Heterologous expression of LSDV immunogenic epitopes as TMV Coat Protein fusions in *Nicotiana benthamiana* plants.

By

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This dissertation has been approved as meeting the required standards of scholarship for partial fulfilment for the degree of Masters of Science (Applied Microbiology), University of Botswana.

Supervisor: Dr S.W. Mpoloka

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Date
DECLARATION

This dissertation is my original work except where due reference is made. It has not been and shall not be submitted for the award of any degree in any other University.

Melitah Motlhale

__________________________  ________________________
Student signature                      Date
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DEDICATION

This is dedicated to my parents, Mr Nonofo Motlhale and Mrs Banyana Motlhale, for their support and encouragement throughout my graduate studies. I could not have made it this far without their love and support.
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LIST OF ABBREVIATIONS

Ab........................................Antibody
AP........................................Alkaline phosphatase
APHIS..................................Animal and Plant Health Inspection Service
APS......................................Ammonium per sulphate
ASM.....................................American Society for Microbiology
BEDIA.................................Botswana Export Development Investment Authority
BSA......................................Bovine serum albumin
CaPVs..................................Capripoxviruses
ChPV....................................Chordopoxvirus
CP.........................................Coat protein
cDNA.................................Complementary deoxyribonucleic acid
DEFRA.................................Department of Environment Food and Rural Affairs
DMSO.................................Dimethyl sulfoxide
DNA.................................Deoxyribonucleic acid
EDTA.................................Ethylenediaminetetraacetic acid
ELISA.................................Enzyme linked immunosorbent assay
EtBr.....................................Ethidium bromide
EtOH.....................................Ethanol
FAO.....................................Food Agriculture Organization
FMD.................................Foot and mouth disease
Gent.........................Gentamicin

GFP..........................Green fluorescent protein

GPV..........................Goat pox virus

HEPES.........................N-2-Hydroxyethylpiperazine-N’-2-ethanesulphonic acid

HPV..........................Human papilloma virus

HRP..........................Horse radish peroxidase

ITR...........................Inverted terminal repeats

Kan...........................Kanamycin

LB............................Luria-Bertani

LSD...........................Lumpy skin disease

LSDV..........................Lumpy skin disease virus

Mab...........................Monoclonal antibody

Min............................minute

NS.............................Non structural

OD............................Optical density

OIE............................Office of International Des Epizooties

O/N............................Overnight

ORF...........................Open reading frame

PAGE..........................Poly acrylamide gel electrophoresis

PBS............................Phosphate buffered saline

PCR............................Polymerase chain reaction
ShPV.................................Sheep pox virus
Rif.................................Rifampicin
RNA.................................Ribonucleic acid
rTMV..............................Recombinant tobacco mosaic virus
rtPCR..............................Reverse transcriptase polymerase chain reaction.
SDS.................................Sodium dodecyl sulphate
ss(+)RNA..........................single stranded positive sense ribonucleic acid
TAE.................................Tris-acetate EDTA buffer
TBS.................................Tris-buffered saline
TMV.................................Tobacco mosaic virus
UTR.................................Untranslated region
UV.................................Ultraviolet light
VIPR...............................Virus Pathogen Resource
VLP.................................Virus-like particle
wtTMV.............................Wild-type Tobacco mosaic virus
ABSTRACT

Lumpy skin disease (LSD) is an economically important disease in Botswana. Currently the disease has no cure. In Botswana this disease is endemic; it has serious economic effects on beef export and the tourism industry. The only way of controlling LSD is by vaccination, separation of healthy animals from the diseased and killing or eliminating the diseased animals. Current strategies in vaccine production use intact or inactivated pathogen strains to induce immunity, as well as subunit vaccines which are commercially produced in yeast or mammalian cell cultures. Vaccine production is expensive. This study aimed at production of a candidate vaccine for lumpy skin disease using tobacco mosaic virus (TMV) as a vector for coat protein fusion production. The major immunodominant region in the lumpy skin disease virus (LSDV) is the P32 protein; two epitopes (LSDV-A and LSDV-B) from this protein were selected and used as antigens for the vaccine production. The epitopes were fused to the CP of TMV then cloned into a TMV based *Agrobacterium* compatible binary vector (pJL TRBO). Six weeks old *Nicotiana benthamiana* plants were agroinfiltrated with the recombinant TMV (rTMV). The rTMV was extracted from the plants three weeks post infection using polyethylene glycol (PEG) precipitation method. Reverse transcription polymerase chain reaction (rtPCR) of raw material from infected leaves showed that the rTMV was not expressed in the plants. The virus extracts showed that the LSDV epitopes were not successfully expressed in the plants; the epitopes were not attached to TMVCP. Western blots and ELISA showed that the rTMV was not able to elicit an immune response by reacting to anti-LSDV bovine sera. The study indicated the potential to develop a system to locally generate cheap and effective vaccines against LSDV even though there are some limitations that are to be eliminated.
Chapter 1

1.0 INTRODUCTION

1.1 Lumpy skin disease

Lumpy skin disease is an acute cattle disease caused by the lumpy skin disease virus. The virus is classified in the genus *Capripoxvirus* (CaPVs) which represents one of eight genera within the chordopoxvirus (ChPV) subfamily of the *Poxviridae* (Buller *et al*., 2005; Diallo and Viljoen, 2007; Woods, 1988). The capripoxvirus genus is currently comprised of lumpy skin disease virus (LSDV), Sheeppox virus (ShPV), and Goatpox Virus (GPV).

These viruses are responsible for some of the most economically significant diseases of domestic ruminants in Africa and Asia (Fenner, 1996). Capripoxvirus infections are generally host specific and have specific geographic distributions (Coetzer *et al*., 1994; Davies, 1991). However, CaPVs are serologically indistinguishable from each other. They are able to induce heterologous cross-protection and in some instances, to experimentally cross-infect (Capstick, 1959; Tulman *et al*., 2001).

Restriction fragment analysis and limited DNA sequence data support a close relationship between the CaPVs (Bhanuprakasha *et al*., 2006; Black *et al*., 1986; Kara *et al*., 2003; Kitching *et al*., 1989; Stram *et al*., 2008). The molecular basis of CaPV host range restriction and virulence remains to be elucidated. The disease is found in Southern and Eastern Africa but in recent years it extended Northwest through the continent into sub Saharan West Africa and into the Middle East (Babiuk *et al*., 2008; House *et al*., 1990; Tuppurainen and Oura, 2012). LSD strain of *Capripoxvirus* is antigenically indistinguishable from strains causing sheep pox and
goat pox (Buller et al., 2005). However, LSD has a different geographical distribution to ShPV and GPV (Figure 2), suggesting that cattle strains of Capripoxvirus do not infect and transmit between sheep and goats (Babiuk et al., 2008; Patel and Heldens, 2009).

Lumpy skin disease is an infectious, eruptive, occasionally fatal disease of cattle of high importance (Coetzer et al., 1994; Davies, 1991; Fenner, 1996). LSD is characterised by fever, enlarged lymph nodes, firm, circumscribed nodules on the skin and ulcerative lesion of the mucous membrane of the mouth and sometimes death (Richmond, 1998). Transient viremias have also been described for cattle infected with LSDV (Carn and Kitching, 1995a; Tuppurainen et al., 2005). Internal lesions are often seen at necropsy, especially in the lungs. Lesions in the trachea, rumen, tongue, kidney, nasal turbinates, and reproductive organs have also been reported (Afshar et al., 1986; Gulbahar et al., 2000; Kitching, 1999). Frequently, the centre of the lesion dies, becomes dry, forms a deep scab, and falls out, leaving a raw area that becomes infected (APHIS, 2003).

LSD has morbidity averaging 10% and mortality averaging 1% in affected herds although mortality rate over 75% have been recorded (Diesel, 1949). Although LSD does not usually have a high mortality rate (<10%), it causes severe economic losses due to reduced productivity in animals; particularly reduced milk production. In dairy herds it causes a sharp drop in milk production, often leading to secondary bacterial mastitis (Ayelet et al., 2014). It also causes damage to hides, abortions in pregnant cows and temporary or permanent sterility in males, Figure 1 (DEFRA, 2008). The deleterious effect that the disease has in affected bulls’ ability to mate and to produce fertile sperm is under appreciated. However it has important economic
implications, especially under subsistence farming and in farming systems where a single sire is used (Coetzer, 2004).

**Figure 1:** The typical clinical symptoms of LSD. Pictures A and D show nodules on the skin, C shows severe weight loss and a foetus carcase on its side as a result of abortion (DEFRA, 2003). B shows a closer magnification of the nodules and increased production of mucus (ASM, 2011).
LSD causes significant economic losses and consequently it is identified as a high priority disease for control. LSD is an Office of International Des Epizooties (OIE) ‘List-A’ disease, which identifies diseases with the potential for rapid spread and severe economic losses (Irons et al., 2005). This disease has a major influence on international trade requiring immediate notification of animal disease. It must be reported to all other member states and the commissioner within 24 hours of an outbreak (OIE, 2008).
Figure 2: Map showing likely global distribution of sheeppox and goatpox (a) and LSD (b) viruses. Recent outbreaks marked with arrows (Babiuk et al., 2008).
1.2 Transmission of LSDV

Transmission of the LSD virus is thought to be predominantly by insects, natural contact transmission in the absence of insect vectors has been shown to be inefficient (Carn and Kitching, 1995b; OIE, 2002). Until 1988, the occurrence of LSD was confined to sub-Saharan Africa, but has since spread into Egypt. Two laboratory-confirmed outbreaks of LSD outside Africa have been documented, one in Egypt and one in Israel in 1989. LSDV is not transmissible to humans (Ireland and Binepal, 1998).

LSDV has been isolated from flies *Stomoxys calcitrans* and from *Biomyia fasciata* (Weiss, 1968). Transmission may occur *via* infected saliva in the absence of an insect vector. Natural capripox infections occur throughout endemic areas as a result of contact between diseased and susceptible animals (Davies, 1976). Though no specific vector has been identified to date, transmission studies suggest that the most prevalent method for the spread of LSD is mechanical transmission by insects such as *S. calcitrans* (Kitching and Mellor, 1986; Mellor *et al*., 1987). Mosquitoes like *Culex mirificens* and *Aedes natrionus* (Chihota *et al*., 2001), and other flies could also play a major role in LSDV transmission (Santi *et al*., 2006). Field observations and supporting evidence indicate that the disease is transmitted by biting flies (Kitching and Mellor, 1986).

Mosquitoes have been shown to be efficient mechanical vectors of LSDV and are able to transmit the virus to a susceptible bovine over a period of 2-6 days post infective feeding. LSDV has been proven to be transmissible to calves through infected milk. LSDV may be excreted for long periods after obvious clinical signs are no longer seen (Irons *et al*., 2005). For clinical
diagnosis, incubation period is approximately 12 days (OIE, 2008). Wildlife does not seem to play any significant role in the clinical epidemiology of the disease although suspect clinical disease has been described in species such as Asian water buffalo, springbok and oryx (antelope species) (Kitching and Mellor, 1986; Young et al., 1970).

There are distinct differences between the geographical distribution of sheeppox, goatpox and lumpy skin disease virus. In the last 50 years the geographic range of sheep pox and goatpox (Figure 2a) has been restricted to mainly Asia and Africa, extending from Africa North of the Equator (Kitching et al., 1989).

1.3 History and epidemiology of LSD

Lumpy skin disease is generally confined to Africa (Figure 2b) with a potential of spreading to some Asian countries, (Babiuk et al., 2008; House et al., 1990). Until the 1980s, this disease was only found south of the Sahara desert and in Madagascar, but in 1988, it spread into Egypt (Woods, 1988). It also occurs in other Middle Eastern countries. In 1989, an outbreak in Israel was eradicated by slaughter and vaccination (OIE, 2008). The economic significance of the disease was demonstrated in the first large-scale outbreaks in South Africa in the late 1940s; over 8 million cattle were affected with a mortality rate of over 75% (Diesel, 1949, Von Backsrom, 1945) Table 1.

Periodic epidemics occur in most African countries. The sub-Saharan region is most commonly affected with morbidity rates generally varying between 1% and 20%. Cows in 1% to 7% of cases may abort (OIE, 2012). In Africa periodic epidemics of LSD outbreaks occur. Outbreaks
were recorded in Ethiopia from January 2007- December 2011 (Ayelet et al., 2014). The disease has a severe impact on the socio-economic status of the continent as the cattle industry is of great importance. There is a real risk of LSDV establishing itself in the Middle East and spreading into Asia and Europe (Kitching and Carn, 2004). Eradicating this disease remains a challenge hence the need for research on possible effective vaccine candidates.

**Table 1**: The earliest LSD outbreaks recorded in Africa and some parts of The Middle East.

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1929</td>
<td>Zambia</td>
<td>(MacDonald, 1931; Morries, 1931; Coetzer, 2004)</td>
</tr>
<tr>
<td>1943</td>
<td>Botswana</td>
<td>(Von Backsrom, 1945)</td>
</tr>
<tr>
<td>1945</td>
<td>Zimbabwe, Mozambique,</td>
<td>(Houston, 1945)</td>
</tr>
<tr>
<td>1945</td>
<td>South Africa</td>
<td>(Diesel, 1949; Thomas and Mare, 1945; Von Backsrom, 1945)</td>
</tr>
<tr>
<td>1947</td>
<td>Lesotho</td>
<td>(De Sousa Dias and Limpo Serra, 1956; Diesel, 1949)</td>
</tr>
<tr>
<td>1954</td>
<td>Madagascar</td>
<td>(Ordner and Lefevre, 1987)</td>
</tr>
<tr>
<td>1957</td>
<td>Kenya, Uganda, Somalia, Tanzania (East Africa)</td>
<td>(MacOwan, 1959; Burdin, 1959)</td>
</tr>
<tr>
<td>Early 1970s</td>
<td>Sudan</td>
<td>(Ali and Obeid, 1977; OIE, 1996)</td>
</tr>
<tr>
<td>1989</td>
<td>Israel and Saudi Arabia</td>
<td>Abraham and Zissman, 1991; Greth et al., 1992; Yeruham et al., 1995</td>
</tr>
</tbody>
</table>

1.3.1 Epidemiology of LSD in Botswana

LSD is endemic and widespread countrywide. **Table 2** shows the annual outbreaks of LSD in Botswana. Outbreaks usually take place during the rainy season. Therefore, farmers are advised
to vaccinate their cattle before the rainy season as a prevention strategy (Quinn et al., 2002). In Botswana, farmers are advised to use tetracycline- or sulphonamide-based drugs on infected animals to treat for opportunistic bacterial infections. The LSD live-attenuated vaccine being used currently in Botswana is imported from South Africa (News, 2011). Botswana Ministry of Agriculture Annual reports on LSD outbreaks indicate that the disease caused severe symptoms and in some cases cattle death (Table 2).

**Table 2: LSD outbreaks in Botswana from the year 2000-2011 (Ministry of Agriculture, 2000-2011).**

<table>
<thead>
<tr>
<th>Year</th>
<th>No. Dead animals</th>
<th>No. Affected animals</th>
<th>Number of risk</th>
</tr>
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<tbody>
<tr>
<td>2000</td>
<td>44</td>
<td>343</td>
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<td>2011</td>
<td>26</td>
<td>103</td>
<td>1704</td>
</tr>
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**1.4 The lumpy skin disease virus**

**1.4.1 The LSDV genome**

The poxvirus genome consists of a linear, double stranded DNA (dsDNA) molecule which is covalently cross linked at the terminal repeats (Gershelin and Berns, 1974; Black et al., 1986). These terminal sequences have been shown to contain repeated sequences that are inverted with respect to one another (Wittek et al., 1978).
Figure 3: Linear map of the LSDV genome. ORFs are numbered from left to right based on the position of the methionine initiation codon. ORFs transcribed to the right are located above the horizontal line; ORFs transcribed to the left are below. Genes with similar functions and members of gene families are colored according to the figure key. ITRs are represented as black bars below the ORF map (Tulman et al., 2001).

The 151-kbp LSDV genome contains 156 putative genes and consists of a central coding region bounded by identical 2.4 Kbp-inverted terminal repeats (Gershon and Black, 1989) (Figure 3). Comparison of LSDV with chordopoxviruses of other genera reveals 146 conserved genes which encode proteins involved in transcription and mRNA biogenesis, nucleotide metabolism, DNA replication, protein processing, virion structure and assembly, viral virulence and host range. In the central genomic region, LSDV genes share a high degree of colinearity and amino acid identity (average of 65%) with genes of other known mammalian poxviruses, particularly
suipoxvirus, yatapoxvirus, and leporipoxviruses. In the terminal regions, colinearity is disrupted and poxvirus homologues are either absent or share a lower percentage of amino acid identity (average of about 43%).

The 156 putative genes in the LSDV genome represent a 95% coding density and encode proteins 53 to 2025 amino acids. LSDV contains the majority of conserved pox viral genes involved in basic replicative mechanisms. These include at least 26 genes encoding RNA polymerase subunits, mRNA transcription initiation, elongation and terminal factors. Enzymes directing post transcriptional processing of viral mRNA are also included (Tulman et al., 2001). LSDV proteins potentially involved in nucleotide metabolism include homologues of thymidine kinase, dUTP pyrophosphatase and the small subunit of ribonucleotide reductase. It encodes at least 30 homologues of pox viral proteins known to be structural or involved in virion morphogenesis and assembly (Davies, 1991).

1.4.2 Virion structure of LSDV

LSD appears by electron microscopy to be oval- or brick-shaped, Figure 4 (Westwood et al., 1964). Early electron microphotographs of the capripoxvirus (Figure 5) revealed the presence of two types of particles; one form was thought to be the complete form, consisting of a nucleoid with surrounding envelope material, and the other incomplete form consisting of an envelope with or without nucleoid components (Nagington and Hornes, 1962). Thin sections of virions reveal a lipoprotein bilayer called the outer membrane, surrounding a central core that encases the viral DNA. The core is surrounded by a palisade layer of rod-shaped molecules (Dale and Pogo, 1981).
The virion contains numerous antigens, most of which are shared by all members of the genus (Fenner et al., 1987). DNA analysis using restriction endonucleases on both field samples and South African Neethling strain showed 80% homology between strains of the capripoxvirus (Black et al., 1986). The Neethling strain is the wild type strain of LSDV (Kitching, 1986). The virions are ovoid and classified as “C” (capsule or “C” form) or “M” if surrounded by a membrane or covered in cordlike elements (mulberry or “M” form) (Kitching and Smale, 1986; Munz and Owen, 1966).
Figure 5: Two distinct infectious virus particles exist; the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). The virus is enveloped and brick-shaped with dimensions; 300 x 270 x 200nm. The surface membrane displays surface tubules or surface filaments (Swiss Institute of Bioinformatics, 2008).

1.4.2.1 P32 antigen

The immunodominant region of the LSDV is the P32 protein. The P32 antigen is a structural protein present in all capripoxvirus isolates and contains a major antigenic determinant (Chand, 1992; Chand et al., 1994). Thus P32 is important in pathogenicity, diagnosis, prevention and control of capripoxvirus (Tian et al., 2010). Most of the differences between the different Capripoxvirus strains involve genes and gene families with likely functions involving viral virulence and host range (Tian et al., 2010).

Although LSDV resembles leporipoxviruses in gene content and organization, it also contains homologues of interleukin-10 (IL-10), IL-1 binding proteins, G protein-coupled CC chemokine receptor, and epidermal growth factor-like protein which are found in other poxvirus genera (Tulman et al., 2001). These data show that although LSDV is closely related to other members
of the *Chordopoxvirinae*, it contains a unique complement of genes responsible for viral host range and virulence.

### 1.4.2.2 Capripoxvirus life cycle

The *Poxviridae* are enveloped unsegmented dsDNA viruses; unlike many dsDNA viruses that replicate in the host nucleus poxviruses encode their own replication machinery and therefore replicate in the cytoplasm. Viral genes are expressed in a bi-phasic manner with early-expressed genes encoding non-proteins involved in a genome replication and late-expressed genes encoding the viral structural proteins (OIE, 2008) Poxviruses assemble large virions that can be seen under the microscope (Chen *et al.*, 2003).

Attachment of the viral protein to host glycosaminoglycans mediates endocytosis of the virus into the host cell. The viral proteins fuse with the plasma membrane to release the core into the cytoplasm. During the early phase early genes are transcribed in the cytoplasm by viral RNA polymerase. Early expression begins minutes post infection, as early expression ends the core is completely uncoated as early expression ends, the genome is now free in the cytoplasm. During the intermediate phase intermediate genes are expressed, triggering genomic DNA to replicate. The late phase constitutes late genes expression producing all structural genes.

Assembly of progeny virion starts in the cytoplasmic viral factories producing a spherical immature particle. This virus particle matures into brick shaped intracellular mature virion (IMV). IMV-virion can be releases upon cell lysis, it can also acquire a second double membrane
from trans-golgi and bud as external enveloped virion (EEV) (OIE, 2010; Moss, 2006; Moss, 2001).

1.4.2.3 LSDV serotypes and subtypes

Available evidence from studies on the South African Onderstepoort Neethling strain suggests that there is only one immunological type of LSDV and only one serotype. Virus isolates collected over an extended period from a large number of natural cases originating from outbreaks of the disease in Kenya, Botswana and Malawi showed complete, cross neutralization with the Neethling strain (OIE, 2010; Voster and Mapham, 2008; Fauquet et al., 2005).

1.4.2.4 Immune response to LSDV

Immunity to capripoxvirus infection is predominantly cell mediated and therefore requires a replicating agent to be stimulated (Carn, 1993). Most progeny viruses remain inside cells with the exception of enveloped viruses which are released into the blood (Boulter and Appleyard, 1973). By spreading from cell to cell, the virus is inaccessible to circulating antibodies. Circulating antibodies against capripoxvirus limit the spread of the virus in experimental sheep, but this does not prevent replication of the virus at the site of infection.

All the viruses in the Capripox genus share a common major antigen for neutralizing antibodies; thus, animals recovered from infection by one virus are protected from infections with the other. This is demonstrated by serological evidence (Davies and Otama, 1978), cross infection and cross protection experiments (Capstick, 1959; Kitching and Taylor, 1985).
Plant-derived vaccines can be administered orally to elicit a mucosal immune response. These vaccines possess dual advantage of preventing antigens from being degraded as they pass through the gastrointestinal tract, simultaneously delivering an antigen into the mucosal immune system (Hefferon, 2012).

1.4.2.5 Control of LSD

In endemic countries, vaccination against LSD is the most effective method for controlling the disease, thus far control of LSD has been only partially successful (OIE, 2012). Where LSD is not present the most important method is restriction of the importation of animals and those animal products that may contain live virus from endemic countries (Carn, 1993). In Botswana there are veterinary cordon fences used mostly during outbreaks but currently more focused on control of foot and mouth disease.

In case of outbreaks, isolation and prohibition of animal movements, slaughtering of infected and in contact animals, and ring vaccination in a radius of 20-50 km should usually be sufficient to eradicate the disease (Carn, 1993; APHIS, 2003). Proper disposal of dead animals, for example incineration, is also beneficial. Other control measures include disinfection of premises, and vector control in premises and on animals. Intensive insect control, including widespread use of insecticides can be used (OIE, 2002).

1.4.2.5.1 Vaccination

1.4.2.5.1.1 Current vaccines
Many poxviruses (including LSDV) have been attenuated by serial passage in either cell cultures or on the chorioallantoic membranes of embryonated chicken eggs to serve as live attenuated vaccines (Van Rooyen et al., 1969; Kirsch, 1969; Taylor and Paoletti, 1988; Winterfield and Reed, 1985). The highly attenuated strain has proven to be safe and effective and to provide long-term immunity although over time the effectiveness has been challenged and more frequent vaccinations are now recommended (Hunter and Wallace, 2001; OIE 2010; Weiss, 1968).

Different vaccines have been widely and successfully used for the prevention of LSD in cattle populations in Africa. In Southern Africa, the Onderstepoort LSD freeze dried G 0110 (Act 36/1947) live attenuated vaccine is used. The Neethling strain of LSD was passaged 50 times in tissue cultures of lamb kidney cells and then 20 times in embryonated eggs (OIE, 2008). The strain proved to be immunogenic for cattle, although local reactions do occur in a high proportion of animals at the vaccination site. It is produced in tissue culture and issued as a freeze-dried product (Weiss, 1968). Immunity conferred lasts up to three years.

The second type is the sheep goat pox virus (SGPV) vaccine for cattle, (Capstick and Coackley, 1961). The use of this vaccine has been restricted to countries where sheep and goat pox viruses are endemic. The heterologous attenuated virus vaccine include sheep goat poxvirus, but may cause severe reaction, therefore this option is not advised in countries free from goat poxvirus (OIE, 2002). In Kenya, an effective vaccine has been produced from a local sheep and goat pox virus. This was shown to immunize cattle against LSD (Capstick and Coackley, 1961; Kitching, 2003).
The SGPV was passaged 16 times in pre-pubertal lamb testis or foetal muscle cell cultures and used for vaccination at this level. Local reactions have not been observed, but some Bos taurus breeds have shown lymphadenitis with signs of mild, generalized LSD-like lesions following vaccination (approximately 0.02%). Neutralizing antibodies to LSDV persist for at least two to three years after vaccination. In other cases, some animals have antibody levels which are too low to be demonstrated but they nevertheless are still resistant to challenge (Carn and Kitching, 1995b; Weiss, 1968).

Control of LSDV in Egypt depends on prophylactic vaccination programs using live attenuated cell cultures adapted sheep pox vaccine (SPV) (Brenner et al., 2006; Michael et al., 1994). Two other strains of SPV have recently been used as a prophylaxis against LSD. The Romanian strain, prepared in the skin of lambs for use against sheep pox, was used in several million cattle in Egypt and was able to illicit an immune response. Another sheep pox strain, the Yugoslavian RM 65 prepared in tissue culture, was used in Israel. No complications have followed the use of these strains in cattle (FAO, 1991). Immunity lasts at least two years.

The use of the live attenuated LSD/SPGV vaccine has been successful in reducing LSD outbreaks (Hunter and Wallace, 2001; Weiss, 1968). However there are several concerns and limitations. These include the need for high containment facility for production of the vaccine making the cost of the vaccine to be high. There is need for improved vaccines which produce rapid protection and can be cheaply produced.
1.4.2.5.2 Plant based vaccines

1.4.2.5.2.1 Simple virus-based vectors

Plants are now gaining widespread acceptance as a general platform for large scale production of recombinant proteins. The first recombinant pharmaceutical proteins are reaching the final stages of clinical evaluation and many more are in the development pipeline (Cummings et al., 2014; Desai et al., 2010; Fischer et al., 2004; Grimsley et al., 1986; Hefferon, 2012; Lindbo, 2007a; Lindbo, 2007b; Mortimer et al., 2015; Paul and Ma, 2011; Penney et al., 2011; Rybicki, 2014). Viral vectors have been used for some time for the expression of foreign proteins or for chimaeric coat proteins in plants (Gleba et al., 2005; Steinmetz et al., 2009; Yusibov et al., 2006).

Economic losses in agriculture caused by viral infections led to the development of genetic systems that allow manipulation of the virus to manage plant diseases; these genetic systems have also led to the development of viruses as beneficial molecular tools (Yusibov et al., 2002). The utility, variety and application of these genetic systems have expanded greatly since the early use of cDNA-derived in vitro-synthesised RNA to infect plants with recombinant tobacco mosaic virus (rTMV) (Yusibov et al., 1999). Examples of some of the vaccine epitopes which have been expressed on TMV include antigens from human papillomavirus, foot and mouth disease virus, HIV-1 and rotavirus (Noris et al., 2011; Turpen et al., 1995; Wigdorovitz et al., 1999; Rybicki, 2009; Yusibov et al., 2011).

One of the first plant-based vaccines described was rTMV with malarial epitopes expressed on the virion surface (Turpen et al., 1995; Koo et al., 1999). Large Scale Biology Corp had the
recombinant TMV technology (Geneware™) as one of their main products and it was successfully used to express patient-specific non-Hodgkin lymphoma tumor-derived single-chain vaccine for FDA approved clinical trial (McCormick and Palmer, 2008; McCormick et al., 1999, 2006). Cottontail rabbit papillomavirus (CRPV) major protein L1 was also produced either transgenesis or via rTMV expression and protected rabbits against CRPV challenge (Kohl et al., 2006). In animals rTMV expressing surface located epitope from CRPV and ROPV (rabbit oral papillomavirus) L2 minor capsid was also used as an injectable vaccine (Palmer et al., 2006).

High-level expression of the introduced genes and the rapid accumulation of appropriate products that are easily purified from the host plant are the primary characteristics that make plant viruses well suited as transient expression vectors. Moreover, plant viruses generally have a wide host range that allows expression of a gene in different plant species using the same vector construct (Yusibov et al., 2002).

The vast majority of viruses that infect plants are single-stranded RNA viruses that replicate in the cytoplasm. Many of these viruses replicate to very high levels in plants and a few have been used to express foreign genes (Scholthof et al., 1996). One of such viruses is TMV. Other plant viruses that have been used successfully for either peptide expression or for whole antigens include the Bamboo mosaic virus (BaMV), Cowpea mosaic virus (CPMV), Papaya mosaic virus (PapMV) and Cucumber mosaic virus (CMV) among others.

There are challenges associated with expression of whole genes using replicating plant virus vectors constructs which spread via natural means in plants. In many cases there is loss of the
transgene during the expression period, especially when expression gets depressed overtime. A cottontail rabbit papillomavirus (CRPV)-L1 protein gene expression in N. benthamiana via rTMV was lost progressively as infected plants developed, leading to lower yields of proteins than were obtained in transgenic tobacco (Kohl et al., 2006). The elegant means of getting around the problem and completely redesigning plant virus involves the use of A. tumefaciens for systemic delivery of viral genomes (Rybicki, 2010).

1.4.2.5.2.2 Agroinfiltration of viral vectors

The most widely used method of generating transgenic plants is the A. tumefaciens-mediated transformation. This technically simple plant transient expression system takes advantage of A. tumefaciens’ molecular machinery to transfer DNA into plant cells (Gleba, et al., 2007; Grimsley et al., 1986; Maclean et al., 2007; Rybicki 2009, 2010, 2014). A. tumefaciens is a soil bacterium that has evolved a natural genetic engineering system; it contains a segment of DNA that is transferred from the bacterium to the plant cells (Snustad and Simmons, 2006) Achieving an A. tumefaciens-mediated transient expression involves infiltration of intracellular spaces in plant leaves with a suspension of the bacterium, using a syringe or vacuum. This should result in mobilization of the T-DNA into the nuclei of a large portion of the cells. Infiltrating more leaves or the whole plant would improve viral recovery.

Because it is effective, easy and inexpensive to use, this system has become the most commonly used for production of proteins in plants. However the approach sometimes expresses very low T-DNA, thus low levels of proteins of interest (Lindbo, 2007a). An agro-infiltration delivered
TMV based vector has also been used to produce many antigens, among others, H5N1 influenza virus haemagglutinin (Hahn et al., 2014; Paul and Ma, 2011; Rybicki, 2010). Several vectors have a host range problem, with amplified expression being limited mainly to *N. benthamiana*. Tobacco among the leafy plants has been widely used as a host because it is easy to transform and regulation of transgene expression is well understood. Being a non-food, non-feed crop, tobacco reduces the risk of contaminating the food/feed chain (Chakauya et al., 2006; Lee and Yang, 2006).

### 1.5 Tobacco mosaic virus (TMV)

#### 1.5.1 TMV genome

TMV is a single stranded positive sense RNA, rod shaped virus. RNA is encapsulated with viral coat proteins. It is approximately 6400 nucleotides long (Grill, 1993; Mims et al., 2004), with dimensions of 300nm x 15-18nm. The TMV has only four open reading frames (Goelet et al., 1982). The 126 kDa protein and 183 kDa read through proteins are translated from the 5’ end of the genomic RNA. The movement protein (MP) (30 kDa) and 17.5 kDa capsid protein are translated from the 3’-coterminal subgenomic mRNAs produced during replication of the viral RNA, **Figure 6** (Hunter et al., 1976; Beachy and Zaintlin, 1977).

![Genetic map of Tobacco mosaic virus (TMV)](image)

**Figure 6:** Genetic map of Tobacco mosaic virus (TMV). The approximately 6,400 nucleotide TMV RNA has a 5’-cap and the 3’-tRNA-like structure. The open reading frames are indicated by boxes. The replicase consists of a 126 kDa protein and, by read-through of an amber stop codon (asterisk), the 183 kDa protein. The replicase proteins are translated from the genomic
RNA. The 30 kDa movement protein (MP) and the 17.5 kDa capsid protein (CP) are expressed from separate subgenomic RNAs (not shown). (Scholthof, 2004).

The MP is necessary for the virus to pass through the plasmodesmatal opening between the cells to support cell-to-cell movement. The capsid protein is the single structural unit protecting the RNA in virions and is required for long-distance movement throughout the plant (Yusibov et al., 2002). The Coat Protein (CP) of TMV is one of the most accumulated proteins in infected plants. The CP can reach up to 10% of leaf dry weight (Copeman et al., 1969; Siegel et al., 1978; Fraser, 1987). The structure of the CP is known (Namba et al., 1989; Butler et al., 1992). Fusion of the viral CP and a foreign protein resulted in one of the first hybrid constructs (Takamatsu et al., 1987; Dawson et al., 1988).

The TMV virions are stable for decades and the virions can also survive heating. A broad range of crop species and weeds mostly Solanaceae, which includes tobacco, tomato, pepper plants, can be infected experimentally (Awram et al., 2002; Knapp et al., 2001).

1.5.2 TMV as a vector expressing rTMV proteins

There are several different methods to genetically alter plants to produce foreign proteins. Generally, these methods introduce foreign DNA sequences into plant cell DNA, which requires time-consuming manipulations. Naturally occurring plant viruses are capable of altering plant genomes such that the plant produces virally encoded products within a matter of days or weeks (Grill, 1993). A recombinant TMV (rTMV), once introduced into a leaf of a tobacco plant is able to replicate and express the foreign gene in the plant within a short time.
Tobacco serves as a good host for foreign protein expression due to the fact that it is one of the highest biomass producing cash-crop in the world. Tobacco under optimal conditions can produce up to 2000 Kg per acre per year (Turpen et al., 1995). However, tobacco plants contain numerous toxic alkaloids and phenolic compounds and this makes purification of heterologously produced recombinant proteins a difficult process (Awram et al., 2002).

*N. benthamiana* is recommended as the best tobacco species to use for expression of foreign proteins because it does not contain high levels of toxic phenolics and alkaloids that are associated with other tobacco species. TMV’s utilization as an expression vector has already been well established and has been used to produce many different kinds of proteins including allergens, antibodies and vaccine candidates (Gleba et al., 2005; Grill, 1993; Grill et al., 2005; Lindbo, 2007a; Smith et al., 2006).

This vector has in turn allowed for the development of economical methods for the expression of foreign products. The amount of foreign product produced depends on different factors. These include the protein being expressed, choice of the expression system and the host plant used. Depending on the TMV based expression vectors used, the infection may result in systemic spread causing high expression of recombinant proteins. Active replication of recombinant virus is characterized by many symptoms; these include leaf curling, mottling, chlorosis, necrosis and growth stunting. The symptoms are not lethal to the plant (Culver et al., 2002).

Fusion of foreign amino acid sequences to the TMV CP has been shown to induce severe chlorosis and necrosis in *N. benthamiana* plants (Turpen et al., 1993; Bendahmane et al., 1999),
even though this is not always the case. The symptoms of infection are not necessarily an indication of production of rTMV, but are instead due to TMV assembly. The CP enables systemic infection; deletion of the TMV CP limits the viral ability to spread systemically throughout the plant. Without the CP the virus is still able to move locally from cell to cell but loses for systemic infection ability (Santi et al., 2006).

1.5.3 TMV transient expression vectors

Genetically modified plant viruses are powerful tools with a variety of applications. Accumulation of specific recombinant proteins in whole plants provides a cost-effective source of therapeutic reagents for human and animal diseases and this application has immediate potential in health care application (Yusibov et al., 2002). TMV and its tobamovirus relatives have advantages for consideration as transient expression vectors.

One advantage is their long history of experimentation. TMV was the first virus to be recognized and purified, and the first virus for which virion structure and the coat protein sequence were determined (Yusibov et al., 2006). TMV represents a reduced biohazard as a recombinant virus in the field, it is only spread mechanically. TMV virions can easily be purified in large quantities by relatively simple procedures and equipment (Yusibov et al., 2002).

The CP is immunogenic in mammals due to its ability to activate T cells (Loor, 1967). This has made this virus a potential vector for expressing foreign epitopes fused to the CP for development and application of novel vaccines (Jiang et al., 2006; Dawson and Lehto, 1990; Lacomme et al., 1998). The CP subunits in the TMV particles are assembled in a highly ordered
fashion, with the N- and C-termini both located on the surface of the TMV particle (Namba et al., 1989). TMV CP fusion would result in a TMV particle displaying the epitope (red) on the outside of the TMV virion (Figure 7). The epitope would be covalently linked to the TMV CP (brown), but otherwise the TMV virion would assemble as normal with the TMV RNA (green) enclosed in the virion.

Thus foreign protein fused to the termini of CP could be displayed outside the virus particle if the fused CP subunits are still able to encapsidate the recombinant viral genome in proper order (Turpen et al., 1995; Sugiyama et al., 1995). However, the limited capacity of typical TMV-based vectors like pJL TRBO, for expressing longer foreign peptides (more than 20 residues), has hobbled its applications to the purpose that required longer peptides (Beachy et al., 1996; Jiang et al., 2006; Wu et al., 2003).

**Figure 7:** Diagram of “decorated” TMV virion expressing a foreign epitope (red).
1.6 Statement of the problem

LSD is a viral disease. LSD clinical disease has been described in species such as Asian water buffalo, springbok and oryx (antelope species) but it mainly affects cattle (VIPR, 2012). The rate at which outbreaks take place in Botswana and in Africa as a whole is a major cause of concern (OIE, 2012). The disease is viral in nature and currently the only means of controlling it is by vaccination and separating healthy animals from the affected ones.

LSD is endemic in Botswana. Its socio-economic effects do not only affect the beef industry (beef export for the Botswana government), but also affects individual farmers who are dependent on animal husbandry for their livelihoods. Cattle population in Botswana fluctuates between 2.5 and 3 million (BEDIA, 2012). Individual farmers have to buy the current vaccine for their cattle. The cost of production of the currently used vaccine is high. Several millions of Botswana Pula are mainly attributed to manufacturing, research and development (BEDIA, 2011).

In Botswana, currently a chemically attenuated live vaccine is the primary option in use. Although live attenuated virus vaccines are generally considered safe, reported cases of reversion to virulence have highlighted the inherent safety problems of live attenuated vaccines (Cizman et al., 1989; Christensen et al., 1992; Minor, 1993; Xie et al., 1998; Huang et al., 1999; Nielsen et al., 2001). Mutations can occur when the vaccine virus replicates within the animal and cause a virulent strain.
There is no guarantee under the right conditions the live attenuated live vaccine might not be able to revert to virulence. The need for cold chain storage for these vaccines makes them costly, especially for farmers in rural areas. The use of eggs or tissue culture for the conventional methods of vaccine production also causes a need for alternative methods, since the vaccine may harbour contaminants like endotoxins or pathogens (Fischer and Emans, 2000; Giddings et al., 2000). All these reasons justify the need for development of a locally produced, cost effective vaccine.

1.7 Objectives of the study

1.7.1 Aim

The main aim was to clone two LSDV epitopes into the TMV binary vector (pJL TRBO) and to investigate the ability of the rTMV to retain the cloned epitopes and cause an infection in N. benthamiana plants using Agrobacterium tumefaciens. Basically, the aim was to develop a method for production of TMV based subunit vaccines against LSDV using a plant based production system.

1.7.2 Specific objectives

- To identify two immunodominant regions (LSDV A and LSDV B) of the LSDV genome.
- To clone the two epitopes into the pJL TRBO plasmid.
- To transform A. tumefaciens with a TMV based binary vector (pJL TRBO).
- To agroinfect N. benthamiana plants with the transformed A. tumefaciens.
- To purify rTMV from infected N. benthamiana leaf material.
To determine immunogenicity of rTMV by ELISA and western blotting.

1.8 Significance of the study

The study pioneers the production of safe, efficient and cost-effective vaccines. The plant-based vaccine production method could be adapted for any disease. This study could potentially equip Botswana with a substantial vaccine production industry and minimise the country’s reliance on imported vaccines.

Currently the Onderstepoort lumpy skin disease vaccine for cattle is being used in Botswana. This vaccine is imported from South Africa, it is to be refrigerated before use, calves from vaccinated cows should be vaccinated at six months of age and an annual booster vaccination should be given. The yearly outbreaks of this disease in Botswana are of major concern, currently the effectiveness and efficacy of the vaccine being used is still questionable. Not all farmers afford to purchase this vaccine and keep it under the right conditions before use.

This would have major implications in Africa, even world-wide especially in countries that have a huge beef and tourism industry. In 2003/04, agriculture contributed 2.3% of Botswana’s gross domestic product (GDP) out of which about 70% to 80% was attributed to the cattle industry (BEDIA, 2012).
1.9 Rationale

Vaccination is useful for eradication of infectious diseases in animals and human beings. The ultimate goal of vaccination is to protect vaccinated animals against re-infection by the same pathogen and provide sterile immunity. Current strategies in vaccine production use intact or inactivated pathogen strains to induce immunity. Subunit vaccines which are commercially produced in yeast or genetically engineered mammalian cell cultures are also used (Andersson, 2000; Turpen, 1999).

Subunit vaccines are composed of one or more subunits of an antigenic protein from a disease causing organism. They are usually produced in bioreactors or fermenters and also tend to require extensive purification prior to use (Korban et al., 2002). Some vaccines, especially parenteral vaccines (vaccines administered by injection), contain preservatives such as formaldehyde, thiomersal (a mercury based compound) and aluminium sulphate. These commercially produced vaccines tend to be ineffective and are costly (Hunter and Wallace, 2001).

The beef industry contributes significantly to the economy. Livestock plays a critical role in terms of income, savings, food security and employment. In Botswana, outbreaks of diseases such as LSD, rabies and foot and mouth disease (FMD) are of great concern since these are endemic in southern Africa. Development and improvement of vaccines is a suitable way to combat infectious endemic diseases in both wild and domestic animals.
Plants offer advantages in terms of production scale, economy, safety, ease of storage and distribution (Canizares et al., 2005; Gidding et al., 2000; Neimer et al., 2014). Use of plants with comparison to the traditional methods eliminates the need for fermentation facilities and overcomes the possible risks of contamination of subunit vaccines by mammalian pathogens (Santi et al., 2006). Pathogens that infect plants do not infect animals whereas mammalian pathogens can infect the animal population (Gidding et al., 2000).

The production of subunit vaccines has been validated using different plant heterologous expression approaches (Grill, 1993; Yusibov et al., 1999; Babiuk, 2002; Kang et al., 2004; Jiang et al., 2006). Plants can express a large variety of proteins and perform the post translational modifications required for proper biological function (Hofbauer and Stoger, 2013; Mason et al., 2004; Sabalza et al., 2014). Hence they offer great potential as production systems for recombinant proteins (Lindbo, 2007a). Recombinant protein expression can reach very high levels (range) of expression in a relatively short time, ranging from 3-14 days post infection, depending on the system used (Canizares et al., 2005; Turpen, 1999). The success of viral vector infection depends on the synthesized construct being stable within the plant (Chakauya et al., 2006).

Several expression vectors have been developed using different types of plant viruses: the most commonly used plant virus is the tobacco mosaic virus (Mason et al., 2004). Plant viral vectors offer several advantages. TMV-based vectors express the highest amounts of foreign proteins in plants (Wagner et al., 2004). The TMV vector produced between 600 and 1200 micrograms of recombinant proteins per gram of infiltrated tissue by 6 days post infiltration (DPI) (Lindbo,
TMV vectors can be used to produce many different kinds of proteins in plants including allergens, antibodies, or antibody fragments and vaccine candidates (Lindbo, 2007a). Plant virus based expression vectors can be used for production of vaccines for infectious diseases such as LSD as they allow for rapid, effective expression of proteins in the whole plant (Lindbo, 2007a).

In this study TMV was used as an expression vector for the immune dominant region of the LSDV genome in *N. benthamiana* plants geared towards the production of a LSD vaccine.
Chapter 2

2.0 MATERIALS AND METHODS

2.1 Experimental design

The experimental work was done in three different stages. Stage one involved, identification and cloning of the LSDV epitopes into pJL TRBO, a TMV based vector (from the Grill Vaccine lab, Ptitzer College). Cloning was done using a series of PCR steps to insert the epitopes into the TMV coat protein (CP). Initially the wild-type CP (wtCP) was amplified from pJL 36 plasmid followed by restriction digestions and ligations to insert the recombinant TMV CP into pJL TRBO. The plasmid carrying the LSDV epitope and the coat protein was transformed into E. coli (DH5α), to bulk up the constructs.

For the second stage, the Agro-infiltration of N. benthamiana with the recombinant pJL TRBO (pJL TRBO containing LSD epitopes) was done as well as extraction of the rTMV from plants. A. tumefaciens was transformed with rpJL TRBO. Agro-induction was done, and Agro infiltration was performed on six weeks old N. benthamiana plants. The success of the infiltration was tracked by monitoring disease symptoms, after viral expression the virus particles were extracted from the plant leaf material.

The third stage involved testing of the plant extracts to verify the expression of the LSDV epitopes. A reverse transcription PCR (rtPCR) was carried out using rTMV extract from infected N. benthamiana leaves. This was to show that the LSDV epitopes were still associated with the TMV coat protein in viral RNA. The extracts were then subjected to SDS PAGE analysis,
western blotting, and ELISAs to confirm the presence of TMV and that the rTMV still had the LSDV antigens capable of eliciting an immune response in animals. **Figure 8** shows a simplified flow diagram of the sequence of events followed during the study.

**Figure 8:** Flow diagram showing the sequence of events in the study.

### 2.2 Stage I

#### 2.2.1 Epitope identification
The major LSDV antigenic sites are located on the P32 structural protein. The protein is found in all *Capripoxviruses*, and contains the major immunogenic determinants. Two particular amino-acid sequences; 92-118 LSDV A (27 amino acids (a.a) and 156-175 LSDV B (20 a.a) (Tian *et al.*, 2010) were identified (see **Table 3**). The consensus sequences for LSDV A and LSDV B are the epitope sequences used in the study. The LSDV A was reduced to 20 amino acids since the plasmid used can only accommodate about 20 amino acids while the LSDV B was used as it is.

The LSDV DNA analysis was carried out online using a programme called Serial Cloner (version 3.1). Primer synthesis was done at Inqaba Biotechnological Industries in Pretoria, South Africa.

**Table 3**: Sequences of the synthesized LSDV epitopes.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Amino acid sequence</th>
<th>Site</th>
<th>Length (a.a)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LSDV A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original LSDV A</td>
<td>EAKSSIAKHFSLWKSYADADIKNSENK</td>
<td>92-118</td>
<td>27 a.a</td>
<td>(Tian <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>Consensus LSDV A</td>
<td>SSIAKHFSLWKSYADADIKN</td>
<td>95-114</td>
<td>20 a.a</td>
<td>(Tian <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>Consensus Sequence</td>
<td>AGC AGC ATT GCG AAA CAT TTT AGC CTG TGG AAA AGC TAT GCG GAT GCG GAT AAA AAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LSDV B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original LSDV B</td>
<td>FHNSNRLFNNQENNNFMYSS</td>
<td>156-175</td>
<td>20 a.a</td>
<td>(Tian <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>Consensus LSDV B</td>
<td>FHNSNRLFNNQENNNFMYSS</td>
<td>156-175</td>
<td>20 a.a</td>
<td>(Tian <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>Consensus Sequence</td>
<td>TTT CAT AAC AGC AAC AGC CGC ATT CTG TTT AAC CAG GAA AAC AAC AAC TTT ATG TAT AGC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.2 Cloning of LSDV epitopes into the Coat Protein by PCR

Primers specific to the sequences generated were designed and the LSDV epitopes were inserted into the TMV CP via three rounds of PCR. The primers used in this study were designed with the method of inserting the epitopes of interest in the CP (see Table 4). The method used to achieve this was adopted from Wu et al., (2003). This method used three rounds of PCR to enable the removal of wtTMV CP from the pJL 36 plasmid, then inserting the epitopes into the cloning site within the TMV CP.

2.2.2.1 Round 1 PCR

The first round of PCR was done to amplify the coat protein from the pJL 36 plasmid and to that end primers were designed to amplify the entire 480bp TMV CP. Primers specific to the TMV CP were used to amplify the TMV CP from pJL 36 and introduce the restriction sites NotI and AvrII. The PCR 1 product was run on an agarose gel and the 480bp amplicon was excised, gel purified and used as the template for the round 2 PCR.

2.2.2.2 Round 2 PCR

In the second round of PCR, the TMV CP forward primer was used. The reverse primer constituted a sequence complementary to the TMV CP just downstream of the insertion site, with the LSDV epitopes extending from the end. This round binds the CP downstream of the insertion sites. The final product constitutes the TMV CP with insert attached but missing the CP sequence.
upstream of the insertion sites. The round 2 PCR product was run on an agarose gel and the
approximately 500bp amplicon was excised, gel purified and used as the template for the PCR 3.

2.2.2.3 Round 3 PCR

In the third round of PCR the reverse primer consisted of a sequence complementary to the end
of the LSDV epitopes and this time the remainder of the TMV CP (Table 4) (sequence after the
insertion site) was hanging from the end. The TMV CP forward primer was used as the forward
primer. The amplicon for PCR 3 is about 600bp in size, it was excised and purified from the
agarose gel.

The reaction mix for all the PCRs (Table 5), first round PCR and the second round PCR used the
same reaction mix (Table 5) and same parameters. Due to the length and GC content of the
reverse primers used in this round, the original PCR programme was no longer effective.
Gradient PCR was done in order to find the optimal annealing temperature for the Round 3
primers, which was finally determined to be 70.4°C (Table 6). All other parameters in the PCR
programme remained unchanged.
Table 4: Properties and sequences of primers used to clone the LSDV epitopes into the TMV CP.

<table>
<thead>
<tr>
<th>Round 1</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward Primer</strong></td>
<td><strong>Reverse Primer</strong></td>
<td></td>
</tr>
<tr>
<td>TMV Forward</td>
<td>TMV Reverse</td>
<td></td>
</tr>
<tr>
<td>5’gggctaggATGCCTTATACAATCAACTCT-3’*</td>
<td>5’ccgcccgggCTAAGTGGCCCGAGGTTG-3’**</td>
<td></td>
</tr>
<tr>
<td>Primer length: 30 Bases</td>
<td>Primer length: 30 Bases</td>
<td></td>
</tr>
<tr>
<td>Melting Temperature (Tm) = 67.37°C</td>
<td>Melting Temperature (Tm) = 76.93°C</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Round 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward Primer</strong></td>
<td><strong>Reverse Primer</strong></td>
<td></td>
</tr>
<tr>
<td><strong>LSDV A</strong></td>
<td><strong>LSDV B</strong></td>
<td></td>
</tr>
<tr>
<td>TMV Forward</td>
<td>TMV Forward</td>
<td></td>
</tr>
<tr>
<td>5’gggctaggATGCCTTATACAATCAACTCT-3’</td>
<td>5’gggctaggATGCCTTATACAATCAACTCT-3’</td>
<td></td>
</tr>
<tr>
<td>Primer length: 30 Bases</td>
<td>Primer length: 30 Bases</td>
<td></td>
</tr>
<tr>
<td>Melting Temperature (Tm) = 67.37°C</td>
<td>Prime</td>
<td></td>
</tr>
</tbody>
</table>
**Table 5**: PCR reaction mix.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (50µL reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfu DNA polymerase (Fermentas)</td>
<td>2 units</td>
</tr>
<tr>
<td>dNTP Mix (2mM each) (Fermentas)</td>
<td>5µL</td>
</tr>
<tr>
<td>Forward Primer (Inqaba)</td>
<td>1 µM</td>
</tr>
<tr>
<td>Reverse Primer(Inqaba)</td>
<td>1 µM</td>
</tr>
<tr>
<td>Template DNA*</td>
<td>1 µL</td>
</tr>
<tr>
<td>10X Pfu buffer with MgSO$_4$ (Fermentas)</td>
<td>5 µL</td>
</tr>
<tr>
<td>Water</td>
<td>35µL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50 µL</strong></td>
</tr>
</tbody>
</table>

* For colony PCR the template DNA is replaced with a bit of the colony sample as the template.

**Table 6**: The temperature regimes used in the PCR.

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Denaturation</td>
<td>94°C</td>
<td>3 min</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94°C</td>
<td>15 sec</td>
</tr>
<tr>
<td>3</td>
<td>Annealing*</td>
<td>56°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>4</td>
<td>Elongation</td>
<td>72°C</td>
<td>45 min</td>
</tr>
<tr>
<td>5</td>
<td>Number of Cycles</td>
<td>Repeat 2-4 30 times</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Elongation</td>
<td>72°C</td>
<td>5 min</td>
</tr>
<tr>
<td>7</td>
<td>Hold</td>
<td>Hold at 4°C</td>
<td></td>
</tr>
</tbody>
</table>

*Annealing temperature for PCR 3 was changed to 70.4°C
Figure 9: A flow diagram of the cloning process.

**ROUND 1 PCR**

The pJL36 plasmid which contains a wtTMV.

The wtTMV CP amplified out of pJL36 and restriction sites added.

The wtTMV with *NotI* and *AvrII* restriction sites.

**ROUND 2 PCR**

Round 2 reverse primers binds to the TMV CP sequence downstream of the insertion sites.

LSDV epitope

The TMV CP with insert attached but missing sequence upstream of the insertion sites.

**ROUND 3 PCR**

The TMV CP with insert and restriction sites on either ends of the sequence.

**Key**

- TMV specific primer with restriction site attached.
- Round 1 primers with the LSDV epitope overhang.
- Round 2 primer with TMV CP sequence downstream of the CP insertion site overhang.
2.2.3 Gel electrophoresis

Successful amplification of all PCRs, as shown in Figure 9, was confirmed by agarose gel electrophoresis. DNA samples, along with appropriate molecular weight markers, 5μL of ThermoScientific O’ Gene Ruler 100bp DNA ladder ready to use (Thermo Scientific) ; 1kb DNA ladder (Promega) or Quick-Load 1kb Extent DNA ladder (Biolabs), were loaded into the wells and ran at 80 volts/cm for approximately 60 minutes in TAE buffer. Bands were visualised using a UV light box and the Gene Snap (Gene Genius Bio Imaging System).

2.2.4 Gel purification

After each round of PCR required for the cloning of the LSDV epitopes into the TMV CP, and after each restriction digest, the DNA was subjected to gel purification. The amplicons and digested materials were run on a 1% (w/v) agarose gel. Long wavelength UV light was used to visualise the bands on the gel and the bands were excised using a sterile razor blade and transferred to a sterile 2mL microcentrifuge tube. The TMV CP amplicon was identified as a 480bp band by comparison with a 100bp DNA ladder (Thermo Scientific). The amplified DNA was purified from the gel using the Zyppy Zymoclean™ Gel DNA Recovery kit (Zymo, California, USA), according to the manufacturer’s instructions. DNA was eluted in 10μL sterile distilled water and quantified using a nanodrop. All DNA gel purifications in this study were performed in this manner.
2.2.5 Plasmid bulking up

2.2.5.1 Preparation of *E. coli* competent cells (DH5α)

The competent cells were made using a method modified from the CaCl₂ method, (Dagert and Ehrlich, 1979; Sambrook and Russell, 2000). A colony from an overnight culture was inoculated into 10mL of 2xYT broth and incubated overnight on a shaker at 37°C. 5mL of the overnight culture was then inoculated into 100mL of fresh, pre-warmed 2x YT broth and incubated at 37°C with shaking till the culture reached an optical density of 1 (OD₆₅₀). The cells were then pelleted by centrifugation at 5000rpm for 5 minutes under refrigerated conditions (4°C).

The supernatant was discarded and the cell pellets were re-suspended in 30mL ice cold 100mM MgCl₂ after which the suspension was allowed to sit on ice for 10 minutes. The cells were re-pelleted under the same conditions as before and re-suspended in the same volume of ice cold 100mM CaCl₂. The suspension was then incubated on ice for 30 minutes after which the cells were pelleted out again under the same conditions and re-suspended in 10mL ice cold 100mM CaCl₂ plus 20% glycerol. The 200µL aliquots were then placed into sterile microcentrifuge tubes and stored at -80°C. The level of competence was determined by transforming the cells with the pUC19 plasmid using the transformation method described above, with kanamycin (50mg/L) being replaced with ampicillin (100mg/L).

2.2.5.2 Preparation of *A. tumefaciens* competent cells (GV3101)

A 10mL overnight culture of *A. tumefaciens* (strain GV3101) in 2x YT broth containing rifampicin and gentamicin was prepared from a single colony. 4mL of the overnight (O/N)
culture was used to inoculate 100mL of 2x YT containing rifampicin (20µg/mL) and gentamicin (50µg/mL) and then incubated with shaking at 30°C. When the culture reached an OD$_{600}$ of between 0.5 and 1.0 it was removed and placed on ice.

The cells were then pelleted by centrifugation at 5000rpm for 5 minutes under refrigerated conditions (4°C) and the supernatant discarded. The cell pellet was re-suspended in 2mL of ice cold 20mM CaCl$_2$. Then 0.1mL aliquots of the suspension were dispensed into pre-chilled microcentrifuge tubes and stored at -80°C. To determine the level of competence the cells were transformed with the pUC19 plasmid using the transformation method described in Section 2.3.2, with kanamycin being replaced with ampicillin as the pUC19 plasmid carries an ampicillin resistance gene instead of a kanamycin resistance gene.

2.2.6 Preparation of recombinant plasmids

2.2.6.1 Restriction digest

To clone the three TMV CP constructs, restriction enzyme digestion was carried out on the purified pJL TRBO plasmid and the purified recombinant TMV CP amplicons carrying the LSDV A and LSDV B epitopes. pJL TRBO plasmid was digested with AvrII and NotI simultaneously according to the manufacturer’s instructions. Samples were then mixed with an appropriate volume of 6X gel loading buffer and analysed by 1.2% (w/v) agarose gel electrophoresis. The linearized plasmid was excised from the gel and purified using the gel purification method, as described previously (Section 2.2.4).
2.2.6.2 Ligation

For this study three different TMV constructs were generated: TMV containing either of the LSDV epitopes (named LSDV-A and LSDV-B), and wild type TMV were made by ligating the appropriate epitope TMV CP fusion to the linearized pJL TRBO. T4 ligase (Fermentas) and ligation buffer were also added to the mixture. The ligation mix was then incubated at room temperature overnight. The ligation mix was used to transform DH5α competent cells and colony PCR was used to select for transformants carrying the correct plasmid. The selected transformants were then used to inoculate LB containing antibiotics and plasmid extractions followed. The final plasmids generated are listed in Table 7.

Table 7: Names assigned to the plasmids generated and their composition.

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLSDV-A</td>
<td>pJL TRBO containing the LSDV-A epitope</td>
</tr>
<tr>
<td>pLSDV-B</td>
<td>pJL TRBO containing the LSDV-B epitope</td>
</tr>
<tr>
<td>pTMV-CP</td>
<td>pJL TRBO containing the wtTMV CP from pJL 36</td>
</tr>
</tbody>
</table>

The constructs were introduced into competent *E. coli* cells by transformation.

2.2.7 Transformation into DH5α *E. coli* cells

Transformation of competent *E. coli* DH5α cells was carried out for DNA amplification of the various plasmids used in the study. To transform the plasmids into competent *E. coli* cells, the standard heat-shock method was used (Sambrook and Russell, 2000). Frozen competent cells
were removed from the -80°C freezer and thawed on ice, then 100ng of plasmid DNA was added and the mixture was incubated on ice for 30 minutes. A heat shocked was performed, for the competent cells to take up the DNA, by placing the reaction mixture in a water bath at 42°C for 45 seconds and immediately back on ice for 5 minutes. Approximately 1mL of warmed 2x YT broth (37°C) was subsequently added and the resulting culture was incubated at 37°C for 3 hours with shaking (200 rpm) to allow for expression.

The cells were then spread on LB agar plates with kanamycin (50µg/L). Plates were then incubated overnight at 37°C and the colonies obtained were inoculated in LB broth containing kanamycin for plasmid (Plasmid containing the construct) isolation from the E. coli culture, using the Zippy plasmid miniprep kit. The isolated plasmids were visualised by agarose gel electrophoresis. Furthermore, as another measure of verification, colony PCR of the isolated plasmid was done, using the Round 3 parameters as outlined in section 2.3.2.1.

2.2.8 Plasmid isolation

To isolate pJL TRBO, DH5α: pJL TRBO was cultured in Luria-Bertani (LB) broth with kanamycin (50µg/mL) in order to isolate pJL TRBO from the bacterial cells. Plasmid isolation was done using the Zippy Plasmid MiniPrep kit (Zymo Research). Isolation of the plasmid of interest was confirmed by agarose gel (1.5%) electrophoresis against a 1kb DNA ladder (Promega) and a Quick-Load 1kb Extent DNA ladder (Biolabs). The concentration of the isolate was determined using a NanoDrop spectrometer (A260).
2.3 Stage II

2.3.1 Growth conditions for the plants

The *N. benthamiana* seeds were planted in individual Styrofoam cups containing organic compost. The plants were grown in a greenhouse with an 18 hour photoperiod at 25-27°C. After 2-3 weeks, seedlings were transplanted into individual cups (1 seedling per cup). The plants were housed in the University of Botswana greenhouse during germination and growth, and were watered daily as required. The plants were fertilized once a week using a commercial hydroponic fertilizer (Nutrifeed starke Ayres- Garden care).

2.3.2 Transformation into *A. tumefaciens* (GV3101).

The *A. tumefaciens* GV3101 was transformed with plasmids purified from *E. coli* using the freeze thaw method (Jyothishwaran *et al.*, 2007). 100µL of *A. tumefaciens* cells were removed from the -80°C freezer and thawed on ice. A bottle of LB media was removed from the fridge and allowed to warm to room temperature. Plasmid DNA (100ng) was added to the thawed cells and the micro-centrifuge tubes were tapped gently several times to mix. The tubes were then placed in liquid nitrogen for 5 minutes. Immediately the tubes were transferred to the 37°C water bath for 5 minutes. About 1mL of room temperature LB broth was added to each tube. The tubes were capped and inverted 6 times to mix.

The tubes were incubated at room temperature for 4 hours. Then the cells were centrifuged at 7000xg for 7 minutes to pellet the cells. Then 0.9mL of the culture was removed and the cell
pellets were resuspended in the remaining media using a P1000 pipette. The entire suspensions were plated on LB containing 50µg/mL kanamycin, 25µg/mL gentamicin and 10µg/mL rifampicin using glass rods. The plates were incubated overnight at 25°C. *A. tumefaciens* was transformed with pTMV, pLSDV-A, and pLSDV-B for use in agro-infection. Colony PCR was done to check that the transformation was successful and that the insert was still present within the vector as indicated below.

2.3.2.1 Colony screening

Confirmation of successful transformation was carried out by colony PCR using a method adapted from Mirhendi *et al.*, (2007). A colony was picked using a sterile micropipette tip (0.5µL) and mixed into the same PCR mix used in normal PCR reactions (Table 5). The same thermocycler conditions (see Table 6), and PCR round 1 primers were used (see Table 5). This method was applied to each recombinant TMV CP transformation in DH5α and *A. tumefaciens* GV3101.

2.3.3 Agro-induction

Individual colonies of *A. tumefaciens* transformed with Ptmv, pLSDV-A and LSDV-B plasmid were inoculated into LB broth (containing rifampicin, gentamicin and kanamycin) and grown to an OD600 of 1.0 in L-MES media at 25°C overnight. The cells were collected by centrifugation at 10Krpm for 5 minutes and re-suspended in agro-induction media (Refer to the appendix for
media/reagent composition), and then the cells were allowed to sit at room temperature overnight.

**2.3.4 Agro-infection**

Six week old *N. benthamiana* plants were infected by infiltrating the agro-induced cells into the abaxial surface of 3 leaves using a 1-3mL needleless syringe. Pressure was applied from the top side of the leaf with a gloved finger, and the syringe was depressed slowly and evenly to inject the liquid culture into the leaf.

The plants were infected with *A. tumefaciens* carrying the pTMV, pLSDV-A, pLSDV-B plasmids; *A. tumefaciens* with no plasmid and water (Table 8). Expression was visible within 2-3 days. Symptoms of TMV infection include, light and dark mottled areas on leaves, infected leaves are often curled and puckered. Virus was extracted within 3 weeks.

**Table 8:** Composition and function of materials used to infect *N. benthamiana* plants.

<table>
<thead>
<tr>
<th>Plants infected with</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTMV</td>
<td>This plasmid constituted the wtTMV CP amplified out of pJL 36 and inserted into pJL TRBO with no LSDV epitope fused to the TMV CP. The virus produced was also used as wtTMV in the ELISAs and western blots</td>
</tr>
<tr>
<td>pLSDV-A</td>
<td>pJL TRBO containing the LSDV-A epitope.</td>
</tr>
<tr>
<td>pLSDV-B</td>
<td>pJL TRBO containing the LSDV-B epitope</td>
</tr>
<tr>
<td>Water</td>
<td>Served as a negative control</td>
</tr>
</tbody>
</table>
2.3.5 rtPCR

Confirmation of successful infection of the *N. benthamiana* plants was by reverse transcription PCR (rtPCR) of the TMV coat protein. Crude extract from leaves were obtained seven days post-inoculation by grinding the leaves in liquid nitrogen and re-suspending in TE buffer with DNase added. rtPCR reactions were performed using an rtPCR kit (First Strand cDNA Synthesis Kit, Fermentas), as described by the supplier, using TMV CP specific primers. The product of the rtPCR was used as the template for the standard PCR using the conditions already described above.

2.3.6 Virus extraction

All virus assays were carried out on semi-pure extracts from infected *N. benthamiana* plants, 3 weeks post-infection; 20g (recorded as fresh weight-A) of systemically infected leaves were harvested from the plants infected with pLSDV-A, pLSDV-B, pTMV, water and *A. tumefaciens* with no plasmid. The leaves were ground using a pestle and mortar until homogenized. The grinding was done in 4 x weight-A volume of virion extraction buffer, which was added in small amounts until the leaves were well macerated. The homogenate was filtered through two layers of cheesecloth into 50mL polypropylene centrifuge tubes, making sure that as much of the liquid was collected while preventing any of the particles from entering the filtrate. The liquid was heated for 5 minutes at 50°C.

The tubes were centrifuged at 12K rpm for 25 minutes to pellet out the green plant material. The supernatant was transferred into a clean 50mL centrifuge tube and the volume was recorded.
The green pellet was re-suspended in about 1mL phosphate buffer and saved for analysis. Then volume-B x 0.12ml of 40% PEG and volume-B x 0.17ml of 5M NaCl were added to the supernatant to precipitate the virus. The supernatant was left over night in a fridge. The following day the supernatant was centrifuged at 15K rpm for 25 minutes at 4°C. After centrifugation a white pellet was visible and the supernatant was poured off. The pellet was re-suspended in 1/10 volume-A of 50mM phosphate buffer (pH 5.7). The virus was stored in the fridge until analysis was completed.

2.4 Stage III

2.4.1 Virus analysis

The extracted virus was subjected to SDS PAGE, western blot, and ELISA analysis to confirm the presence of TMV, to check if the LSDV epitopes are covalently bonded to the TMV virion and to check if the LSDV/TMV display particle was capable of eliciting an immune response in animals.

2.4.1.1 SDS-PAGE

Equal volumes of viral extract of each treatment were combined with SDS-PAGE loading dye. The samples were loaded on a 12% separating gel with a 4% stacking gel (Laemmli, 1970). The PROTEAN® II xi 2-D (BIO-RAD) system was used to prepare and run the gel according to the manufacturer’s instructions.

2.4.1.2 Western blotting
One piece of nitrocellulose membrane and 20 sheets of filter paper, the size of the gel, were cut and soaked in transfer buffer for 20 min. After the electrophoresis, the membrane was blotted using a semi-dry electro-blotter (Fisher Scientific). 10 sheets of filter paper were placed on the anode followed by the nitrocellulose membrane, gel and the remaining filter paper. The cathode was then placed on top. A constant voltage of 30V was run through the setup for 45 minutes to effect blotting of the SDS gel onto the membrane.

Upon completion of the transfer, the membrane was washed twice in Tris-buffered saline (TBS) to remove the methanol from the transfer buffer then it was placed in freshly made blocking buffer with 5% milk TBS-T (Tris-buffered saline- Tween/Triton), and shaken for an hour at room temperature. The membrane was then washed twice with TBS-Tween/Triton, then twice with TBS at room temperature, 15 min each. The membrane was then probed with either goat anti-TMV (polyclonal anti TMV, Dr I Becker, University of Cape Town, South Africa) or bovine anti LSDV (polyclonal anti-LSDV, OVI, South Africa), diluted in 3% BSA (in TBS) for 4 hours at room temperature with shaking.

After the four hours had elapsed the membrane was washed as before then probed with the appropriate secondary antibody (anti-Goat/Sheep monoclonal IgG, alkaline phosphatase conjugate, Sigma; or anti-Bovine monoclonal IgG alkaline phosphatase conjugate, Santa-Cruz Scientific) diluted in 3% BSA for 4 hours at room temperature with shaking. The membrane was then washed four times with TBS-Tween/Triton for 15 minutes each at room temperature. To develop the membrane a tablet of NBT/BCIP (Sigma) was diluted in 200mL of sterile water and added to the membrane and then allowed to sit in the dark till sufficiently dark bands appeared.
The reaction was stopped by washing away the NBT/BCIP with sterile distilled water (QIAGEN, 2002).

2.4.1.3 ELISA

A 96-well plate (number of wells used varied depending on the number of samples available) was coated with 50μl of capture antibody in carbonate buffer overnight at 4°C. For LSDV-TMV sandwich ELISAs, anti LSDV antibodies (bovine anti LSDV polyclonal sera obtained from the OVI, South Africa) were used as the capture antibody while for the TMV ELISA, anti-TMV antibody (goat anti TMV polyclonal sera, Dr I Becker, University of Cape Town) was used. The plate was covered with cling wrap to prevent drying.

The wells were then emptied and washed 5 times with phosphate-buffered saline (PBS) and then dried by tapping gently on paper towel to remove all the liquid in the wells. 200μl ELISA blocking buffer was then added and the plate was incubated for 1 hour at 37°C. To each well 50μl of purified TMV extracted from agro-infected plants in glycerol was added and the plate was incubated for 30 minutes at room temperature.

The sample was then removed and the plate washed 3 times with 200μl PBS and dried as before, after which 50μl of the sandwich antibody (goat anti TMV polyclonal sera, Dr I Becker, University of Cape Town), diluted 1:10000 in blocking buffer, was added to each well. The plate was covered and incubated for 2 hours at room temperature. The antibody was removed and the plate washed 4 times with PBS and dried as before. 50μl secondary antibody; alkaline phosphatase conjugated monoclonal anti goat/ sheep IgG (Sigma); diluted in blocking buffer to
the appropriate concentration was added to each well and the plate covered and incubated for 1 hour at room temperature. The antibody was removed and washed off 4 times with PBS after which 100µl NBT/BCIP solution was added to each well and colour allowed to develop for 30 minutes then the plate was photographed. **Figure 10** shows the flow diagram for the ELISA design.

**Figure 10**: The ELISA design.
Chapter 3

3.0 RESULTS

3.1 Plasmids bulking up

3.1.1 Transformation efficiency of E. coli (DH5α)

The competent cells were prepared successfully. This was seen as the newly prepared competent cells were able to take up the different plasmid and grow on nutrient agar containing ampicillin (for pUC19) and rifampicin, kanamycin and gentamycin. The transformation of the plasmids into DH5α was also successful. However, the transformation efficiency of the competent cells seemed to drop when the TMV based plasmids (pJL TRBO and pJL 36) were used to transform the cells.

3.1.2 Transformation efficiency of A. tumefaciens (GV3101)

The A. tumefaciens competent cells were able to take up the different plasmids and grow on nutrient agar containing with specific antibiotics.

3.1.3 Plasmid isolation

The isolation of pJL 36 and pJL TRBO plasmids from DH5α cells was successful as shown by bands electrophoresing between the 9413bp and 23130bp marker. This corresponds to the expected 11578bp for pJL 36 and 10606bp for pJL TRBO (Figure 11). Two bands are visible on the gel photograph which is not uncommon with plasmids. One band represents the super coiled
form of the plasmid while the other is the normal plasmid. The super coiling did not interfere with downstream processes in the study.

![Agarose gel electrophoresis of plasmid extracted from E. coli. Lane 1: Quick load 1Kb Extend ladder (New England BioLABS). Lane 2: Blank. Lane 3: pJL36. Lane 4: Blank. Lane 5: pJL TRBO. Lane 6: Blank.]

**Figure 11:** Agarose gel electrophoresis of plasmid extracted from *E. coli*. Lane 1: Quick load 1Kb Extend ladder (New England BioLABS). Lane 2: Blank. Lane 3: pJL36. Lane 4: Blank. Lane 5: pJL TRBO. Lane 6: Blank.

### 3.2 Cloning epitopes into pJL TRBO

#### 3.2.1 PCR

The three rounds of PCR used to introduce the epitope into the TMV CP were successful. In the first round of PCR the TMV CP was amplified out of pJL36 to give a band 480 bases long (Figure 12a Lane 2). The second round resulted in a construct of 540 bases long (Figure 12a Lane 3 and 5 for LSD A and LSD B respectively) while the final round resulted in an amplicon 560 base pairs long (Figure 12a Lane 4 and 6 for LSD A and LSD B respectively).
Figure 12: a) Agarose gel electrophoresis of products of three rounds of PCR. Lane 1: Quick load DNA Ladder. Lane 2: TMV CP (PCR 1). Lane 3: 2\textsuperscript{nd} round PCR product of LSD A. Lane 4: 3\textsuperscript{rd} round of PCR product for LSD A. Lane 5: 2\textsuperscript{nd} round PCR product of LSD B. Lane 6: 3\textsuperscript{rd} round PCR product for LSD B. b) Restriction enzyme digests of pJL TRBO. Lane 1: Quick load 1Kb Extend Ladder. Lane 2: Blank. Lane 3: pJL TRBO uncut. Lane 4: Blank. Lane 5: pJL TRBO cut using NotI and AvrII. Lane 6: Blank. c) Plasmids isolated from \textit{E. coli} after ligation of pJL TRBO and TMV CP constructs. Lane 1: Quick load 1 Kb Extend Ladder. Lane 2: pLSDV-A. Lane 3: Blank. Lane 4: pLSDV-B. Lane 5: Blank. Lane 6: pTMV. d) Colony PCR results for \textit{E. coli} colonies carrying the TMV CP. Lane 1: Quick load DNA Ladder. Lane 2: pLSDV-A. Lane 3: Negative control (water). Lane 4: pLSDV-B. Lane 5: Blank. Lane 6: pTMV.
3.2.2 Restriction digest

In order to insert the recombinant TMV coat protein constructs, pJL TRBO (Figure 12b) and the TMV CP constructs (Figure 12a), were subjected to restriction endonuclease activity (NotI and AvrII). Since only a few bases are removed due to the digestion, the TMV CP constructs appear to be the same length on the agarose gel (Figure 12b). There is only a slight difference between the linearised pJL TRBO and the circular form of the plasmid (Figure 12a). In lane 3 (Figure 12a), the pJL TRBO has been linearised by digestion with both NotI and AvrII and is shown to be electrophoresing slightly lower than the circular and super coiled forms of the plasmid.

3.2.3 Ligation

The linearised pJL TRBO was ligated to the different coat protein constructs to form plasmids named pTMV, LSDV-A and LSDV-B, depending on the epitope fused to the TMV coat protein (Table 5). The electrophoresis of these plasmids showed bands running between the 9413bp and 15000bp markers as expected from the plasmids that are approximately 11150bp in length (Figure 12b). The pJL TRBO (Figure 12b lane 2) plasmid was used as a control to compare the newly constructed plasmids and it shows that the plasmids are of the expected size based on rate of electrophoresis.
3.2.4 Screening of the TMV CP transformants in *E. coli*.

A colony PCR was carried out on the colonies that grew on LB agar containing kanamycin. These colonies were carrying the plasmid containing kanamycin resistance. Colony PCR was carried out to further confirm the presence of the plasmid which would also be carrying the TMV CP as well as the epitopes of interest. Gel electrophoresis of the DNA product from the colony PCR showed successful amplification of the DNA falling between the 500bp and the 600bp band. The TMV CP is expected to be about 490bp in length, thus the pTMV was about the expected size (*Figure 12d*: Lane 6); whereas fragments obtained for the LSDV-A and LSDV-B (*Figure 12d* lane 2 and 4 respectively) were slightly bigger than the pTMV as expected. There was no product for the negative control where water was used as the template (*Figure 12d*: lane3).

3.3 Screening of TMV CP transformants of *A. tumefaciens*.

Colonies that grew on LB containing kanamycin, rifampicin and gentamicin were assumed to be carrying the plasmid containing kanamycin resistance since the *A. tumefaciens* (GV3101) already contains resistance to gentamycin and rifampacin. A colony PCR was done to confirm the presence of the plasmid which would also contain the TMV CP and the epitopes of interests. Gel electrophoresis of the amplicons from the colony PCR revealed a successful amplification of the DNA fragment falling between the 500bp and the 600bp. For the negative control there was no amplicon (*Figure 13*: lane 3). The pTMV (*Figure 13*: lane 6) was slightly smaller in size as compared to LSD A (*Figure 13*: lane 2) and LSD B (*Figure 13*: lane 4).
Figure 13: Colony PCR results for *A. tumefaciens* colonies carrying the TMV CP. Lane 1: 100bp Quick load DNA Ladder (New England BioLabs). Lane 2: pLSD A. Lane 3: Negative control (water). Lane 4: pLSD B. Lane 5: Blank. Lane 6: pTMV.

3.4 Confirmation of infection of *N. benthamiana* plants.

3.4.1 Visual confirmation

Two weeks post infiltration typical symptoms of TMV infection were not observed these include curling, mottling and chlorosis of leaves (Figure 14b). Leaf necrosis was clearly visibly in all infiltrated leaves Figure 14c and d.
Figure 14: Comparison of a healthy and Agro-infected *N. benthamiana* plants. a) A healthy *N. benthamiana* plant. b) Typical symptoms of TMV infected *N. benthamiana* plant, arrows showing curling and molting leaves. c) and d) Leaf necrosis indicated by arrows was visible in the agro infiltrated leaves.

3.4.2 rtPCR of Agro-infiltrated plants.

The raw plant material was used to carry out rtPCR using the TMV CP specific primers to amplify the DNA fragments. The negative controls being plants infiltrated with pure water and plants infiltrated with clean *A. tumefaciens* (with no plasmid) are blank as TMV CP was not present in the plant material. The pLSDV A and pLSD B did not have amplicons, the pTMV did show an amplicon between the 500bp and 600bp as expected.
3.5 Virus extraction and analysis

The nature of the extracted virus was determined by subjecting the extracts to SDS analysis, western blotting and ELISA.

3.5.1 SDS PAGE and western blot

The TMV CP has a molecular weight of 17.6 kDa. The pTMV ran between the 15k Da and 20 kDa markers in the SDS PAGE gel. The pLSDV-A and pLSDV-B could not be detected on the SDS PAGE gel. The positive control being the pJL 24 was detected running at about 17.6 kDa. The negative controls (water and *A. tumefaciens* without plasmid) did not show any bands.

The western blots of gels loaded with the same material and same order as the SDS PAGE gels showed that only pTMV was detected with goat anti-TMV antibodies. Refer to Appendix 1: Figure 15 for the SDS PAGE and the western blot. The pLSDV-A and pLSDV-B were not detected as expected using bovine anti-LSDV antibodies. The negative controls did not show any bands in the western blots (data not shown due to poor picture quality).

3.5.2 ELISA

To test the potential antigenicity of the viral extract a sandwich ELISA was used. The negative controls; water *A. tumefaciens* with no plasmid pTMV, pLSDV-A and pLSDV-B were used as samples for the ELISA. The plates were coated with goat anti-TMV antibodies (Row A) and anti-LSDV sera (Row B). The samples were then captured on the plates and the anti-TMV
antibodies were used as secondary antibodies. AP conjugated rabbit anti goat antibody was used in conjunction with NBT/BCIP to visualize the reaction. The ELISA showed that the viral extract pTMV reacted with the anti-TMV antibody, refer to Appendix 1: Figure 16. LSDV constructs did not react with the anti-LSDV sera. The pTMV did not react with the LSDV sera since it was not carrying an LSDV epitope. The negative controls from plants infiltrated with water and A. tumefaciens without plasmid tested negative for the LSDV (data not shown).
Chapter 4

4.0 DISCUSSION

Administration of vaccines is by far the most humane and cost effective method of combating the spread of diseases (Streatfield, 2005). Several systems have been used to produce vaccines including chemical synthesis, fermentation and development of transgenic plants (Wigdorovitz et al., 2004). Production costs, bulk up capacity and stable storage still remain a great challenge in vaccine production.

Plant based vaccines offer potential remedies to several disadvantages of conventional vaccines (Fischer et al., 2012; Streatfield and Howard, 2003; Rybicki, 2014; Tiwari et al., 2009; Tremblay et al., 2010). In this study the possibility of using a TMV-based vector to produce epitopes of LSDV as TMV CP fusions in N. benthamiana was explored, in order to develop a candidate vaccine for LSDV. The P32 antigen is important in pathogenicity, diagnosis, prevention and control of capripoxvirus (Tian et al., 2010). The study mainly focused on the P32 structural protein, and attempted to establish a cost effective method of vaccine production for LSDV in a country that is heavily reliable on the cattle industry.

4.1 Epitope design

The epitopes selected and used in this study, had to be suitable to elicit effective and lasting protection against LSDV thus the use of part of the structural protein 32 (P32). P32 was used because it is the immunodominant region of LSDV. The consensus sequences generated were
derived from the sequences studied before (Tian et al., 2010); these included the amino acids 95-114 and 156-175 of the structural protein P32.

The choice of epitopes was limited, because there is a size limit associated with the CP fusions (Mortimer et al., 2015). A maximum of 25 amino acids may be inserted into the TMV CP without disrupting the proper folding of the TMV CP. Thus the epitopes used were 20 amino acids in length to fit into the acceptable range for TMV CP fusions. The epitopes used were synthesized as opposed to being amplified out of the LSDV genome, and this ensured that the epitopes used were of the right sequence as the ones generated from the consensus sequence.

The coat protein of several plant viruses, including TMV (Hamamoto et al., 1993), cow pea mosaic virus (Porta et al., 1994; Usha et al., 1993), tomato bushy stunt virus and alfalfa mosaic virus have been used successfully as carrier molecules. For this study TMV CP was used. Plant virus CP fused to a foreign peptide can self assemble into particles. It is increasingly being used in the production of antigenic peptides in plants.

In addition to the safe and inexpensive production environment provided by plants, in-frame fusion of peptides to plant virus CP provides a means of easy and low cost purification, an important aspect of large scale peptide manufacturing (Belanger et al., 2000; Fahad et al., 2015; Verma and Daniell, 2007).
4.2 Cloning of LSDV epitopes into pJL TRBO

The LSDV epitopes were successfully inserted into the TMV CP using three rounds of PCR. Due to the size of reverse primers used in PCR 2 and 3, respectively large sizes of products were formed. Pure amplicons of the right sizes were produced by gel purifying the product using the Zyppy Zymoclean™ Gel Recovery kit.

From PCR 1 to the 3rd PCR the product size increased gradually as expected (Figure 14). This was due to the LSDV epitope sequence being added to the second round of PCR and only a small piece of the TMV CP being excised. In the 3rd round PCR, the excised part of TMV CP is re-introduced therefore increasing the length of the TMV CP sequence. The primers also added the restriction sites (NotI and AvrII) corresponding to the enzymes found in the pJL TRBO multicloning site to the end of the TMV CP. This aids easy insertion of the constructs (the modified TMV CP) into the pJL TRBO.

The coat proteins carrying the different constructs were cloned successfully into the binary vector pJL TRBO. The vector was designed without the TMV CP, for high protein expression in plants (Lindbo, 2007a). The CP is not essential for cell to cell transport but is for efficient systemic movement in N. benthamiana and other host species (Dawson et al., 1988; Saito et al., 1990; Spitsin et al., 1999). The use of recombinant TMV virion as a vaccine has advantages over the whole virus or peptides alone (McCormick et al., 2006; McCormick and Palmer, 2008).

The pJL TRBO containing the different constructs was successfully transformed into E. coli (DH5α). The plasmid carrying the constructs was then extracted and purified from E. coli and
used to transform *A. tumefaciens* (GV3101). The transformation was successful as shown by colony PCR. Due to time constrains the amplicons from the colony PCR were not sequenced. The colonies that carried the constructs were used for Agro-infiltration.

### 4.3 Agro-infiltration of *N. benthamiana*

Bacterial expression systems have the advantage of speed and abundant production. However, they are limited in many instances by their inability to produce properly folded proteins. In this study, Agro-infection was not successful as the typical TMV infection symptoms were not observed in *N. benthamiana* plants post infiltration. However, the leaves showed signs of necrosis. Different TMV CP substitutions, deletions and peptide additions have also been shown to induce necrotic response in plants independent of any known host resistance response (Bendahmane *et al*., 1999; Culver, 2002; Dawson *et al*., 1988; Lu *et al*., 1998; Saito *et al*., 1989).

Although the native CP was expressed the recombinants were not expressed and this could be due to a number of factors. In some cases, epitopes expressed on the surface of plant viruses interfere with the normal virus infection cycle. Such results were observed with TMV and CPMV where the insertion of certain foreign sequences resulted in interference of viral assembly (Bendahmane *et al*., 1999). Those foreign epitopes aided loss of systemic infection, and or induction of a necrotic response in the host (Takamatsu *et al*., 1990; Portal *et al*., 1994; Beachy *et al*., 1996). Such effects limit the use of viruses for production of peptides, including their use as vaccines (Bendahmane *et al*., 1999).
The CP units in the TMV particles are assembled in a highly ordered fashion, with the N- and C-termini both located on the surface of the TMV particles (Namba et al., 1989; Durham et al., 1971). The epitopes fused to the CP could only be displayed outside the virus particles if the fused CP subunits are still able to encapsidate the recombinant viral genome properly (Turpen et al., 1995; Sugiyama et al., 1995). In this study, there were problems when it comes to the assembly of virus-like particle in the plant cells. This resulted in unsuccessful expression of the epitopes of interest.

4.4 Virus extraction and analysis

4.4.1 Virus extraction

In this study the virus expressing the epitopes of interest was not obtained in the extraction made from the infiltrated leaf material. Agro-infiltration was repeated on several occasions and the expression was still unsuccessful. The generated constructs were not stable in the plants. However, unsuccessful expressions of certain short epitopes are often experienced, the corresponding rTMV genomes become less infectious (Takamatsu et al., 1990).

It has been reported that a group of rTMV genomes encoding short peptides with transmembrane domain induce necrotic lesions (Li et al., 2006). The display of foreign peptides on the surface of TMV has been proven to be an effective way to produce valuable peptide medicines using tobacco. However, some foreign peptides have been severely affected by the viral infectivity, viral particle formation and even symptom appearance on susceptible hosts (Bendahmane et al., 1999; Portal et al., 1994; Takamatsu et al., 1990; Li et al., 2006).
It has been suggested that various criteria such as the amount of the amino acid residues (Wu et al., 2003), pI/charge value of foreign peptides (Bendahmane et al., 1999) and fusion sides (Fischen et al., 1995; Hamamoto et al., 1993; Sugiyama et al., 1995; Takamatsu et al., 1990; Turpen et al., 1995) in the TMV CP correlate with the infectivity and stability of the rTMV in tobacco plants. Unfortunately, there is no single criterion that could correlate the host response to the virus infection (Li et al., 2006).

Therefore, for successful epitope expression in *N. benthamiana* plants there are several factors to consider. These include; stability of the constructs after being cloned into the vector and the presence of transmembrane domain encoded by the constructs (Bendahmane et al., 1999). Any possible transmembrane sequence in foreign peptide must be avoided in the mass expression of foreign peptides in tobacco plants using a TMV-based vector (Li et al., 2006).

### 4.4.2 Serology

To enable the rTMV to elicit an immune response when used as a vaccine, the epitopes fused to the TMV CP would have to be expressed on the surface of TMV virion. In this study the epitopes were not successfully expressed on the TMV surface as CP fusions since could not be detected using ELISAs and western blots. The TMV CP used in this study was approximately 480bp (160a.a long). Estimation of the molecular weight of the pTMV is 17.6 kDa so the expectation was that the pLSDV constructs would run between 15 and 20kDa. The bands were not visualized on SDS and even on western blot.
The use of the anti-LSDV sera also showed that the LSDV antigens were not synthesised in the plants. The negative controls (water and *A. tumefaciens* with no plasmid) did not show any reaction to sera indicating that the antibodies were not reacting non-specifically to unknown plant derived antigens. The wtTMV CP did not react to the anti-LSDV antibodies. This clearly indicated that anti-LSDV antibodies were not reacting to TMV CP. The covalent association between the TMV CP and LSDV epitopes used in this study was not achieved. This was further confirmed with a sandwich ELISA, for both pLSDV-A and pLSDV-B constructs were not attached to the TMV CP. The controls tested negative when probed with anti-LSDV antibodies.

### 4.5 Limitations

The TMV CP can only be fused with a maximum of 24 amino acids. Larger epitopes compromise the folding of the CP and result in unsuccessful TMV virion formation (Wu *et al.*, 2003; Beachy *et al.*, 1996). As a result the sizes of the epitopes used in this study were limited. The size also limits the number of epitopes that can be fused to the TMV CP simultaneously. Only linear epitopes can be fused to the TMV CP but in some cases the non-linear epitopes are the major immunodominant regions. Instability of the epitopes in the viral genome can also present some problems (Scholthof *et al.*, 1996)

### 4.6 Conclusion

Previous studies showed that subunit vaccines can be generated using TMV CP fusions in *N. benthamiana* plants. However, for this study the epitopes of interest were not successfully
expressed in *N. benthamiana* plants. This clearly indicates that epitope design should be thoroughly investigated to successfully express the epitopes in plants. It was noted that not all epitopes can be expressed on the surface of TMV. The agro infiltration method is quick and cost effective. It takes 3 weeks from infiltration to harvest as compared to the traditional methods which takes at least 6 months. This method can be optimized and used in plant vaccine production systems. The success of this method would result in better, more specific and cheap vaccine production.

4.7 Recommendations

In order to improve this study the following recommendations were made:

- The use of an alternative cloning method for insertion of duplex epitopes into modified pJL TRBO vectors like linker, helical linker or CP154. The method is appropriate for insertion of epitopes that are more than 20 amino acids in length. These vectors are already being tested by the Grill lab in Pitzer College Plant Vaccine Group (unpublished data).

- The PCR amplification of the TMV genome outside of the CP gene to determine whether it was in fact present where the peptide and or the CP was not.

- The use of transformed plants expressing native TMV CP. The use of plants that already express the native TMV CP should help stabilize TMV virions with longer epitopes, since each virion should be a mixture of native CP subunit and CP subunits with an epitope attached.
- The use of multiple epitopes and selecting the best expressed epitopes to use for vaccine production. Concoction or a mixture of epitopes should be tested to see which would give the best and long lasting protection against LSDV.

- Sequencing and analysis of the amplicons after the colony PCR from *A. tumafaciens* and after the rtPCR of the plant extracts can also be done to confirm if the LSDV epitopes were still attached to the TMV CP.

- The use of longer epitopes using a different vector can also be investigated to compare how short epitopes fare compared to longer epitopes.
Chapter 5

5.0 REFERENCES


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Chapter 6

6.0 APPENDICES

6.1 Appendix 1: Virus extraction and analysis

Figure 15: SDS PAGE and western blot analysis of viral extracts. 

a) Coomassie stained SDS gel

b) The western blot was carried out using anti TMV. The gel and the blot were loaded as follows; lane 1: the pre-stained marker (biorad). Lane 2: water. Lane 3: pLSDV-A. Lane 4: water. Lane 5: A. tumefaciens without plasmid. Lane 6: pLSDV-B. Lane 7: water. Lane 8: pLSDV-B. Lane 9 pTMV. Lane 10: pJL 24.
Figure 16: Sandwhich ELISA of the extracted TMV constructs. Row A: coated with anti-TMV antibodies, Row B: anti-LSDV sera. Column A: 1 and 2: coated with water, Column 3 and 4: coated with *A. tumefaciens* without plasmid. Column 5 and 6: coated with pTMV.
6.2 Appendix 2: Plasmid Maps

Figure 17: pJL 36 vector map.

Binary vector, Kanamycin resistance marker. T-DNA borders not shown in map. Clone gene of interest into \textit{PacI-AvrII-NotI} multiple cloning site. (Lindbo, 2007a)
Multiple cloning Site (MCS)

Figure 18: pJL TRBO

The binary plasmid, replicates in *E. coli* or *A. tumefaciens*. It has Kan resistance selection (50μg/mL). The T-DNA borders are not shown in map. This TMV based replicon is used for high level gene expression in plants. The gene of interest is cloned into *PacI-AvrII-NotI* polylinker [ttaattaa, cctagg, gcggccgc] (see MSC sequence map). This replicon does NOT have coat protein gene, so the replicon will NOT move systemically, and will not form virion particles (Lindbo, 2007b).
6.3 Appendix 3: Buffers and solutions

30% Acrylamide

29g Acrylamide
1g N,N’-methylenebisacrylamide
Dissolve in 60mL of dH2O
Adjust volume to 100mL with dH2O
Filter sterilise (0.45 micron pore size)
Store at 4°C

50mg/mL Rifampicin

1g Rifampicin
2mL DMSO
Filter sterilise
Store in 500µL aliquots at -20°C

100mg/mL Ampicillin

1g Ampicillin sodium salt
Add dH2O up to 10mL
Filter sterilise (0.22 micron pore size)
Aliquot 500µL into microcentrifuge tubes
Store at -20°C

10% APS

1g Ammonium per sulphate (MW 228.2)
Dissolve in 10mL dH2O
Store in 200µL aliquots at -20°C

10X PBS

80g NaCl
2g KCl
11.5g Na2HPO4
2g KH2PO4
Make up to 1L. Autoclave.

50mg/mL Kanamycin

1g Kanamycin sulphate
2mL H2O
Filter sterilise
Store in 500µL aliquots at -20°C

1% Agarose

1g Agarose
Make up to 100mL with 1X TAE

25mg/mL Gentamicin

1g Gentamicin
4mL H2O
Filter sterilise
Store in 500µL aliquots at -20°C

0.5µg/mL (200mL) Ethidium Bromide
Add 10μl of 10mg/mL Ethidium Bromide (BioRad) to 200ml ddH2O. Wrap in foil and store at 4°C

6X Gel loading dye
25mg Bromophenol Blue
3mL Glycerol
10mL dH2O

Coomassie stain
250mL Isopropanol
100mL Glacial acetic acid
1g Coomassie Blue
650mL dH2O

Destain
250mL Isopropanol
100mL Glacial acetic acid
650mL Water

Transfer buffer (pH 9.2) (Semi-dry)
5.82g Tris Base
2.93g Glycine
200mL Methanol
Make up to 1L with dH2O

Induction Media
2mL 0.5M MES pH5.7
2mL 0.5M MgCl2
200μL 0.1M Acetosyringone
Make up to 100mL with sterile d H2O

LB broth (1L)

L-MES media
50mL LB broth
1mL 0.5M MES pH 5.7
10µl Acetosyringone
50µl Kan, 25µl Gent and 10µl Rif

NBT/BCIP solution
1 tablet in 20mL dH2O
(Sigma Aldrich)

2X YT broth (1L)
10g Yeast extract powder
16g Triptone
5g NaCl
Autoclave

TBS (500mL)
50mL 1M Tris-Cl
4.5g NaCl
pH to 7.8

TBS-Tween/Triton
50mL 1M TRIS-CL
14.6g NaCl
0.25mL Tween 20
1mL Triton-X 100
pH to 7.8
Make up to 500mL with dH2O

100mM MgCl2
50mL 1M MgCl2
450mL dH2O
Autoclave

100mM CaCl2
50mL 1M CaCl2
450mL dH2O
Autoclave

80% Glycerol v/v
80mL Glycerol
20mL H2O
Autoclave

100mM CaCl2 and 20% Glycerol
50mL 1M CaCl2
100mL Glycerol
350mL dH2O
Autoclave

3% BSA (Blocking buffer)
3g BSA
100mL TBS
20mM MgCl$_2$
10mL 1M MgCl$_2$
490mL dH$_2$O
Autoclave

20% PEG (8000)
20g PEG 8000
100mL sterile dH$_2$O
Filter sterilise

5M NaCl
146g NaCl
Make up to 1L with dH$_2$O

20% SDS
20g SDS
100mL dH$_2$O

0.5M MES pH5.7
19.52g MES
60mL sterile dH$_2$O
Adjust pH to 5.7 with 10M NaOH
Make up to 100mL with sterile dH$_2$O
Filter sterilize into sterile bottle

Sample treatment buffer (10mL)
2.5mL 4x Stacking Buffer
4mL 10% SDS

4% PEG (8000)
4g PEG 8000
100mL sterile dH$_2$O
Filter sterilise
2mL glycerol
0.2mL β-mercaptoethanol (1M)
0.2mg bromophenol blue
Store as 500µL aliquotes at -20°C.

Carbonate buffer (50mM, pH 9.6)
Make a stock solution of 1M NaHCO$_3$
(84.01g/L of water)
Dilute 1:20 before use and adjust pH to 9.6
with NaOH.
Store at 4°C.

4x Resolving buffer (200mL)
36.3g Tris-base
pH 8.8, adjusted with HCl.
Autoclave.

4x Stacking buffer
3g Tris-base
Make up to 50mL with dH$_2$O
pH 6.8, adjusted with HCl.
Autoclave.

20% SDS
10g SDS
Make up to 50mL with dH₂O

Autoclave.

0.5M MgCl₂

10.16g MgCl₂.6 H₂O

Make up to 100mL with sterile distilled water

Filter sterilize into sterile bottle

10x Running buffer

30.3g Tris-base
144.2g glycine
10g SDS

Water to 1L

pH 8.5, adjusted with HCl.

12% SDS PAGE gel

10.2 mL H₂O
7.5 mL 4X Resolving Buffer
0.15 mL 20% SDS
12.0 mL 30% Acrylamide
0.15 mL 10% APS
0.02 TEMED

Extraction buffer

50Mm NaAcetate pH 5.0 (2.05g)
Adjust pH to 5.0
0.1% Na metabisulfite