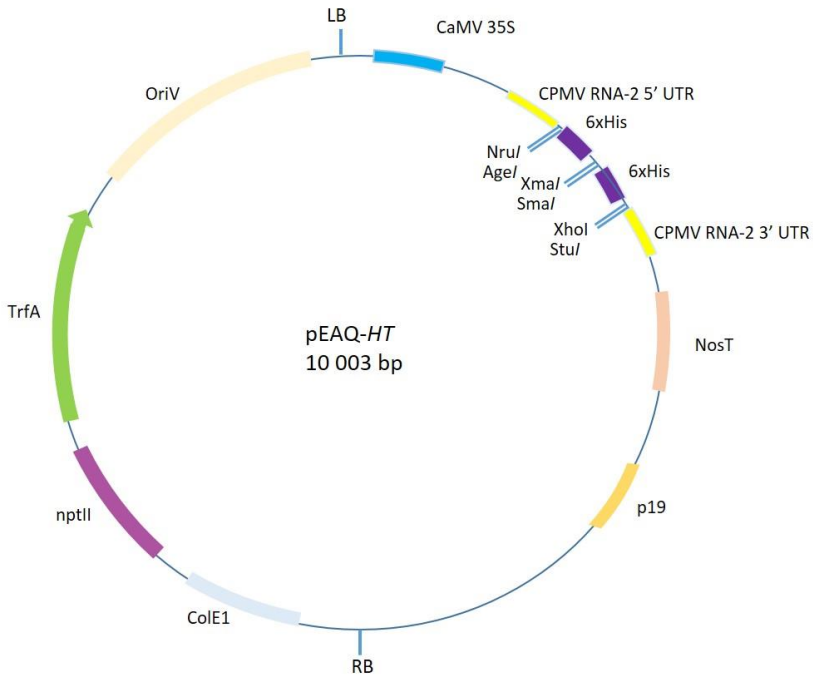

Figure 2.6: Hypertranslatable *Agrobacterium* pEAQ-*HT* vector. LB and RB, left and right borders for T-DNA integration; CaMV 35S, cauliflower mosaic virus promoter, CPMV RNA-2 UTRs; NosT nopaline synthase terminator; OviV, pRK2 replication origin, ColE1, pBR322 origin of replication, npt II, neomycin phosphotransferase; 6xhis, 6 x histidine tag, P19, silencing suppressor; TrfA, replication essential locus (Sainsbury et al., 2009a).

2.11.4 Tobamovirus-based expression vector

Tobacco mosaic virus (TMV) is the type species of genus *Tobamovirus*, family *Virgaviridae*, which are rod-shaped, single stranded positive sense RNA viruses. Numerous TMV-based vectors have been used for protein expression in plants (Dawson et al., 1985; Donson et al., 1991; Sugiyama et al., 1995). The TMV-based MagnICON system is the first of a series of deconstructed virus-based systems, made with the objective of obtaining high recombinant protein yields (Marillonnet et al., 2004). This system comprises of 3 modules which contain the gene of interest in place of the sequence of the viral coat protein along with the *nos* terminator; the 5' module contains the *Arabidopsis* actin 2 (ACT2) promoter along with the tobamovirus RNA polymerase and movement protein (MP) genes, while the recombinase module containing the PhiC31 integrase gene (*Streptomyces* phage C31) acts to fuse the 3' and 5' modules together in the nucleus to allow the formation of a complete tobamovirus-based replicon, with the gene of interest under the control of the sub-genomic promoter present within the MP sequence. When *Agrobacterium* strains containing these constructs are co-infiltrated into plant cells, recombinase-mediated

recombination takes place at the recombinant sites, resulting in viral expression vector. Expression of genes in these vectors generates desired recombinant protein and MP facilitates cell to cell movement of the vector as well as modified TMW replicons which increases the levels of recombinant protein produced. Selection is carried out with ampicillin in *E. coli* and kanamycin in *Agrobacterium* (Marillonnet et al., 2004; Marillonnet et al., 2005).

As proof of concept, yields of green fluorescent protein (GFP) as high as 5 g/kg of fresh weight (FW) were obtained from *N. benthamiana* (Marillonnet et al., 2004). This system has been used for the production of plague bacillus antigens, which reached yields of 2 g/kg FW (Santi et al., 2006); Hepatitis B virus core VLPs at 2.4 g/kg FW (Huang et al., 2006) and Norwalk virus VLPs at 800 mg/kg FW (Santi et al., 2008).

2.12 Codon optimisation

Proteins are often difficult to express outside their original context. They might contain codons that are rarely used in the desired host, come from organisms that use non-canonical genetic code, or contain expression-limiting regulatory elements within the coding sequence (Gustafsson et al., 2004). Various strategies such as the modification of translation initiation regions, alteration of mRNA structural elements and use of different codon biases can improve protein expression. The DNA sequence used to encode a protein in one organism is often different from the sequence that would be used to encode the same protein in a different organism, as the degeneracy of the genetic code enables many alternative nucleic acid sequences to encode the same protein. The frequency at which different codons are used varies significantly between different organisms, between proteins expressed at high or low levels within the same organism, and sometimes within the same operon in bacteria (Gouy and Gautier, 1982; Webster et al., 2016). The more codons that a gene contains that are rarely used in the expression host, the less likely it is that the heterologous protein will be expressed at reasonable levels (Kane, 1995). Codon usage bias is positively correlated with gene transcription levels (Wang and Roossinck, 2006).

Codon optimisation has been widely used to enhance protein expression in heterologous systems (Batard et al., 2000; Perlak et al., 1991; Rouwendal et al., 1997;

Zhou et al., 2015). Even though codon optimisation plays an essential role in gene expression, the choice of expression vectors and transcriptional promoters as well as signal peptides are also important. The nucleotide sequence around the N-terminal region of the protein is sensitive to rare codons (Deana et al., 1998; Hoekema et al., 1987) and to codons immediately adjacent to the initiation AUG codon (Sato et al., 2001).

2.13 Signal peptide fusions

The success of transient expression is also dependant on intercellular organs to which the protein of interest is targeted; this is directed by the appropriate signal peptide fused to the gene of interest which targets recombinant proteins to plant tissue compartments such as the ER or chloroplast (Maclean et al., 2007). Such targeting could lead to higher levels of accumulation of recombinant proteins in the secretory pathway or within specific organelles (Yoshida et al., 2004). The plant's vesicular transport system is valuable for the production of recombinant proteins that require post-translational modifications for their activity, such as antibodies and glycoproteins. Secretion of heterologous protein becomes possible by the attachment of a signal peptide at the N-terminus of the proteins that allows entry into the vesicular transport system. The secretory pathway directs the accumulation of recombinant proteins in the cell wall region, apoplastic space or ER. Varying the signal peptide of recombinant proteins can affect the degree of protein production (Yoshida et al., 2004). Signal peptides vary in their amino acid composition and length, but they all contain a positively charged N-terminus of one to five residues, a central hydrophobic core of 6-15 amino acids, and a polar region of three to seven amino acids that serves as a recognition site for signal peptidase, which cleave the signal peptide from the protein which it is fused to. The signal peptide ensures effective direction of the expressed protein across the membrane of the ER (Kjærulff and Jensen, 2005). The ER targeting efficiency increases with signal peptide hydrophobicity (Milstein et al., 1972; Perlmann et al., 1967).

2.14 Final Remarks

The agricultural and health problems associated with RVF due to the lack of a safe, easy to produce and affordable vaccine as well as an affordable reagent which is DIVA complaint. This allows for more research and development on RVF using the plant production platform which is easy to use and can be applied on varies products.

Chapter 3: Expression strategies for RVFV *GnGc* genes in *N. benthamiana* to identify suitable candidates for vaccines or reagent development

3.1 Introduction

RVF is an emerging disease that is rapidly expanding its geographical range, and which may quickly become a worldwide threat. The spread of the disease could be prevented by vaccination against RVFV: however, despite much effort having been made in developing safe and efficacious vaccines, none have yet been released for general use (Bird et al., 2008; Mandell et al., 2010b; Morrill et al., 1987; Muller et al., 1995; Naslund et al., 2009; Schmaljohn et al., 1989).

Vaccination is an important and effective public-health measure for control and prevention against infectious diseases. RVFV vaccines have been developed in expression systems such as bacteria (Keegan and Collett, 1986; Wallace et al., 2006) and mammalian cell cultures (Bird et al., 2008; Mandell et al., 2010b). Cell cultures can be difficult to maintain and require expensive equipment and media. In downstream processing, oncogenic sequences or viral contamination must be removed if *in vivo* applications are anticipated. While bacterial cultures on the other hand do not produce glycosylated proteins, contaminating endotoxins are difficult to remove and recombinant proteins often form inclusion bodies, making labour and cost intensive *in vitro* refolding necessary to recover functional proteins (Fischer et al., 1999a). Plants offer an alternative cost-effective approach for mass production of recombinant proteins due to their high biomass and ability to carry out posttranslational modifications similar to other higher eukaryotes (Rybicki, 2009; Rybicki, 2010).

The RVFV M segment RNA has one open reading frame that encodes the two envelope glycoproteins Gn and Gc with an additional upstream NSm region. Gene translation depends on the alternative usage of five in-frame ATG codons. Translational products of the M segment have been characterised *in vitro* and consist

of polyproteins that are expressed from different ATG codons (Kakach et al., 1988; Suzich et al., 1990; Suzich and Collett, 1988). After transcription of the viral genes, GnGc is translated as a polyprotein in the endoplasmic reticulum (ER) where it is N-glycosylated and is then cleaved by signal peptidase in the ER into Gn and Gc glycoproteins (Gerrard and Nichol, 2007; Suzich et al., 1990). Both Gn and Gc localise to the Golgi apparatus when expressed as a polyprotein. The Golgi localization signal is found within the Gn protein transmembrane domain (Gerrard and Nichol, 2002; Schmaljohn and Hooper, 2001).

Glycoproteins of RVFV (Gn and Gc) are found on the outermost surface of the virus and are the most exposed parts of the virus; they have therefore been tested by different approaches for competence to induce neutralising antibodies, which can protect against the disease. The production of recombinant RVFV proteins as vaccines has been made possible by the mapping of their protective determinants using vaccinia virus expressing GnGc and a series of truncated Gn (Dalrymple, 1989). Three neutralizing epitopes have been mapped in Gn (Keegan and Collett, 1986). Animals immunised with Gn produced neutralising antibodies against RVFV (Schmaljohn et al., 1989). Subsequently, there have been several other examples of the efficacy of recombinant vaccines: Gorchakov et al., (Gorchakov et al., 2007) showed that mice were protected from wild type ZH501 RVFV challenge following immunisation with Venezuelan equine encephalitis virus (VEEV) replicon expressing Gn. Holman et al., 2009 (Holman et al., 2009) developed a complex adenovirus system encoding RVFV Gn and Gc with human CD4 signal peptides under the human cytomegalovirus promoter for human vaccines. Mice were protected from the wild type ZH501 RVFV challenge. A recombinant Newcastle disease virus LaSota strain expressing Gn (NDFL-Gn) (Kortekaas et al., 2010b) and GnGc (NDFL2-GnGc) (Kortekaas et al., 2010a) were developed and characterised. Animals immunised with the recombinant protein produced neutralising antibodies and were protected from a challenge.

The aim of the work reported in this chapter was to test different cloning strategies for the expression of the RVFV vaccine candidates GnGc, and Gn alone. The expression

of wild type, plant- and human-codon optimised *GnGc* was tested using several different plant expression vectors to compare which one would result in the highest levels of recombinant protein: these were a self-replicating pRIC3.0 vector, the cytoplasm-targeting pTRAc vector, the ER-targeting pTRAc-ERH vector and the CMPV based hypertranslatable pEAQ-*HT* vector.

3.2 Materials and Methods

3.2.1 Rift Valley fever virus (RVFV) *GnGc* genes

The Rift Valley fever (RVF) wild type strain (ZH548 M12) *GnGc* gene was kindly provided by Prof A Williamson (IIDMM, UCT) in the pSCRV6 plasmid (Figure 3.1 A). The RVF *GnGc* from South African isolate M35/74 (GenBank accession number JF 784 387.1) was both *Nicotiana* sp. (Figure 3.1 B) and human-codon optimised (Figure 3.1 C). Both genes were synthesised by GenScript (GenScript Biotechnologies, Piscataway, NJ, USA) inclusive of the 4th ATG.

Figure 3.1: RVFV *GnGc* genes of: **A**) wild type RVFV strain ZH548 M12, **B**) plant-codon optimised South African isolate M35/74 synthesised inclusive of the 4th ATG, **C**) human codon optimised South African isolate M35/74 synthesised inclusive of the 4th ATG.

3.2.2 Plant expression vectors

The plant expression vectors used for these studies are shown in Table 3.1 and depicted schematically in Figure 3.2.

Table 3.1: Plant expressed vectors used in this study

Plasmid	Description	Reference
pEAQ-*HT*	CPMV-based hypertranslatable expression vector	(Sainsbury et al., 2009a)
pTRAc	Cytoplasm-targeting vector	(Maclean et al., 2007)
pTRAkc-ERH	Endoplasmic reticulum-targeting vector	(Maclean et al., 2007)
pRIC3.0	Self-replicating vector	(Regnard et al., 2010)
pTRAc-HT	Cytoplasm- targeting vector with 5' *6xhis* tag	[a]BRU-Culture Collection

[a] Biopharming Research Unit, Department of Molecular and Cell Biology, UCT

3.2.3 Bacterial and plant growth conditions

Chemically competent *Escherichia coli* DH5α™ cells (E. cloni™, Lucigen, WI) were grown in Luria Bertani medium (LB) (0.5% yeast, 1% tryptone, 0.5% sodium chloride) (Sambrook et al., 1989) supplemented with either 100 µg/mL kanamycin (kan) (for pEAQ-*HT*) or 100 µg/mL ampicillin (amp) (for pTRAc vectors and pRIC3.0) as the selection agent and incubated at 37 ˚C for 16 hours (h), shaking at 180 rpm.

Agrobacterium tumefaciens LBA4404 was grown in LB supplemented with 10 mM MES, 30 µg/mL kan, 50 µg/mL rifampicin (rif) and 2 mM $MgSO_4$ to prevent clumping of the cells and incubated for 2 - 3 days at 27 ˚C, shaking at 180 rpm. *A. tumefaciens* GV3101::pMP90RK was grown in LB supplemented with 10 mM MES, 30 µg/mL kan, 50 µg/mL rif, and 50 µg/mL carbenicillin (carb) and incubated for 2 – 3 days at 27 ˚C, shaking at 180 rpm. In addition, the *Agrobacterium* LBA4404 (pBIN-NSs) strain obtained from Marcel Prins (Laboratory of Virology, University of Wageningen) was also cultured. This strain contains a silencing suppressor gene of tomato spotted wilt virus (TSWV) to prolong and increase recombinant gene expression.

Nicotiana benthamiana plants were grown in a plant growth room, at 22 ˚C with a 16:8 h light/dark photoperiod, light intensity of 60 – 80 µE/m2/s and 45% relative humidity.

Plants were supplemented with a commercially available organic fertilizer weekly (Chemicult® hydroponic nutrient powder).

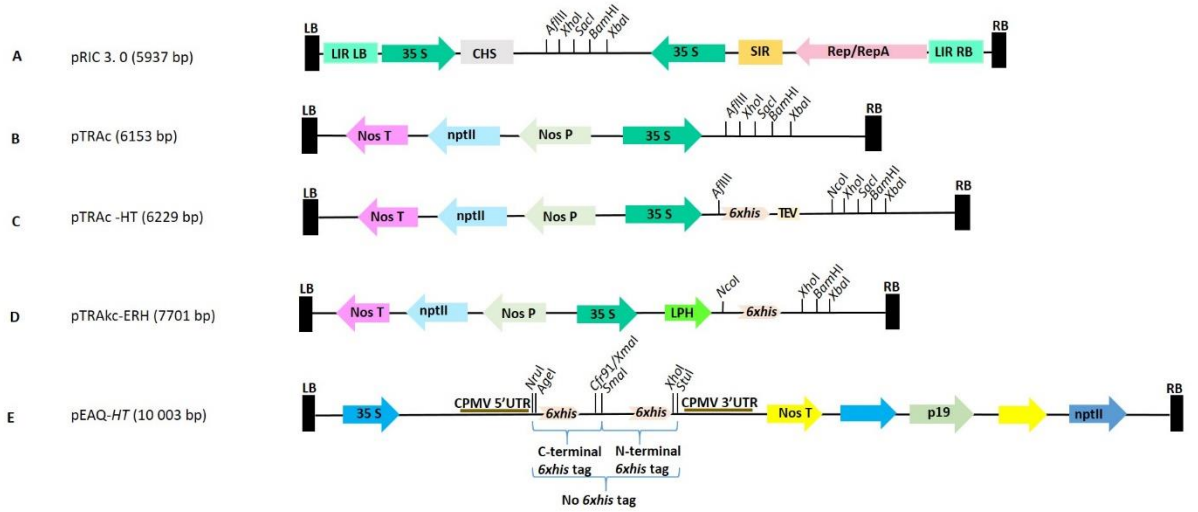

Figure 3.2: Plant expression vectors. (**A**) **pRIC3.0 vector** - refer to figure 2.5 for vector elements (**B-D**) **pTRA vectors** – refer to figure 2.4 for vector elements (**E**) **pEAQ-*HT* vector** – refer to figure 2.6 for vector elements.

3.2.4 DNA manipulation and analysis

Recombinant plasmids were transformed into chemically competent *E. coli* DH5α cells (E. cloni™, Lucigen, WI) according to Sambrook et al., (Sambrook, 1989), unless otherwise stated. The transformed cells were plated out onto LB agar plates with appropriate antibiotics and incubated overnight at 37 °C. Recombinant single colonies were picked and grown overnight as described in section 3.2.3. Plasmid DNA was isolated with the QIAprep® Spin Miniprep kit (Qiagen Gnbh, Hilden, Germany) according to the manufacturer's instructions. DNA concentrations were obtained using the NanoDrop (ND1000 Spectophotometer). All DNA sequencing was carried out with appropriate primers by Macrogen Inc. (Netherlands) and sequences were analysed with CLC Main Workbench 6.0 (CLC Bio) or SnapGene Viewer (GSL Biotech LLC). Plasmid integrity was confirmed by restriction enzyme digestion for 1h at 37 °C with 1U appropriate restriction enzyme (Fermentas, Hanover, MD) unless otherwise stated. Digested DNA fragments were separated on 0.8% (w/v) Tris-borate-EDTA (TBE) (89 mM Tris base, 89 mM boric acid and 2 mM EDTA) agarose gels containing 2.5 mg/mL

ethidium bromide. The gels were visualised either under short wavelength ultraviolet (UV) (245 nm) or long-wavelength (360 nm) illumination depending on requirements. O'GeneRuler™ 1kb DNA ladder (Fermentas, Hanover, MD) was used as a molecular weight marker on all agarose gels. Digested DNA fragments were gel-purified using the QIAquick® Gel Extraction kit (Qiagen Gnbh, Hilden, Germany) and de-phosphorylated using 1U rAPid Alkaline Phosphatase kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Insert DNA was ligated into a plant expression vector in a 1:3 vector: insert ratio using the Fermentas ligate kit (Fermentas, Hanover, MD) according to manufacturer's instruction. The ligation mix is transformed into *E. coli* cells as describe in section 3.2.4. Putative positive colonies were screened by PCR with vector specific primers, with the PCR profile in section 3.2.5. Recombinant plasmids were confirmed by restriction enzyme digest mapping.

3.2.5 Gene amplification by PCR

DNA sequences were amplified by PCR using primers listed in Chapter 8 Appendix B. PCR reactions were conducted in the BioRad Biocycler automated temperature cycler. All PCR reactions were carried out in 25 µL with 0.5 Units (U) of KAPA *Taq* DNA polymerase (KAPA Biosystems, South Africa), 1x buffer, 200 µM dNTPs, 2 mM $MgCl_2$, 200 µM of each primer and 1-5 ng of DNA template. Cycles were performed using the following parameters: denaturation at 95 ˚C for 2 min, followed by 30 cycles of denaturation at 95 ˚C for 30 s, primer annealing for 30 s and extension at 72 ˚C for 30 s. A final elongation step at 72 ˚C for 5 min was performed. The annealing temperature was adjusted depending on the primer set used.

3.2.6 Sub-cloning of RVFV glycoprotein genes for sequencing

Purified recombinant DNA was sub-cloned using a 1:3 vector:insert ratio into the pGEM®-T-Easy sequencing vector (Promega Corporation, Madison) according to the manufacturer's instructions. Ligated plasmid DNA was transformed into chemically competent *E. coli* DH5α cells (E. cloni ™, Lucigen, WI). Transformed cells were

selected on LB agar plates with 100 µg/mL amp, 80 µg/mL X-gal (5-bromo-chloro-3-indolyl-β-D-galactopyranoside) and 0.5 mM IPTG (Isoprophylthio-β-D-galactoside).

Positive colonies were screened by PCR with M13 universal primers using the PCR profile described in 3.2.4, annealing at 65 ˚C. Plasmid DNA from positive colonies was isolated, confirmed by restriction enzyme digest mapping and sequenced with M13 universal primers as described in 3.2.4.

3.2.7 Cloning of glycoprotein genes into plant expression vectors

Plant expression vector DNA was prepared for cloning by digestion with appropriate restriction enzymes, gel-purified and de-phosphorylated as described in 3.2.4.

3.2.8 Cloning of WT strain RVFV *GnGc* glycoprotein gene

3.2.8.1 Cloning of GnGc into pGEM®-T-Easy for sequencing

The WT RVFV *Gn* and *GnGc* sequences were amplified from pSCRV6 by PCR using G1 Fw and G1 Rev as well as G1 Fw and G2 Rev gene-specific primers, respectively (primers sequences found in Table 1, Appendix B), annealing at 54 ˚C with the PCR profile described in section 3.2.5. The DNA was resolved on an agarose gel and gel-purified as described in section 3.2.4. The purified DNA was ligated into pGEM®-T-Easy as described in section 3.2.6. Plasmid DNA was purified and recombinant pGEM®-T-Easy–Gn and pGEM®-T-Easy–GnGc clones confirmed by restriction enzyme digest mapping with *Eco*RI and *Sal*I.

3.2.8.2 Modification of Gn and GnGc glycoprotein for cloning into plant expression vectors

The *Gn* and *GnGc* gene fragments were amplified from pGEM®-T-Easy-Gn and pGEM®-T-Easy-GnGc, respectively. Gene amplification was performed by PCR with the following different pairs of primers (primer sequences in Table 1, Appendix B) to introduce the appropriate restriction enzyme sites for cloning into pRIC3.0 (Figure 3.2 A) and pEAQ-*HT* (Figure 3.2 E) plant expression vectors: Gn *Nru*I Fw and Gc *Xma*I

Rev; Gn N*ruI* Fw and Gn *Xma*I Rev; Gn *Nru*I Fw and Gc *Xho*I Rev; Gn *Nru*I Fw and Gn *Xho*I Rev; Gn *Nco*I Fw and Gn *Xho*I Rev as well as Gn *Nco*I Fw and GnGc *Xho*I Rev respectively. Gene amplification was performed using the PCR profile described in section 3.2.5 with the annealing temperature set at 55 ˚C. Amplified DNA was gel-purified as described in section 3.2.4.

Gel-purified DNA was sub-cloned into pGEM®-T-Easy as described in section 3.2.6. The ligated plasmid DNA was transformed into *E. coli* C43 cells (Lucigen, WI) according to (Sambrook and Russell, 2001). The cells were made competent according to Draper et al., 1988. The resulting plasmids are found on Table 3.2. Recombinant plasmids were confirmed by restriction digest and sequenced with M13F and M13R universal primers as well as gene internal primers Appendix B, Table 1 (G1 Fw979, G1 R1023, G2 Fw2693, G2 Rev2749).

3.2.8.3 Cloning into pEAQ-HT

The pEAQ-*HT* vector (Figure 3.1 E) was linearized by restriction enzyme digest with *Nru*I and *Xma*I (for a C terminal *6xhis* tag), *Sma*I and *Xho*I (for an N terminal *6xhis* tag) and *Nru*I and *Xho*I (for no *6xhis* tag). *Gn* and *GnGc* were excised from pGEM®-T-Easy-GnNruI, pGEM®-T-Easy-GnSmaI, pGEM®-T-Easy-GnXhoI, pGEM®-T-Easy-GnGcNruI, pGEM®-T-Easy-GnGcSmaI, and pGEM®-T-Easy-GnGcXhoI clones (Table 3.1) with corresponding restriction enzymes and cloned into pEAQ-*HT* as described in section 3.2.7. Putative positive colonies were screened by PCR using gene-specific primers pEAQ Fw and pEAQ Rev (primers sequences found in Table1, Appendix B) and PCR cycle parameters described in section 3.2.5, primer set annealing at 55 ˚C Recombinant DNA was isolated from bacterial cultures (see section 3.2.4) and the recombinant pEAQ-*HT*-his-Gn (c), pEAQ-*HT*-his-Gn (n), pEAQ-*HT*-Gn, pEAQ-*HT*-his-GnGc (c), pEAQ-*HT*-GnGc (n) and pEAQ-*HT*-GnGc (see Table 3.2) plasmids were confirmed by restriction digest mapping with *Afl*III (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

3.2.8.4 Cloning into pRIC3.0

The pRIC3.0 geminivirus-derived replicating vector (Regnard et al., 2010) was linearized with *Afl*III and *Xho*I (Roche Diagnostics GmbH, Mannheim, Germany), gel-purified and de-phosphorylated (section 3.2.4). *Gn* and *GnGc* was excised from pGEM®-T-Easy-GnNcoI and pGEM®-T-Easy-GnGcNcoI with *Nco*I and *Xho*I. The insert DNA was cloned into pRIC3.0 as described in section 3.2.7. The ligation reactions were transformed into competent *E. coli* C43 cells as described 3.2.8.2. Putative positive colonies were screened by PCR using pTRAc-specific primers (Appendix B, Table1) using the PCR profile described in section 3.2.5. The recombinant pRIC3.0-Gn and pRIC3.0-GnGc plasmids were verified by restriction enzyme digest mapping using *Bam*HI and *Eco*RI, respectively.

3.2.9 Cloning of human and *Nicotiana sp.* codon optimised RVFV *GnGc*

The RVFV *Nicotiana* sp. (p) codon optimised pCC1-pGnGn (Figure 3.1 B) and human (h)-codon optimised pUC57-hGnGc (Figure 3.1 C) plasmids were transformed into chemically competent *E. coli* DH5α cells (E. cloni™, Lucigen, WI) as described in section 3.2.4.

3.2.9.1 Modification of codon optimised Gn and GnGc genes for cloning into plant expression vectors

pCC1-pGnGc was used as a template to amplify *pGn* and *pGnGc* for cloning into pEAQ-*HT* and pRIC3.0. Enzyme cloning sites for inserting *pGn* into plant expression vectors were introduced by PCR with GnNcoI Fw and GnStuI pair and GnNcoI Fw and Gc Rev primer (primer sequences listed in Appendix B, Table 3,). The PCR cycle profile is described in section 3.2.5, primers annealing at 53 ˚C and 50 ˚C, respectively. Amplified DNA was gel purified as described in section 3.2.4 and sub-cloned into pGEM®-T-Easy as described in section 3.2.6. Recombinant plasmids pGEM®-T-Easy-pGnAgeI, pGEM®-T-Easy-pGnNcoI, and pGEM®-T-Easy-pGnGcNcoI were confirmed by restriction digest with *Age*I and *Stu*I and sequenced with M13F and M13R universal primers as well as gene internal primers Appendix B, Table 2, (Fw961, Fw2161, Rev2221, Rev1012).

3.2.9.2 Cloning codon-optimised RVFV Gn and GnGc into pEAQ-HT

RVFV *pGn* encoding sequence was excised from pGEM®-T-Easy-pGnAgeI (Table 3.3), while the full length *pGnGc* and *hGnGc* were excised from pCC1-pGnGc and pUC57-hGnGc respectively, using appropriate restriction enzymes. pEAQ-*HT* was linearized with the *Age*I and *Stu*I restriction enzymes respectively. *pGn*, *pGnGc* and *hGnGc* were cloned into pEAQ-*HT* as described in section 3.2.7 to generate pEAQ-*HT*-pGn, pEAQ-*HT*-pGnGc and pEAQ-*HT*-hGnGc (Table 3.3). Recombinant plasmids were verified by PCR using pEAQ Fw and pEAQ Rev primers and confirmed by restriction digest mapping with *Age*I and *Stu*I.

3.2.9.3 Cloning codon optimised RVFV Gn and GnGc into pRIC3.0

pGn, and *pGnGc* sequences were subsequently excised from the pGEM®-T-Easy-pGnNcoI, and pGEM®-T-Easy-pGnGcNcoI vectors, while *hGnGc* was directly cloned from pUC57-hGnGc with *Nco*I & *Xma*I and gel purified. These were cloned into pRIC3.0 linearized with *Afl*III and *Xma*I as described in section 3.2.7. Positive colonies were verified by PCR using pTRAc Fw and pTRAc Rev primers with the PCR profile in section 3.2.5. Plasmid integrity of pRIC3.0-pGn by digest with *Eco*RI, and pRIC3.0-hGnGc was confirmed by digestion with *Eco*RV while pRIC3.0-pGnGc was confirmed by digestion with *Bam*HI.

3.2.9.4 Cloning of codon optimised RVFV GnGc into the pTRAc and pTRAc-ERH plant expression vectors

Full length *hGnGc* was directly cloned from pUC57-hGnGc into the pTRAc (Figure 3.2 B) and pTRAc-ERH (Figure 3.2 D) vectors as described in section 3.2.7. The vectors were linearized with *Afl*III or *Nco*I and *Xho*I restriction enzymes respectively. Positive colonies were verified by PCR with pTRAc vector-specific primers as described in section 3.2.5. Recombinant pTRAc-hGnGc and pTRAc-ERH-hGnGc were confirmed by restriction mapping with *Eco*RI.

3.2.10 *Agrobacterium* transformation

A. tumefaciens LBA4404 and GV3101::pMP90RK cells were made electrocompetent using the method described by Shen and Forde (1989) (Shen and Forde, 1989). One

hundred ng of the recombinant plasmid DNA was electroporated using a GenePulser (BioRad) into *A. tumefaciens* LBA4404 and GV3101::pMP90RK cells, respectively in 1 mm gap electroporation cuvettes (Molecular BioProducts, USA) as described by Mclean et al., 2007 (Maclean et al., 2007). Transformed *Agrobacterium* cells were plated on LB agar plates and grown as in section 3.2.3. Recombinant *Agrobacterium* was verified by PCR using the PCR profile described in section 3.2.5 using vector specific primers listed in Table 1 (Appendix B).

3.2.11 Transient expression of recombinant proteins

Recombinant *A. tumefaciens* was grown in LB supplemented with the appropriate antibiotics to maintain selection of transformants. Cultures containing the recombinant plasmids were cultured in induction medium (LB broth, 10 mM MES, 20 µM acetosyringone and 2 mM $MgSO_4$, pH 5.6) containing antibiotics at 27 °C overnight. Bacteria were cultured to exponential phase (OD_{600} = 0.5) and centrifuged at low speed (4000 x g). The pellet was re-suspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$, 150 µg acetosyringone/mL and 2% sucrose) and incubated at room temperature for two h. The recombinant *A. tumefaciens* GV3101::pMP90RK and LBA4404 (pBIN-NSS) cells were diluted with infiltration medium to generate a final OD_{600} (UltraSpec™ 10) of 0.25 for each strain (total *Agrobacterium* OD_{600} of the suspension was 0.5). The recombinant *Agrobacterium* LBA4404 was diluted to generate a final OD_{600} of 0.5.

Six week old *N. benthamiana* plants were infiltrated with a 1 mL needleless syringe directly into the abaxial air spaces with recombinant *Agrobacterium* cultures. Infiltrated leaves were harvested from 1 – 9 days post infiltration (dpi).

3.2.12 Small scale protein extraction

Four leaf discs (~ 8mm diameter) were harvested using an Eppendorf vial lid as a cutter, and ground in liquid nitrogen. The protein was extracted with 250 µL buffer (100 mM Tris/HCl pH 7.5 and 1% Triton X-100), by vortexing for 1 min and incubation on ice for 10 min. Crude plant extracts were clarified by centrifugation at 14 000 rpm for 5 min using a benchtop centrifuge.

3.2.13 Protein expression analysis

For western blot analysis, the plant crude extracts were boiled at 95 ˚C for 5 min in 5x sample application buffer (5x SAB) (940 uL 10% SDS, 470 uL 1 M Tris/Cl pH 7.5 (250 mM), 95 uL 100 mM EDTA, 205 uL β-mercaptoethanol, 2.45 mL glycerol, 0.2 mg Bromophenol blue (0.1%), 545 uL dH_2O). An equal amount of total soluble protein (TSP) were loaded in each well and separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). For all western blots PageRuler™ Prestained Protein Ladder (Thermo Scientific, MA, USA) was used as a molecular weight marker.

The proteins were transferred by semi dry blotting on nitrocellulose blotting membrane (Amersham™ Protran™ Premium 0.45 µm NC) using a Trans-blot®SD semi dry transfer cell (BioRad Power Pack 200) at 15 volts (V) for 1.5 h. Following the transfer, membranes were blocked in blocking buffer (1 x PBS [137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 2 mM KH_2PO_4] at pH 7.4, 0.1% Tween-20 and 5% fat free milk). After blocking, the membranes were incubated with polyclonal rabbit antibodies raised in rabbits inoculated with KLH-linked synthetic peptides derived from Gn (374-CFEHKGQYKGTMDSGQTKRE-393) (GenScript, Piscataway, NJ, USA) as described by de Boer et al., 2010 (de Boer et al., 2010) or incubated with *6xhis* antibody. The membranes were washed four times with blocking buffer for 15 min each and subsequently incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody (1: 7000, Sigma Aldrich) or alkaline phosphatase-conjugated goat anti-mouse antibody (1:7000, Sigma Aldrich) in blocking buffer for 1 h at 37 ˚C. This was followed by four washes, 15 min each, with wash buffer (1x PBS pH 7.4, 0.1% Tween-20). Blots were developed using 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium chloride (BCIP/NBT 1-component, KPL) as a substrate. Protein expression, extraction and western blot analysis were repeated at least three times to confirm the expression of the RVFV glycoproteins.

3.3 Result

3.3.1 Cloning of WT *Gn* and *GnGc*

RVFV WT *GnGc* was successfully amplified from pSCRV6 and cloned into the pGEM®-T-Easy to yield pGEM®-T-Easy-GnGc. The 3885 nucleotide sequence was confirmed to be 100% homologous to the ZH548 M12 strain from Egypt in 1977 (GenBank accession no NC_014396) as expected.

In addition, *Gn* was amplified from pSCRV6 and sub-cloned into pGEM®-T-Easy to yield pGEM®-T-Easy-Gn (Table 3.2). *Gn* and *GnGc* were subsequently successfully amplified in order to attach appropriate restriction sites for cloning into pEAQ-*HT* and pRIC3.0 plant expression vectors. Amplified *Gn* and *GnGc* fragments were cloned into the pEAQ-*HT* vector using different restriction enzyme sites such that both *Gn* and *GnGc* could be expressed with a N terminal *6xhis* tag (pEAQ-*HT*-GnGc (n); pEAQ-*HT*-Gn (n)), a C terminal *6xhis* tag (pEAQ-*HT*-GnGc (c); pEAQ-*HT*-Gn (c)) or no *6xhis* tag (pEAQ-*HT*-GnGc; pEAQ-*HT*-Gn) – Table 3.2. Amplified *GnGc* and *Gn* fragments were also successfully cloned into pRIC3.0 resulting in pRIC3.0-GnGc and pRIC3.0-Gn, respectively (Table 3.2). Recombinant clones were confirmed successfully by restriction enzyme digest mapping.

Table 3.2: Wild type Rift Valley Fever ZH548 M12 strain *Gn* and *GnGc* glycoprotein encoding plasmids. Refer to the Appendix B (Table 1) for primer sequences.

Plasmid name	Gn or GnGc polyprotein	*6xhis* tag	Sub-cloning	Primers
pGEM®-T-Easy-GnGc		N/A	pSCVR6-GnGc	G1 Fw and G2 rev
pGEM®-T-Easy-Gn		N/A	pSCVR6-GnGc	G1 Fw and G1 Rev
pEAQ-*HT*-his- Gn (c)	NruI XmaI	C- terminal *6xhis* tag	pGEM®-T-Easy-GnNruI	GnGc *Nru*I Fw & Gn *Xma*I Rev
pEAQ-*HT*-his- Gn (n)	SmaI XhoI	N- terminal *6xhis* tag	pGEM®-T-Easy-GnSmaI	Gn *Sma*I Fw & Gn *Xho*I Rev
pEAQ-*HT*- Gn	NruI XhoI	No *6xhis* tag	pGEM®-T-Easy-GnXhoI	GnGc *Nru*I Fw & Gn *Xho*I Rev
pEAQ-*HT*-his- GnGc (c)	NruI XmaI	C- terminal *6xhis* tag	pGEM®-T-Easy-GnGcNruI	Gn *Sma*I Fw & GnGc *Xma*I Rev
pEAQ-*HT*-his- GnGc (n)	SmaI XhoI	N- terminal *6xhis* tag	pGEM®-T-Easy-GnGcXhoI	GnGc *Nru*I Fw & GnGc *Xho*I Rev
pEAQ-*HT*-GnGc	NruI XmaI	No *6xhis* tag	pGEM®-T-Easy-GnGcXmaI	GnGc *Nru*I Fw & GnGc *Xho*I Rev
pRIC3.0-Gn	NcoI XhoI	No *6xhis* tag	pGEM®-T-Easy-GnNcoI	Gn NcoI Fw & Gn *Xho*I Rev
pRIC3.0-GnGc	NcoI XhoI	No *6xhis* tag	pGEM®-T-Easy-GnGcNcoI	Gn NcoI Fw & GnGc *Xho*I Rev

3.3.2 Expression of WT *Gn* and *GnGc* in *N. benthamiana*

All plant expression constructs made in Section 3.3.1 were infiltrated into *N. benthamiana* and plants were sampled from 2 to 9 days post infiltration (dpi) in order to screen for recombinant *Gn* and *GnGc* expression by western blotting of crude leaf extracts. The Gn and GnGc glycoprotein transient expression time trial showed no physiological or morphological differences observed between the experiment and the control plants. However, the expected protein band sizes of 60.9 kDa for WT Gn and 112.0 kDa for WT GnGc could not be detected with Gn-specific antibodies or the *6xhis* antibody.

3.3.3 Cloning of human (h) and *N. benthamiana* (p) codon optimised *Gn* and *GnGc*

It was thought that the reason for the lack of detection of WT Gn or GnGc in crude *N. benthamiana* extracts may have been because the gene sequence used was not codon optimised for optimal host expression. Thus, 2 new gene sequences, namely a *Nicotiana* sp. and a human-codon optimised gene, were synthesised, cloned into various plant expression vectors and screened for recombinant protein expression after infiltration of constructs.

3.3.3.1 Analysis of Nicotiana sp. and human-codon optimised GnGc

RVFV *GnGc* of the South African M35/74 strain was selected as it was considered to be more relevant in the South African context and sequence analysis showed 95% protein homology to the WT RVFV ZH548 strain, with 11 aa differences. The 3225 bp sequence was *Nicotiana* sp. codon optimised and human-codon optimised. Sequence analysis showed that *Nicotiana* sp. codon optimised *GnGc* was 79.50% homologous to the WT M35/74 strain, while human-codon optimised *GnGc* was 77.31%. Plant-codon optimisation resulted in a lower GC content of *GnGc* compared to the WT M35/74 *GnGc*, decreasing from 50% to 41%. Human-codon optimisation resulted in a higher GC content of GnGc increasing from 50% to 56.39%. Both genes were successfully assembled and cloned into pCC1 and pUC57 respectfully by GenScript (GenScript Biotechnologies, Piscataway, NJ, USA). *GnGc* and *Gn* were subsequently cloned into the following plant expression vectors to determine and compare expression: there were pEAQ-*HT*, pRIC3.0 and pTRAc.

3.3.3.2 Cloning of Gn and GnGc into the pEAQ-HT expression vector

Nicotiana sp. and human-codon optimised *GnGc* were successfully cloned directly into pEAQ-*HT* to yield pEAQ-*HT*-pGnGc and pEAQ-*HT*-hGnGc (Table 3.3). The plant-codono ptimised *pGn* was amplified by PCR to introduce enzyme cloning sites for the vector and sub-cloned into pGEM®-T-Easy successfully to yield pGEM®-T-Easy-pGn. Restriction enzyme mapping and sequencing confirmed the construct. *pGn* was subsequently cloned successfully into pEAQ-*HT* to yield pEAQ-*HT*-pGn (Table 3.3). Restriction enzyme digest mapping of the final plasmids gave the expected DNA banding pattern. pEAQ-*HT*-pGnGc resulted in DNA bands of the expected sizes of 9947 and 3222 bp (Figure 3.3 A), while pEAQ-*HT*-pGn showed the expected banding pattern of 9947, 1698, 73 bp (Figure 3.3 B). Digestion of pEAQ-*HT*-hGnGc resulted in the expected DNA band sizes of 3069, 2639, 2580, 2345, 1414, 1005 and 122 bp (Figure 3.3 C).

Table 3.3: Codon optimised RVFV glycoproteins from South Africa M35/74 strain encoding plasmids primer sequences found in the Appendix B, Table 2.

Plasmid name	Size	RVFV polyprotein	Cell compartment	Sub-cloning	Primers[a]
pEAQ-*HT*-pGnGc	3204 bp (116.3 kDa)	AgeI — pGnGc — XmaI	Cytoplasm	pCC1-pGnGc	N/A
pEAQ-*HT*-pGn	1716 bp (62.1 kDa)	AgeI — pGn — StuI	Cytoplasm	pGEM®-T-Easy-GnAgeI	Gn Fw & Gn *Stu*I Rev
pRIC3.0-pGnGc	3208 bp (112.0 kDa)	NcoI — pGnGc — XmaI	Cytoplasm	pGEM®-T-Easy-GnGcNcoI	Gn *Nco*I Fw & Gc rev
pRIC3.0-pGn	3218 bp (60.9 kDa)	BspHI — pGn — XmaI	Cytoplasm	pGEM®-T-Easy-GnNcoI	Gn *Nco*I Fw & Gn *Stu*I Rev
pEAQ-*HT*-hGnGc	3204 bp (116,3 kDa)	AgeI — hGnGc — XhoI	Cytoplasm	pUC57-hGnGc	N/A
pRIC3.0-hGnGc	3204 bp (116,3 kDa)	NcoI — hGnGc — XhoI	Cytoplasm	pUC57-hGnGc	N/A
pTRAc-hGnGc	3204 bp (116,3 kDa)	AflIII — hGnGc — XhoI	Cytoplasm	pUC57-hGnGc	N/A
pTRAc-ERH-hGnGc	3204 bp (116,3 kDa)	NcoI — hGnGc — XhoI	Endoplasmic Reticulum	pUC57-hGnGc	N/A

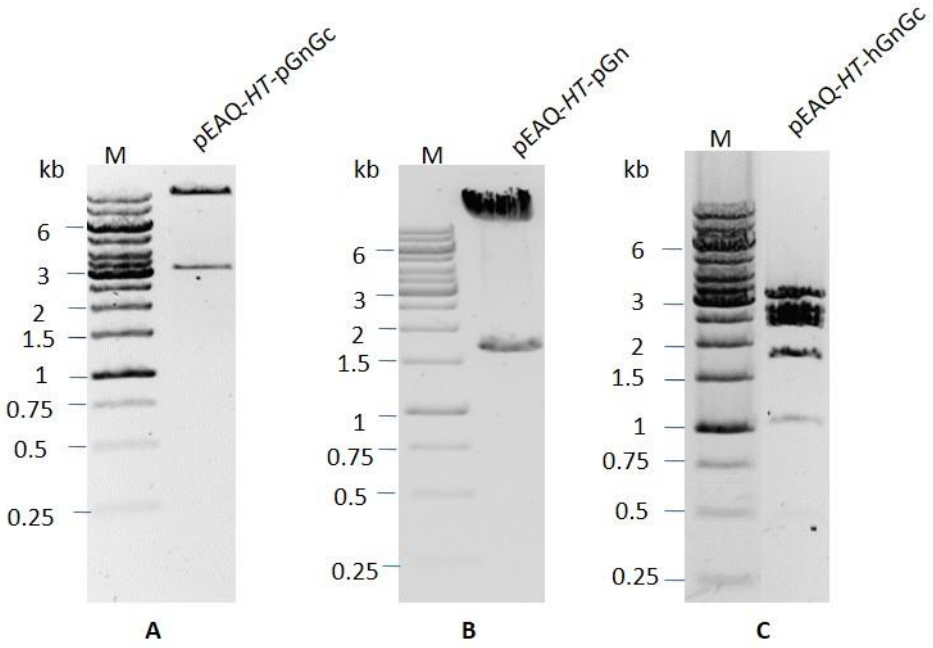

Figure 3.3: Restriction enzyme digest mapping of codon optimised RVFV *Gn* and *GnGc* with *Eco*RV in the CPMV-based hyplerslatable expression vector**.** **A**) pEAQ-*HT*-pGnGc (9947, 3222 bp), **B**) pEAQ-*HT*-pGn (9942, 1698, 17 bp), **C**) pEAQ-*HT*-hGnGc (3069, 2639, 2580, 1414, 1005, 122). Lane M represents the 1 kb O'GreenGene ruler used as a DNA ladder. DNA was resolved on 0.8% (w/v) TBE agarose gel.

3.3.3.3 Cloning of Gn and GnGc into the pRIC3.0 plant expression vector

Human-codon optimised *hGnGc* was directly cloned into the vector resulting in pRIC3.0-hGnGc. Restriction enzyme digest mapping gave the expected DNA banding pattern of 8159, 953 bp (Figure 3.4 A). *pGn* and *pGnGc* were amplified by PCR to

introduce enzyme cloning sites for the vector and sub-cloned into the sequencing vector and successfully cloned into pRIC3.0. Restriction digest mapping gave the expected DNA banding pattern for pRIC3.0-pGn (6544, 1080 bp) (Figure 3.4 B) and pRIC3.0 – pGnGc (5452, 2495, 727, 362 bp) (Figure 3.4 C).

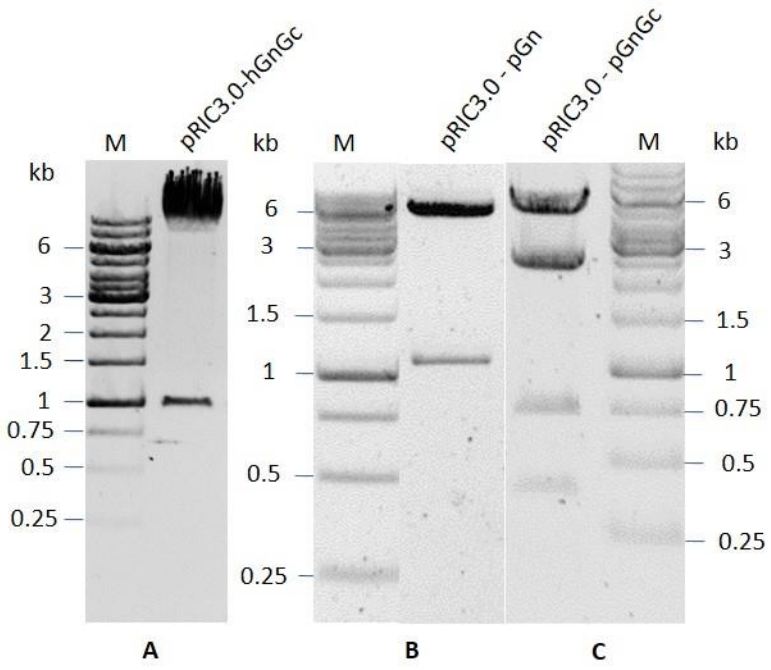

Figure 3.4: Verification of codon optimised *Gn* and *GnGc* in the self-replicating pRIC3.0 plasmids. **A**) pRIC3.0-hGnGc (8159, 953 bp) with *Eco*RV, **B**) pRIC3.0-pGn (6544, 1080 bp) *Eco*RI and **C**) pRIC3.0-pGnGc (5452, 2495, 727, 362 bp) digested with *Bam*HI. Resolved on 0.8% (w/v) TBE agarose gel. The 1 kb O'GreenGene ruler was used as a DNA ladder is presented by lane M.

3.3.3.4 Cloning of GnGc into the pTRAc vectors

hGnGc was directly cloned, successfully from pUC57-hGnGc into pTRAc, the cytoplasm targeting and pTRAc-ERH, the endoplasmic reticulum (ER) organelle targeting vectors (Table 3.3). Restriction enzyme digest mapping confirmed the constructs. Expected DNA band sizes for digestion of pTRAc-ERH-hGnGc (6766, 1633, 1405, 1012, 19 bp) (Figure 3.5A) and pTRAc-hGnGc (8412, 953 bp) (Figure 3.5 B).

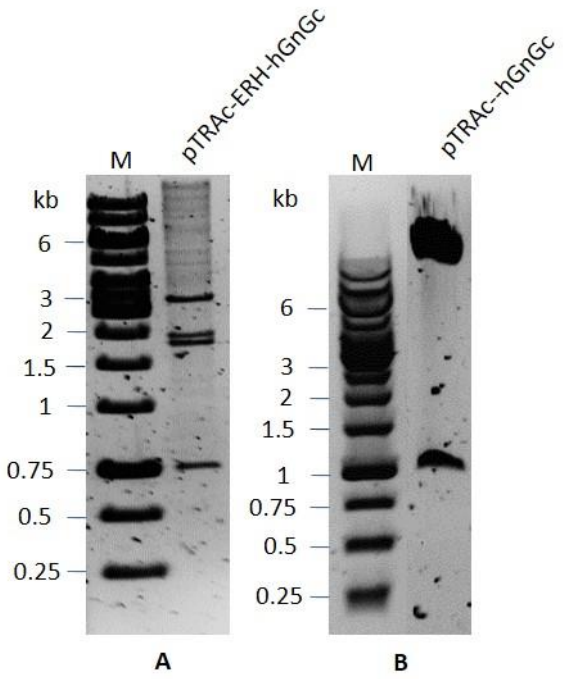

Figure 3.5: Restriction enzyme digest mapping with of pTRAc plasmids with *Eco*RV of human-codon optimised RVFV polyproteins expression plasmids. **A**) mapping in non-replicating expression vector which targets protein expression in the ER. **B**) mapping in a non-replicating vector pTRAc which allows expression in the cytoplasm. 1 kb O'GreenGene ruler (M) was used as a DNA ladder. DNA was resolved on 0.8% (w/v) TBE agarose gel.

3.3.3.5 Expression analysis of recombinant Gn and GnGc

All recombinant expression plasmids were successfully electroporated into the appropriate *A. tumefaciens* cells. *N. benthamiana* leaves were infiltrated with recombinant *Agrobacterium* cultures and expression was determined by western blotting of crude leaf extracts harvested at various dpi and probing with the appropriate antibodies. However, bands corresponding to the Gn and GnGc proteins of expected sizes of ~60 and 116.3 kDa, respectively, still could not be detected at any time points with any of the Gn or GnGc constructs tested.

3.4 Discussion

RVFV VLPs have been made in mammalian and bacterial expression systems as candidate vaccines by expressing Gn and GnGc (de Boer et al., 2010; Kortekaas et al., 2012; Wallace et al., 2006). This is the first time an attempt has been made to

express RVFV GnGc glycoproteins in tobacco. An attempt has been made recently to express a mutated Gn in *Arabidopsis* (Kalbina et al., 2016). Compared to the other expression systems used, plants are easy to use, inexpensive, are scalable and do not require biosafety containment (Rybicki, 2009; Rybicki, 2010). These advantages are very suitable for production of recombinant protein products such as vaccines in African countries, where low costs are desirable and they are often the source of target diseases which are mainly endemic. In addition, there have been several examples of successful VLP production in plants.

Initially, I tested whether a readily available WT RVFV ZH548 M12 strain *GnGc* could be first, expressed transiently in *N. benthamiana* when cloned into 2 different plant expression vectors, pEAQ-*HT* and pRIC3.0 and second, if so, whether it assembled into VLPs. The reason for testing 2 different vectors was because these vectors direct expression in different ways, and it has been shown that optimising recombinant protein expression *in planta* is highly empirical. *Gn* was also tested for expression, as it has been shown by de Boer et al. 2010 (de Boer et al., 2010; Wallace et al., 2006) that Gn on its own can be appropriately immunogenic. Western blotting was the technique used for detection, since it was suggested that if expression occurred, albeit low, that western blotting would be sensitive enough for detection of any expressed protein. It was suggested that the lack of detection could be due to antibody not binding to any of the expressed protein. Hence, the genes were cloned into pEAQ-*HT* such that they resulted in being fused to a 6x*his* tag, either on the 5' terminus or the 3' terminus. This was to facilitate detection of expressed protein using an anti-6xhis antibody. Attaching the histidine tag at the N- or C-terminal was to ensure that the tag does not prevent correct protein folding which might result in lack of protein expression or detection. However, recombinant GnGc or Gn protein expression was still not detected using this strategy either.

The lack of recombinant protein detection could also be due to the possibility that the plasmid encoding the gene of interest was no longer present in the host *Agrobacterium* cells. On carrying out PCR on DNA extracted from the cells, this was found to be the case. They were all negative; the plasmids could not be detected by PCR or restriction

enzyme digest. The recombinant gene seemed to be toxic to bacterial culture. This could be a result of gene toxicity to the cells, as it has been previously demonstrated that the full length GnGc is toxic to *E. coli,* even in the absence of detectable protein expression (Kakach et al., 1988).

One of the reasons for lack of expression could be the lack of use of codon optimised sequences. Proteins are generally difficult to express outside their host since they might contain codons that are rarely used in the desired expression system or expression limiting regulatory elements within their coding sequence. It was thus decided to address this problem of lack of expression in *N. benthamiana* by testing codon optimised *GnGc* and *Gn* instead.

The degeneracy of the generic code allows different codon sequences to encode the same amino acids (Gouy and Gautier, 1982), making codon-use optimisation possible. Codon optimisation has been widely used to enhance protein expression in heterologous systems by exchanging codons which are rarely found in the host organism, with frequently used codons. This was demonstrated by (Maclean et al., 2007) when they expressed plant- and human-codon optimised HPV 16 and found that the human-codon optimised gene gave much higher expression levels than the plant-codon optimised gene. Gao et al., 2004 (Gao et al., 2004) revealed that the main obstacle to human immunodeficiency virus DNA-based vaccines is the low expression levels of the genes, which is attributed to rare codon usage and AU-rich elements. The frequencies at which the various codons are used varies significantly between different organisms, between proteins expressed at high or low levels within the same organisms (Knight RD et al., 2001). Codon usage is involved in the control of transcription, splicing and RNA structure (Cannarozzi et al., 2010; Gu et al., 2010; Tuller et al., 2010).

For this study, it was decided to synthesise a RVFV *GnGc* gene from a South African strain, making it more relevant to the South African situation. The RVFV isolate M35/74 was codon optimised for both the *Nicotiana* sp. expression host as well as for

human expression, since it has been shown in some cases that increases in expression levels are not necessarily attributed to expression host, but to GC content. Despite being a different strain to that originally tested, I did not expect this to influence the development of the revised strategy. The *N. benthamiana* sp. codon optimised gene shared 79.5% of nucleotide sequence identity with the WT gene. A total of 754 nucleotides were changed and the G+C content decreased from 50% to 41.6%. The human optimised gene shared 77.31% nucleotide sequence identity with the WT gene. A total of 709 nucleotides (see Chapter 8, Appendix A) were changed and the G+C content increased from 50% to 56.39%. The plant- and human-codon optimised genes shared 71.37% nucleotide identity. The WT gene employs tandem rare codons that can reduce the efficiency of translation or even disengage the translation machinery (Webster et al., 2016). The codon usage bias was optimised with a codon adaptation index, which measures the degree of preference at which the host/organism prefers specific codon (Carbone et al., 2003), to be 0.87. The stem-loop structures, which impact ribosomal binding and stability of mRNA, were disrupted, which may be an advantage.

For the plant-codon optimised genes, *Gn* and *GnGc* were cloned into pEAQ-*HT* and pRIC3.0 while human-codon optimised *GnGc* encoding genes were successfully cloned into three different plant expression vectors. The pTRAc suite of plant expression vectors which allow protein expression and subsequent targeting to different plant organelles such as the ER or cytoplasm may have an effect in protein expression. The expression and targeting of heterologous proteins to different plant organelles has been previously demonstrated to significantly enhance protein expression (Maclean et al., 2007; Mortimer et al., 2012). The use of pRIC3.0, which allows vector replication *in planta* (Regnard et al., 2010), also resulted in the lack of recombinant protein expression. The genes were cloned into the CPMV based pEAQ-*HT* plant expression vector which enhances recombinant protein expression when they are cloned downstream of the UTR (Sainsbury et al., 2008; Sainsbury et al., 2009a). However, gene codon optimisation and compartmentalisation seemed to have made no difference in recombinant Gn/Gc protein translation and expression in plants.

The native signal peptide is required for the proteolytic processing of the GnGc precursor molecules into mature envelope glycoproteins. The Gn hydrophobic domain acts as a signal peptide when translation initiates at the second, fourth or fifth ATG by leaky scanning of the host translational mechanism (Gerrard and Nichol, 2002). The native signal peptide might be poorly recognised by the plant's mechanism, which could result in lack of or impaired ribosomal binding for protein translation, thus no protein expression. The secretion of foreign proteins is often enhanced by fusion of a signal peptide at the N-terminus of a protein that allows entry into the vesicular transport system of the specific host. The complete lack of expression of any RVFV GnGc recombinant proteins led to the decision to work only with Gn, in order to troubleshoot the lack of expression problem. Gn was also selected as it has been shown to be immunogenic on its own, and to elicit protection (de Boer et al., 2010; Kortekaas et al., 2014).

Chapter 4: The modification and expression of RVFV Gn as a candidate subunit vaccine

4.1 Introduction

Viral glycoproteins are often synthesised as precursors containing a 5' signal peptide or leader sequence. Signal peptide sequences contain 15 – 30 amino acid residues, which are involved at the start of the secretion pathway (Milstein et al., 1972). Signal peptide sequences are not conserved, but they share certain structural features, such as a positively charged N-terminal region, a central hydrophobic core and a polar carboxyl-terminal region with a peptidase cleavage site (Milstein et al., 1972; Perlmann et al., 1967). They usually direct the proteins across the membrane of the ER and are cleaved off within the lumen by a signal peptidase. Translocation of proteins from the cytosol into the ER mediated by the signal peptide is an imperative step in protein secretion. The secretion process leads to the accumulation of recombinant protein in different plant organelles such as the cell membrane, apoplastic space or the ER (Yoshida et al., 2004). Different signal peptides for the same recombinant proteins can significantly affect the amount of protein produced: this has been demonstrated successfully in various production systems (Chen et al., 2016; Haryadi et al., 2015; Herrera-Estrella et al., 1990; Herrera et al., 2000; Shahryari et al., 2013; Singh et al., 2013; Tamura et al., 2008). For example, the use of a murine signal peptide sequences (Fischer et al., 1999b; Fischer et al., 1999c; Shahryari et al., 2013) for recombinant single-chain antibody production *in planta* has been successful; the secretion of HIV type 1 envelope glycoprotein gp 120 in mammalian cells was improved when the native signal sequence was replaced with a leader sequence from pJW4303 vector (Wang and Roossinck, 2006), honeybee mellitin or murine interleukin (Li et al., 1994); the use of tissue plasminogen activator and *Gaussia* luciferase signal peptides were also successfully demonstrated in the expression and secretion of E1 and E2 envelope glycoproteins of hepatitis C virus in mammalian cells (Eddy and Peters, 1980; Niklasson, 1982; Wen et al., 2011).

RVFV GnGc has a native signal peptide which is required for proteolytic processing of the GnGc precursor molecules into mature envelope glycoproteins (Gerrard and Nichol, 2002). In this work, in an effort to develop a Gn candidate vaccine for RVFV, two modifications were carried out to *Gn* to determine whether it could be expressed *in planta*. The first involved the removal of the native 5' signal sequence and substitution with an alternative one; the second involved the removal of the transmembrane domain and cytosolic tail (TMD/CT) region of Gn. For substitution of the N-terminal signal sequence of Gn, two different heterologous signal peptides were selected (Table 4.1): these were the protein disulphide isomerase (PDI) signal peptide of *Medicago sativa* L. (Shorrosh and Dixon, 1991; Shorrosh and Dixon, 1992), which is an ER luminal protein that aids the formation of disulphide bonds of nascent polypeptide chains in the lumen (Houston et al., 2005); and a plant-codon optimised murine mAb24 heavy chain signal sequence (LPH) (Fischer et al., 1999b; Vaquero et al., 1999), which targets proteins into the ER.

Table 4.1: Rift Valley fever wild type and heterologous signal peptides substituted at the N-terminus of Gn

Source of signal	Organism	Amino acid sequence	Amino acids	Signal peptide
Murine	mAb24	MEWSWIFLFLLSGTAGVHS	19	LPH
Medicago sativa. L	Alfalfa	MAKNVAIFGLLFSLLLLVPSQIF	23	PDI
Bunyaviridae	RVFV WT	MAGIAMTVLPALAVFALAPVVF	22	homologous

The second modification involved truncating Gn by deleting the TMD/CT sequences. Gn is a type 1 integral membrane protein: it spans the viral membrane in such a manner that the C-terminal (cytosolic) tail points towards the intraviral space, while the N-terminal part is in contact with the outer environment (ecto-domain) (Strandin et al., 2013). Correct folding, heterodimerization and trafficking of the enveloped glycoprotein is important for Golgi-located assembly and budding of virus particles (Gerrard and Nichol, 2002).

This chapter describes the testing of the different heterologous signal peptides for expression of South African isolate M35/74 Gn in *N. benthamiana* with a view to developing it as a candidate subunit vaccine. The expression of modified Gn with PDI or LPH signal peptide with and without a TMD/CT were tested using different plant expression vectors. *N. benthamiana* plants were syringe-infiltrated with recombinant *Agrobacterium* encoding the different genes, and their expression was determined by western blotting. The modified recombinant protein was enriched for by ammonium sulphate precipitation and purified by ion exchange chromatography. Protein concentration was quantified by using bovine serum albumin as a standard.

4.2 Materials and Methods

4.2.1 Cloning and sequencing of constructs

All bacterial and plant growth conditions were as described in section 3.2.3. All DNA manipulation and analysis was carried out as described in section 3.2.4. Gn modifications were made by PCR, with the PCR profile described in section 3.2.5. All PCR primer sequences are listed in Table 2, Appendix B. The modified RVFV Gn (*pGn* and *hGn*) genes were sub-cloned into pGEM®-T-Easy vector as described in section 3.2.6. The constructs were verified by restriction enzyme mapping and sequencing with M13 universal primers (Macrogen Inc.). The modified genes were subsequently cloned into plant expression vectors (pTRAc, pTRAc-ERH and pEAQ-*HT*) as described in section 3.2.7.

4.2.2 Modification of *N. benthamiana* codon optimised *pGn* and construction of plasmids

4.2.2.1 Removal of native 5' signal peptide from pGn to form ∆pGn

∆*pGn* was amplified from pGEM®-T-Easy-pGnNcoI (section 3.2.8.4) using GnΔSP Fw and GnStuI Rev primers (Table 3, Appendix B) annealing at 55 ˚C and sub-cloned into pGEM®-T-Easy to yield pGEM®-T-Easy-ΔpGn. The construct was verified by restriction enzyme digest using *Not*I. ∆*pGn* was subsequently cloned from pGEM®-T-Easy-ΔpGn using restriction enzymes *NcoI* and *Xho*I into pTRAc *Afl*II and *Xho*I

restriction enzyme cloning sites, to yield pTRAc-ΔpGn. The construct was verified by restriction enzyme digest mapping with *Eco*RV.

4.2.2.2 Truncation of transmembrane domain and cytosolic tail (TMD/CT) from pGn to form truncated pGn (ptGn)

ptGn was amplified by PCR from pGEM®-T-Easy-pGn using GnNcoI Fw and tGnXhoI Rev using a 55 ˚C annealing temperature. Amplified DNA was sub-cloned into pGEM®-T-Easy to yield pGEM®-T-Easy-ptGn. This construct was verified by restriction enzyme digest using *Not*I. *tGn* was subsequently cloned from pGEM®-T-Easy-ptGn using restriction enzymes *Nco*I and *Xho*I into pTRAc *Afl*III and *Xho*I restriction enzyme cloning sites to yield pTRAc-ptGn. The recombinant plasmid was verified by restriction enzyme digest mapping with *Eco*RV.

4.2.2.3 Truncation of TMD/CT from ptGn to form ΔptGn

Δ*ptGn* was amplified from pGEM®-T-Easy-ptGn using GnΔSP Fw and tGnXhoI Rev primers at 50 ˚C annealing temperature and sub-cloned into pGEM®-T-Easy to yield pGEM®-T-Easy-ΔptGn. The construct was verified by restriction enzyme digest with *Not*I. Δ*ptGn* was subsequently cloned from pGEM®-T-Easy-ΔptGn using restriction enzymes *Nco*I and *Xho*I into pTRAc *Afl*III and *Xho*I restriction enzyme cloning sites to yield pTRAc-ΔptGn. The recombinant construct was verified restriction enzyme digest mapping with *Eco*RV.

4.2.2.4 Addition of PDI signal peptide to ΔpGn and ΔptGn to form PDI-ΔpGn and PDI-ΔptGn

PDI-ΔpGn and *PDI-ΔptGn* were constructed by overlap extension PCR (Figure 4.1). The signal peptide of PDI from *Medicago sativa L.*, was amplified from a pre-existing construct in the laboratory (pUC57-3ABC synthesised by GenScript) with Primer 1 Fw and PDI rev Table 3, Appendix B) primers and an annealing temperature of 55 ˚C resulting in *PDI* with Δ*pGn* 5’ overhang. Δ*pGn* with *PDI* 3’ overhangs were amplified by with Gn_PDI Fw and Gn Rev/ tGnXhoI Rev primers, and annealing at 50 ˚C. Amplified *PDI* with Δ*pGn* 5’overhangs were fused with Δ*pGn* with *PDI* 3’ overhangs by

overlapping PCR with Primer 1 Fw and GnStuI Rev, annealing at 55 ˚C. Amplified *PDI-ΔpGn* was sub-cloned into pGEM®-T-Easy to yield pGEM®-T-Easy-PDI-ΔpGn and pGEM®-T-Easy-PDI-ΔptGn. The constructs were verified with *Not*I restriction enzyme.

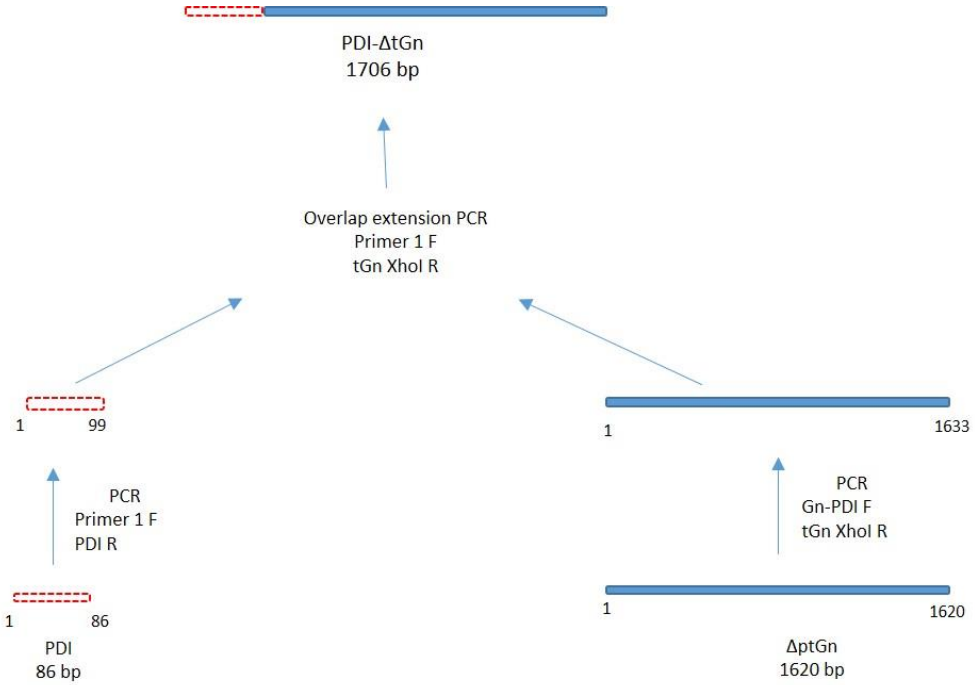

Figure 4.1: Schematic diagram for amplification and overlap extension of PDI signal peptide with modified *Gn.*

PDI-ΔpGn and *PDI-ΔptGn* were subsequently cloned from pGEM®-T-Easy-PDI-ΔpGn and pGEM®-T-Easy-PDI-ΔptGn with *Nco*I and *Xho*I into pTRAc with *Afl*III and *Xho*I restriction enzyme cloning sites, as well as with *Cfr*91 and *Xho*I into pEAQ-*HT Age*I and *Xho*I restriction enzyme sites to yield pTRAc-PDI-ΔpGn, pTRAc-PDI-ΔtpGn, pEAQ-*HT*-PDI-ΔpGn and pEAQ-*HT*-PDI-ΔptGn, respectively. Recombinant pTRAc-PDI-ΔpGn, pTRAc-PDI-ΔtpGn plasmid integrity was confirmed by restriction enzyme digest mapping with *Eco*RV and pEAQ-*HT*-PDI-ΔpGn and pEAQ-*HT*-PDI-ΔptGn integrity was confirmed with *Nru*I and *Xho*I restriction enzymes.

4.2.2.5 Addition of LPH signal peptide sequence to ∆pGn and ∆ptGn to form LPH-∆pGn and LPH-∆ptGn

∆*pGn* and ∆*ptGn* were cloned from pGEM®-T-Easy-∆pGn and pGEM®-T-Easy-∆ptGn into pTRAc-ERH, introducing the LPH signal peptide on the 5' terminus using *Nco*I and *Xho*I restriction enzymes. This resulted in pTRAc-LPH-∆pGn and pTRAc-LPH-∆ptGn. The integrity of the recombinant plasmids was confirmed by restriction enzyme mapping with *Eco*RV.

In order to make pEAQ-*HT*-LPH-∆pGn and pEAQ-*HT*-LPH-∆ptGn (Figure 4.2), ∆*pGn* and ∆*ptGn* were excised from pGEM®-T-Easy-∆pGn and pGEM®-T-Easy-∆ptGn with *Nco*I and *Xho*I restriction enzymes. The fragments were ligated into pUC18-LPH which was linearized with *Nco*I and *Xho*I, to yield pUC18-LPH-∆pGn and pUC18-LPH-∆ptGn plasmids which were confirmed by PCR with M13 primers annealing at 65 ˚C and restriction enzyme mapping with *Age*I and *Sal*I.

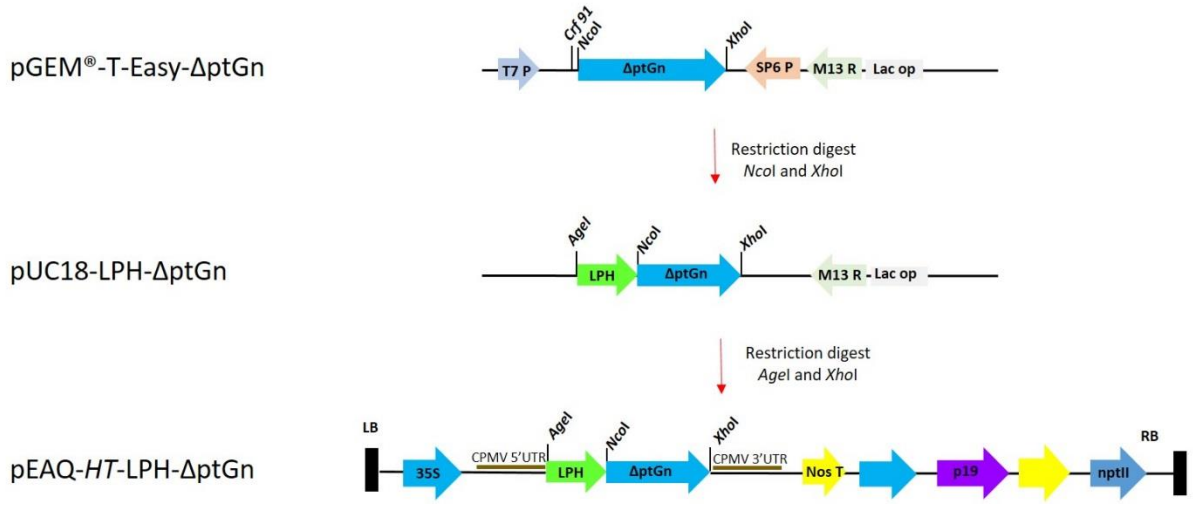

Figure 4.2: Schematic diagram representing the construction of pEAQ-*HT* plasmids with LPH fused to ∆ptGn and ∆htGn.

LPH-∆pGn and *LPH-∆ptGn* DNA was subsequently excised from pUC18-LPH-∆pGn and pUC18-LPH-∆ptGn with *Age*I and *Xho*I restriction enzymes. The pEAQ-*HT* vector was linearized with *Age*I and *Xho*I restriction enzymes. The insert DNA was cloned

into pEAQ-*HT* to yield pEAQ-*HT*-LPH-ΔpGn and pEAQ-*HT*-LPH-ΔptGn. The integrity of the recombinant vectors was confirmed with *Age*I and *Stu*I.

4.2.3 Modification of human-codon optimised *Gn* (*hGn*) and construction of plasmids

4.2.3.1 Substitution of 5' signal peptide sequence and truncation of TMD/CT

Δ*htGn* was amplified from human-codon optimised *GnGc* (pUC57-*hGnGc*) using ΔhGn Fw and htGn Rev primers, annealing temperature at 50 ˚C. Amplified DNA was sub-cloned into pGEM®-T-Easy to yield pGEM®-T-Easy-ΔhtGn. The construct was confirmed by restriction enzyme mapping with *Eco*RV.

In order to make pTRAc-LPH-ΔhtGn, Δ*htGn* was excised from pGEM®-T-Easy-ΔhtGn using *Nco*I and *Xho*I restriction enzymes and cloned into pTRAc-ERH, introducing the LPH signal peptide, using *Nco*I and *Xho*I restriction enzyme cloning sites to yield pTRAc-LPH-ΔhtGn. The recombinant clone was confirmed by restriction enzyme digest with *Eco*RV according to the manufacturer's instruction.

In order to make pEAQ-*HT*-LPH-ΔhtGn (Figure 4.2), Δ*htGn* was excised from pGEM®-T-Easy-ΔhtGn using *Nco*I and *Xho*I restriction enzymes into pUC18-LPH, resulting in pUC18-LPH-ΔhtGn. The plasmid was screened by PCR with M13 universal primers, annealing at 65 ˚C and restriction enzyme digest with *Age*I and *Sal*I. Insert DNA was excised from pUC18-LPH-ΔhtGn with *Age*I and *Sal*I restriction enzymes. The pEAQ-*HT* vector was linearized with *Age*I and *Xho*I restriction enzymes and cloned into pEAQ-*HT* to yield pEAQ-*HT*-LPH-ΔhtGn. The integrity of the recombinant plasmid was confirmed by restriction enzyme mapping with *Age*I and *Stu*I.

4.2.4 *Agrobacterium* transformation and recombinant protein expression

The recombinant plasmids were transformed by electroporation into *A. tumefaciens* GV3101::pMP90RK and *A. tumefaciens* LBA4404 respectively, as described in section 3.2.10. A preliminary expression time trial was conducted by syringe infiltration as described in section 3.2.11. Crude plant extract was obtained as described in section 3.2.12. The concentration of total soluble protein (TSP) in the crude extract

was determined by Bradford assay using BSA (Sigma-Aldrich, MO, USA) as a standard. Protein expression was analysed by western blotting as described in section 3.2.13.

4.2.5 Optimal buffer composition

Four different buffers were tested for protein extraction efficiency from infiltrated leaves:

1. 100 mM Tris/HCl, pH 7.5 + 1% Triton X-100
2. 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl
3. 20 mM Na_2HPO_4, 150 mM NaCl
4. 100 mM Tris/HCl pH 7.5

Crude plant protein was extracted as described in section 3.2.12 and protein expression was analysed by western blotting as described in section 3.2.13.

4.2.6 Large scale protein expression

Recombinant *Agrobacterium* cultures were grown in LBB medium (trytone 2.5 g/L, yeast extract 12.5 g/L, NaCl 5 g/L) supplemented with 10 mM MES, pH 5.6, 20 µM acetosyringone, 50 µg/mL kan and 2 mM $MgSO_4$ until they reached an OD_{600} of between 3 and 4. *Agrobacterium* cultures were subsequently diluted to an OD_{600} of 0.25 in resuspension medium (10 mM $MgCl_2$ and 5 mM MES, pH 5.6). Six week old whole plants of *N. benthamiana* were placed upside down in the bacterial suspension culture in an airtight steel tank under vacuum of -90 to -100 kPa. Following vacuum infiltration the plants were returned to the plant growth room for 5 days incubation period until harvest.

4.2.7 Protein purification of pEAQ-*HT*-LPH-ΔptGn

4.2.7.1 Ammonium precipitation

Plant material was harvested at 5 dpi. Crude plant protein was extracted with 100 mM Tris/HCl pH 7.5 in 1:2 w/v plant to buffer ratio with a food blender (HBF500S Series, Hamilton Beach) and filtered twice through two layers of Miracloth™ (EMD Millipore Corp., Billirica, MA USA). The crude extract was clarified by centrifugation at 13 000 rpm, for 20 min at 4 ˚C four times or until the pellet was no longer visible at the bottom

of the tube. Ammonium sulphate precipitation was performed with series of increasing $(NH_4)_2SO_4$ (0 – 40%, 40 – 60% and 60 – 80%) (Englard and Seifter, 1990). The amount of solid $(NH_4)_2SO_4$ required was calculated using the online tool available at http://ww.encorbio.com/protocols/AM-SO4.htm. The solid $(NH_4)_2SO_4$ was added slowly to the crude protein and kept at 4 ˚C, stirring for 1 hr. The protein was pelleted by centrifugation at 13 000 rpm, 20 min at 4 ˚C. The protein pellet was re-suspended with half the original buffer volume with 50 mM NaOAc, pH 5.6 and was dialysed O/N at 4 ˚C using dialysis tubing with a molecular weight cut-off of 10 kDa (Thermo Fischer Scientific, USA) in 5L against the same buffer. The extract was clarified by centrifugation at 13 000 rpm, 4 ˚C, for 20 min. Clarification was repeated four times or until the pellet disappeared.

4.2.7.2 Ion Exchange Chromatography

The partially purified LPH-ΔptGn was filtered firstly through a 0.45 µm and subsequently through a 0.2 µm membrane filter (Merck Millipore). The protein was loaded on a 20 mL HiPrep SP XL 16/10 cation column (GE Healthcare) and purified with an automated fast protein liquid chromatography (FPLC) system ÄKTA Purifier Plus 10 (GE-Healthcare Life Sciences). Non-specific proteins were washed off the column with 10 column volumes (CV) of buffer B (50 mM NaOAc pH 5.6). Thereafter, 10 CV of buffer C (50 mM NaOAc, pH 5.6, 1M NaCl) was applied to elute the target protein. Five mL fractions were collected with a fraction collector and absorbance readings at 280 nm of each fraction was determined. The protein containing fractions were dialysed O/N at 4 ˚C using dialysis tubing with a molecular weight cut-off of 10 kDa (Thermo Fischer Scientific, USA) in 1L sterile 100 mM Tris/HCl pH 7.5. The fractions were collected and stored at -80 ˚C for further analysis. A non-infiltrated plant was included as a negative control.

Endotoxin levels were measured using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript Biotechnologies, Piscataway, NJ, USA) as per the manufacturer's instructions. The presence of contaminant bacteria was tested for by plating samples on LB agar plate with no antibiotics and incubating O/N at 37 ˚C.

4.2.8 Protein expression analysis

Purified protein was resolved on SDS-PAGE gels and analysed by western blot as described in section 3.2.13. Proteins were also resolved on SDS-PAGE gel and stained with Coomassie-blue (0.1% Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid). Total soluble protein present in the collected fractions was determined using the Bio-Rad Protein Assay according to the manufacturer's instructions. Bovine serum albumin (BSA, Sigma-Aldrich) was used as protein standard and the absorbance was read at 750 nm using a Bio-Tek Powerwave XS spectrophotometer.

4.2.9 Liquid chromatography - mass spectrometry (LC-MS)

Following Coomassie blue staining of SDS-PAGE gels, the bands of interest were excised and sent to the Centre for Proteomic and Genomic Research (Cape Town, South Africa) for LC-MS analysis. The proteins were enzymatically digested with trypsin alongside a BSA standard and the resulting peptides were separated by reverse-phase high performance liquid chromatography. The peptides were injected into a Thermo Q-Exactive mass spectrometer (Thermo Fisher Scientific) and the resulting spectra were analysed using Byionic Software (Protein Metrics USA) using reviewed sequences available from UniProt (www.uniprot.org). Gel-digested samples were compared against a merged database comprised of *N. benthamiana, N. tabacum, A. tumefaciens* and *Bunyaviridae*.

4.2.10 Immunisation of mice

Female BALB/c mice were bred by the Animal Unit at the Health Science Faculty, University of Cape Town and were housed in filter top micro-insulator cages under Biosafety Level 2 (BSL 2) conditions. Approval for this study was granted by the Animal Research Committee at the University of Cape Town (AEC# 015-048). Female mice (8-10 weeks old) were immunised with the plant-produced vaccine candidate RVFV LPH-ΔtGn sub-unit vaccine. A non-infiltrated plant was used as a negative control.

The immunogenicity study was carried out in duplicate (experiment 1 and 2), with each experiment containing a total of 10 mice, divided into 2 groups (group 1 - vaccine and group 2 – negative control) of 5 mice each. The TSP of the vaccine candidate and negative control was determined using the Bio-Rad DC Protein Assay Kit. The vaccine candidates were prepared to contain 5 µg antigen in 100 µL Tris/HCl pH 8. Pre-bleeds were collected from each mice via the saphenous vein three days prior (Day -3) to vaccination. The vaccine candidates were administrated by subcutaneous injection into both left and right flanks (50 µL each) on Day 0. The mice were boosted on day 13 and 27 with doses containing 10 µg of appropriate antigen. Final bleeds were obtained via cardiac puncture at Day 58. Serum was collected from the blood and stored at -80 ˚C.

4.2.11 Indirect ELISA detection of anti-Gn antibodies in mouse sera

The anti-Gn responses were determined by ELISA. 96-well Maxisorp® microtiter plates (Nunc) were coated with 100 µL/well (1 µg) of *N. benthamiana* produced human-codon optimised RVFV LPH-ΔtGn, diluted in 10 mM Tris/HCl pH 8) coating buffer and incubated O/N at 4 ˚C. The plates were blocked with TBP blocking buffer (5% non-fat dry milk in 1 x TBS [50 mM Tris, NaCl, pH 7.5]) for 2 h at RT after which they were washed four times with 1 x TST buffer (1x PBS, pH 7.5, 0.05% Tween®20).

To evaluate the anti-Gn immune response elicited by each mouse, the pre- and final bleed sera were diluted 1:100 in 100 µL TBS blocking buffer and added to each well, after which the plates were incubated for 2 h, RT. Blank wells contained no antibody we included as background control. The plates were washed four times with 1x TST buffer and 100 µL of anti-goat anti-mouse IgG alkaline phosphatase conjugates (1:10 000, Sigma) diluted in blocking buffer was added to each well and incubated for 1 h at 37 ˚C. After incubation, the plates were washed four times with 1x TBS, pH 9 and 200 µL SIGMAFAST™ p-Nitrophenyl phosphate (pNPP, Sigma) was added to each well. The plates were developed in the dark for 30 min after which absorbance was detected at 405 nm on BOI-TEK® Powerwave XS microtiter plate reader.

To determine the anti-Gn binding titres, mouse sera from each group were pooled into vaccine groups (5 mice/vaccine) for analysis. Final bleed sera were diluted in TBS locking buffer in a 4-fold series in triplicate ranging from 1:50 to 1: 51 200. Mouse sera from the mice vaccinated with non-infiltrated plant protein served as a negative control. Blank wells with no antibody were included for background control in all the ELISA's.

The serum was pre-absorbed by lunchbox immunoabsorbent technique. Briefly, on a nitrocellulose blotting membrane (Amersham™ Protran™ Premium 0.45 µm NC) ~25 mL volume of crude plant extract was added, and incubated at 37 ˚C for 1 hr. This was followed by then blocking in blocking buffer (1 x PBS [137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 2 mM KH_2PO_4] at pH 7.4, 0.1% Tween-20 and 5% fat free milk). Sera to be absorbed was diluted to 1:50 in blocking buffer and agitated on the nitrocellulose membrane at RT for 2 hrs. The serum was then used for further analysis.

4.3 Results

4.3.1 Cloning of the recombinant modified Gn constructs into plant expression vectors
The South African isolate M35/74 RVFV Gn modified constructs shown in Figure 4.3 were all successfully made. All recombinant plasmids were confirmed by restriction enzyme mapping, giving the expected banding patterns (results not shown) as well as by sequencing, confirming gene orientation and no mutations were introduced by PCR. The recombinant modified genes were successfully cloned into the appropriate plant expression vectors as depicted in Figure 4.4 (A-K). The sizes (bp) of the recombinant plasmids and their expected protein band sizes (kDa) are listed in Table 4.2.

Figure 4.3: RVFV Gn truncation and substitution of signal peptide. **A**) the full-length pGn/hGn glycoprotein, **B**) pGn without the native signal peptide, **C**) pGn with LPH substitution of WT RVFV signal peptide, **D**) pGn with PDI substitution of RVFV WT signal peptide, **E**) pGn without the native signal peptide and transmembrane domain (TMD), **F**) pGn with native signal peptide and no TMD/CT, **G**) pGn/hGn with LPH substitution of WT RVFV signal peptide and no TMD/CT, **H**) pGn with PDI substitution of WT RVFV signal peptide and no TMD/CT.

The plant- and human-codon optimised full length *Gn* (Figure 4.3 A) were cloned successfully into pTRAc resulting in plant expression plasmid represented by Figure 4.4 A. The truncated *ptGn*, Δ*pGn* and Δ*ptGn* (Figure 4.3 B, C and D) encoding genes were successfully cloned into pTRAc, resulting in the plant expression plasmids represented by Figure 4.4 B, C and D. Δ*pGn* and Δ*ptGn*/Δ*htGn* were successfully cloned into pTRAc-ERH, introducing the LPH signal sequence, resulting in the plant expression plasmids in Figure 4.4 E and H, which targets the protein to the ER. *PDI*-Δ*pGn* and *PDI*-Δ*ptGn* (Figure 4.3 D and H) gene encoding sequences were successfully cloned into pTRAc and pEAQ-*HT* plant expression vectors resulting in plasmids in Figure 4.4 F, G, J and K. The plant- and human-codon optimised RVFV Δ*tGn* were fused to the LPH signal peptide at the N-terminus (Figure 4.3 G) and cloned successfully into pEAQ-*HT* resulting in the construct represented by Figure 4.4 I. Recombinant plasmids were successfully confirmed by restriction enzyme digest mapping (results not shown).

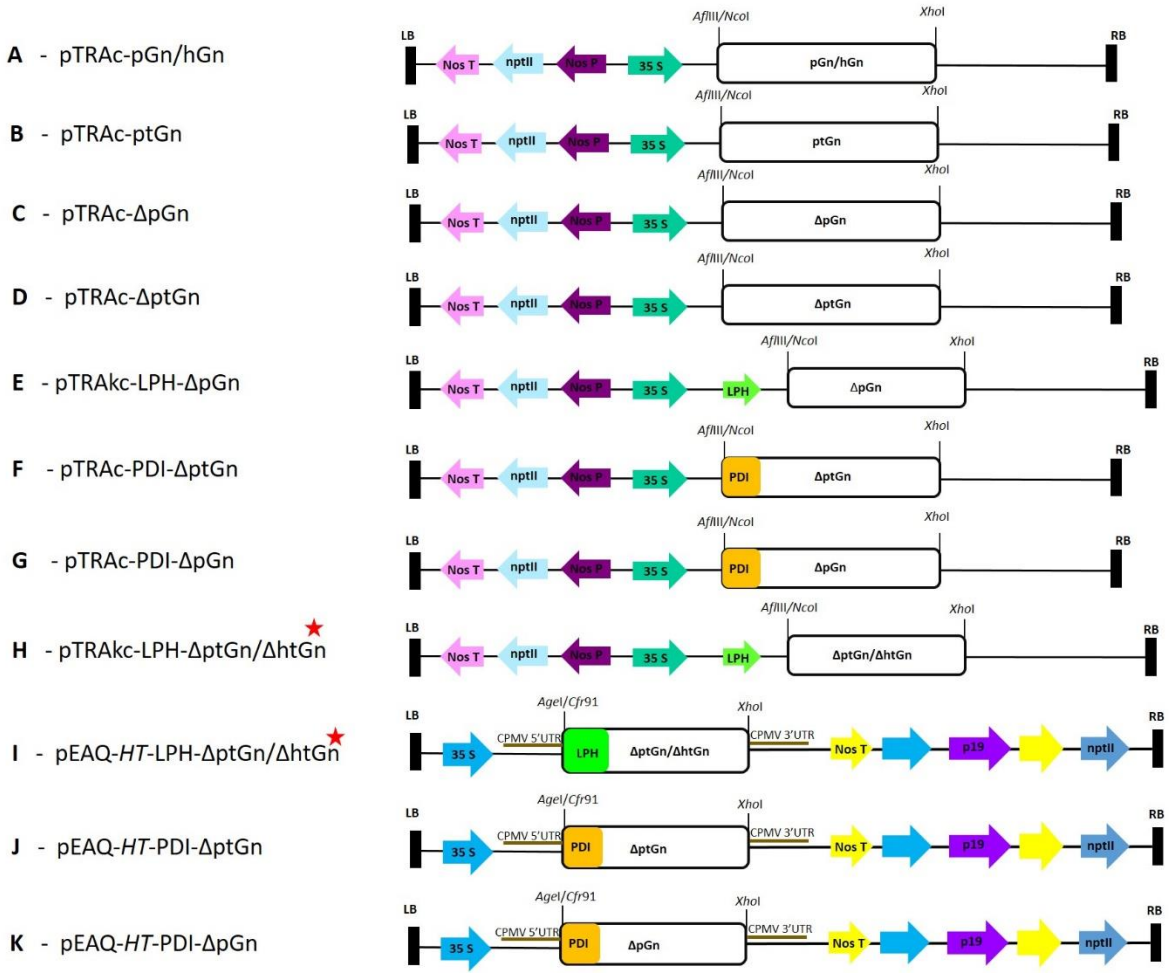

Figure 4.4: Schematic presentation of recombinant RVFV Gn expression plasmids. **A**) Full length RVFV plant (pGn)- and human (hGn)-codon optimised glycoprotein, **B**) pGn lacking transmembrane domain and cytosolic tail (TMD/CT) - ptGn, **C**) pGn lacking the native signal peptide - ΔpGn, **D)** pGn lacking the native signal peptide and TMD/CT - ΔptGn, **E)** native signal sequence substituted with a plant-codon optimised murine mAb24 heavy chain signal sequence (LPH) - LPH-ΔpGn, **F**) native signal sequence substituted with PDI (protein disulphide isomerase) signal sequence and no TMD/CT - PDI-ΔptGn, **G**) pGn with LPH signal sequence and no TMD/CT region in pTRAc - ΔpGn, **H**) Gn with LPH signal sequence and lacking TMD/CT region in pEAQ-*HT* – plant (ΔptGn)- and human (ΔhtGn)-codon optimised, **I**) *pGn* with PDI signal sequence and lacking TMD/CT region - PDI-ΔptGn. Red stars indicate the plant – and human-codon optimised genes in pTRAc and pEAQ-HT that expressed detectable protein in *N. benthamiana*.

Table 4.2: Expected sizes of the modified RFV Gn plasmids

Recombinant plasmids	Base pairs (bp)	Insert Position	Insert (bp)	Amino acids (aa)	Size (kDa)
pTRAc-pGn	7 841	1783-3462	1680	559	60.9
pTRAc-hGn	7 841	1783-3462	1680	559	60.9
pTRAc-ptGn	7 517	1783-3150	1368	455	49.1
pTRAc-ΔpGn	7 772	1783-3393	1611	536	58.7
pTRAc-ΔptGn	7 448	1783-3081	1299	432	46.9
pTRAc-PDI-ΔpGn	7 844	1783-3465	1683	560	61.3
pTRAc-PDI-ΔptGn	7 520	1783-3153	1371	456	49.5
pTRAc-LPH-ΔpGn	9 242	1653-3452	1800	599	66.3
pTRAc-LPH-ΔptGn	8 918	1851-3141	1301	432	46.9
pTRAc-LPH-ΔhtGn	8 918	1653-3140	1488	495	54.5
pEAQ-*HT*-LPH-ΔptGn	11 323	1301-2656	1356	451	49.0
pEAQ-*HT*-LPH-ΔhtGn	11 323	1301-2656	1356	451	49.0
pEAQ-*HT*-PDI-ΔpGn	11 653	1304-2986	1683	560	61.3
pEAQ-*HT*-PDI-ΔptGn	11 329	1304-2674	1371	456	49.5

4.3.2 Expression and detection of recombinant protein in crude leaf extracts

Recombinant protein expression was assessed with small scale expression time trials and western blotting of crude leaf extracts harvested at different days post infiltration (dpi). Recombinant protein expression could not be detected using anti-Gn antibody on samples from leaves infiltrated with *Agrobacterium* strains harbouring plasmids depicted in Figure 4.4 A – G and J - K (results not shown). However, recombinant protein expression was successfully detected in leaf extracts from plants infiltrated with pTRAc-LPH-ΔptGn i.e. truncated *Gn* with a substituted LPH signal sequence, showing an expected protein band size of 46.3 kDa (432 amino acids). Figure 4.5 shows these results compared to samples from leaves infiltrated with the full length *Gn* gene including the TMD/CT region and the heterologous LPH signal peptide (pTRAc-LPH-ΔpGn) which had the expected band size of 66.3 kDa (599 aa), but did not show detectable recombinant protein expression. The protein was detectable from 1 dpi, with day 5 showing the highest level of recombinant protein expression over the 5 days tested. A protein band of ~55 kDa was detected in the empty vector negative control plant which is indicative of RuBisCo protein.

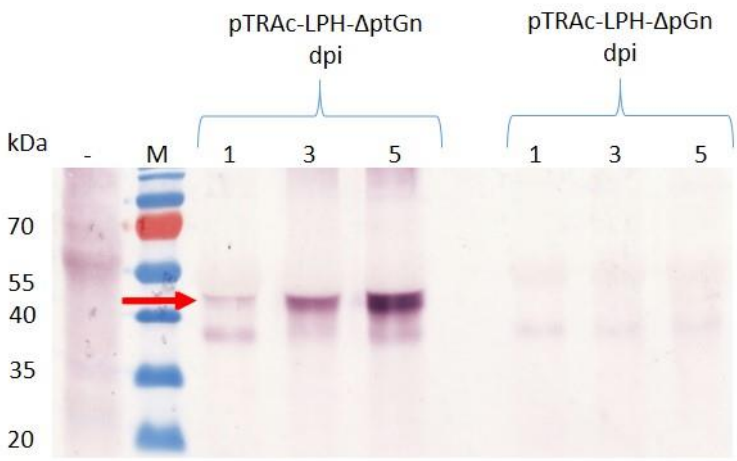

Figure 4.5: Western blot comparing expression levels of recombinant protein extracted from leaves infiltrated with pTRAc-LPH-ΔptGn lacking the TMD/CT and pTRAc-LPH-ΔpGn with the TMD/CT. 30 µg was loaded in each well. The time trial was conducted at OD_{600} of 0.5. Lanes 1, 3 and 5 denote the number of days harvested post infiltration (dpi). Lane "-", – negative control represented by extract from leaves infiltrated with an empty vector. Lane M contains PageRuler™ Prestained protein ladder (Thermo Scientific, MA, USA). The red arrow indicated the ΔptGn expected band.

Due to the successful expression of protein from pTRAc-LPH-ΔptGn, the construct encoding the cognate human-codon optimised gene (LPH-ΔhtGn) cloned into pTRAc-ERH (pTRAc-LPH-ΔhtGn) was also tested for expression in *N. benthamiana*. Expression of LPH-ΔhtGn and LPH-ΔptGn after infiltration at optical densities of 0.25 and 0.5 and harvesting at 1-7 dpi is shown Figure 4.6 A and B. TSP of crude leaf extracts indicated that an OD_{600} of 0.25 was better for infiltration compared to an OD_{600} of 0.5 for *Nicotiana* sp. codon optimised gene, while the TSP of crude leaf extracts for the human-codon optimised gene indicated that an OD_{600} of 0.5 was better for infiltration when compared to an OD_{600} of 0.25. The recombinant protein was expressed from days 1 to 3 with the highest level of recombinant protein expression at 3 dpi, after which protein expression levels decreased and were not detectable by day 7. Due to the successful expression of these constructs, *LPH-Δptgn* and *LPH-ΔhtGn* were similarly successfully cloned into pEAQ-*HT* and tested for expression in *N. benthamiana*. An expression time trial for LPH-ΔptGn (Figure 4.6 C) and LPH-ΔhtGn (Figure 4.6 D) was carried out with an infiltration OD_{600} of 0.25 and 0.5. TSP of crude leaf extracts indicated that an OD_{600} of 0.5 gave lower protein expression levels when compared to that of an OD_{600} of 0.25 which showed the highest expression levels. Recombinant plant produced protein was detectable from day 1 up to 7 dpi.

Samples from day 5 showed the highest level of recombinant protein expression, after which the expression levels decreased.

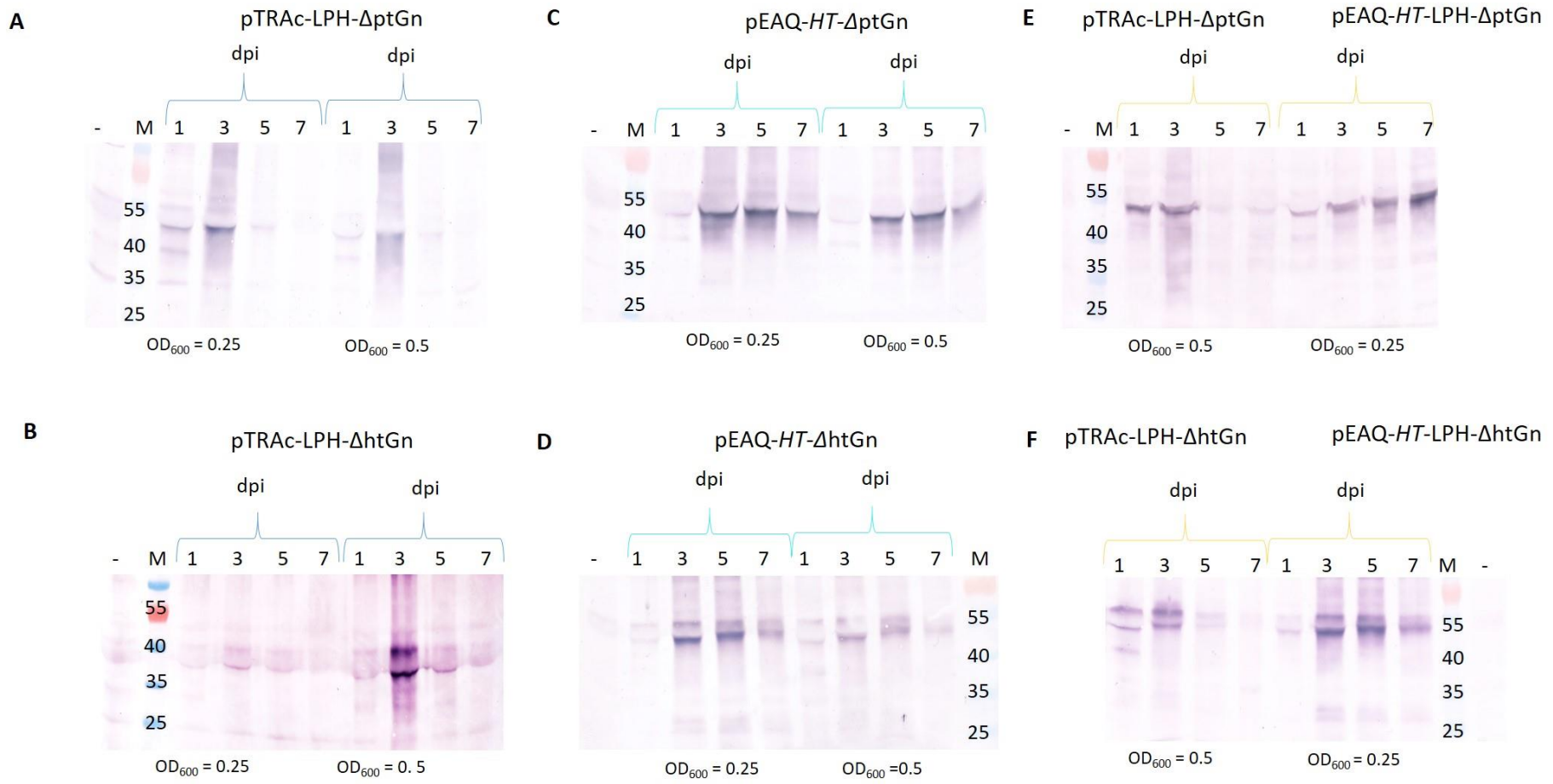

Figure 4.6: Expression time trial of plant- and human-codon optimised Δ*tGn* fused with LPH signal peptide. The top panel represents plant-codon optimised genes, the bottom represents the human-codon optimised gene. 30 µg of TSP for **A**) pTRAc-LPH-ΔptGn (removed the SEKDEL sequence), **B**) pTRAc-LPH-ΔhtGn, **C**) pEAQ-*HT*-LPH-ΔptGn, **D**) pEAQ-*HT*-LPH-ΔhtGn, **E**) pTRAc-LPH-ΔptGn vs pEAQ-*HT*-LPH-ΔptGn, **F**) pTRAc-LPH-ΔhtGn vs pEAQ-*HT*-LPH-ΔhtGn was loaded in each lane. Lane - indicates is the negative control, a plant infiltrated with pEAQ-*HT* empty vector. Lane M contains PageRuler™ Prestained protein ladder (Thermo Scientific, MA, USA). DPI denote day 1, 3, 5 and 7 post infiltration. The time trials were at OD_{600} of 0.25 and 0.5 respectively. The protein was detected with anti-Gn antibody.

Having established that the highest levels of expression were obtained at 0.25 for pEAQ-*HT* and 0.5 for pTRAc, to determine which expression vector (pEAQ-*HT* or pTRAc) gave the highest levels of recombinant protein (ΔhtGn and ΔptGn) expression, (Figure 4.6 E and F). pTRAc-LPH was infiltrated at an OD_{600} of 0.5 and pEAQ-*HT*-LPH-ΔptGn/ ΔhtGn was infiltrated at an OD_{600} of 0.25. pTRAc-LPH-ΔptGn showed expression at 1 and 3 dpi, with the highest expression levels at 3 dpi. Specific protein expression was barely detectable for 5 and 7 dpi. pEAQ-*HT*-LPH-ΔptGn/ ΔhtGn showed expression from day 1 to 7, with day 5 giving the highest level of recombinant expression. These results demonstrated that pEAQ-*HT*-LPH-ΔptGn/ ΔhtGn expressed the recombinant protein exceptionally well, giving the highest protein levels for plant- and human-codon optimised modified *Gn* genes. The pEAQ-*HT*-LPH-ΔptGn/ ΔhtGn recombinant plasmids were selected for further analysis.

Having established that the pEAQ-*HT* vector yielded the highest levels of recombinant protein, the two constructs encoding plant- and human-codon-optimised tGn (ΔptGn and ΔhtGn) were used to determine whether the use of different protein extraction buffers could improve protein yields. Four different extraction buffers were assessed for protein extraction efficiency. Comparisons of protein levels by western blots probed with anti-Gn antibody showed that 100 mM Tris/HCl pH 7.5 buffer was the most effective in yielding higher yields as determined by density of protein bands on western blot (results not shown).

Finally, to determine which codon preference yielded the highest levels of recombinant protein in *N. benthamiana*, expression was compared between crude leaf extracts sampled from plants infiltrated at an OD_{600} of 0.25 with pEAQ-*HT*-LPH-ΔhtGn and pEAQ-*HT*-ΔptGn (Figure 4.7). The highest recombinant protein levels were detected using the *Nicotiana sp*. (ΔptGn) codon optimised gene. Thus, the pEAQ-*HT*-LPH-ΔptGn construct was selected for further work including scaled-up infiltration at an OD_{600} of 0.25 and harvesting at 5 dpi for purification by ion exchange chromatography.

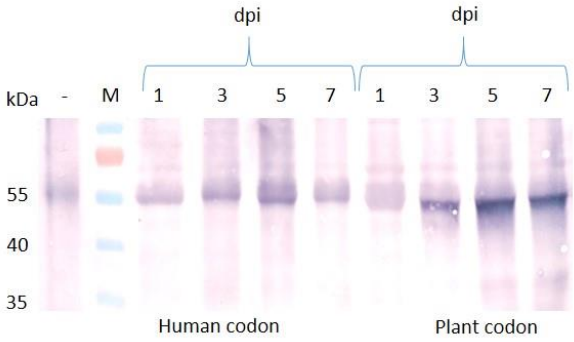

Figure 4.7: Comparison between plant- and human-codon optimised RVFV recombinant modified protein: 30 µg of TSP for pEAQ-*HT*-LPH-ΔhtGn and pEAQ-*HT*-LPH-ΔptGn infiltrated at OD_{600} = 0.25. The protein was detected with anti-Gn (1:1000). "–"indicates a negative control, plant infiltrated with pEAQ-*HT* empty vector. Lane M contains PageRuler™ Prestained protein ladder (Thermo Scientific, MA, USA). 1, 3, 5 and 7 indicate the number of days post infiltration (dpi).

4.3.3 Purification of pEAQ-*HT*-LPH-ΔptGn product

For large scale purification, ΔptGn was successfully partially purified by $(NH_4)_2SO_4$ precipitation, with the majority of the protein being found in the 40 – 60% fraction.

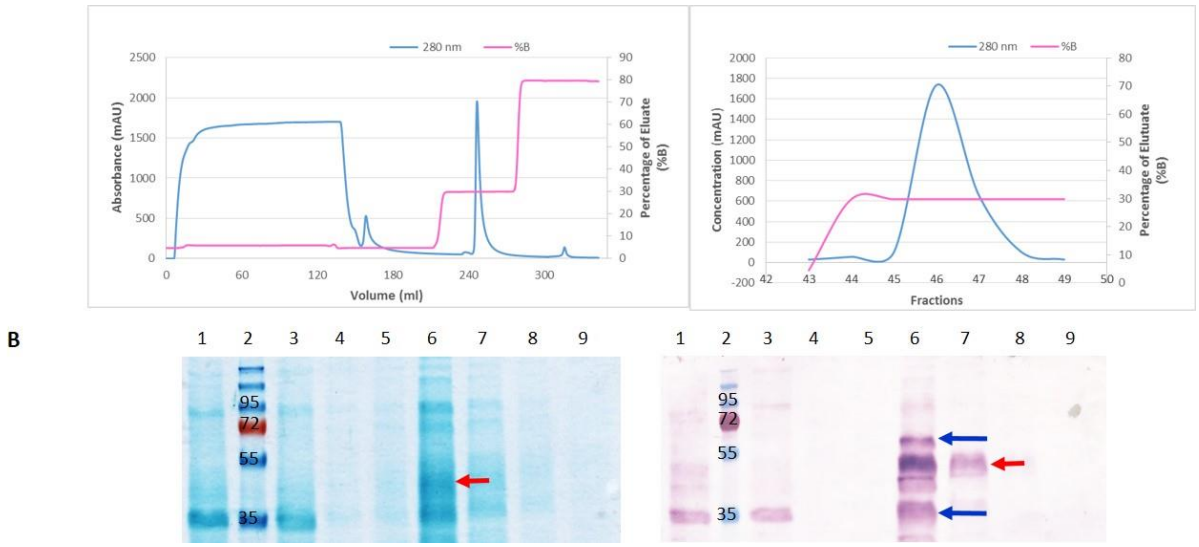

Figure 4.8: Purification of pEAQ-*HT*-LPH-ΔptGn with ion exchange chromatography. **A**) chromatographic trace showing LPH-ΔptGn elution from the HiPrep SP XL 16/10 cation column with increasing salt concentration (Pink line). **B**) Coomassie staining and western blot analysis of affinity chromatography fractions collected. Lane 1 contains crude plant extract, lane 2; contained PageRuler™ Prestained protein ladder plus (Thermo Scientific, MA, USA), lane 3 contained 40 – 60% $(NH_4)_2SO_4$ precipitate, lane 4 contained unbound wash fraction, lane 5 - 8 contained fractions 45 – 48 and lane 9 contained fraction 68. The protein was detected with 1:100 anti-Gn primary antibody and 1:5000 anti-rabbit secondary antibody. The red arrows indicate the LPH-ΔptGn expected size. The blue arrows indicate the bands analysed by LC-MS.

Recombinant RVF LPH-ΔptGn protein was calculated to have a pI of 8.05, and was thus purified using ion exchange chromatography (IEX). The protein was eluted off the column with a 30% salt concentration, corresponding to a protein peak visualised from fractions 45 to 48 (Figure 4.8 A). Western blot analysis and Coomassie gel staining of the fractions showed that the expected protein of 46.3 kDa was mainly present in fraction 46 (Figure 4.8 B; lane 6). The additional protein band sizes of ~70 kDa and 36 kDa were detected, which correspond to *Nicotiana* sp. ATP synthase unit beta, chloroplastic and methionine synthase respectively, as demonstrated by LC-MS.

4.3.4 Total soluble protein (TSP) quantification

Coomassie-stained SDS-PAGE gel analysis showed other contaminating plant proteins were co-purified with recombinant LPH-ΔtGn on the column. Due to the bands smearing, TSP of the fraction was determined using the Bio-Rad DC Protein Assay Kit. A standard curve was plotted using the average absorbance values obtained for the BSA standards. Using the equation on the chart, the TSP was calculated to be ~1.23 mg/mL in a total volume of 5 mL. The final protein yield was calculated to be ~56.81 mg TSP per kilogram of fresh leaf material.

4.3.5 Immunisation of mice with LPH-ΔtGn and animal serum analysis

Two experiments in mice were conducted to determine whether the modified RVFV Gn vaccine candidate produced in tobacco was immunogenic. The analysis of the final bleed sera of the individual mice showed the presence of Gn-specific antibodies, therefore mouse sera from each group were pooled (5 mice/group) for the analysis of anti-Gn binding (Figure 4.9 A). The antibody binding titres were expressed as a reciprocal of the maximum serum dilution containing absorbance values that were three times greater than the corresponding pre-bleed serum at 1:100. Preliminary data analysis for the presence of anti-Gn specific antibodies was analysed by indirect ELISA using plant-produced Gn protein as coating antigen. No binding was detected using the pre-bleed serum (Figure 4.9 B); however, binding was seen for antiserum raised against the candidate vaccine as well as for the negative plant control (Figure 4.9 B). No significant differences were observed in the absorbance readings between vaccine candidate and the negative plant control, despite the antiserum having been

pre-absorbed against a crude plant extract. Antiserum titration analysis revealed an apparently stronger antibody binding affinity to the vaccine candidate as compared to the negative plant control (Figure 4.9 C). The indirect IgG ELISA at serum dilution of 1:50 showed an 8-fold difference between the vaccine candidate and the negative plant control (Figure 4.9 D).

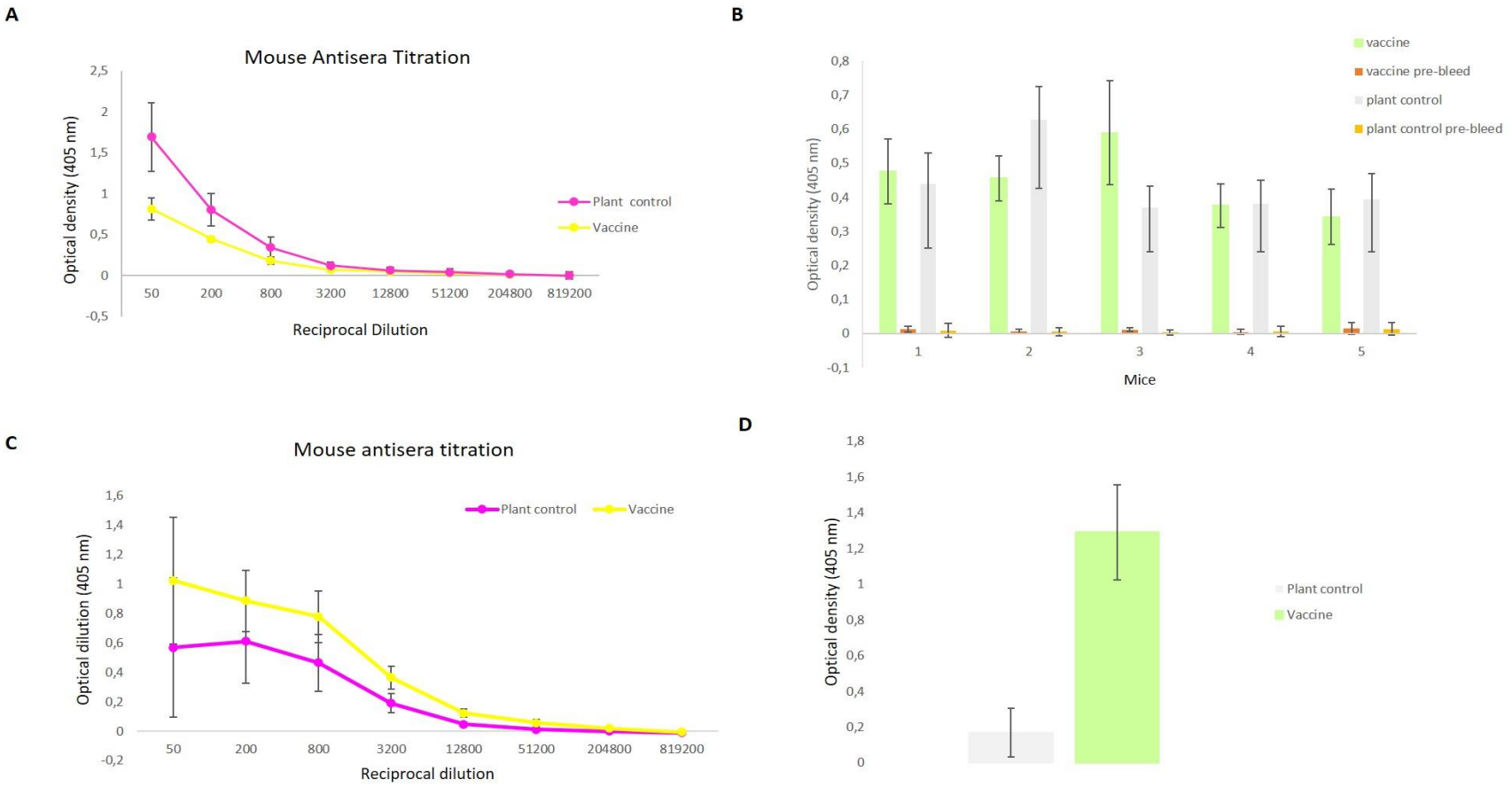

Figure 4.9: Analysis of antibody response in mice by vaccination with LPH-ΔtGn vaccine candidate. **A**) Titration of mice antiserum against the LPH-ΔtGn vaccine candidate (yellow) and plant negative control (pink). The absorbance readings are shown as an average (n =10). **B**) Absorbance values of pre- and final -bleeds at 1:100 reciprocal dilution. **C**) Titration of pre-absorbed mice antiserum against the LPH-ΔtGn vaccine (yellow) and plant negative control (pink). **D**) Absorbance values of the vaccine candidate (green) and plant negative (grey) control from pre-absorbed mice antiserum. The error bars indicate standard deviation.

4.4 Discussion

In this study an attempt was made to develop a RVFV subunit candidate vaccine by modifying the immunogenic RVFV *Gn* glycoprotein gene. I focused on using codon optimised genes since gene-codon optimisation has been shown to significantly enhance gene expression in plants (Maclean et al., 2007). This phenomenon has also been demonstrated with RVFV-encoded genes when it was shown that 293-gag cells transfected with expression plasmids encoding native RVFV or mammalian-codon optimised RVFV *GnGc* and *Gn* resulted in the enhanced expression of chimaeric VLPs using the codon optimized genes (Mandell et al., 2010c).

In this work, RVFV plant- and human- codon optimised (*pGn* and *hGn*) genes were successfully cloned into the pTRAc suite of plant expression vectors. However, the expression of recombinant protein from these genes was not detectable by western blots probed with a specific anti-Gn antibody. Faburay et al (2013) showed similar difficulties with expressing full-length RVFV Gn in Sf9 cells which resulted in no or very low expression of Gn (Faburay et al., 2013). In eukaryotes, translation efficiency is thought to be controlled by the rate of initiation. Initiation is known to follow a scanning mechanism whereby the 40S ribosomal unit together with co-factors such as eIF2, eIF3, eIF4, met tRNA and GTP, bind to the 5' cap of mRNA and then descend through the untranslated leader scanning for the first AUG codon (Kozak, 1989). The lack of RVFV Gn protein expression could be due to the lack of ribosome translocation during protein translation in plant host cells. Since rare codons or stable secondary structure in mRNA might cause pausing of the ribosome translocation it results in premature termination of transcription (Angenon et al., 1990).

In this project, efforts to promote RVFV Gn protein expression were made mainly using the plant-codon optimised gene (*pGn*). It was further thought that the presence of the native RVFV *Gn* signal peptide on the 5' terminus of the gene may not be suitable for initiation of transcription. Although mRNA and protein levels are generally correlated, the nucleotide sequence flanking the translation start codon and specific features of

the 5' and 3' un-translated regions (UTR) contribute to translation efficiency. In eukaryotes, translation has been shown to initiate efficiently when the AUG is in the context of a leader sequence (RNNAUGG, where R is A or G). When the AUG is not in this context, the ribosome will continue scanning in the 3' direction until an AUG in the preferred context is located (Kozak, 2002). Thus, in our case the *Gn* gene was modified, by removing the native signal peptide and replacing it with heterologous PDI (PDI-ΔpGn) or LPH (LPH-ΔpGn) signal peptides which target translation into the ER where post-translation modifications occur (Gomord and Faye, 2004). The reason for this was to ensure correct protein trafficking through the lumen of the ER, where glycosylation enzymes and signal peptidase are located (Gerrard and Nichol, 2002; Gerrard and Nichol, 2007). Alone, changing the signal peptide did not affect protein expression as no expressed Gn was detected.

It was subsequently thought that perhaps the TMD/CT was interfering with expression. This has been shown with the HIV type 2 envelope glycoprotein with a carboxy terminal residue that did not allow the formation of infectious retroviral particles. Expression of HIV-2 envelope protein with 7 cytoplasmic amino acids was more efficient compared to HIV-2 with a slightly longer (11 amino acids) cytoplasmic tail which had reduced expression levels (Höhne et al., 1999). Due to the similarities between HIV-2 and RVFV glycoproteins which are type 1 transmembrane proteins, the RVFV Δ*Gn* genes were further modified by truncating the TMD/CT resulting in *PDI-*Δ*ptGn* and *LPH-*Δ*ptGn*. Unfortunately, there was still no protein expression from the recombinant plasmid *PDI-*Δ*ptGn* using the PDI signal peptide. However, when *LPH-*Δ*ptGn* cloned into the pTRAc vector was infiltrated into plants, expression of ΔptGn was successfully detected. Expression was detected with infiltration OD_{600} of 0.25 from 1 dpi, with the highest expression levels detected at 3 dpi. This suggests that both the 5' signal peptide and TMD/CT have a significant effect on gene translation and expression *in planta*. These results are similar to those reported for expression of RVFV Gn in insect cells. De Boer and co-workers expressed the Gn ectodomain (Gn-e) (Gn lacking TMD/CT) with the *Drosophila* BiP secretion signal in *Drosophila* Schneider (S2) cells (de Boer et al., 2010). In addition, the same gene Gn-e was also fused with a Flag-tag and expressed with three Strep-tags and successfully expressed in S2 cells (Kortekaas et al., 2012). Recombinant Gn expression was also

demonstrated when Gn was fused to a 54 nucleotide sequence beginning from the 5th ATG of the RVFV M segment (Faburay et al., 2013) and expressed without the TMD/CT in Sf9 cells (Faburay et al., 2014; Faburay et al., 2013).

With this work, the successful expression of the plant-codon optimised Δ*tGn* in the pTRAc vector, fused to the LPH signal peptide, lead to the construction of a cognate construct encoding the human-codon optimised truncated *Gn* (Δ*htGn*). Optimal protein expression conditions were determined and shown to be different to those used for the Δ*ptGn* (infiltration OD_{600} of 0.5 and expression at 3 dpi compared to 0.25 at 3 dpi).

To test whether expression levels could be increased, the recombinant modified genes were cloned into the CPMV-based hypertranslatable expression vector, pEAQ-*HT*. A comparison of protein expression levels between the pTRAc and pEAQ-*HT* vector, showed that the pEAQ-*HT* vector yielded higher expression levels than the pTRAc vector and thus pEAQ-*HT* vector was selected for continuation of scaled up infiltration and purification.

To determine whether codon optimisation had a significant effect on expression a comparison of the codon optimised genes revealed that plant-codon optimisation for *Nicotiana* sp. gave higher expression levels compared to human-codon optimisation. The advantages of codon optimisation for protein expression has previously been demonstrated by Maclean and co-workers. They revealed that human-codon optimisation was had higher protein expression levels than plant-codon optimisation for HPV16 L1 *in planta* (Maclean et al., 2007). However, in this study, I discovered the opposite: plant-codon optimised genes were expressed at higher levels at a higher optical density of *Agrobacterium* compared to those of the human-codon optimised genes. Since the human-codon optimised gene required higher ODs to achieve similar expression levels as the *Nicotiana* sp. codon optimised gene, this suggests that RVFV WT gene codons might affect transcription in a heterologous expression system. The plant-codon optimised gene was selected for further analysis.

This is the first time to our knowledge that the RVFV Gn protein has been able to be produced in *N. benthamiana*. While Kalbina et al., (2016) were able to show that putative transgenic *Arabidopsis* plant lines had Gn-specific mRNA expression by RT-PCR, they were unable to demonstrate recombinant Gn expression (Kalbina et al., 2016). I managed to successfully express recombinant RVFV LPH-ΔptGn protein and purify it using ammonium sulphate precipitation and subsequent cation exchange chromatography. Protein yields of 56.81 mg/kg fresh weight, with a soluble protein concentration of 1.25 mg/mL were obtained. These yields are comparable to those that have been achieved from recombinantly-produced *E. coli* (6 mg) (El Salam, 2014) and mammalian cell (3.6 µg/mL) production systems (Mandell et al., 2010b). A few contaminating plant proteins with a similar pI to LPH-ΔptGn co-eluted off the column at the same salt concentration, as shown by protein analysis (see Figure 4.8 B). It is possible that the protein can be further purified by size exclusion chromatography to separate Gn from the contaminating proteins.

The plant-produced RVFV Gn candidate vaccine was preliminarily assessed for immunogenicity in BALB/c mice by subcutaneous vaccination. Detection of antibodies in mouse antisera was determined by indirect ELISA using plant-produced LPH-ΔtGn as a coating antigen. The pre-bleed sera gave no detectable antibody binding responses, as expected. For both the vaccine and plant negative control, however, antibodies that bound to the coating antigen were detected. Similar antibody titres were observed for both vaccine and negative plant controls. This is almost certainly due to the co-purified plant contaminating proteins, which would be present in both vaccine inocula and coating antigen, thus making it difficult to accurately determine if the antibodies detected in sera elicited using the vaccine candidate was purely due to the vaccine antigen. Therefore, the antisera were pre-absorbed against plant crude extract to absorb the anti-plant antibodies from the sera, with the hope that this would allow me to demonstrate a positive difference between reactions with the vaccine candidate and with the negative control plant material. This was previously demonstrated for a plant virus (Rybicki, 1984). The pre-absorbed serum showed significant differences in binding between the vaccine candidate and the negative plant control, indicating that the recombinant protein is weakly immunogenic. These results

are far more promising compared to the vaccine candidate produced in transgenic *Arabidopsis* which acted as a booster in mice (Kalbina et al., 2016).

In conclusion, gene codon optimisation seems to have played a significant role in improving yield, since both codon optimisations allowed for expression of modified Gn but at different ODs of *A. tumefaciens*. The substitution of the homologous signal peptide alone also seems to not have influenced protein expression, since no Gn protein was detected with just substituting the native signal peptide. The removal of the TMD/CT, however, played a significant role when ∆ptGn was fused to a mouse LPH signal peptide. Expression of constructs lacking the TMD/CT coding sequence resulted in successful expression of the ∆ptGn protein, while fusion with a plant PDI signal peptide, with or without the TMD/CT, resulted in no expression. This shows that the choice of heterologous signal peptide played a significant role in the expression of recombinant proteins in a heterologous expression system. Protein expression was further improved by the choice of expression vector. Chromatography allowed for the purification of the protein and a weak specific antibody response was detected in the immunogenicity studies. Since no adjuvant was used in this study, a chimaeric vaccine candidate might give a higher immune response.

Chapter 5: The modification and expression of a chimaeric RVFV virus-like particle candidate vaccine

5.1 Introduction

Recombinant protein vaccines in their monomeric forms are generally weakly immunogenic despite the use of adjuvants to enhance their efficacy. Particulate vaccines such as VLPs, however, provide a much better immune response due to the presence of multiple epitopes which resemble the virus in shape and morphology (Kushnir et al., 2012; Roldao et al., 2010). VLPs are more immunogenic than protein immunogens or subunit vaccines because their proteins are displayed in a more authentic conformation (Garcea and Gissmann, 2004; Meyers et al., 2014). The VLP vaccine platform is a viable option for the development of a safe and efficient RVFV vaccine, since VLPs are free of viral genetic material and therefore cannot be encumbered by the possible safety-related drawbacks of live-attenuated virus vaccines such as reversion, recombination and reassortment (Mandell et al., 2010b). They have also been shown to stimulate T-cell independent B cell-mediated immune responses and are highly effective at stimulating CD4 proliferative and cytotoxic T lymphocyte responses (Paliard et al., 2000; Schirmbeck et al., 1996).

Several different RVFV VLP expression systems have been successfully developed using *Spodoptera* (Liu et al., 2008), *Drosophila* (de Boer et al., 2010) and mammalian cells (Habjan et al., 2009; Mandell et al., 2010a; Naslund et al., 2009). These VLPs were made up primarily of Gn and Gc, which display the external virion immunogenic determinants, or a Gn and Gc with nucleocapsid (N) protein recruited inside the core (Habjan et al., 2009; Liu et al., 2008). Some of these have been tested in animals and shown to be immunogenic (Kortekaas et al., 2010a; Naslund et al., 2009). Studies have demonstrated that antibodies to Gn alone are sufficient for virus neutralization (Naslund et al., 2009; Oreshkova et al., 2013), and such antibodies were found to be fully protective in mice (de Boer et al., 2010). Gn expressed with N-protein on the same plasmid was found to be fully protective in lambs (Oreshkova et al., 2013) and

mice (Pichlmair et al., 2010). In another study with VLPs containing the N-protein, 56% of the mice survived a challenge, compared to VLPs without the N-protein, where 19% of the mice survived (Mandell et al., 2010a).

Since I previously showed that I was unable to express GnGc in *N. benthamiana* using plant expression vectors available in our laboratory (Chapter 3), I looked at alternative strategies for making particles with present Gn (the most immunogenic RVFV protein). RVFV GnGc, influenza virus HA (White et al., 2008), Ebola virus GP (Mohan et al., 2015) and human immunodeficiency virus (HIV) Env are all type 1 transmembrane glycoproteins having C-terminal TMD/CT. They are presented on the virion envelope as homotrimeric spikes. It has been previously found that HIV envelope (Env) protein is poorly incorporated into VLPs compared to other enveloped glycoproteins; this problem was to some extent resolved by substituting the native TMD/CT with various heterologous TMD/CT sequences including that of the avian influenza virus hemagglutinin (HA) protein. The study showed that the heterologous TMD/CT significantly increased the incorporation of HIV Env into the VLPs due to the length of the heterologous TMD/CT being shorter than the long native TMD/CT (Wang et al., 2007). Each HA monomer is made up of two subunits: these are HA1, which contains the receptor binding and major antigenic sites, and HA2, which is primarily responsible for fusion. HA2 contains an NH_2-termination fusion peptide, a 27 aa transmembrane domain (TMD) and a 10 aa cytosolic tail (CT) (Armstrong et al., 2000).

I reasoned that by substituting the native TMD/CT of RVFV Gn with that of the influenza HA TMD/CT, that this may similarly encourage the formation of chimaeric RVFV VLPs. Part of this chapter describes the production of chimaeric RVFV Gn VLPs by expressing the modified recombinant LPH-ΔtGn construct fused to the highly pathogenic H5N1 avian influenza HA TMD/CT (LPH-ΔtGnHA) in plants.

In addition, unlike other enveloped viruses such as influenza and HIV, RVFV lacks the structural matrix protein linking the viral envelope with the virus core. Instead, there is a direct interaction between the RVFV glycoprotein CT and the RNP complexes.

The bridging density complexes observed among capsomers of RVFV may help with assembly and stabilisation of the virus particles (Freiberg et al., 2008). Matrix proteins are the driving force of assembly of enveloped proteins. Their main function is to interact with and polymerise at cellular membranes and link other viral components to the matrix-membrane resulting in individual particles. They ensure the integrity of the viral particles by interacting with membranes and providing a link between the CT of the glycoprotein and N-protein (Baudin et al., 2001; Noton et al., 2007). The influenza virus matrix protein (M1) is composed of 252 amino acids (~28 kDa) and consists of an N-terminal (1 to 164 aa) linker, middle and C-terminal domains (Harris et al., 2001; Noton et al., 2007). It plays a role in viral assembly and budding. It interacts with the cytoplasmic tail of the virus transmembrane thus contributing to the formation of the budding particle, by forming a virion protein matrix in the inner surface of the viral envelope (Gomez-Puertas et al., 2000; Latham and Galarza, 2001).

Therefore, to further encourage more stable chimaeric VLP formation, the recombinant LPH-ΔtGnHA construct was co-expressed with two other proteins: the H5N1 avian influenza M1 protein and RVF N-protein. It was reasoned that the influenza M1 may interact with the influenza TMD/CT and contribute to the formation of more stable RVFV chimaeric VLPs; also, the RVF N-protein could possibly be recruited into the ΔtGn and assist with stabilisation of VLPs. The RVFV Gn endodomain is known to interact with N-protein and the interaction is important for genome packaging (Ferron et al., 2011).

This chapter describes experiments involving the fusion of RVFV ΔtGn with the TMD/CT of avian influenza H5N1 HA glycoprotein, cloning into different plant expression vectors, and testing for expression in *N. benthamiana* as well as monitoring for particle assembly. In addition, leaves previously infiltrated with this construct were subsequently infiltrated with constructs either encoding RVF N-protein or influenza M1 in order to determine whether this enhanced the production/stability of particles presenting Gn. Putative recombinant protein-based particles were purified by discontinuous gradient ultracentrifugation and tested for immunogenicity in mice.

5.2 Materials and Methods

5.2.1 Genes

The RVFV genes used to make the chimaeric VLP constructs were *ptGn* (*Nicotiana* sp. codon optimised *Gn* with a native signal peptide and no native TMD/CT) and Δ*ptGn* (*Nicotiana* sp. codon optimised *Gn* lacking the native signal peptide of the 5' terminus and no native TMD/CT) the generation of which are described in 4.2.2.3. The influenza HA TMD/CT was amplified from a human-codon optimised construct previously made in our laboratory encoding avian influenza H5N1 A/Vietnam/1194/2004 (GenBank accession number AY651333). Δ*ptGn* and *ptGn* modifications were made by PCR, using the PCR profile described in section 2.2.5. All PCR primer sequences are listed in Table 3, Appendix B. All DNA manipulation and analysis was carried out as described in section 2.2.4. All bacteria and plant growth conditions used were carried out as described in section 2.2.3.

Table 5.1: Plasmids used in this study

Plasmid	Description	Reference
pTRAc-ptGnHA	*ptGnHA* cloned in pTRAc vector	This study
pTRAkc-LPH-ΔptGnHA	Δ*ptGnHA* cloned in pTRAc-ERH vector	This study
pEAQ-*HT*-LPH-ΔptGnHA	*LPH*-Δ*ptGnHA* cloned into pEAQ-*HT* vector	This study
pEAQ-*HT*-his-N	*N* cloned in pEAQ-*HT* vector	Chapter 6
pRIC3.0-M1	*M1* cloned into pRIC3.0 vector	[a]BRU-Culture Collection

[a] Biopharming Research Unit, Department of Molecular and Cell Biology, UCT

5.2.2 Fusion of *Nicotiana* sp. codon optimised glycoprotein (ΔtGn and tGn) with avian influenza transmembrane domain and cytosolic tail of HA

The HA TMD/CT was fused to *tGn* or Δ*tGn* by overlap extension PCR as described in section 4.2.2.4 using HA-tGn For and HA TMD Rev and annealing at 55 ˚C, resulting in *TMD/CT* with *tGn* 3' overhangs. *tGn* and Δ*tGn* with HA TMD/CT 3' overhangs were amplified with Gn For/ ΔGn Fw and tGnHA Rev with an annealing temperature of 55 ˚C. This resulted in *tGn* with HA TMD/CT 5' overhangs, as well as Δ*tGn* with HA TMD/CT 5' overhangs.

The amplified *tGn* and ∆*tGn* with *HA TMD/CT* 5' overhangs were fused with *HA* with *tGn*/∆*tGn* with HA 3' overhangs. Final full length fragments were assembled by PCR with Gn1 Fw /GnΔSP NcoI Fw and HA TMD Rev, with a primer annealing temperature of 55 ˚C. This resulted in DNA fragments ∆*tGnHA* and *tGnHA*.

5.2.3 Cloning of ∆*tGnHA* and *tGnHA* into pTRAc and pTRAkc-ERH

The amplified ∆*tGnHA* and *tGnHA* insert DNA was digested with *Nco*I and *Xba*I restriction enzymes and directly cloned into plant expression vectors pTRAkc-ERH and pTRAc using *Nco*I/ Afl*III* and *XbaI* (Roche Diagnostics, GmbH, Mannheim, Germany), respectively. The recombinant plasmids were confirmed by PCR using vector-specific pTRAc primers as well as restriction enzyme digestion with *Eco*RV. This resulted in constructs pTRAc-tGnHA with the (native signal peptide and HA TMD/CT) and pTRAc-LPH-∆tGnHA (LPH signal peptide and HA TMD/CT) recombinant vectors.

5.2.4 Cloning of LPH-∆tGnHA into pEAQ-*HT* plant expression vector

LPH-∆*tGnHA* was cloned into pEAQ-*HT* as described in section 4.2.2.5, resulting in pEAQ-*HT*-LPH-∆tGnHA.

5.2.5 *Agrobacterium* transformation and recombinant protein expression

The pTRA- and pEAQ-recombinant plasmids were transformed by electroporation into *A. tumefaciens* GV3101::pMP90RK and *A. tumefaciens* LBA4404 respectively, as described in section 3.2.10. A preliminary expression time trial was conducted by syringe infiltration as described in section 3.2.11. Crude plant extract was prepared from infiltrated leaves as described in 3.2.12. Protein expression was analysed by western blotting as described in section 3.2.13, loading equal amounts of total soluble protein (TSP) as determined by Bradford assay using BSA (Sigma-Aldrich, MO, USA) as a standard.

5.2.6 Large scale expression of pEAQ-*HT*-LPH-ΔtGnHA

Large scale expression by vacuum infiltration of pEAQ-*HT*-LPH-ΔtGnHA was conducted as described in section 4.2.6.

5.2.7 Staggered infiltration with M1 and N-protein

In order to favour the formation of particles, plants infiltrated with pEAQ-*HT*-LPH-ΔtGnHA were re-infiltrated with pEAQ-*HT*-his-N or pRIC3.0-M1 at 3 days post the first infiltration, with an infiltration OD_{600} of 0.5. The plants were incubated for a further 2 days of pEAQ-*HT*-LPH-ΔtGnHA (and harvested at 5 days post first infiltration).

5.2.8 Purification of pEAQ-*HT*-LPH-ΔtGnHA chimaeric particles

Plant material was harvested at 5 dpi. Crude plant protein was extracted using a high pH buffer, 100 mM Tris/HCl pH 7.5 or a low pH buffer 100 mM NaOAc pH 5.6, in a 1:2 mass:volume ratio as described in 4.2.7.1. The protein was purified by sedimentation through a 20% iodixanol cushion onto a 60% iodixanol cushion using a SW32Ti rotor in a Optima™ L-100 ultracentrifuge (Beckman Coulter) at 32 000 rpm for 2 h at 4 ˚C. Two mL fractions from the interface at the 20%/60% iodixanol boundary were collected, pooled, layered onto a 20%, 30%, 40% and 50% 2 mL step gradient, and re-centrifuged as described above for 16 h. One ml fractions were collected from the gradient and stored at -80 ˚C for further analysis.

5.2.9 Protein expression analysis

Protein expression analysis was by western blot and Coomassie Blue gel staining as described in section 4.2.8. Endotoxin levels were measured using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript Biotechnologies, Piscataway, NJ, USA) as per the manufacturer's instructions. The presence of contaminant bacteria was tested for by plating samples on LB agar with no antibiotics and incubating O/N at 37 ˚C.

5.2.10 Transmission electron microscopy (TEM)

Copper-coated grids (mesh size 200) were made hydrophilic by glow discharging at 25 mA for 30 s using a Model 900 SmartSet Cold Stage Controller (Electron Microscopy Sciences). Three uL of sample was aliquoted onto grids, incubated for 30 s and washed twice with sterile water. The samples were negatively stained for 30 s with 3% (w/v) uranyl acetate and viewed using a Technai G2 transmission electron microscope (FEI). Particles were measured using the Ruler tool in Adobe.

5.2.11 Immunisation of mice

Eight to ten week old female BALB/c mice were obtained from South African Vaccine Producers (SAVP, Johannesburg, South Africa) and housed in filter top micro-isolator cages under Biosafety Level 2 (BSL-2) conditions in the Research Animal Facility at the Heath Science Faculty, UCT. Approval for this study was granted by the Animal Research Committee at the University of Cape Town (AEC# 015-048). The study was carried out as described in 4.2.9 with five mice per group.

5.2.12 Indirect ELISA detection of anti-Gn antibodies in mouse sera

The anti-Gn responses were determined by ELISA as described in 4.2.10. To evaluate the anti-Gn immune response elicited by each mouse, the pre- and final bleed sera were diluted 1:1000 in 100 µL.

5.3 Results

5.3.1 PCR fusion of *Nicotiana* sp. codon optimised glycoprotein (∆tGn and tGn) with avian influenza TMD/CT of HA

To further optimise the truncated Gn to enhance protein expression and formation of chimaeric particles, the native 418 bp TMD was removed (section 4.2.2.4). The plant-codon optimised RVFV ∆*tGn* was successfully fused with 108 bp of the influenza HA TMD/CT at its C- terminal position 1298. Sequencing confirmed the gene fusion and that no mutations were introduced by PCR. ∆*tGnHA* and *LPH-*∆*tGnHA* were

successfully cloned into plant expression vectors pTRAc, pTRAkc-ERH and pEAQ-*HT* (Figure 5.1 A-C) respectively.

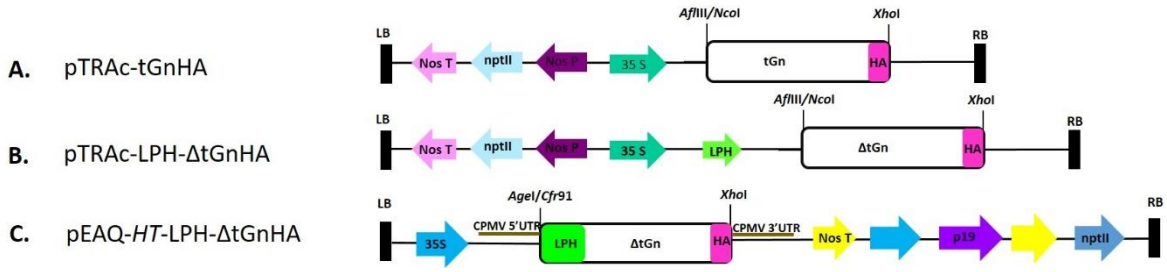

Figure 5.1: The schematic presentation of plasmids with *Nicotiana* sp. RVFV ΔtGn fused with avian influenza TMD/CT (HA). **A**) pTRAc-ΔtGnHA, ΔtGn with the native signal peptide and influenza TMD/CT, **B**) pTRAc-LPH-ΔtGnHA, ΔtGn with the LPH signal peptide and influenza TMD/CT and **C**) pEAQ-*HT*-LPH-ΔtGnHA.

Restriction enzyme digest mapping confirmed the plasmid integrity as depicted in Figure 5.2 A, giving the expected banding pattern for pTRAc-ΔtGnHA (6657 and 884 bp), pTRAkc-LPH-ΔtGnHA (5011, 1652, 1405 and 943 bp) and Figure 5.2 B representing pEAQ-*HT*-LPH-ΔtGnHA (9945 and 1788 bp).

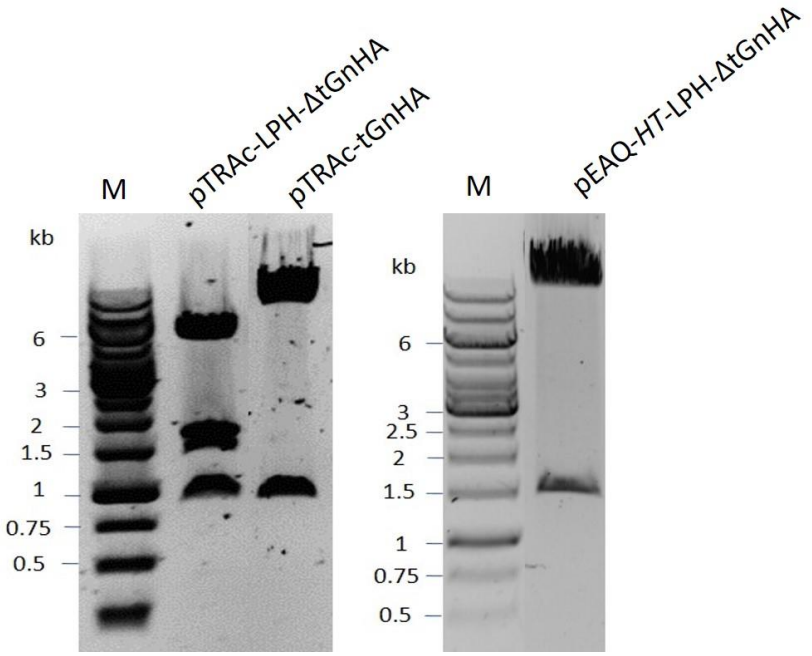

Figure 5.2: Restriction digest mapping confirming the recombinant expression plasmids. **A**) pTRAc suite vectors digested with *Eco*RV (Fermentas, Hanover, MD) restriction enzyme, **B**) pEAQ-*HT* vector digest with *Age*I and *Stu*I restriction enzymes. The bands represent the expected banding pattern. The marker lane (M) contains the O'GeneRuler™ 1kb DNA ladder (Fermentas, Hanover, MD) was used as molecular weight marker.

5.3.2 Transient expression of recombinant proteins in *Nicotiana benthamiana*

The recombinant plasmids were successfully electroporated into *A. tumefaciens* cells. Infiltration of *N. benthamiana* plants was conducted at culture OD_{600} values of 0.25 and 0.5. Recombinant protein expression was assessed with small scale expression time trials and western blotting of crude leaf extracts harvested at 1, 3, 5 and 7 dpi. Thirty µg of TSP was loaded in each well. Recombinant protein expression could not be detected in the samples from leaves infiltrated with recombinant *Agrobacterium* strains harbouring plasmids pTRAc-tGnHA and pTRAc-LPH-ΔtGnHA (results not shown). However, protein expression was successfully detected in leaf extract from plants infiltrated with pEAQ-*HT*-LPH-ΔtGnHA (Figure 5.3). Western blotting showed a 64.5 kDa band corresponding to the expected protein size (591 aa) in samples from 1, 3, 5 and 7 dpi at OD_{600} of 0.25 and 0.5. Expression at OD_{600} of 0.25 showed the highest expression levels at 3 dpi and gradually decreased to 7dpi. Expression of OD_{600} of 0.5 gradually increased from 3 dpi to day 7 showing the overall highest expression levels. These results also showed that the highest expression levels were

obtained using an OD_{600} of 0.5 compared to 0.25. Thus, parameters of pEAQ-*HT*-LPH-ΔGnHA infiltrated at OD_{600} of 0.5, and leaves harvested at 7 dpi were used for further experimentation.

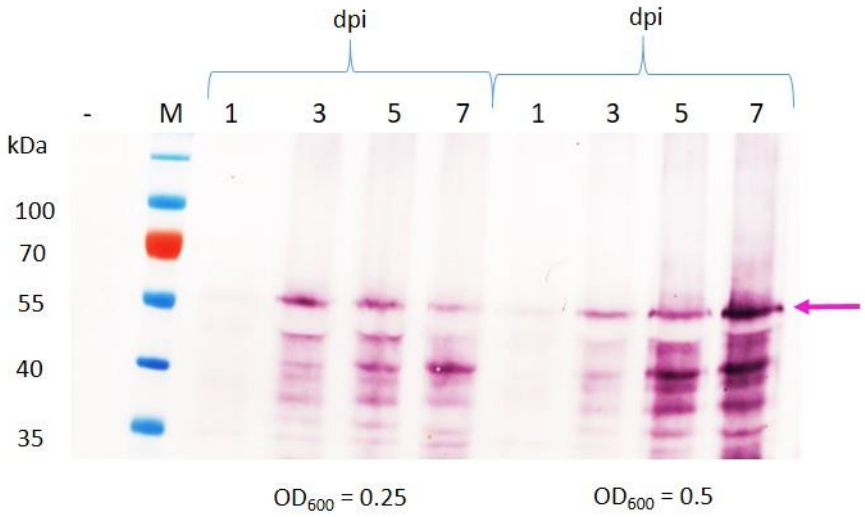

Figure 5.3: Expression time trial of *Nicotiana* sp. RVFV ΔtGn fused with Influenza TMD/CT. pEAQ-*HT*-LPH-ΔtGnHA, expressed at two different OD_{600} of 0.25 and 0.5, TSP of 30 µg was loaded in each lane for 1, 3, 5 and 7 dpi. The recombinant protein was detected with 1: 1000 anti-Gn primary antibody. Lane "-"indicates the un-infiltrated negative control, Lane M contains PageRuler™ Prestained protein ladder (Fermentas, Hanover, MD). The arrow indicated the expected band size of 64.5 kDa.

5.3.3 Protein purification and characterisation of LPH-ΔtGnHA

In order to determine whether LPH-ΔtGnHA assembled into particles, crude leaf extracts from plants infiltrated with pEAQ-*HT*-LPH-ΔtGnHA were centrifuged through a 20% iodixanol cushion onto a 60% cushion. Two mL of sample was collected from the interface (Figure 5.4 A). Western blot analysis confirmed the recombinant protein was mainly in the interface (F1), although detectable both in F2 and F3 fractions. However, there were also other plant proteins detected in fractions F2 and F3 as detected by their binding to the anti-Gn serum antibodies (Figure 5.4 B). Plant proteins were present in all fractions as demonstrated by results with the pEAQ-*HT* empty vector control. The protein collected in the interface (F1) was characterised by TEM for particle formation. Protein aggregates were observed ranging from 15 - 19 nm in diameter (Figure 5.4 C), these protein aggregtes were not observed in the pEAQ-*HT* empty vector control (Figure 5.4 D).

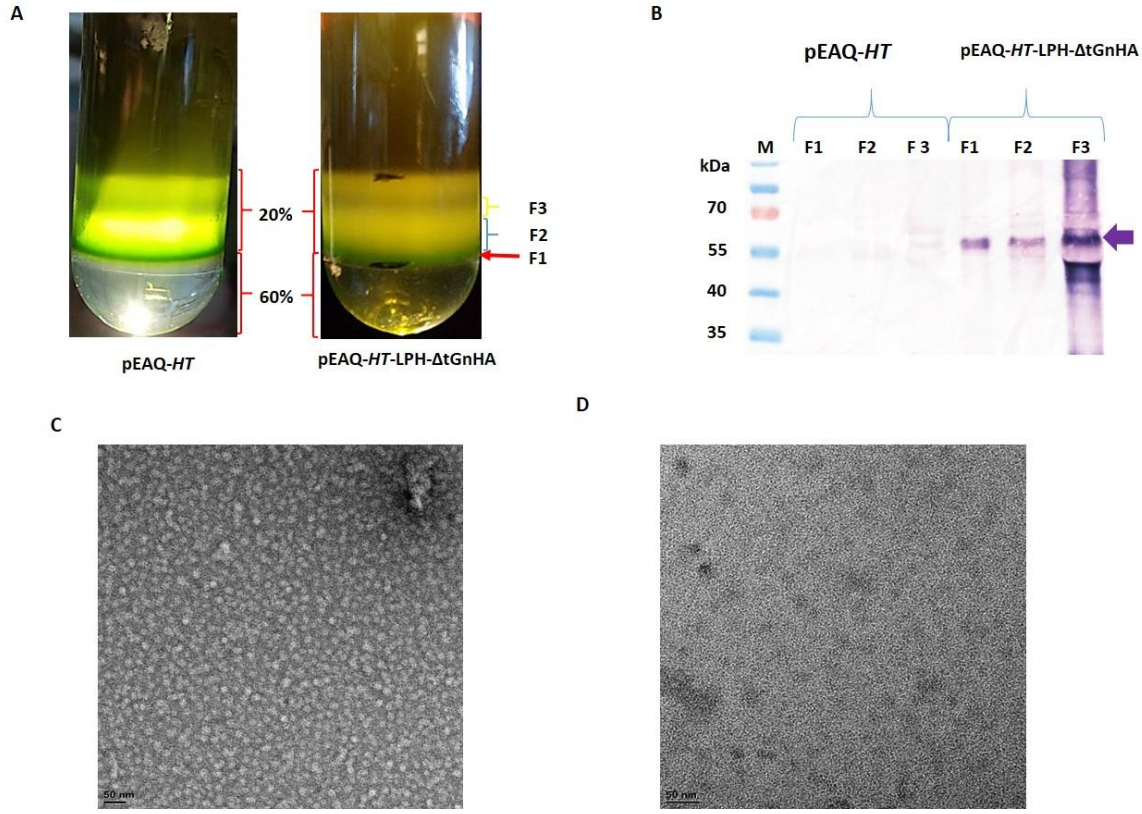

Figure 5.4: Purfication of LPH-ΔtGnHA by sedimentation through a 20% iodixanol cushion and onto a 60% iodixanol cushion. **A**) Tube after sedimentation. The red arrow indicates the interface **B**) western blot analysis of different fractions using anti-Gn antibody. F1 represents the interface, F2 is a 2 mL layer above the interface and F3 is 2 mL above F2. Lane M contains PageRuler™ Prestained protein ladder. **C**) Protein aggregates observed in the 20%/60% interface. **D**) pEAQ-*HT* empty vector control observed in the 20%/60% interphase. Scale bar indicates 50 nm.

5.3.4 Staggered infiltration of LPH-ΔtGnHA with M1 and N-protein

In order to promote better particle assembly rather than simply having protein aggregates, the LPH-ΔtGnHA protein was co-expressed with RVF N-protein or with the influenza M1 protein. Co-expression of LPH-ΔtGnHA with M1 or N-protein-encoding constructs at infiltration ratios of 2:1 or 1:1 ratio (LPH-ΔtGnHA:M1/N) resulted in high expression levels of the co-expressed proteins M1 and N-protein and low expression level of LPH-ΔtGnHA as determined by western blot (Figure 5.5 A and B). It was thought that possibly the higher expression of either M1 or N-protein may be hindering the expression of the LPH-ΔtGnHA and that staggering the days of infiltration by infiltrating M1 and N-protein 3 days after infiltration of LPH-ΔtGnHA may increase the initial expressed levels LPH-ΔtGnHA. This was subsequently carried out at an infiltration ratio of 1:1 which resulted in detectable protein expression levels of

all the proteins (Figure 5.5 C and D). Infiltration at OD of 0.5 and co-infiltration (re-infiltrating the same plant) at 3 dpi of LPH-ΔtGnHA and harvesting at 5 dpi of LPH-ΔtGnHA were the optimal co-expression conditions selected for further experimentation.

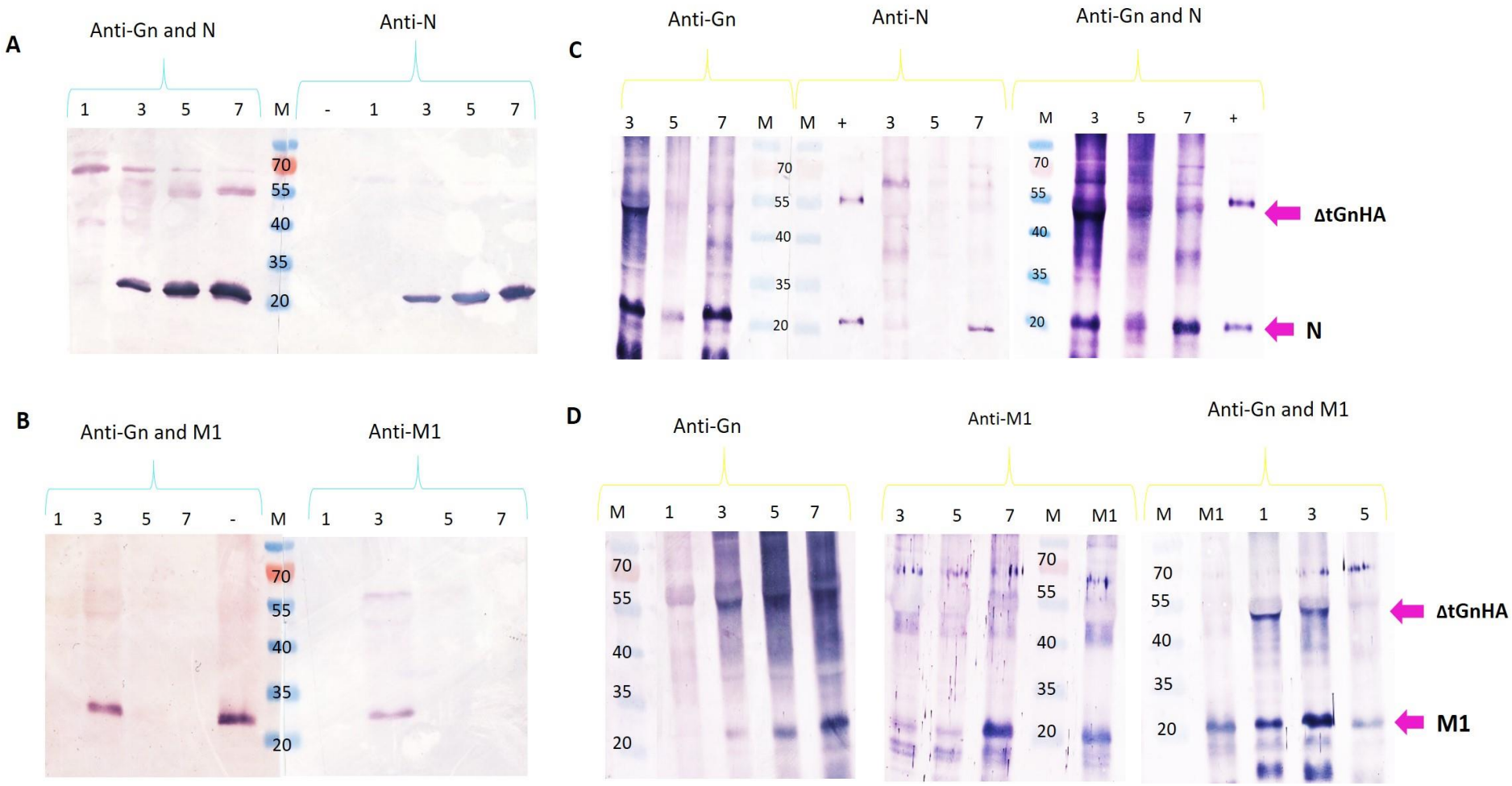

Figure 5.5: Western blotting of crude leaf extracts infiltrated with ΔtGnHA co-expression and subsequent staggered expression of N and M1. **A**) Represents the co-expression of ΔtGnHA with N-protein at 1:1 ratio, the left panel detected with anti-Gn and N at 1:100, the right panel with anti-N at 1:100, **B**) represents co-expression of ΔtGnHA with M1 at 1:1 ratio, the left panel detected with anti-Gn at 1:100 and M1at 1:2000, the right panel with anti-M1 at 1:2000, **C**) staggered expresstion of ΔtGnHA with N-protein, **D**) staggered expression of ΔtGnHA with M1. The pink arrows indicate the expected protein bands.

High levels of contaminating plant proteins were present, therefore a low pH extraction buffer was tested to get rid of the excess. This phenomenon was also observed with other proteins analysed in our laboratory. The protein was extracted with a low pH buffer (100 mM NaOAc pH 5.6) and the routine high pH (100 mM Tris/HCl pH7.5) buffer, and the two extracts were compared. Putative particles were purified by centrifugation with a double cushion as described in Figure 5.5. Low pH buffer extracts did not show appropriately sized protein bands on western blots for all the recombinant proteins tested (Figure 5.5 A and B, 5.6 A and B, 5.7 A and B), yet protein aggregates were visible using TEM although samples had fewer contaminating proteins than those extracted with high pH buffer. However, Gn, N and M1 protein were detectable on western blots when leaves were processed with high pH buffer (Figure 5.5 A and B, 5.6 A and B, 5.7 A and B).

Recombinant LPH-ΔtGnHA was co-expressed with RVF N-protein: N-protein alone and LPH-ΔtGnHA alone were expressed as positive controls. This was to confirm that the N-protein was expressed in the staggered infiltration. The expected N-protein band size of 27 kDa was detected with the anti-N-protein specific antibody (Figure 5.5 A), confirming the expression of N-protein on its own at 2 dpi and when co-infiltrated with LPH-ΔtGnHA with high pH buffer extraction were successfully expressed. The LPH-ΔtGnHA was detected with the anti-Gn specific antibody (Figure 5.6 B). pRIC3.0-M1 was used as a negative control. A protein band of ~55 kDa was detected in the pRIC3.0-M1 negative control plant which is indicative of the RuBisCo protein. TEM showed protein aggregates for protein extraction in the high pH buffer (Figure 5.6 C); these aggregates seemed to be smaller (~20 nm) in low pH buffer-extracted samples (Figure 5.6 D). The purified protein extract had less protein contaminants with the low pH buffer as compared with the high pH buffer. No particles were observed in the pRIC3.0-M1 (Figure 5.6 E) negative control.

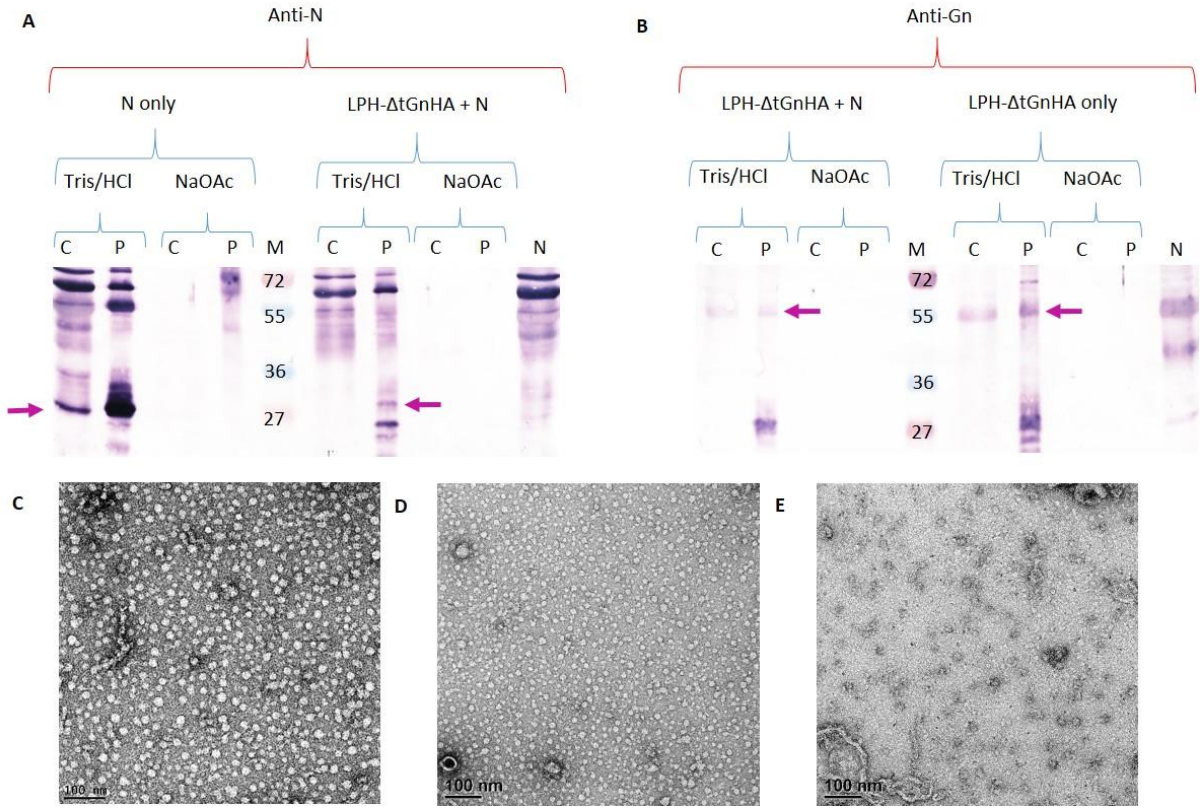

Figure 5.6: Western blotting and TEM representing the co-expression of LPH-ΔtGnHA with RVFV N-rotein, confirming the expression of N-protein in the staggered expresssion. **A**) Left panel represents pEAQ-*HT*-his-N only, the right panel presents pEAQ-*HT*-LPH-ΔtGnHA co-expressed with pEAQ-*HT*-his-N. The protein was detected with 1:100 anti-N. **B**) Left panel presents pEAQ-*HT*-LPH-ΔtGnHA co-expressed with pEAQ-*HT*-his-N, the right panel presents pEAQ-*HT*-LPH-ΔtGnHA only. Protein detection with 1:100 anti-Gn. C represents the crude sample, P represents the interface and N is the pRIC3.0-M1 used as a negative control. The pink arrow indicates the protein of interest. **C**) Recombinant protein extracted with high pH buffer, **D**) Recombinant protein with low pH buffer, **E**) represents pRIC3.0-M1 used as a negative control extracted with high pH buffer. The grids were stained with 3% uranyl acetate. Scale bar represents 100 nm.

Recombinant LPH-ΔtGnHA was co-expressed with avian influenza M1; M1 on its own and LPH-ΔtGnHA alone were expressed as positive controls. This was to confirm that the M1 protein was expressed in the staggered infiltration. The expected band size of 64.5 kDa was detected with the anti-Gn specific antibody (Figure 5.7 A). A band size of 55 kDa indicative of RuBiSco was detected in the negative control lane (N). The expected protein band size of 27 kDa and dimer 54 kDa were detected with the anti-M1-specific serum (Figure 5.7 B), confirming the expression of M1 protein on its own at 2 dpi and when co-infiltrated with LPH-ΔtGnHA with high pH buffer extraction. pEAQ-*HT*-his-N was used as a negative control. TEM showed protein aggregates that

varied in sizes (~20 – 30 nm) (Figure 5.7 C and D). Figure 5.6 E displays pEAQ-*HT*-his-N negative control.

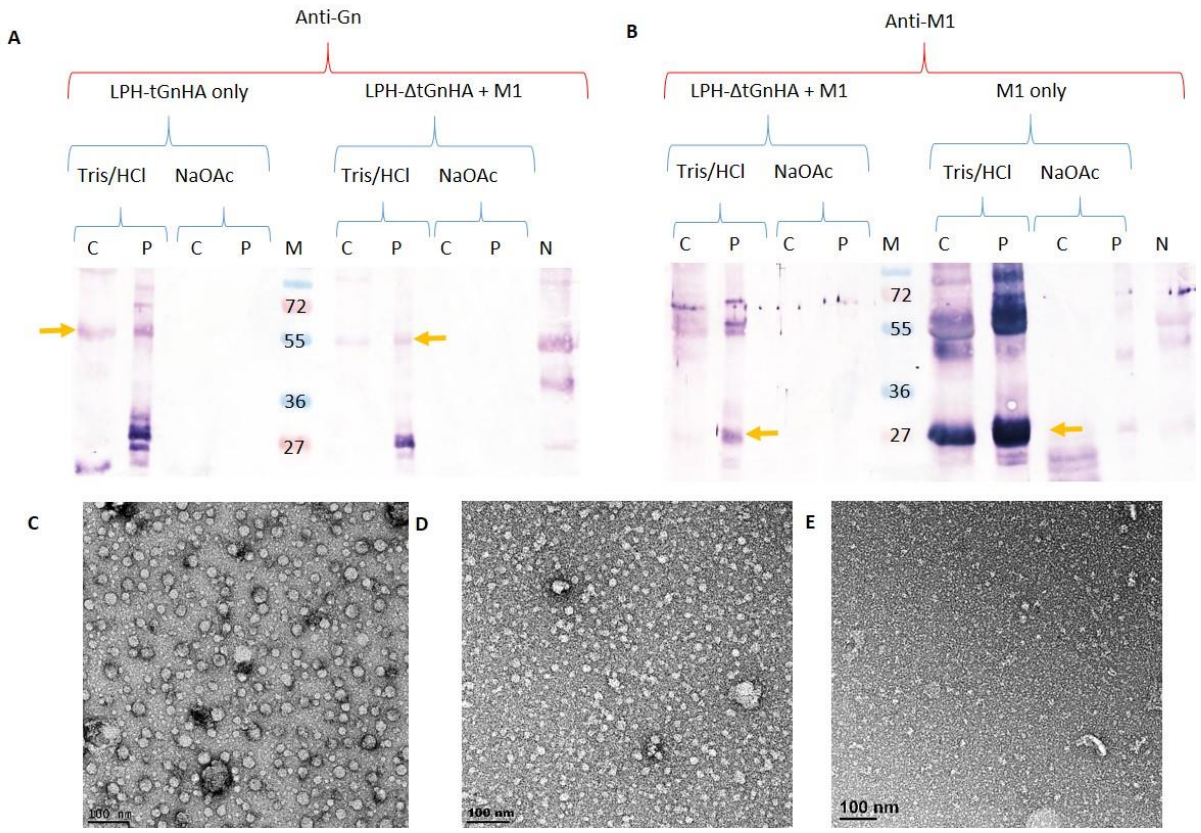

Figure 5.7: Western blotting and TEM representing the co-expression of LPH-ΔtGnHA with influenza the M1 protein confirming the expression of M1 in the staggered infiltration **A**) Left panel represents pEAQ-*HT*-LPH-ΔtGnHA only, the right panel presents pEAQ-*HT*-LPH-ΔtGnHA co-expressed with pRIC3.0-M1. The protein was detected with 1:100 anti-Gn. **B**) Left panel presents pEAQ-*HT*-LPH-ΔtGnHA co-expressed with pRIC3.0-M1, the right panel presents pRIC3.0-M1 only. Protein detection with 1:2000 anti-M1 serum. C presents the crude sample, P is the interface and N is the pEAQ-*HT*-his-N used as a negative control. The orange arrow indicates the protein of interest. TEM of **C**) Recombinant protein extracted with high pH extraction buffer, **D**) Recombinant protein with low pH buffer, **E**) represents pEAQ-*HT*-his-N used as a negative control extracted with high pH extraction buffer. Grids were stained with 3% uranyl acetate. Scale bar are 100 nm.

RVFV LPH-ΔtGnHA expressed on its own, its co-expression with N-protein (Figure 5.8 A) and with M1 (Figure 5.8 B) were used as controls. This was to confirm that the RVFV ΔtGnHA recombinant protein was expressed in the staggered infiltration. The recombinant protein was detected with anti-Gn specific antibody. The high pH extraction buffer consistently showed particles ranging from 49 – 60 nm (Figure 5.8 C) in diameter while the low pH extraction buffer gave protein aggregates or smaller

particles which range from 15 - 19 nm (Figure 5.8 D). These experiments were conducted in triplicate. Since particles of similar size were consistently viewed by TEM, the production of LPH-ΔtGnHA alone was selected for further studies rather than when co-expressed with M1 and N-protein.

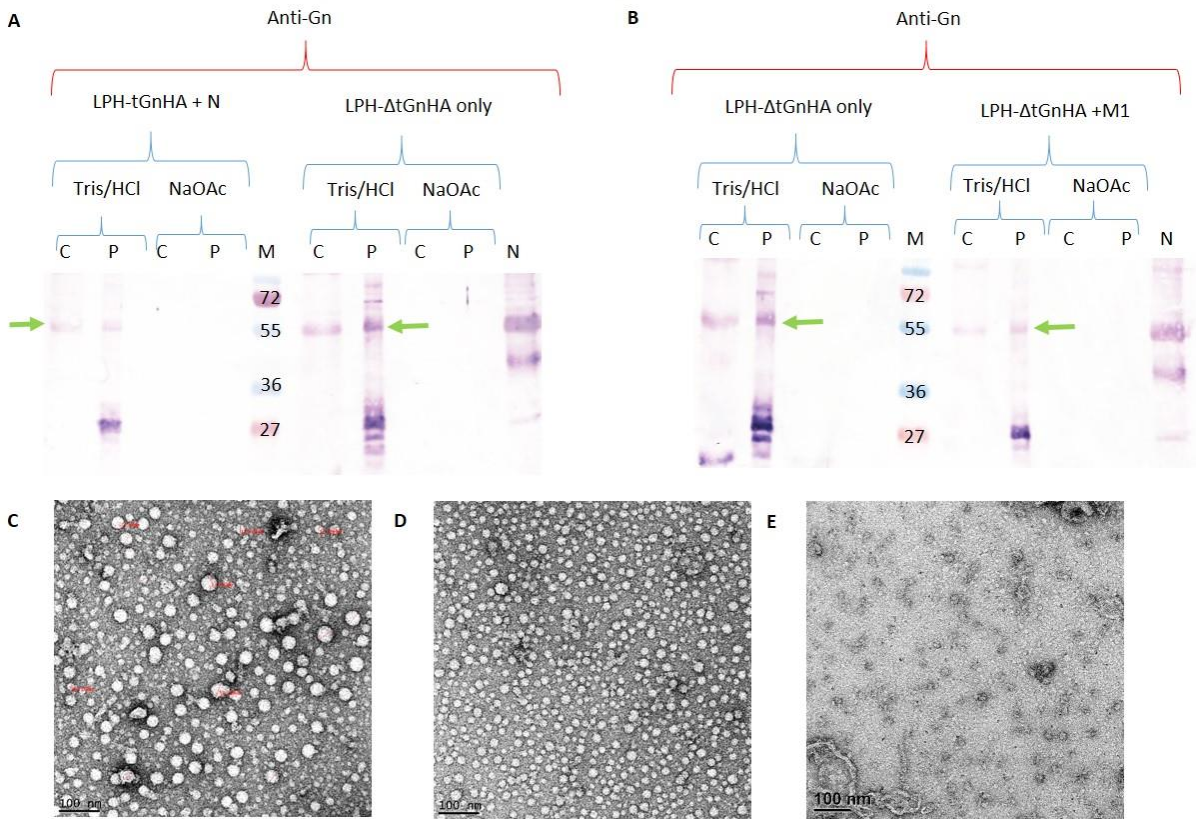

Figure 5.8: Western blotting and transmission electron microscope representing the expression of RVFV LPH-ΔtGnHA in the staggered infiltration. **A**) Left panel presents pEAQ-*HT*-LPH-ΔtGnHA co-expressed with pEAQ-*HT*-his-N, the right panel presents pEAQ-*HT*-LPH-ΔtGnHA only. N presents pRIC3.0-M1 used as negative control. The protein was detected with 1:100 anti-Gn. **B**) Left panel represents pEAQ-*HT*-LPH-ΔtGnHA only, the right panel presents pEAQ-*HT*-LPH-ΔtGnHA co-expressed with pRIC3.0-M1. N presents pEAQ-*HT*-his-N used as negative control. Protein detection with 1:100 anti-Gn. C presents the crude sample and P is the interface. The green arrow indicates the protein of interest. TEM of **C**) Recombinant protein extracted with high pH buffer, **D**) Recombinant protein with low pH extraction buffer, **E**) represents pEAQ-*HT*-his-N used as a negative control extracted with high pH extraction buffer. Grids were stained with 3% uranyl acetate. Scale bar represents 100 nm.

5.3.5 Large-scale production of chimaeric particles

For large-scale expression, LPH-ΔtGnHA was expressed on its own. Putative chimaeric VLPs were purified by sedimentation through a 20% iodixanol cushion onto

a 60% cushion. Two mL was collected from the interface (Figure 5.4 A) and subsequently centrifuged through the discontinous gradient and quantified by gel densitometry. The protein was further purified by differential centrifugation. One mL fractions were collected from the bottom of the tube and analysed by western blotting with anti-Gn specific antibody (Figure 5.9 A). and SDS-PAGE gel staining (Figure 5.9 B).

The recombinant protein was detected as a doublet, and was seen mainly in sample from the 40% and 50% iodixanol layers (Figure 5.9 A, F2 & 3). RuBisCo, the most abundant protein in plants partially co-purified with the Gn protein and was detected mostly in the 50% iodixanol fraction. TEM showed that most of the fractions contained protein aggregates and what looked like capsomers, with apparent VLPs in the 30% iodixanol fraction (Figure 5.9 F5); these chimaeric VLPs (Figure 5.7 C) were similar to those produced by de Boer et al 2010 in baculovirus (Figure 5.9 E). However, these particles were present at very low concentrations.

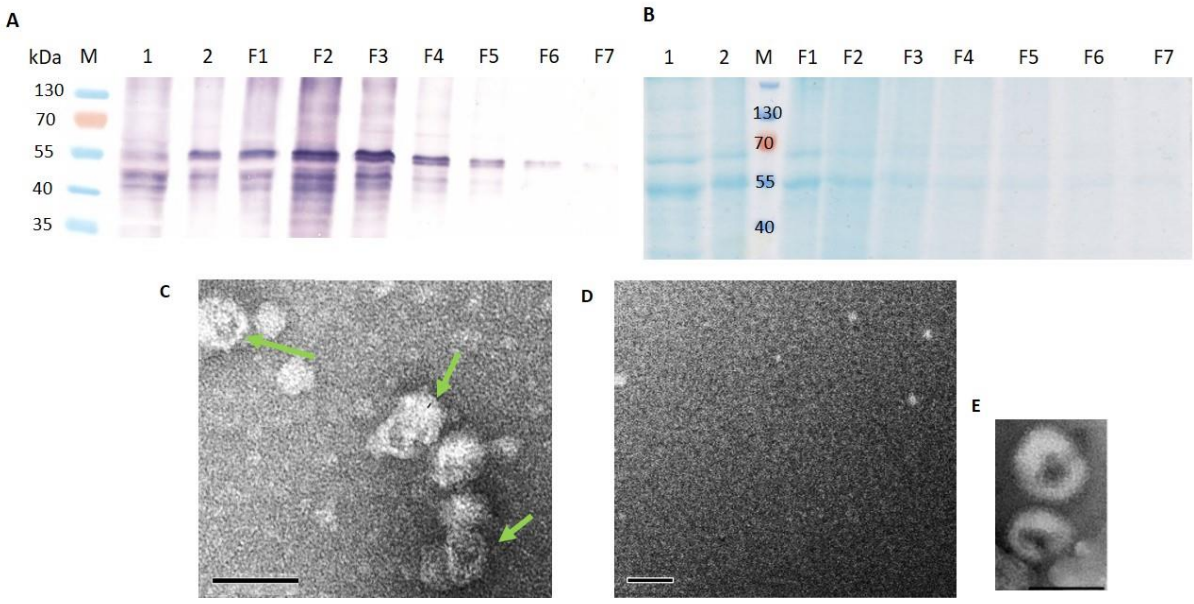

Figure 5.9: Western blot analyis of fractions collected from a step iodixanol gradient and transmission electron microscope of pEAQ-*HT*-LPH-ΔtGnHA. **A**) protein detected with anti-Gn specific antibody, **B**) coomasie gel staining of the collected fractions. Lane M contains PageRuler™ Prestained protein ladder. Lane 1 contains crude recombinant protein Lane 2 contains partially purified with double cushion protein. F1 and 2 represents 50%, F3 and 4 represent 40%, F5 and 6 represents 30% and F7 represents 20% iodixanol. **C**) TEM of F5 stained with 3% uranyl acetate. Green arrows indicate VLPs,

scale bar is 100 nm. **D**) TEM of pEAQ-*HT* empty vector stained with 3% uranyl acetate, scale bar is 50 nm. **E**) TEM of baculovirus expressed GnGc VLPs (de Boer et al., 2010).

5.3.6 Immunisation and animal serum analyisis

Due to the low protein concentration levels obtained from discontinous gradient purification, mice were immunised with protein purified by sedimentation through the 20% iodixanol cushion onto a 60% cushion. Two mL collected from the interface was used for immunisation. Two experiments in mice were conducted to determine if the modified RVFV Gn vaccine candidate produced in *N. benthamiana* was immunogenic. The analysis of the pre- and final bleed sera of the individual mice showed the apparent presence of specific antibodies in the final bleed sera, therefore sera from each group were pooled (5 mice/group) for the analysis of anti-Gn binding (Figure 5.10 A). The antibody binding titres were expressed as a reciprocal of the maximum serum dilution containing absorbance values that were three times greater than the corresponding pre-bleed serum at 1:1000. Preliminary data analysis for the presence of anti-Gn specific antibodies was analysed by indirect ELISA using plant-produced Gn protein as coating antigen.

No binding to coating antigen was detected in the pre-bleed serum (Figure 5.10 B). Binding was seen for sera raised against the candidate vaccine as well as the negative plant control, however (Figure 5.10 C). From the grouped individual mice sera, no differences in binding were observed between sera to the vaccine candidate and to the plant extract negative control. The mice antisera were accordingly pre-absorbed against a crude plant extract, as described previously (Chapter 4, section 4.2.11). Antiserum titration analysis revealed a stronger antibody binding for the vaccine candidate sera compared to sera to the negative plant control (Figure 4.9 C). The indirect IgG ELISA at serum dilution of 1:800 showed a 2-fold difference between the vaccine candidate and the negative plant control (Figure 4.9 D).

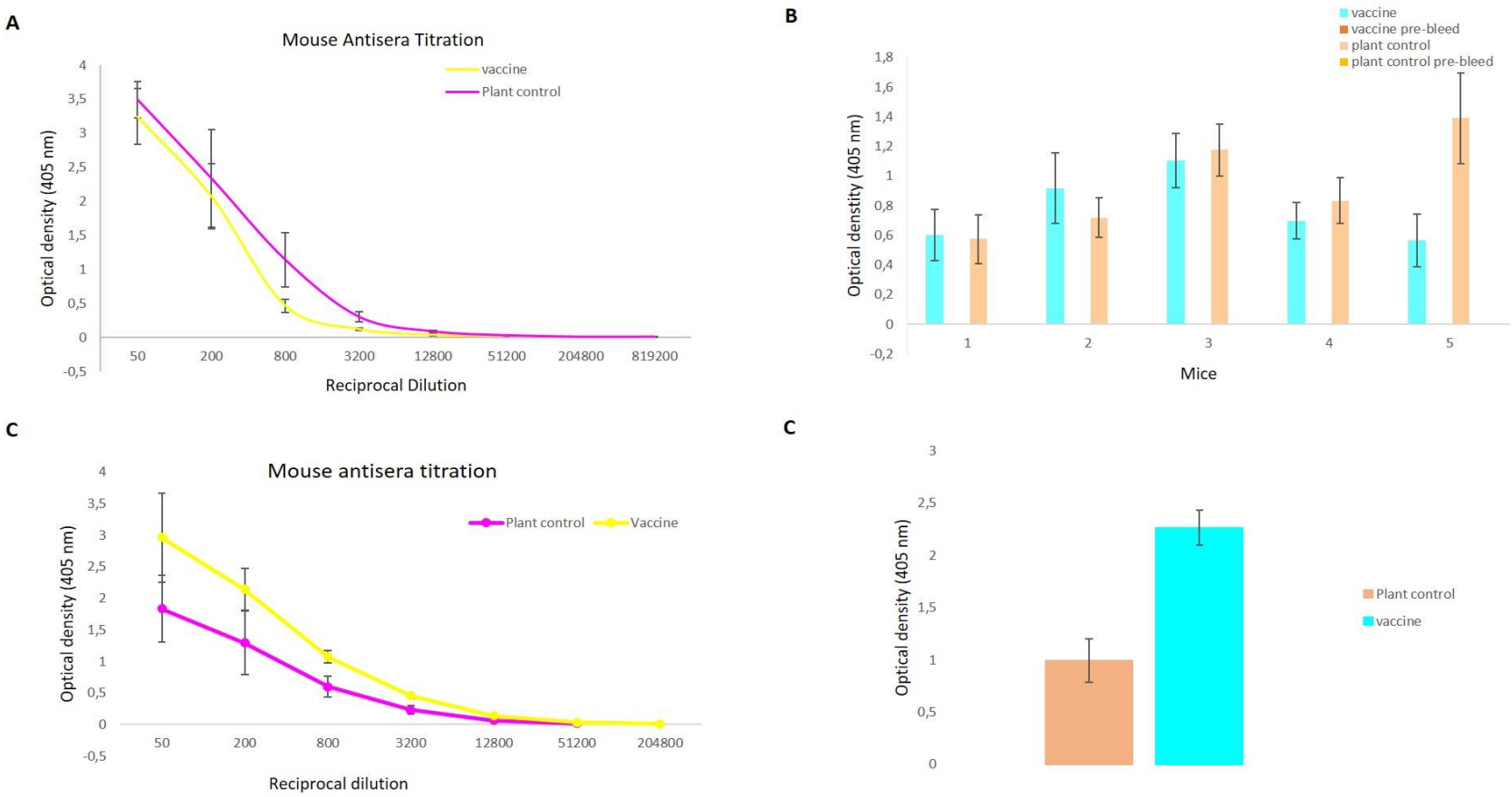

Figure 5.10: Analysis of antibody response in mice after vaccination with LPH-ΔtGnHA vaccine candidate. **A**) Titration of mice antiserum against the LPH-ΔtGnHA vaccine candidate (yellow) and plant negative control (pink). The absorbance readings are shown as an average (n =10). **B**) Absorbance value of pre- and final bleeds at reciprocal dilution of 1:1000. **C**) Titration of pre-absorbed mice antiserum against the LPH-ΔtGnHA vaccine (yellow) and plant negative control (pink). The error bars indicate standard deviation. **D**) Absorbance values of the vaccine candidate (blue) and plant negative (orange) control from pre-absorbed mice antiserum. The error bars indicate standard deviations

5.4 Discussion

I previously showed that RVFV *GnGc* could not be detectably expressed in *N. benthamiana* with our available plant expression systems. I therefore tried a different approach in an effort to develop a particulate vaccine, by fusing part of the immunogenic RVFV *Gn* gene to the influenza HA TMD/CT. The modified plant-codon optimised (LPH-ΔtGn) gene sequence was fused to the influenza HA TMD/CT (108 bp) to encourage the formation of chimaeric particles displaying RVFV Gn. The resultant LPH-ΔtGnHA was cloned successfully into the pTRAc suite of the plant expression vectors.

However, recombinant Gn-derived protein was not detected in leaves infiltrated with the recombinant pTRAc constructs by western blotting with a specific anti-Gn antibody. This reaffirms what was discussed in Chapter 4, which is that the native signal peptide and TMD/CT significantly affected the expression of the full length RVFV Gn *in planta*. Even use of a heterologous TMD/CT with a native signal peptide resulted in no detectable gene expression. Irrespective of which TMD/CT is on the Gn gene, it seems not to allow for successful protein expression. Moreover, using the heterologous LPH signal peptide with the HA TMD/CT also did not show recombinant Gn expression: the reason for this is unknown, since our laboratory has previously been able to successfully express the H5N1 avian influenza HA and soluble HAtr at high concentration using the pTRAc suite *in planta* (Mortimer et al., 2012).

To determine whether the expression vector promoter elements could affect expression, the gene was cloned between the CPMV untranslated regions (UTRs) in the plant expression vector pEAQ-*HT*, resulting in pEAQ-*HT*-LPH-ΔtGnHA: this resulted in successful expression of LPH-ΔtGnHA in *N. benthamiana*. The choice of vector is known to be important when expressing recombinant proteins, since there are various elements on the vector which play a role in driving the expression of the protein. It has been previously demonstrated that flanking the gene of interest with the modified 5' UTR and the 3' UTR from CMPV with the hypertranslatable (*HT*) leader

sequence region significantly increases recombinant protein expression levels (Sainsbury and Lomonossoff, 2008; Sainsbury et al., 2009b). The highest expression levels were obtained at OD_{600} of 0.5 at 7dpi. Therefore, the choice of expression vector pEAQ-*HT* allowed for successful expression of the recombinant LPH-ΔtGnHA.

Recombinant Gn-derived protein expression was subsequently scaled up by use of vacuum infiltration, and the putative particle fraction purified by centrifugation with a double Optiprep™ cushion. TEM analysis showed the presence of abundant protein aggregates. The RVFV Gn C-terminal tail interacts directly with ribonucleoproteins (RNPs) during the natural budding process to ensure the proper packaging of the RNPs. The interaction between RNP and the GnGc stabilises the RVFV virions and drives assembly (Huiskonen et al., 2009). Since in this case the glycoprotein C-terminal tail has been removed, in order to try and stabilise VLP formation, the LPH-ΔtGnHA protein was co-expressed with M1 and N-protein. Co-expression at a 2:1 or 1:1 ratio resulted in high expression levels of the co-expressed proteins; however LPH-ΔtGnHA was expressed at low levels. This could be due to the small M1 and N proteins (28 kDa), which are the most abundant proteins in their virions, exceeding the production rate of the large 64.5 kDa LPH-ΔtGnHA glycoprotein.

TEM analysis of the co-expressed proteins showed inconsistency in results between extractions, and variability in particle formation. It was hoped that co-expression with influenza M1 would favour better VLP formation, with the influenza TMD/CT interacting with M1 as it does in influenza virus virions. The M1 protein is the most abundant structural protein of the influenza particle, and it plays a role in the assembly and release of the particles (Gomez-Puertas et al., 2000). It has the ability to form core-like particles with an average diameter of 30 nm (Baniasadi and Lal, 2014). However, we have not been able to see such particles (personal communication, Francisco Pêra), since they form at low pH (4.0) (Bui et al., 1996; Shtykova et al., 2013). Co-expression with the RVF N-protein was anticipated to facilitate the formation of VLPs, since formation of RVFV VLPs has been demonstrated by transfection of animal cells with plasmids encoding GnGc as well as co-transfection of GnGc with RVF N-protein (de Boer et al., 2010; Habjan et al., 2009; Mandell et al., 2010a).

Expression and analysis of LPH-ΔtGnHA expressed alone and fractionated on a discontinuous gradient resulted in virus-like particles consistently being found, together with abundant protein aggregates. These protein aggregates were also observed by Huiskonen et al., (Huiskonen et al., 2009). This could be due to weak bonds between the proteins leading to formation of subviral particles. Stabilisation of the VLPs can achieved by fixation of the particles with 0.5% glutaraldehyde prior to purification to preserve the fragile complexes (de Boer et al., 2010; Huiskonen et al., 2009). Discontinuous gradient purification showed particles and apparent capsomer-like aggregates in the various iodixanol fractions. VLPs varying in size from 49 – 60 nm were detected in the 30% fraction. The native RVFV virion surface is covered by a glycoprotein shell of 122 capsomers with a diameter of 101 to 106 nm (Huiskonen et al., 2009). While the VLPs were not morphologically identical to those obtained in other systems – via recombinant baculovirus expression in insect cells, for example (de Boer et al., 2010) – their consistent recovery indicates that the strategy of using H5N1 TMD/CT as a particle-forming display vehicle was successful, which is a highly novel finding.

Preliminary immunogenicity studies of the candidate chimaeric vaccine was carried out in BALB/c mice. The purified fraction from 30% iodixanol had low protein concentration levels. With the objective of increasing the probability of an immune response, the protein that was purified by sedimentation through the 20% iodixanol cushion onto a 60% cushion interface was used for immunisation. However, this fraction included a lot of native plant proteins. Antibody binding to putative RVFV-specific coating antigen was detected at similar levels in indirect ELISA for the vaccine- and plant negative control-specific sera. Pre-absorption of the antiserum with plant extract, however, revealed a significant difference in binding between the sera to the vaccine candidate and to the plant negative control. The chimaeric RVFV Gn candidate vaccine was therefore immunogenic.

In conclusion, in an attempt to produce a VLP based vaccine candidate, I successfully fused the modified Gn with influenza TMD/CT and cloned it into both the pTRAc suite and pEAQ-*HT* plant expression vectors. Successful recombinant protein expression

was attributed to our choice of vector. Protein purification was optimised and consistent recovery of Gn particles confirmed the novelty of using the influenza TMD/CT as a particle-forming carrier to be a qualified success. These chimaeric Gn particles were found to be immunogenic.

Chapter 6: Production of Rift Valley fever virus N-protein in plants for use as a potential diagnostic antigen

6.1 Introduction

The requirement for monitoring of RVFV outbreaks has become ever more urgent due to global warming, which has expanded the regions of vector distribution. This in turn has increased the demand for diagnostic assays which are reliable and rapid, to implement the control of spread of RVFV outbreaks. There are several different ways of diagnosing RVFV infections. Quantitative real-time PCR can be used to detect viraemic animals (Bird et al., 2007; Chengula et al., 2014; Drosten et al., 2002; Garcia et al., 2001). In addition, a real-time reverse transcription loop-mediated isothermal amplification (LAMP) test for rapid detection of RVFV has been developed (Euler et al., 2012; Le Roux et al., 2009; Peyrefitte et al., 2008). Most recently, a method for rapid inactivation of virus and single step rRT-PCR for detection on RVFV RNA has been developed (Drolet et al., 2012). However, although viremia in individuals infected with RVFV reaches high levels, it is short-lived, which makes monitoring of the spread of the virus in the field using PCR inadequate. In addition, PCR requires specialised laboratory equipment and well trained personnel which are additional drawbacks when an outbreak occurs in remote areas.

Serological testing of samples is more appropriate, and the ELISA is considered a rapid and reliable diagnosis tool. Development of this tool led to a highly sensitive and specific indirect assay for the detection of IgG and IgM antibodies to RVFin animal and human serum, using purified, inactivated sucrose-acetone extracted RVFV protein as the coating antigen (Niklasson et al., 1984; Paweska et al., 2003a; Paweska et al., 2005a; Paweska et al., 2005b; Paweska et al., 2003b). However, there are several disadvantages to using inactivated antigens: they tend to bind poorly to plates (Jansen van Vuren et al., 2007), and production of the antigen requires the use of a biosafety containment facility which increases the cost of production as well as the risk of exposure to laboratory personnel (Fafetine et al., 2013; Jansen van Vuren et al.,

2007). In addition, these ELISAs cannot be used in RVFV non-endemic areas due to the presence of virus-derived antigen and the risk of viral escape.

The N-protein is highly conserved among various RVFV isolates (Bird et al., 2007) is the most abundant protein in the virion and highly immunogenic (Boshra et al., 2011; Faburay et al., 2014; Lagerqvist et al., 2009). It has led to the development of the production of recombinant RVF N-protein in several different expression systems: RVF N-protein has been successfully produced in *Aedes pseudoscaullaris* mosquito cell lines (Zaki et al., 2006); Trichoplusia ni (Tn5) insect cells (Fukushi et al., 2012) as well as *E. coli* (Fafetine et al., 2013; Jansen van Vuren et al., 2007; McElroy et al., 2009; van der Wal et al., 2012). At least two of the commercially available RVFV ELISAs now incorporate recombinant *E. coli*-produced N-protein for the detection of RVFV antibodies in animal serum (Kortekaas et al., 2013). A double antigen ELISA for the simultaneous identification of IgM and IgG of RVFV antibodies, using N-protein produced in *E. coli,* has also been developed and validated using animal serum (Ellis et al., 2014).

Despite the success of *E. coli*-produced N-protein in ELISA, there are some disadvantages: *E. coli* recombinant proteins are often incorrectly folded and/or often insoluble or aggregated, which may influence functionality in an ELISA. Recombinant proteins produced in plants, on the other hand, are normally correctly folded and post-translationally modified as the expression host is also eukaryotic, and the production system is immune to contamination by animal-infecting agents. In addition, the production of recombinant proteins in plants is deemed to be more cost-effective than other expression platforms (Fischer et al., 2015; Rybicki, 2010).

RVF N-protein has been recently produced in transgenic *Arabidopsis* (Kalbina et al., 2016). The work described in this chapter explored the potential for transient production in *Nicotiana benthamiana* plants as a cost-effective and safe RVFV N antigen for diagnostic use.

6.2 Materials and Methods

6.2.1 Growth conditions and DNA manipulation

All bacterial and plant growth conditions were carried out as described in section 3.2.3. All DNA manipulation and analysis was carried out as described in section 3.2.4.

6.2.2 Cloning of *N*-protein into pEAQ-*HT*

The RVF nucleoprotein (*N*)-encoding DNA sequence of South African isolate M35/74 (GenBank accession number JF784 388) was human-codon optimised, synthesised by GenScript (GenScript Biotechnologies, Piscataway, NJ, USA) and cloned into pUC57 to yield pUC57-N. This was transformed into *E. coli* DH5α (E. cloni ™, Lucigen, WI). *N* was cloned from pUC57-N using restriction enzymes *Age*I and *Stu*I into pEAQ-*HT Age*I/ *Cfr*91 and *Stu*I cloning sites respectively, to yield pEAQ-*HT*-N and pEAQ-*HT*-his-N

6.2.3 *Agrobacterium* transformation and recombinant protein expression analysis

The recombinant plasmids were transformed by electroporation into *A. tumefaciens* LBA4404 as described in section 3.2.10. A preliminary expression time trial was conducted by syringe infiltration as described in section 3.2.11. The protein was extracted from *N. benthamiana* leaves by grinding up leaf samples in 250 µL extraction buffer (100 mM Tris/HCl pH 7.5 and 1% Triton X-100), vortexing and subsequent incubation on ice for 10 min. The crude extracts were clarified by centrifugation at 14 000 rpm for 5 min in a bench top centrifuge. The concentration of total soluble protein (TSP) in the crude extract was determined by Bradford assay using BSA (Sigma-Aldrich, MO, USA) as a standard. Recombinant protein expression analysis was conducted as described in section 3.2.13 using anti-N antibody (keyhole limpet haemocyanin-linked anti-N peptide (NKPRRMMMKMSEKEG); GenScript, Piscataway, NJ, USA) diluted to 1: 5000.

6.2.4 Large scale protein expression

Large scale protein expression was conducted by vacuum infiltration as described in section 4.2.6. One hundred and ninety-seven grams of plant material was harvested at 3 dpi.

6.2.5 Protein purification

6.2.5.1 Ammonium sulphate precipitation

Crude plant protein was extracted with 100 mM Tris/HCl pH 7.5, 1% Triton X-100 in a 1:2 plant mass: buffer volume ratio, with an Ultra-Turrax® homogeniser (IKA® Works Inc., NC, USA) and filtered twice with four layers of Miracloth™ (EMD Millipore Corp., Billirica, MA USA). The crude extract was clarified by centrifugation at 13 000 rpm, 20 min, 4 ˚C four times or until there was no longer a pellet visible at the bottom of the tube. Ammonium sulphate precipitation was performed as described in section 4.2.7.1. The 0 – 40% precipitated pellet was re-suspended with half the original volume of buffer (50 mM Na_2HPO_4, 0.5 M NaCl, pH 8) and was dialysed O/N at 4 ˚C using dialysis tubing with a molecular weight cut-off of 10 kDa (Thermo Fischer Scientific, USA) in 2L against the same buffer. The extract was clarified by centrifugation at 13 000 rpm, 4 ˚C, for 20 min. This was repeated four times or until the pellet became invisible.

6.2.5.2 Nickel affinity chromatography

The crude extract was filtered through a 0.45 µm and subsequently a 0.2 µm filter. The protein was loaded on a 5 mL HIS-chelating affinity column (Sigma-Aldrich, St. Louis, USA) and purified with an automated fast protein liquid chromatography (FPLC) system (ÄKTA Purifier Plus, GE-Healthcare Life Technologies) at a flow rate of 2.5 min/mL. The column was equilibrated with binding buffer (50 mM Na_2HPO_4, 0.5 M NaCl and 20 mM imidazole, pH 8). N-protein was eluted with elution buffer (50 mM Na_2HPO_4, 0.5 M NaCl, 500 mM imidazole, pH 8). Five mL fractions were collected with a fraction collector and an absorbance reading at 280 nm determined for each fraction. Purified N-protein was used for further analysis.

6.2.6 Protein expression analysis

Fractions corresponding to the protein peak were analysed for the presence of N-protein. Samples from the 5 mL fractions were resolved on SDS-PAGE gels and analysed by western blotting as described in section 3.2.13 and staining as described in section 4.2.8. Protein concentration of the N-protein was quantified by gel densitometry of a coomassie blue strained gel using a Gene Genius Bioimaging System – GeneTools (version 3.07.03) (Syngne) and BSA (Roche Diagnostics, Germany) was used to construct a linear standard curve.

6.2.7 Liquid chromatography - mass spectrometry (LC-MS)

LC-MS analysis was conducted as described in 4.2.9

6.2.8 Transmission electron microscopy (TEM)

The purified protein fractions were put onto copper-coated grids (mesh size 200) made hydrophilic by glow discharging at 25 mA for 30 s using a Model 900 SmartSet Cold Stage Controller (Electron Microscopy Sciences). Three uL of sample was aliquoted on the grid for 30 s and washed twice with sterile water. The samples were negatively stained for 30 s with 3% (w/v) uranyl acetate and viewed using a Technai G2 transmission electron microscope (FEI). Particles were measured using the Ruler tool in Adobe.

6.2.9 IgG ELISA assays

6.2.9.1 Serum samples

To determine whether the purified RVF N-protein was functionally active and able to detect IgG antibody against RVF, the antigen was reacted against known RVFV-positive and RVFV-negative sera from animals using ELISA. A group of 24 seronegative sheep were previously experimentally infected with RVFV strain M35/74 by Williams et al., as part of their validation of their assay at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) (Williams et al., 2011). A group of negative reference sera from sheep was included.

6.2.9.2 Preparation of mock antigen

Mock antigen was prepared by *Agrobacterium*-mediated infiltration of pEAQ-*HT* empty vector into *N. benthamiana.* Crude extract was prepared in the same manner as the purified N-protein as described in section 6.2.5, precipitated with $(NH_4)_2SO_4$ at 0-40% fraction, followed by affinity chromatography.

6.2.9.3 Indirect RVF IgG ELISA

Fractions containing the purified N-protein were analysed with a standard protocol (VT_ME_016-03) for RVF indirect IgG ELISA (Ellis et al., 2014; Williams et al., 2011) at the ARC-OVI. The test is accredited by the South African National Accreditation System (SANAS). Briefly, Nunc Polysorp plates (Nunc, Rosklilde, Denmark) were coated with purified recombinant plant-produced N-protein, followed by the addition of different sera and detection of binding by addition of secondary antibody (Williams et al., 2011). A total of 40 serum samples were tested (in duplicate) - twenty positive and twenty negative for RVF antibodies (Williams et al., 2011). *E. coli* produced N-protein was included as a positive control.

Optical density (OD) values were read at 405 nm. The net OD_{405} for each serum tested was calculated by subtracting the OD_{405} reading of the mock antigen from the cognate reading of that obtained using the plant-produced N antigen to account for any background absorbance in ten separate experiments. OD readings were converted to PP values (percentage of positive control serum) (Williams et al., 2011) using the following equation:

$$\%PP = \frac{\text{Mean OD of test sample} - \text{Mean OD of negative control}}{\text{Mean OD of positive control} - \text{Mean OD of negative control}}\ x\ 100$$

6.2.10 Protein stability assays

To determine the protein stability of the plant-produced N-protein, 30 uL aliquots of purified protein were stored at RT, 4 ˚C, -20 ˚C and -80 ˚C. Stability was assessed at

four week intervals by monitoring of protein integrity on Coomassie-stained SDS polyacrylamide gels and western blotting as described in section 6.2.6.

6.3 Results

6.3.1 Cloning of Rift Valley fever nucleocapsid (N) protein gene

The *N* gene is 750 nucleotides long with an open reading frame of 254 aa. The N-encoding gene was directly cloned with two different restriction enzymes between the CPMV untranslated region (UTRs) in the plant expression vector pEAQ-*HT*, resulting in the plasmids pEAQ-*HT*-his-N (N terminal *6xhis* affinity tag) (Figure 6.1.A) and pEAQ-*HT*-N (Figure 6.1 B). These recombinant plasmids were confirmed by restriction digest mapping (Figure 6.2) giving the expected banding pattern of 9947 and 773 bp for pEAQ-*HT*-his-N and 9947 and 746 for pEAQ-*HT*-N.

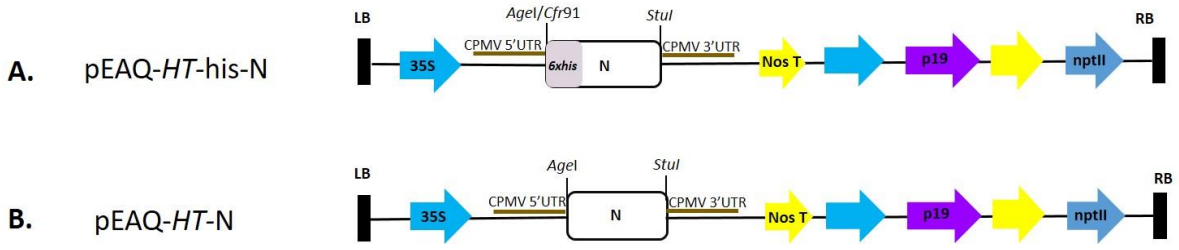

Figure 6.1: Schematic presentation of recombinant RVFV N-protein in pEAQ-*HT* vector. **A**) N protein with N-terminal *6xhis* tag, **B**) N protein without the *6xhis* tag. **pEAQ-*HT* vector components**: RB and LB: right and left borders for T-DNA integration, 35S promoter from Cauliflower mosaic virus (CaMV), 5'UTR: modified 5' UTR from CPMV RNA-2, 3'UTR from CPMV RNA-2, NosT: nopaline synthase terminator, P19: suppressor of gene silencing from TBSV, 35S terminator from CaMV, *npt*II: kanamycin resistance gene, OriV: pRK2 origin of replication, TrfA: replication essential locus and ColEI; the pBR322 *E. coli* origin of replication (Sainsbury et al., 2009a).

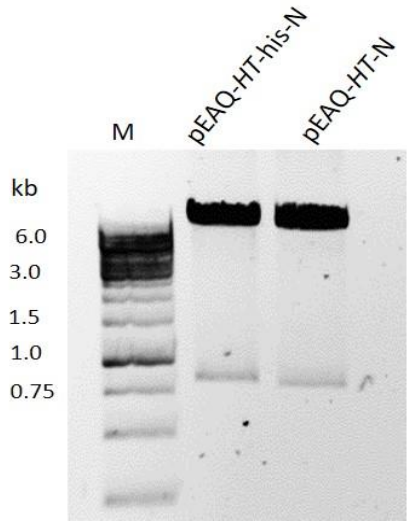

Figure 6.2: Confirmation of pEAQ-*HT*-his-N (9947 and 773 bp) and pEAQ-*HT*-N (9947 and 746 bp) by restriction enzyme digest mapping with *Nru*I and *Stu*I, 1 kb O'GreenGene ruler (M) was used as a DNA ladder. DNA was resolved on 0.8% (w/v) TBE agarose gel.

6.3.2 Transient expression and detection of recombinant N-protein in crude leaf extracts

The recombinant plasmids were successfully electroporated into *A. tumefaciens* LBA 4404 cells. Recombinant protein expression was assessed with small scale expression time trials and western blotting of crude leaf extracts harvested at 1-4 days post infiltration (dpi). Recombinant protein expression of N-protein in leaves infiltrated at culture optical densities (OD_{600}) of 0.25, 0.5 and 1 was determined by western blots probed with anti-N antibody (Figure 6.3 A-C); TSP (30 µg) was loaded in each well.

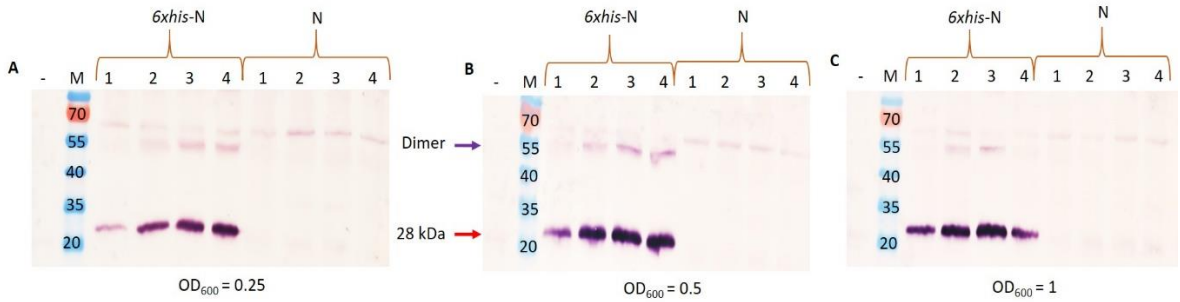

Figure 6.3: Western blot analysis of transient expression time trial of N-protein in *N. benthamiana* via *Agrobacterium* mediated transfer. **A**) infiltration at OD_{600} of 0.25, **B**) infiltration at OD_{600} of 0.5, **C**) infiltration at OD_{600} of 1. Lanes 1-4 denote the number of days post infiltration (dpi). Lane "-", – negative control represented by extract from leaves infiltrated with an empty pEAQ-*HT* vector. Lane M contains PageRuler™ Prestained protein ladder (Thermo Scientific, MA, USA). The protein was detected with 1:5000 anti-N primary antibody and 1:5000 anti-rabbit secondary antibody.

The 28 kDa band representing N-protein could not be detected in crude extracts from leaves infiltrated with pEAQ-*HT*-N (i.e.: construct lacking the *6xhis* tag) at an OD_{600} of 0.25 and 0.5. There was a very faint band visible from samples prepared from leaves infiltrated at OD_{600} of 1 from 2 to 4 dpi. However, the expected band size of 28 kDa, as well as a dimer of 56 kDa, were detected from leaf samples infiltrated with pEAQ-*HT*-his-N from 1 dpi. These progressively increased in concentration with the incubation period until 4 dpi. These bands were not present in the negative control. The highest protein expression levels, viewed as the darkest protein band, were visualised for conditions of *Agrobacterium* OD_{600} = 0.5 at 3 and duration of 4 dpi. The optical density of 0.5 was thus selected for further experimentation. Since speed or processing would be important when producing this antigen commercially, the shorter 3 dpi period of incubation was selected for further analysis of the N-protein.

6.3.3 Purification of recombinant protein

In order to scale up the production of N-protein, 40 plants were vacuum-infiltrated at an OD_{600} of 0.5 and harvested at 3 dpi. N-protein was enriched for by $(NH_4)_2SO_4$ precipitation and subsequent nickel affinity column chromatography. The protein was detected in the 0 - 40% fraction, and was purified by *6xhis* affinity chromatography. The protein was eluted from the column with an increase in imidazole concentration, corresponding to a protein peak visualised in fractions 33 - 38 (Figure 6.4 A). The

recombinant protein was detected by western blot analysis as monomers (28 kDa) and dimers (56 kDa) (Figure 6.4 B and C). No other plant-contaminating proteins were detected on Coomassie-stained gels (Figure 6.4 C). Two further batches of protein were purified and eluted off the column at 30% (150 mM) imodazole concentration and peak fractions 23 – 26 were analysed. The protein was mainly detected in fraction 24. The protein eluted off the column with other plant contaminating proteins. Peak fractions 36 (Batch 1) and 24 (Batch 2 and 3) were used for further analysis.

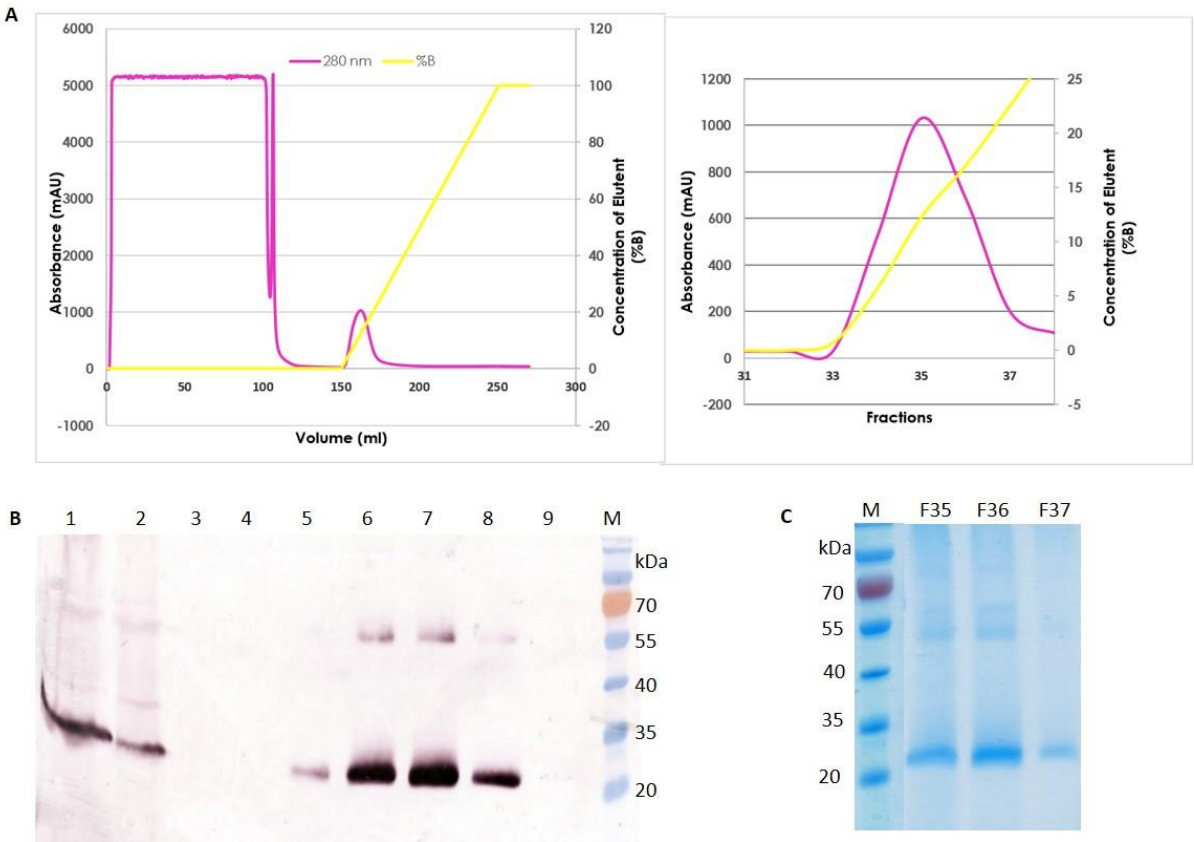

Figure 6.4: Purification of the N-protein using nickel affinity chromatography. Chromatographic trace showing *6xhis*-N elution from the HIS-chelating affinity column with increasing imidazole concentration (Yellow line). **B**) western blot analysis of collected fractions. Lane 1 contains crude plant extract, lane 2 0 – 40% $(NH_4)_2SO_4$ precipitate, lane 3 contained unbound wash fraction, lane 4-9 contained fractions 33 – 38 and lane 10 contained PageRuler ™ Prestained protein ladder (Thermo Scientific, MA, USA). The protein was detected with 1:5000 anti-NP primary antibody and 1:5000 anti-rabbit secondary antibody. **C**) coomassie gel staining of fractions 35, 36, and 38 containing the purified protein. Lane M contained PageRuler ™ Prestained protein ladder.

The maximum protein yield of RVF N-protein was calculated to be ~500-558 mg/kg of fresh weight leaf material. An average total protein concentration of 0.450 mg/mL was obtained. I also measured the OD_{260nm}/OD_{280nm} ratio for the fractions to determine the presence of bound nucleic acids. Fractions 35 and 36 were pooled and had an OD_{260nm}/OD_{280nm} ratio of 1.9. The identity of the protein was confirmed by LC-MS resulting in 87.35% coverage and 264 unique peptides (Figure 6.5 A).

A

Description: tr|A2T003|A2T003_RVFV NP
Database: Bunyaviridae
Score: 1094.60
Coverage %: 87.35
Unique peptides: 264
Taxonomy: Rift Valley Fever nucleocapsid protein

1 DITGMDNYQE LAIQFAAQAV DRNEIEQWVR EFAYQGFDAR RVIELLKQYG GADWEKDAKK
61 MIVLALTRGN KPRRMMMKMS KEGKATVEAL INKYKLKEGN PSRDELTLSR VAAALAGWTC
121 QALVVLSEWL PVTGTTMDGL SPAYPRHMMH PSFAGMVDPS LPEDYLRAIL DAHSLYLLQF
181 SRVINPNLRG RTKEEVAATF TQPMNAAVNS NFISHEKRRE FLKAFGLVDS NGKPSAAVMA
241 AAQAYKTAA* RPDI

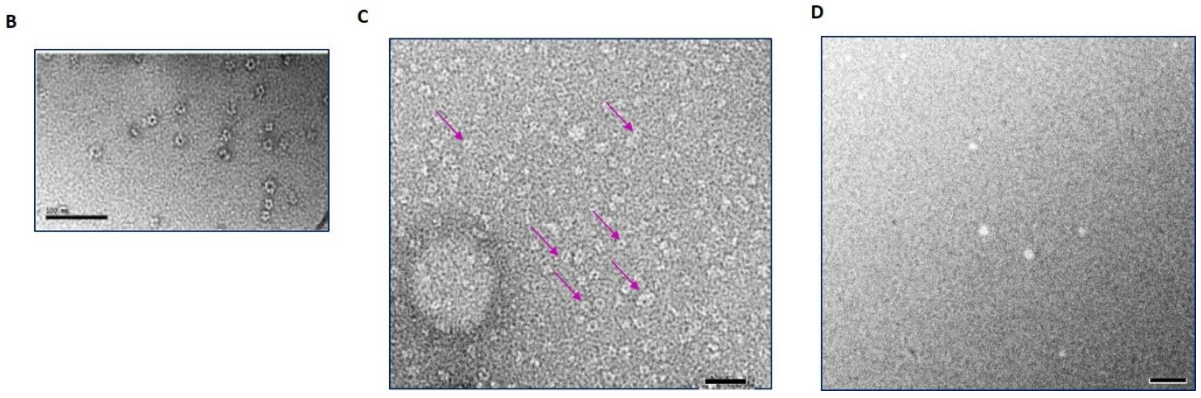

Figure 6.5: Recombinant protein confirmation and visualisation. **A**) Mass spectrophotometry of N-protein recovered from SDS-PAGE analysis of a nickel affinity chromatography purified protein from infiltrated plant leaf material. Unique peptide sequences highlighted in red. **B**) TEM of *E. coli* produced N-protein by Ferron et al 2011, (Ferron et al., 2011). **C**) TEM of plant-produced N-protein (indicated by pink arrow), **D**) represents an empty pEAQ-*HT* infiltrated plant was used as the negative control. Scale bar represents 100 nm

Previously, purified N-protein expressed in a bacterial system (Ferron et al., 2011) , has been shown using TEM to form distinct ring-shaped particulate structures which

are approximately 10 nm in diameter (Figure 6.5 B). Samples of affinity–purified plant-produced N-protein were therefore subjected to TEM and shown to contain similar-shaped particles of similar magnitude (Figure 6.5 C). This particulate matter was not observed in the negative control (Figure 6.5 D).

6.3.4 IgG capture ELISA

To determine whether the purified plant-produced RVFV N antigen was functionally active and able to bind IgG antibody directed against RVFV, the antigen was reacted against known RVFV-positive and RVFV-negative sera from animals using ELISA. Plant-produced mock antigen was simultaneously tested as a negative control. Checkerboard titrations of the antigen were used to optimise the antigen dilution used for the ELISA protocol. The optimal antigen dilution of 1:200 was used. Two replicates of positive and negative RVF control sera as well as a conjugate control were tested. The mean OD values calculated for the 3 different batches of the positive control tested ranged from 0.146 to 1.8635, while the negative control values ranged from 0.0635 to 0.143. The cut-off value for the distinction between positive and negative results for a positive result in IgG capture ELISA was calculated as the percentage of high-positive control serum (PP) values greater than 7.0. PP values less than 4.0 were regarded as negative and PP values between 4.0 and 7.0 were regarded as suspect.

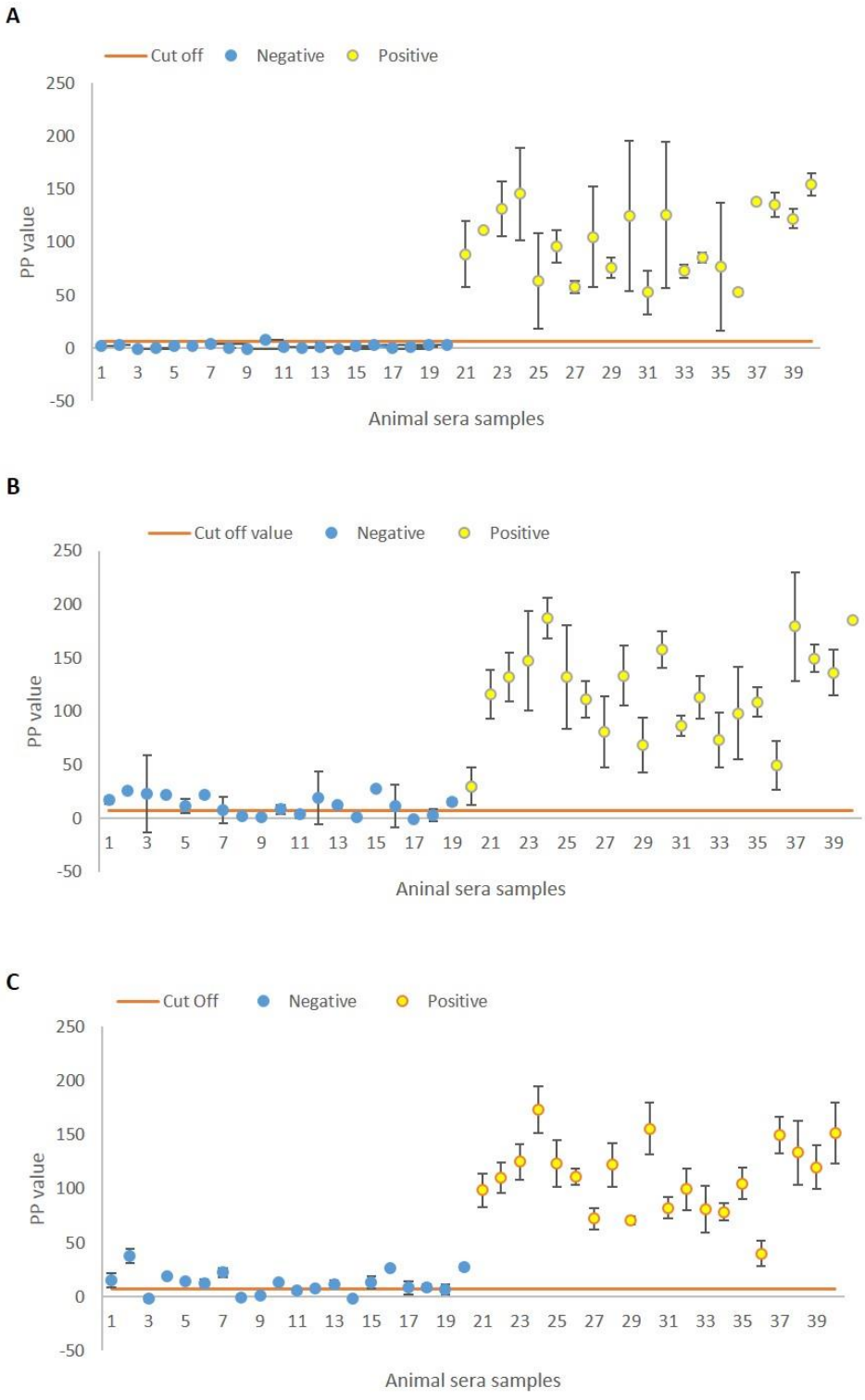

Figure 6.6: Detection of anti-RVFV IgG antibodies in sheep sera by ELISA using plant produced N-protein eluted off the HIS-chelating affinity column, **A**) batch 1, **B**) batch 2 and **C**) batch 3. Sample 1 – 20 represent PP values calculated for sera from RVFV negative sera and 21 – 40 represents PP values calculated for RVFV positive sera. The error bars illustrate ± mean standard deviation calculated from each PP value. PP values less than 4.0 regarded as negative, PP values between 4.0 and 7.0 regarded as suspect and PP values greater than 7.0 are considered positive.

The mean PP values for the positive sera ranged from 53.05 to 186.883, while the negative sera ranged from -1.51513 to 37.7862 (Figure 6.6 A-C). From the first purified protein batch (6.6 A), all positive sera tested positive with PP values ranging from 53.05 to 138.025; 19 negative sera tested negative with a PP value range of -0.525 to 3.75 with 1 false positive having a PP value of 7.75. For the ELISA using the second N-protein batch (Figure 6.6 B) all positive sera tested positive with PP values ranging from 49.49 to 189.88; six negative sera tested negative with PP values ranging from -0.917 to 3.36 with13 false positive samples with PP values ranging from 7.5 to 27.32. For the third N-protein batch tested (Figure 6.6 C), all positive sera tested positive with PP values ranging from 78.63 to 155.75; five negative samples tested negative with PP value ranges of -1.5 to 5.45 with 15 false positive samples with PP values ranging from 7.05 to 37.78.

6.3.5 Protein stability

The potential for use of the N-protein as a reagent in a diagnostic ELISA would call for antigen stability, particularly at temperatures close to ambient temperature which would make it easier for transportation and use in areas which do not have cold storage facilities. I conducted a preliminary set of experiments to assess the stability of the N-protein stored at various temperatures over a fixed period (36 weeks). SDS-PAGE staining and western blot analysis of purified N-protein after 4 to 36 weeks showed the presence of the usual 2 bands representing the monomeric (28 kDa) and dimeric (56 kDa) forms of N-protein (Figure 6.5 A and B) when stored at RT, 4 ˚C, -20 ˚C and -80 ˚C. However, in addition to these, a third band corresponding to a hexameric form of N-protein (140 kDa) became visible, over the fixed storage period at RT, 4 ˚C, -20 ˚C and -80 ˚C (Figure 6.5 A and B) (not visible on day 0, refer to Figure 6.4 B and C). This large protein aggregate became visible at RT within four weeks. A similar pattern gradually occurred over time with storage at other temperatures. Hexamer formation at -20 ˚C and -80 ˚C occurred at a much slower rate (Figure 6.5 B). The protein was mainly stable at 4˚C and could still be detected at -80 ˚C after 8 months' storage (Figure 6.5 C).

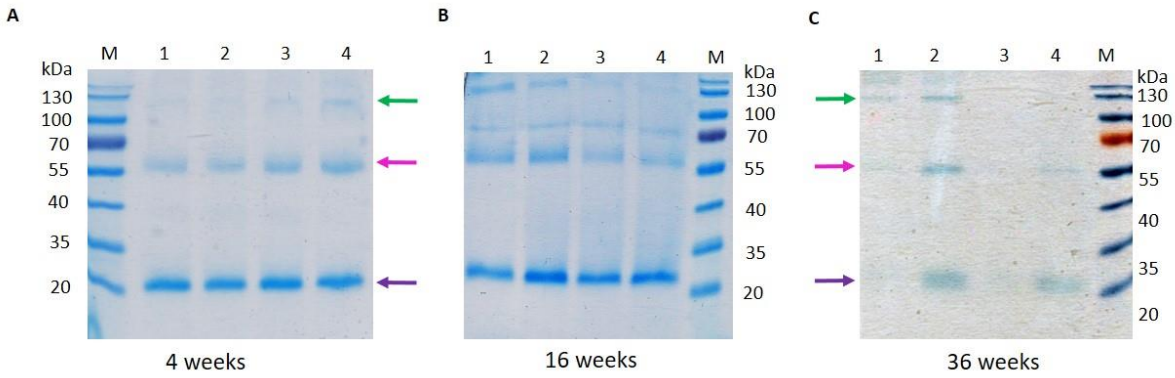

Figure 6.5: Coomassie blue staining presenting N-protein stability at varies storage temperatures over a period of time. **A**) recombinant protein stored for 4 weeks, **B**) recombinant protein stored for 16 weeks, **C**) recombinant protein stored for 34 weeks, lane 1, room temperature (RT), lane 2; 4 ˚C, lane 3; - 20 ˚C, lane 4; - 80 ˚C and lane M contains PageRuler ™ Prestained protein ladder. The arrows indicate protein, monomers (28 kDa) (purple arrow), dimers (56 kDa) (pink arrow) and hexamers (140 kDa) (green arrow).

6.4 Discussion

Rift Valley fever virus is regarded as an emerging virus due to the increasing number of mosquito vectors that can spread the virus into non-endemic areas as a result of recent global warming (Paweska et al., 2008). Monitoring of the spread of the disease is imperative in both endemic and non-endemic areas, and consequently requires reliable diagnostic tools. The development of a safe and cost-effective diagnostic tool is important, especially for South Africa as well as other developing countries where RVFV disease is prevalent and also where high levels of surveillance/monitoring for the disease are conducted (Rybicki et al., 2012).

This study looked at the possibility of using plants to express RVFV N-protein as a low-cost antigen for use in an ELISA which can be used to distinguish between animal serum containing and lacking anti-RVFV antibodies. RVF N-protein is highly conserved amongst other RVFV strains (Pepin et al., 2010; Saijo et al., 2002; Zaki et al., 2006) and it is the main immunodominant viral protein in other members of the *Bunyaviridae* family (Schwarz et al., 1996; Swanepoel et al., 1986b). Although N-protein is highly immunogenic, antibodies against N-protein do not exhibit neutralising activity (Boshra et al., 2011; Faburay et al., 2014; Lagerqvist et al., 2009). Since

recombinant N-protein lacks infectivity and is very stable, it is a suitable candidate for use as an antigen in an ELISA (Fafetine et al., 2007; Jansen van Vuren et al., 2007; Paweska et al., 2007).

The RVFV gene encoding the N-protein of a South African RVFV strain (SA M35/74) was human-codon optimised and synthesised. It was cloned into the pEAQ-*HT* plant expression vector, and I successfully expressed the recombinant RVF N-protein transiently in *N. benthamiana* by *Agrobacterium*-mediated infiltration. An expression time trial revealed that the N-protein without the *6xhis* tag was poorly expressed compared to the N-protein with the *6xhis* tag. The *6xhis*-N was expressed well at ODs of 0.25, 0.5 and 1, with the OD of 0.5 giving the highest protein expression levels. This could be due to the *6xhis* tag having some kind of stabilising effect on the recombinant protein. Reed et al (2005) have shown that N-terminus *6xhis* tag fusions have been found to improve the stability of ricin B-chain in tobacco (Reed et al., 2005).

The production of the *6xhis*-tagged N-protein was scaled up by vacuum infiltration of plants and crude extracts treated for purification of N-protein by using ammonium sulphate precipitation and subsequent affinity chromatography. The protein was detected as monomers (28 kDa), dimers (56 kDa) and (140 kDa) hexamers after being stored. This correlates with work done by Le May et al (Le May et al., 2005) who showed similar results with RVF N-protein expressed in mammalian cells. When Liu et al., (Liu et al., 2008) expressed it in recombinant baculovirus cells and Ferron et al., (Ferron et al., 2011) expressed the protein in *E. coli*, they also demonstrated that N-protein interacts with itself forming dimers and hexamers. The high protein concentration drives the small units of N-protein to assemble into larger stable oligomers (Ferron et al., 2011).

Plant production resulted in protein yields ranging from 500 – 558 µg/g fresh leaf material. These yields are much higher than those obtained in transgenic *Arabidopsis* 3.8 µg/g of fresh weight (Kalbina et al., 2016). An average soluble protein concentration of 0.450 mg/mL was obtained. These yields are comparable to those

that have been achieved from recombinantly-produced in *E. coli* - 0.34 – 0.71 mg/mL (Fafetine et al., 2007; Jansen van Vuren et al., 2007). Moreover, the plant-produced N-protein was soluble and stable, and apparently much more suitable than other products for use in the validated ELISA.

Mass spectrometry confirmed the monomeric protein to be RVF N-protein. The variability in oligomeric state of N-protein is consistent with previous studies of the protein expressed in *E. coli*, mammalian cells or baculovirus cells. The OD_{260nm}/OD_{280nm} ratio of the protein fractions was 1.9, indicating that the protein co-eluted with nucleic acids, presumably with RNA from the expression host. TEM images revealed distinct 10 nm ring shaped structures similar to those visualised in an *E. coli* expression system; this phenomenon has not been previously demonstrated in plants. The N-protein associates with RNA to form stable ring-shaped structures (Ferron et al., 2011).

The ability of plant-produced N-protein to differentiate between infected and uninfected sera was assessed by testing samples of RVFV-infected or uninfected sheep (Williams et al., 2011). The objective of the cut-off value is to be able to distinguish between serum from animals or individuals which are infected from those that are not infected with RVFV. The plant-produced N-protein could detect 100% of the known positive sera with a rage of PP values from 53.05 – 186.88 and 40% of the known negative sera as negative with PP value rage of -1.5 – 3.36 and 60% were regarded as false positive with PP values range from 4.05 – 37.78. The first protein batch which contained very few plant contaminating proteins showed 100% specificity for the positive sera and 95% of the negative sera. The recombinant antigen bound readily to the ELISA plates and generated minimal background. The second and the third batches of recombinant N-protein showed 100% specificity in detecting the positive sera and 30% and 20%, respectively, of the negative sera as negative, thus conferring low sensitivity on the assay. False positives possibly arise from the background absorbance of the plant proteins, which may react with plant-specific antibodies in the sheep serum. One of the limitations of the *6xhis* tag is co-purification of the target protein with contaminating proteins which have an external His residue. The presence

of co-purified contaminating plant proteins with batches 2 and 3 of N-protein could explain the false positives from the non-infected animal sera. This phenomenon was shown to be a major obstacle in the purification of human SERCA2a cardiac isoform expressed in *Saccharomyces cerevisiae* (Antaloae et al., 2013), as well as the purification of Gn ectodomain in insect cells which resulted in co-purification of contaminating proteins (de Boer et al., 2010). This was resolved by replacing the C-terminal 6xhis tag with three Strep-tags (van der Wal et al., 2012).

My preliminary data indicates that the antigen could differentiate clearly between most of the sera from infected and non-infected animals – see Figure 6.6 A. This could be attributed to the purity of the N-protein preparation, as protein purity significantly reduced the sensitivity of plant-produced N-protein to the negative sera. Similarly to other recombinantly produced N-protein in other expression systems, purity of the antigen affects sensitivity and specificity of the ELISA. High protein purity is required for high specificity and sensitivity (Paweska et al., 2005a). Results of ELISA from recombinant N-protein with high purity levels have been shown to display high sensitivity and specificity (Fafetine et al., 2007; Jansen van Vuren et al., 2007). However, this preliminary study indicated that the plant-produced N-protein is certainly able to detect RVFV-specific antibodies. The problem of contaminating protein in this case can be resolved by eluting the protein off the column with increase in imidazole concentration (see Figure 6.6 A) instead of elution at 30% imidazole during batch purification. The problem of raised PP values for negative sera could be potentially addressed by absorbing all serum samples with a standard clarified soluble plant extract prior to testing with plant-produced N-protein, as this has proved highly effective in (for example) detection of antibodies to plant viruses (Rybicki, 1984 – PhD Thesis).

Recombinant proteins lack infectivity and are generally very stable (Wade-Evans et al., 1993), and are therefore good candidates for use as reagents. With a view to the plant-produced N-protein being a potential candidate for use as a reagent in a routine ELISA, the stability of the protein over time was assessed at various temperatures by assessing the degree of degradation on a western blot. Viewed as monomeric and

dimeric bands, the N-protein remained stable at various temperatures with 4˚C being the optimal storage temperature for 8 months. Interestingly, hexamers were formed over time when stored at RT and 4˚C, viewed as a 140 kDa band. The protein remained mainly as monomers and dimers when stored at -20˚C and -80˚C but formation of hexamers was detected at a later stage compared to samples stored at RT and 4°C.

An advantage of a plant-produced antigen is that it does not have biosafety requirements for its production, which is important for diagnostic laboratories that are not equipped with high levels of biosafety containment facilities. Other advantages of using the plant-produced N-protein as an antigen include stability, as the antigen could still accurately distinguish between infected and non-infected sera after one year of storage. The ELISA technique is ideal for developing countries as they are low-cost, sensitive and not time consuming, thus suitable for general surveillance as well as monitoring during large RVFV outbreaks. In addition, this ELISA is DIVA-compliant. These advantages make a significant contribution to the One Health approach towards controlling RVFV.

Chapter 7: Conclusion

7.1 Conclusion

Although RVFV is preventable by vaccination, there is currently no commercially available vaccine for use outside endemic areas, as these vaccines are comprised of inactivated or live-attenuated virus, and the possibility of live virus escaping remains a problem. It is therefore imperative that we continue to search for safe and economically viable alternative vaccine candidates, especially in Africa. An ideal vaccine candidate would be in line with the One Health approach; thus, it should be able to prevent the spread of the disease by protecting animals and humans, and must be DIVA compliant. Various recombinant vaccines have been developed in different expression systems (Bird et al., 2008; Bird et al., 2011; Boshra et al., 2011; Dulal et al., 2016; Dungu et al., 2010; Kortekaas et al., 2014; Liu et al., 2013b). However, even though these vaccine candidates have been developed and shown to be immunogenic, they are not commercially available. The research conducted in this study was successful in achieving its primary aims, which was to identify recombinant RVFV gene/s suitable for the development of a transiently-produced candidate vaccine in plants. The initial immunogenic candidate/s tested however, were wild type RVFV GnGc or Gn genes expressed in plants, which expression was not successful; further investigation showed that the expression of these particular genes required extensive modification in order to facilitate expression in *N. benthamiana.*

The RVFV glycoproteins have proven to be extremely difficult proteins to work with. Initially, I was unable to express the wild type RVFV GnGc as the gene seemed to be toxic in bacterial host cells; this could possibly be attributed to low-level leaky transcription and translation of toxic protein products.

New strategies were developed to encourage or facilitate protein expression, those included testing expression of codon optimised genes instead, as previously

demonstrated by de Boer et al., who showed that RVFV gene expression in insect cells was only achieved using insect cell codon optimised genes (de Boer et al., 2010) and by Mandell et al who showed that mammalian cell codon optimised genes expressed in mammalian cells (Mandell et al., 2010b). Nonetheless, expression of the full-length *GnGc in planta* was unsuccessful, irrespective of codon optimisation and choice of plant expression vector, which are known to play a role in recombinant protein expression. Even with the focus solely on the immunogenic Gn glycoprotein, recombinant protein expression with the native signal peptide and TMD/CT was also unsuccessful. It was postulated that the TMD/CT might be interfering with the expression because of incompatibility with plant secretion machinery, and therefore expression of Gn with either a heterologous LPH or PDI signal peptide without its native TMD/CT (truncated) was tested. Interestingly, the construct encoding truncated Gn with the plant-derived PDI signal peptide did not result in detectable expression, but the construct encoding truncated Gn with the mouse-derived LPH signal peptide was successful in directing expression of the protein. This was probably due to a mouse-derived signal sequence allowing for sufficient ribosomal translocation during protein translation in plant host cells, thus being more compatible with accumulation of the protein. Plant-codon optimisation was also favoured more than human-codon optimisation as far as enhancement of expression levels was concerned.

Recombinant protein expression levels can be significantly increased by the choice of expression vector. Thus, the recombinant *A. tumefaciens* with modified Gn constructs were transiently expressed in *N. benthamiana* and compared the pEAQ-*HT* vector which targets protein expression to the cytoplasm and with the pTRAc-ERH vector which targets expression ER. Expression was further optimised by investigating longer expression times. Highest expression was found to be at 5 dpi, the pEAQ-*HT* vector gave higher protein expression levels compared to pTRAc vector, and it was thus selected for further studies. An affinity chromatography purification protocol was subsequently developed, which gave sufficient protein yields for testing in animals. Immunisation studies revealed that the subunit Gn-derived vaccine candidate was weakly immunogenic in mice.

In order to investigate whether a chimaeric RVFV vaccine candidate could be made in plants, the H5N1 influenza virus HA TMD/CT was fused to the plant-codon optimised modified Gn. Recombinant protein was transiently expressed by *Agrobactrium*-mediated transfer in plants, expression was detectable using the pEAQ-*HT* vector and no expression was observed in the pTRAc suite vectors; this confirmed again that the native signal peptide and TMD/CT of Gn affects recombinant protein expression, since no protein expression was detectable in the constructs with the native signal peptide and TMD/CT. The choice of vector played a significant role, allowing for successful expression of recombinant protein. Discontinuous iodixanol gradients were investigated for the purification of putative chimaeric RVFV Gn particles. Particle characterisation by TEM showed what appeared like capsomers, and consistent recovery of particles which were morphologically different to those produced in other expression systems. The heterologous influenza TMD/CT apparently allowed for the formation of chimaeric RVFV particles. Immunisation studies revealed that the chimaeric vaccine candidate was highly immunogenic compared to the soluble protein vaccine candidate. Despite the immunogenicity of the candidate vaccines being observed, the yields of the vaccines were very low, which meant that doses were low (~5 µg). It is possible that the immune response could be increased by higher doses and use of adjuvant.

The RVFV N-protein intended for use as a diagnostic reagent was human-codon optimised and successfully transiently produced in *N. benthamiana* by *Agrobacterium*-mediated transfer. Optimal expression levels were at three dpi. Recombinant protein was purified by affinity chromatography and characterised by TEM. TEM demonstrated ring-shaped particles of ~10 nm range produced *in planta*. Preliminary data from ELISAs indicates that the reagent is functional and it is highly successful at differentiating between infected and non-infected animal serum, and that this could be a viable product.

To the best of our knowledge, this is the first study demonstrating the successful production by *Agrobacterium*-mediated transient heterologous expression in *N. benthamiana* of recombinant RVFV Gn and chimaeric Gn particles as vaccine

candidates, as well as of N-protein as a diagnostic reagent. The vaccine candidates were tested in mice and were shown to be immunogenic. The N-protein antigen was functional as it could distinguish between infected and non-infected animal sera.

7.2 Future work

Having successfully managed to produce recombinant plant-produced RVFV glycoprotein vaccine candidates, it is possible that protein yields could be significantly improved by fusing the modified *Gn* with histidine or strep tags to facilitate purification. A recombining *E. coli* plasmid can be made by cloning the modified *Gn* gene into a bacterial expression vector. This would allow for further analysis of humoral antibody response from the mice sera.

Now that I have shown that *Gn* can be expressed on its own, in order to produce a vaccine candidate with the full GnGc polyprotein, modified *Gn* and *Gc* could be cloned into pEAQ-*HT* and simultaneously expressed by co-expression. Alternatively, these could be co-expressed using the pEAQ-*HT* dual expression system. Expression could also potentially be enhanced by co-expression with chaperones.

With the preliminary ELISAs using the plant-produced N-protein antigen showing functionality, the possibility of using this protein as a diagnostic reagent appears bright. The antigen needs to be validated further, however, by testing larger panels of positive and negative animal serum.

Chapter 8: Appendix

Appendix A: Gene alignment between wild type, plant- and human-codon optimised sequences

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CLUSTAL O(1.2.1) multiple sequence alignment


GnGc Human      ATGG----------------CTGGAATCGCTATGACCGTGCTGCCTGCTCTGGCCGTGTTC
GnGc WT         ATGGCTGGAATCGCTATGACTGTGCTTCCAGCTCTTGCTGTGTTTGCTCTTG--------
GnGc Plant      ATGGCTGGAATCGCAATGACTGTTCTCCCTGCTC------TCGCTGTTTTCG--------
                ****               ***   *  *           *   ** * * *

GnGc Human      GCTCTGGCTCCTGTCGTGTTCGCTGAAGACCCCCACCTGCGGAACAGGCCAGGCAAAGGG
GnGc WT         -CTCCAGTT------GTGTTCGCTGAGGATCCACATCTTAGGAACAGACCAGGTAAGGGC
GnGc Plant      -CTCTCGCTCCTGTTGTTTTCGCTGAAGATCCTCACCTTAGGAATAGACCTGGAAAAGGT
                 ***  * *      ** ******** ** ** ** **  **** ** ** ** ** **

GnGc Human      CACAATTACATCGACGGCATGACACAGGAGGATGCCACTTGCAAGCCCGTCACCTATGCA
GnGc WT         CACAACTACATCGATGGCATGACTCAAGAGGATGCTACTTGCAAGCCAGTGACTTACGCT
GnGc Plant      CATAACTATATTGATGGAATGACTCAAGAGGATGCTACTTGTAAGCCAGTGACTTACGCT
                ** ** ** ** ** ** ***** ** ******** ***** ***** ** ** ** **

GnGc Human      GGGGCCTGTAGCTCCTTCGACGTGCTGCTGGAAAAGGGAAAATTCCCCCTGTTTCAGTCC
GnGc WT         GGTGCTTGCTCCTCTTTCGATGTGCTTCTTGAGAAGGGCAAGTTCCCACTCTTCCAGTCT
GnGc Plant      GGAGCATGCTCTTCATTTGATGTTCTTTTGGAGAAGGGTAAATTTCCTTTGTTCCAGTCT
                ** ** **    ** ** ** ** **  * ** ***** ** ** **  * ** *****

GnGc Human      TACGCCCACCATCGCACCCTGCTGGAGGCTGTGCACGATACAATCATTGCTAAGGCAGAC
GnGc WT         TACGCTCATCACAGGACTTTGCTTGAGGCTGTGCACGATACAATTATCGCTAAGGCTGAT
GnGc Plant      TATGCACATCACAGGACATTACTCGAAGCTGTTCATGATACTATTATCGCTAAAGCAGAT
                ** ** ** **  * **  * ** ** ***** ** ***** ** ** ***** ** **

GnGc Human      CCCCCTTCATGCGATCTGCAGAGCGCCCACGGCAACCCTTGTATGAAGGAGAAACTGGTC
GnGc WT         CCACCATCCTGCGATCTTCAATCTGCTCATGGAAACCCATGCATGAAAGAAAAGCTCGTG
GnGc Plant      CCACCTTCCTGTGATTTGCAATCTGCTCACGGAAATCCTTGCATGAAGGAAAAACTTGTG
                ** ** ** ** *** * **    ** ** ** ** ** ** ***** ** ** ** **

GnGc Human      ATGAAAACACACTGCCCAAATGACTACCAGTCCGCTCATTATCTGAACAATGATGGCAAG
GnGc WT         ATGAAGACTCACTGCCCAAACGATTACCAGTCCGCTCACTACCTCAACAACGATGGCAAG
GnGc Plant      ATGAAAACACATTGTCCAAACGATTATCAGAGCGCACACTACTTGAATAACGATGGAAAG
                ***** ** ** ** ***** ** ** ***  *** ** **  * ** ** ***** ***

GnGc Human      ATGGCATCTGTGAAATGTCCACCCAAGTACGAACTGACTGAGGACTGCAACTTTTGTCGG
GnGc WT         ATGGCTTCTGTGAAGTGCCCACCAAAGTACGAGCTTACTGAGGATTGCAACTTCTGCAGG
GnGc Plant      ATGGCTAGTGTTAAATGCCCACCTAAGTACGAGTTGACAGAAGATTGTAACTTCTGCAGA
                *****   *** ** ** ***** ********  * ** ** ** ** ***** **  *

GnGc Human      CAGATGACCGGCGCTTCCCTGAAGAAAGGGTCTTATCCTCTGCAGGATCTGTTCTGCCAG
GnGc WT         CAGATGACTGGCGCTTCTCTTAAGAAGGGATCTTACCCTCTCCAGGATCTCTTCTGCCAG
GnGc Plant      CAAATGACTGGAGCATCACTTAAGAAAGGTAGTTACCCATTGCAAGATTTGTTTTGTCAG
                ** ***** ** ** ** ** ***** **   *** **  * ** *** * ** ** ***

GnGc Human      TCTAGTGAAGACGATGGAAGCAAGCTGAAAACTAAGATGAAAGGAGTGTGCGAAGTGGGA
GnGc WT         TCCTCTGAAGATGATGGCTCCAAGCTCAAGACTAAGATGAAGGGCGTTTGCGAGGTTGGA
GnGc Plant      AGTAGCGAGGATGATGGATCAAAGCTTAAAACAAAGATGAAAGGAGTTTGTGAAGTTGGT
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                      ** ** *****    ***** ** ** ******** ** ** ** ** ** **

GnGc Human       GTCCAGGCCCTGAAGAAATGTGACGGCCAGCTGTCCACCGCACACGAGGTGGTCCCCTTC
GnGc WT          GTGCAGGCTCTCAAGAAATGTGATGGCCAGCTTTCCACTGCTCACGAGGTTGTGCCATTC
GnGc Plant       GTGCAAGCTTTGAAGAAATGCGATGGACAGTTATCTACTGCACATGAAGTTGTGCCTTTT
                 ** ** **  * ******** ** ** *** * ** ** ** ** ** ** ** ** **

GnGc Human       GCCGTGTTTAAGAACTCTAAGAAAGTCTACCTGGACAAACTGGATCTGAAGACCGAGGAA
GnGc WT          GCTGTGTTCAAGAACTCCAAGAAGGTGTACCTCGATAAGCTCGATCTCAAGACTGAGGAA
GnGc Plant       GCTGTTTTCAAAAACTCTAAGAAAGTGTATTTGGATAAACTCGATCTTAAGACTGAAGAG
                 ** ** ** ** ***** ***** ** **  * ** ** ** ***** ***** ** **

GnGc Human       AATCTGCTGCCTGATAGCTTCGTGTGCTTTGAACATAAAGGCCAGTATAAGGGGACTATG
GnGc WT          AACCTCCTCCCAGATTCCTTCGTTTGCTTCGAGCATAAGGGACAGTACAAGGGCACTATG
GnGc Plant       AACCTTTTGCCAGATTCATTTGTTTGTTTCGAGCACAAAGGACAATACAAGGGTACTATG
                 ** **  * ** ***   ** ** ** ** ** ** ** ** ** ** ***** ******

GnGc Human       GACAGCGGCCAGACCAAAAGAGAGCTGAAGTCCTTTGATATCTCTCAGTGCCCAAAGATT
GnGc WT          GATTCCGGACAGACTAAGCGTGAGCTTAAGTCCTTCGATATCTCCCAGTGCCCAAAGATT
GnGc Plant       GATAGTGGTCAGACTAAAAGGGAACTTAAGTCATTCGATATCAGTCAATGTCCTAAGATA
                 **    ** ***** **  * ** ** ***** ** ******   ** ** ** *****

GnGc Human       GGCGGGCACGGATCAAAGAAATGTACTGGCGACGCCGCTTTCTGCAGCGCATACGAGTGT
GnGc WT          GGAGGACACGGCTCTAAAAAGTGCACTGGTGATGCTGCTTTCTGCTCCGCTTATGAGTGC
GnGc Plant       GGAGGTCATGGATCCAAGAAATGCACTGGAGATGCTGCATTTTGTTCTGCATATGAATGC
                 ** ** ** ** ** ** ** ** ***** ** ** ** ** **    ** ** ** **

GnGc Human       ACCGCCCAGTACGCCAACGCTTATTGCAGTCATGCTAATGGATCAGGCGTGGTCCAGATC
GnGc WT          ACTGCTCAGTACGCTAACGCTTACTGCTCTCACGCTAACGGATCTGGTGTGGTGCAGATT
GnGc Plant       ACTGCTCAGTATGCTAATGCATACTGTAGCCACGCAAACGGATCCGGTGTTGTGCAAATT
                 ** ** ***** ** ** ** ** **    ** ** ** ***** ** ** ** ** **

GnGc Human       CAGGTGTCCGGAGTCTGGAAGAAACCACTGTGCGTGGGCTATGAACGAGTGGTCGTGAAA
GnGc WT          CAAGTTTCCGGCGTGTGGAAGAAGCCACTCTGCGTTGGATATGAGAGGGTTGTGGTGAAG
GnGc Plant       CAGGTTTCTGGAGTGTGGAAGAAACCTTTGTGCGTGGGTTACGAGAGAGTTGTGGTTAAA
                 ** ** ** ** ** ******** **  * ***** ** ** **  * ** ** ** **

GnGc Human       CGGGAGCTGTCTGCCAAGCCTATCCAGCGGGTGGAACCATGCACCACATGTATTACAAAA
GnGc WT          AGGGAACTCTCCGCTAAGCCAATTCAAAGGGTTGAGCCATGCACTACTTGCATCACTAAG
GnGc Plant       AGGGAACTTTCAGCTAAGCCTATTCAAAGAGTTGAGCCATGTACTACATGCATCACAAAA
                  **** ** ** ** ***** ** **  * ** ** ***** ** ** ** ** ** **

GnGc Human       TGCGAGCCCCACGGCCTGGTCGTGAGAAGTACTGGGTTCAAGATCTCAAGCGCAGTGGCA
GnGc WT          TGCGAGCCACACGGACTTGTTGTGAGGTCTACTGGCTTCAAGATCTCCTCTGCTGTTGCT
GnGc Plant       TGTGAACCACATGGATTGGTGGTTAGGTCTACTGGTTTTAAGATTTCCTCTGCTGTTGCA
                 ** ** ** ** **  * ** ** **   ****** ** ***** **    ** ** **

GnGc Human       TGCGCTTCAGGCGTGTGCGTGACCGGAAGTCAGTCACCTAGCACCGAGATCACACTGAAA
GnGc WT          TGCGCTTCTGGTGTTTGCGTTACAGGATCTCAGTCCCCATCCACTGAGATCACTCTTAAG
GnGc Plant       TGTGCTTCTGGAGTTTGCGTGACTGGTAGCCAATCCCCTTCTACTGAAATAACACTTAAG
                 ** ***** ** ** ***** ** **    ** ** **    ** ** ** ** ** **

GnGc Human       TACCCAGGAATTAGCCAGTCCTCTGGAGGCGACATTGGCGTGCACATGGCACATGACGAT
GnGc WT          TACCCTGGCATCTCCCAGAGTTCCGGTGGTGATATTGGAGTTCACATGGCTCACGATGAT
GnGc Plant       TATCCAGGAATCTCACAGTCAAGTGGAGGAGATATTGGTGTTCATATGGCACACGATGAT
                 ** ** ** **    ***      ** ** ** ***** ** ** ***** ** ** ***

GnGc Human       CAGTCCGTGAGTTCAAGATCGTCGCCCACTGCCCTCCACAGGATCCCTGTCTGGTGCAC
GnGc WT          CAGTCCGTGTCCAGTAAGATTGTGGCTCATTGCCCACCACAGGATCCATGCCTTGTTCAT
```

```
GnGc Plant      CAAAGTGTTAGCTCCAAGATTGTGGCTCATTGTCCACCTCAGGATCCATGCCTTGTTCAT
                **    **       ***** ** ** ** ** ** ** ******** ** ** ** **

GnGc Human      GGATGCATCGTCTGTGCCCACGGCCTGATTAACTACCAGTGCCATACAGCACTGTCCGCC
GnGc WT         GGATGTATTGTGTGCGCTCACGGCCTCATTAACTACCAGTGTCACACTGCACTCTCCGCA
GnGc Plant      GGATGTATAGTGTGCGCTCACGGTCTCATTAATTATCAATGTCACACAGCACTTTCTGCT
                ***** ** ** ** ** ***** ** ***** ** ** ** ** ** ***** ** **

GnGc Human      TTTGTCGTGGTCTTCGTGTTTAGCTCCGTCGCTATCATTTGCCTGGCAATCCTGTATAAG
GnGc WT         TTCGTTGTGGTGTTCGTGTTCTCCTCCGTGGCTATTATTTGCCTCGCTATCCTCTACAAG
GnGc Plant      TTTGTGGTTGTGTTTGTTTTCTCTTCAGTGGCTATTATTTGTCTCGCTATCCTTTACAAG
                ** ** ** ** ** ** **    ** ** ***** ***** ** ** ***** ** ***

GnGc Human      GTCCTGAAATGTCTGAAGATTGCCCCCAGGAAGGTGCTGGACCCTCTGATGTGGATCACC
GnGc WT         GTGCTCAAGTGCCTCAAGATCGCTCCTAGGAAGGTGCTCGATCCACTCATGTGGATCACT
GnGc Plant      GTGTTGAAGTGCCTCAAGATTGCTCCTAGAAAAGTTCTCGATCCACTTATGTGGATCACT
                **  * ** ** ** ***** ** ** ** ** ** ** ** ** ** ***********

GnGc Human      GTGTTCATTCGCTGGGTCTACAAGAAAATGGTGGCCAGAGTGGCAGATAACATCAATCAG
GnGc WT         GTGTTCATCCGTTGGGTGTACAAGAAAATGGTGGCTAGGGTGGCAGATAACATCAACCAG
GnGc Plant      GTTTTTATTAGATGGGTGTATAAGAAAATGGTTGCAAGGGTGGCTGATAATATCAACCAA
                ** ** **  * ***** ** ********** ** ** ***** ***** ***** **

GnGc Human      GTGAACCGCGAAATTGGATGGATGGAGGGAGGACAGCTGGCCCTGGGAAATCCAGCTCCT
GnGc WT         GTGAACCGTGAGATCGGATGGATGGAAGGTGGACAACTTGCTCTTGGAAATCCAGCTCCA
GnGc Plant      GTTAATAGGGAGATAGGATGGATGGAAGGAGGACAGTTGGCATTAGGTAACCCAGCTCCT
                ** **  * ** ** ********** ** *****  * **  * ** ** ********

GnGc Human      ATCCCACGGCACGCTCCCATTCCTAGATACTCCACTTATCTGATGCTGCTGCTGATCGTG
GnGc WT         ATTCCAAGGCACGCTCCTATCCCAAGGTACTCTACTTACCTCATGCTCCTTTTGATCGTG
GnGc Plant      ATTCCAAGACATGCACCTATCCCAAGGTACTCTACATACTTGATGCTCCTCCTTATAGTT
                ** *** * ** ** ** ** ** ** ***** ** **  * ***** **  * ** **

GnGc Human      TCATATGCAAGCGCCTGCTCCGAACTGATCCAGGCCAGCAGCCGGATTACTACCTGCAGC
GnGc WT         TCCTACGCTTCTGCTTGCTCTGAGCTTATCCAGGCTTCCTCCAGGATTACTACTTGCTCT
GnGc Plant      TCCTACGCATCTGCTTGTTCAGAGCTTATACAAGCTAGTAGCAGAATTACTACATGTTCT
                ** ** **    ** ** ** ** ** ** ** **      * * ******** **

GnGc Human      ACAGAGGGGGTGAATACTAAGTGTCGACTGAGCGGAACCGCCCTGATTCGAGCTGGATCC
GnGc WT         ACTGAGGGCGTGAACACAAAGTGCAGGTTGTCTGGAACTGCACTCATCAGGGCTGGATCT
GnGc Plant      ACAGAAGGAGTTAATACTAAGTGCAGATTATCTGGTACTGCACTCATTAGGGCTGGATCA
                ** ** ** ** ** ** *****  *  *    ** ** ** ** **  * ********

GnGc Human      GTCGGAGCTGAAGCATGCCTGATGCTGAAAGGCGTGAAGGAGGACCAGACAAAATTTCTG
GnGc WT         GTTGGAGCTGAAGCTTGCCTTATGCTTAAGGGTGTGAAAGAGGATCAGACTAAGTTCCTT
GnGc Plant      GTGGGTGCAGAGGCTTGTCTCATGCTTAAGGGAGTTAAGGAAGATCAAACAAAGTTCTTG
                ** ** ** ** ** ** ** ***** ** ** ** ** ** ** ** ** ** **  *

GnGc Human      AAGATCAAAACCGTGAGCAGCGAACTGAGCTGTCGGGAGGGGCAGTCCTACTGGACCGGA
GnGc WT         AAGATCAAGACTGTGTCCTCCGAGCTTTCTTGCAGAGAGGGACAGTCTTATTGGACTGGC
GnGc Plant      AAGATTAAAACTGTTTCCTCTGAGTTATCTTGCAGAGAAGGACAGTCATATTGGACAGGT
                ***** ** ** **   *   **  *    **  * ** ** ***** ** ***** **

GnGc Human      TCCTTCTCTCCTAAGTGCCTGTCCTCTCGGAGATGTCACCTGGTGGGGGAATGCCATGTC
GnGc WT         TCCTTCAGTCCAAAGTGCCTCTCATCTAGAAGGTGCCACCTTGTTGGAGAGTGCCACGTT
GnGc Plant      TCTTTTTCTCCTAAGTGTCTCTCAAGTAGAAGGTGCCATCTTGTTGGAGAGTGTCACGTG
                ** **   *** ***** ** **   * * ** ** ** ** ** ** ** ** ** **

GnGc Human      AACAGATGTCTGTCCTGGAGGGATAATGAAACAAGTGCCGAGTTCTCATTTGTGGGCGAG
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GnGc WT          AACAGATGCCTCAGTTGGAGGGATAACGAGACTTCCGCTGAGTTCTCATTCGTGGGCGAG
GnGc Plant       AATAGATGCTTGTCTTGGAGGGATAACGAGACTAGCGCTGAATTTTCCTTCGTTGGTGAA
                 ** *****  *    ********** ** **    ** ** ** ** ** ** ** **

GnGc Human       TCTACAACTATGAGAGAAAACAAGTGCTTTGAGCAGTGTGGCGGGTGGGGGTGCGGATGT
GnGc WT          TCTACTACTATGCGTGAGAACAAGTGTTTCGAGCAGTGTGGTGGTTGGGGATGCGGATGC
GnGc Plant       TCTACTACAATGAGGGAGAATAAGTGTTTTGAACAATGCGGAGGTTGGGGATGTGGTTGC
                 ***** ** *** * ** ** ***** ** ** ** ** ** ** ***** ** ** **

GnGc Human       TTCAACGTGAATCCATCTTGCCTGTTTGTCCACACCTATCTGCAGAGTGTGCGCAAAGAA
GnGc WT          TTCAATGTTAACCCTTCTTGCCTCTTCGTGCACACTTACCTTCAGTCTGTGAGGAAAGAG
GnGc Plant       TTTAATGTTAACCCAAGTTGTTTGTTCGTGCATACTTACTTACAGAGCGTTAGAAAAGAG
                 ** ** ** ** **   ***  * ** ** ** ** ** **  * ***   **  * *****

GnGc Human       GCCCTGCGAGTCTTCAATTGTATCGACTGGGTGCATAAGCTGACTCTGGAGATTACCGAC
GnGc WT          GCTCTCAGGGTTTTCAACTGCATCGATTGGGTGCACAAGCTCACTCTCGAGATCACTGAT
GnGc Plant       GCTTTGAGGGTGTTTAATTGCATCGATTGGGTTCACAAGTTGACTCTCGAAATCACAGAT
                 **  *  * ** ** ** ** ***** ***** ** *** * ***** ** ** ** **

GnGc Human       TTTGATGGCAGCGTGAGCACCATCGACCTGGGGGCTAGTTCAAGCAGGTTCACAAACTGG
GnGc WT          TTCGATGGCTCCGTGTCTACTATCGATCTCGGCGCTTCTTCCTCCCGTTTCACTAATTGG
GnGc Plant       TTCGATGGAAGCGTGTCCACAATTGATCTTGGTGCAAGCTCCTCTAGGTTTACTAATTGG
                 ** *****   ****   ** ** ** ** ** ** **    **     * ** ** ** ***

GnGc Human       GGCAGCGTGTCCCTGTCTCTGGATGCAGAAGGCATCAGTGGGTCAAATAGCTTCTCCTTT
GnGc WT          GGATCCGTGTCCTTGTCTCTCGATGCTGAGGGAATCTCTGGCTCCAACTCCTTCTCCTTC
GnGc Plant       GGATCTGTTTCACTCAGTCTTGATGCTGAGGGAATCAGCGGTTCCAACTCTTTTTCATTC
                 **    ** **  *   *** ***** ** ** ***   ** ** **    ** ** **

GnGc Human       ATTGAGAGCCCTGGCAAAGGGTACGCCATCGTGGATGAACCATTCTCCGAGATTCCCAGA
GnGc WT          ATCGAATCACCTGGAAAGGGCTACGCTATCGTGGATGAGCCATTCTCTGAGATTCCTAGG
GnGc Plant       ATAGAATCACCTGGAAAAGGTTATGCTATAGTTGATGAGCCTTTTAGTGAAATTCCAAGA
                 ** **    ***** ** ** ** ** ** ** ***** ** **    ** ***** **

GnGc Human       CAGGGATTTCTGGGCGAAATTAGGTGCAACTCTGAGTCCTCTGTGCTGAGCGCCCACGAG
GnGc WT          CAGGGATTCCTCGGAGAGATTAGGTGCAACTCTGAGTCCTCTGTGTTGTCCGCTCATGAG
GnGc Plant       CAAGGATTCTTAGGAGAGATTAGGTGTAATTCAGAATCAAGTGTTCTTTCTGCACATGAA
                 ** *****  * ** ** ******** ** ** ** **   ***  *    ** ** **

GnGc Human       TCCTGTCTGCGAGCACCAAACCTGATCAGCTATAAGCCCATGATTGACCAGCTGGAATGC
GnGc WT          TCTTGTCTTAGGGCTCCAAACCTCATCTCCTACAAGCCAATGATTGATCAGCTCGAGTGC
GnGc Plant       TCATGCCTTAGAGCTCCTAATTTGATCTCTTACAAGCCAATGATAGATCAACTCGAGTGT
                 ** ** **  * ** ** **  * ***   ** ***** ***** ** ** ** ** **

GnGc Human       ACCACAAATCTGATCGATCCCTTCGTGGTCTTTGAGAGGGGCTCTCTGCCTCAGACCCGC
GnGc WT          ACTACTAACCTCATCGATCCATTCGTGGTTTTCGAGAGGGGATCTTTGCCACAGACTAGG
GnGc Plant       ACTACAAACCTTATTGATCCTTTTGTTGTGTTCGAAAGAGGTTCTCTTCCACAGACAAGG
                 ** ** ** ** ** ***** ** ** ** ** ** ** ** ** *** * ** *****  *

GnGc Human       AACGACAAAACATTCGCAGCCAGTAAGGGGAATAGAGGAGTCCAGGCATTTTCTAAGGGC
GnGc WT          AACGATAAGACTTTCGCTGCTTCCAAGGGAAACAGGGGAGTTCAGGCTTTCTCTAAGGGA
GnGc Plant       AATGATAAAACTTTTGCTGCATCTAAGGGAAACAGGGGTGTGCAAGCATTCAGTAAGGGA
                 ** ** ** ** ** ** **    ***** ** ** ** ** ** ** **   ******

GnGc Human       AGTGTGCAGGCCGACCTGACACTGATGTTCGACAATTTTGAAGTGGATTTCGTCGGGGCT
GnGc WT          TCTGTGCAGGCTGATCTCACTCTCATGTTCGATAATTTCGAAGTGGATTTCGTGGGAGCT
GnGc Plant       AGCGTTCAGGCTGATTTGACACTCATGTTCGATAACTTCGAGGTTGATTTTGTGGGTGCT
                    ** ***** **  * ** ** ******** ** ** ** ** ***** ** ** ***
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GnGc Human      GCAGTGTCATGCGATGCCGCTTTTCTGAACCTGACTGGGTGCTACAGCTGTAATGCCGGC
GnGc WT         GCTGTGTCTTGTGATGCAGCATTCCTTAACCTCACTGGCTGCTACTCTTGCAATGCTGGT
GnGc Plant      GCAGTGTCATGTGATGCTGCATTCCTCAATCTTACTGGATGTTATAGTTGCAACGCAGGT
                ** ***** ** ***** ** ** ** ** ** ***** ** **    ** ** ** **

GnGc Human      GCCAGAGTGTGCCTGTCAATCACTAGCACCGGAACAGGCACTCTGTCTGCCCACAACAAA
GnGc WT         GCTAGGGTTTGCCTCTCCATCACTTCTACTGGAACTGGCACACTCTCTGCACACAACAAG
GnGc Plant      GCTAGAGTTTGCCTCTCCATTACTTCTACAGGAACTGGTACATTGAGTGCTCATAATAAG
                ** ** ** ***** ** ** ***   ** ***** ** **  *   *** ** ** **

GnGc Human      GACGGAAGTCTGCATATTGTGCTGCCCTCAGAGAATGGCACAAAGGATCAGTGCCAGATC
GnGc WT         GATGGTTCTCTCCACATCGTGCTCCCATCTGAGAACGGAACTAAGGATCAGTGCCAGATC
GnGc Plant      GATGGAAGCTTGCACATCGTTTTACCTTCCGAAAACGGTACAAAGGATCAATGTCAGATT
                ** **     * ** ** **  * ** ** ** ** ** ** ******** ** *****

GnGc Human      CTGCATTTCACTGTGCCAGAAGTCGAGGAAGAGTTTATGTATTCCTGTGACGGCGATGAG
GnGc WT         CTCCACTTCACTGTTCCAGAAGTGGAAGAGGAATTCATGTACTCATGTGATGGTGATGAA
GnGc Plant      TTGCATTTTACTGTTCCAGAGGTGGAAGAGGAATTCATGTACTCATGCGATGGAGATGAA
                 * ** ** ***** ***** ** ** ** ** ** ***** ** ** ** ** *****

GnGc Human      CGCCCACTGCTGGTGAAGGGGACCCTGATCGCCATTGACCCCTTCGACGATAGGCGCGAA
GnGc WT         CGTCCACTCCTCGTGAAGGGTACTCTCATTGCTATCGATCCTTTCGATGATAGGCGTGAG
GnGc Plant      AGACCATTGTTAGTTAAAGGTACACTTATCGCAATAGATCCATTTGATGATAGAAGGGAG
                 * *** *  * ** ** ** ** ** ** ** ** ** ** ** ** ** *****  * **

GnGc Human      GCTGGAGGCGAGAGCACAGTGGTCAACCCTAAATCTGGCAGTTGGAATTTCTTTGATTGG
GnGc WT         GCTGGTGGTGAGTCTACTGTTGTTAACCCAAAGTCCGGCTCCTGGAACTTCTTCGATTGG
GnGc Plant      GCTGGTGGTGAATCTACTGTTGTGAATCCTAAAAGTGGAAGCTGGAACTTTTTCGATTGG
                ***** ** **    ** ** ** ** ** **    **    ***** ** ** ******

GnGc Human      TTCTCTGGGCTGATGAGTTGGTTTGGGGGACCACTGAAGACCATCCTGCTGATTTGCCTG
GnGc WT         TTCTCTGGACTTATGTCCTGGTTCGGAGGCCCTCTTAAGACTATTCTCCTCATTTGCCTC
GnGc Plant      TTTAGTGGTTTGATGAGCTGGTTCGGAGGTCCATTAAAGACTATTCTCCTTATCTGTCTC
                **   ***  * ***   ***** ** ** **  * ***** ** ** ** ** ** **

GnGc Human      TACGTGGCCCTGAGCATCGGCCTGTTCTTTCTGCTGATCTACCTGGGGAGAACTGGGCTG
GnGc WT         TATGTGGCTCTCTCTATCGGCCTCTTCTTCTTGCTTATCTACCTCGGAAGGACTGGCCTC
GnGc Plant      TATGTTGCACTTTCTATTGGTTTGTTTTTCTTGCTCATCTACTTAGGAAGAACAGGTCTC
                ** ** ** **    ** **  * ** **  **** ****** * ** ** ** ** **

GnGc Human      TCCAAAATGTGGCTGGCCGCTACAAAAAAGGCTTCATGACCCGGGGAGCTCCTCGAG
GnGc WT         TCCAAGATGTGGCTTGCTGCTACTAAGAAGGCTTCC----------------------
GnGc Plant      TCAAAGATGTGGCTTGCTGCAACTAAGAAAGCTAGTTAAAGGCCTCCCGGGAA----
                ** ** ******** ** ** ** ** ** ***
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Figure 1: Alignment of nucleotide sequence between Human-codon optimised (upper), native (middle) and plant-codon optimised genes. Mismatched nucleotides are marketed with …

Percent Identity Matrix - created by Clustal2.1

```
     1: GnGc Human  100.00   77.31   71.37
     2: GnGc WT      77.31  100.00   79.50
     3: GnGc Plant   71.37   79.50  100.00
```

Appendix B: Polymerase chain reaction primer sequences

Table 1: Primer sequences for WT strain RVFV Gn and GnGc glycoprotein gene

Primers name	Primer sequence (5' – 3')	Tm (˚C)	Base pairs	Enzyme site
G1 Fw	ATGGCAGGGATTGCAATGACA	50.9	21	-
RVFV Rev	TGATGCATATGAGACAATCAA	56	21	-
G2 Rev	ACATATTACTTTTACACGTAC	43.0	22	-
G1 Fw979	GGGGTTCAAGCACACAAAAAG	50.9	21	-
G1 Rev1023	CTTTGAGTTCTTAAACACTGC	47.0	21	-
G2 Fw2693	ATCTCAGGCTCAATAGCTT	46.3	20	--
G2 Rev2749	GAACCCTTGCCGAGGAATTTC	52.8	21	-
Gn SmaI Fw	AA**ACCCGGG**ATGGCAGGGATT	54	22	*Sma*I
Gn XmaI Rev	AA**CCCCGGG**TGATGCATATGA	50.4	22	*Xma*I
GnGc XmaI Rev	TT**CCCGGG**ACATATTACTTTTACACGTAC	63.3	29	*XmaI*
Gn XhoI Rev	TT**CTCGAG**TGCTGATGCATATGAGACAATCAATA ATAACAT	59.3	41	*Xho*I
Gc XhoI Rev	TT**CTCGAG**ACATATTACTTTTACACGTA	51.7	28	*Xho*I
GnGc XhoI Rev	TT**CTCGAG**ACATATTACTTTTACACGTA	58.8	28	*XhoI*
GnGc NruI Fw	AA**TCGCGA**ATGGCAGGGATTGCAATGACA	59.4	29	*NruI*
Gn NruI Fw	AA**TCGCGAA**TGGCAGGGATTGCAATGACA	50.4	29	*Nru*I
Gn XmaI Rev	TT**CCCGGG**TGCTGATGCATATGAGACAATCAATA ATAACAT	61.3	41	*Xma*I
Gc XmaI Rev	TT**CCCGGG**ACATATTACTTTTACACGTAC	56.2	29	*Xma*I
GnStuI Rev	TTCCCGGGAGGCCTTTAAGATGCGTAGGAAA	64.3	31	*StuI*
Gn NcoI Fw	AA**CCATG**GGCAGGGATTGCAATGACAGT	69.9	28	*Nco*I

Gc AgeI Fw	TTTACCGGTGCCTAGGATGTCAGA	66.9	27	*AgeI*
M13 Fw	CGCCAGGGTTTTCCCAGTCACGAC		24	
M13 Rev	GAGCGGATAACAATTTCACACAGG		24	
pEAQ Fw	GACGAACTTGGAGAAAGATTGTTAAGC	61.2	27	
pEAQ Rev	GACCGCTCACCAAACATAGAAATG	60.6	24	
pTRAc Fw	AACCATGGCAGTGTGGCTGTCTACG	58.9	25	
pTRAc Rev	AATCTAGACTCGAGTTAAGTACGTCTCTTGCGTTTAGATG	61.3	40	

Table 2: Primer sequence for plant- and human-codon optimised Gn and GnGc glycoproteins

Primers	Primer sequence (5' to 3')	Tm (˚C)	Bases pairs	Enzyme site
GnNcoI Fw	TTTTT**CCATGG**CTGGAATCGCAATGACTGTT	57.7	31	*Nco*I
Fw961	AAGTTTCCGGCGTGTGGAAG	60.2	21	-
Fw2161	CTCTCAGGGTTTTCAACTGC	56.2	20	-
Rev2221	AGTAGACACGGAGCCATCGA	59.5	20	-
Rev1012	TGGCTTAGCGGAGAGTTCCC	60.6	20	-
Gn Fw	AA**CCATGG**ACCGGTGCCACCTAGGATGGCTGAA	66.6	33	*NcoI & AgeI*
Gc Rev	TT**CCCGGG**AGGCCTTAACTAGCTTTCTTA	59.0	30	*XmaI & AgeI*
GnStuI Rev	TT**CCCGGG**AGGCCTTTAAGATGCGTAGGAAA	61.7	31	*XmaI & AgeI*

Table 3: Primer sequences for removing the native signal peptide and PDI peptide fusion primers

Primer name	Primer sequence (5' – 3')	Tm (˚C)	Base pairs	Enzyme site
GnΔSP NcoI Fw	AAAACCATGGATCCTCACCTTAGGAATA (removing SP)	54.6	28	*Nco*I
PDI Rev	TAAGGTGAGGATCCTCAGCGAAGATCTGAGAAGGAACC	64.6	38	-
Gn_PDI Fw	GATCTTCGCTGAGGATCCTCACCTTAGGAATAGACCTGGA	65.4	40	-

Gn Rev	CCCGGGAGGCCTTTAAGATGC	61	21	*Xma*I
Gn-PDI.Rev	TAAGGTGAGGATCCTCAGCGAAGATCTGAGAAGGAACC	67	38	
PDI_Gn Fw	GATCTTCGCTGAGGATCCTCACCTTAGGAATAGACCTGGA	67	40	
∆hGn Fw	TTACCGGTACCATGGACCCCCACCTGCGGAA	58	31	*AgeI &* *Nco*I
htGn Rev	AACTCGAGTTATGCTGTATGGCACTGGTAGTT	57	32	*Xho*I
hGn Rev	TAACTCGAGGCTTGCATATGACACGATCAGCAGC	62	34	*Xho*I
tGn XmaI Fw	AACCCGGGATGGCTGGAATCGCAATG	60.5	26	*XmaI*
tGn XhoI Rev	TTCTCGAGTTATGCTGTGTGACATTGATA	54.8	29	*XhoI*
Primer 1 Fw	TTCCCGGGACCATGGCGAAAAACGTT	58.9	26	

Chapter 9: References

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9.2 Web page

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