Development of plant-produced Bluetongue virus vaccines

By

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“It always seems impossible until it’s done.”

~Nelson Mandela

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Abstract

Bluetongue is a disease of domestic and wild ruminants caused by Bluetongue virus (BTV). It has caused several serious outbreaks, the most recent occurring in Northern Europe in 2006 during which high mortality rates of livestock were reported. The only vaccines currently approved and commercially available for use are live-attenuated or inactivated virus strains and although these are effective, there is the risk of reversion in the case of live-attenuated strains to more virulent forms by recombination. Another drawback associated with the use of live-attenuated virus vaccines is that they are not DIVA (**d**ifferentiate **i**nfected from **v**accinated **a**nimals) compliant, this means that naturally infected animals cannot be distinguished from vaccinated animals. Recombinantly produced vaccines would be preferable to minimize the risks associated with live-attenuated virus vaccines and also enable the development of candidate vaccines that are DIVA-compliant.

A number of recombinant vaccine candidates have been developed against BTV, with the most promising vaccine consisting of BTV virus-like particles (VLPs). BTV VLPs were successfully produced in insect cells by the co-expression of the four BTV capsid proteins (VP2, VP3, VP5 and VP7). Sheep vaccinated with insect cell-produced BTV VLPs were shown to be protected against challenge with wild type virus. However, the high costs associated with the production and scale-up of BTV VLPs in insect cells has possibly limited their widespread application. Plants – such as *N. benthamiana* – provides a safe, efficient and cost effective system for the production of recombinant proteins.

In this study the best plant expression vector with which to co-express the four BTV serotype 8 (BTV-8) VPs – which direct formation of BTV-8 VLPs – was identified. Expression and purification of the BTV-8 VLPs was optimised with the aim of producing a VLP-based vaccine for BTV-8. It was further undertaken to develop two novel second generation plant-produced protein body (PB) vaccines that are DIVA compliant. Mice were immunised with the plant-produced VLP and PB vaccines in order to analyse their ability to elicit humoral immune responses.

Agrobacterium-mediated transient heterologous co-expression of the BTV-8 capsid proteins in *N. benthamiana* was optimised by comparing co-expression profiles obtained using different plant expression vectors that were available to us. Successful co-expression of the four capsid proteins was achieved using the pEAQ-*HT* vector. Expression of BTV-8 VPs using recombinant pEAQ-*HT* constructs was further optimised and it was shown to direct the assembly of VLPs which were successfully purified using discontinuous gradient ultracentrifugation. *In situ* localisation of assembled particles was investigated by using transmission electron microscopy (TEM) and it was shown that a mixed population of core-like particles (CLPs, consisting of VP3 and VP7) and VLPs were present as paracrystalline arrays in the cytoplasm of plant cells co-expressing all four capsid proteins.

Although VLPs are effective vaccine candidates that lack the viral genome and therefore the inherent drawbacks associated with live-attenuated virus vaccines, the BTV-8 VLPs are not DIVA compliant using current diagnostic techniques. Moreover, these VLPs provide protection against only one of the 26 serotypes of BTV. A second approach to BTV vaccine design was applied by initiating the development of two novel second generation PB plant-produced vaccines, Zera®-VP2 and Zera®-VP2ep. Zera®-VP2 contained the full-length BTV-8 VP2 codon-optimised sequence and Zera®-VP2ep contained predicted B-cell epitope sequences of multiple BTV serotypes that were predicted for VP2. In addition to fulfilling the DIVA requirement of a vaccine, Zera®-VP2ep was aimed at being multivalent with the ability to stimulate an immune response to several BTV serotypes. Both these candidate vaccines were successfully expressed in *N. benthamiana* and *in situ* TEM analysis showed that the expressed proteins accumulated within the cytoplasm of plant cells in dense membrane-defined PBs called StorPro® organelles. It was shown that the multi-epitope included in Zera®-VP2ep contained regions that were able to bind to the antibodies produced against native VP2. It was further shown that the PBs were easy to purify due to their dense nature and more importantly they were extremely stable, making them ideal vaccine candidates.

Preliminary immunogenicity studies of the VLP and PB candidate vaccines were carried out in BALB/c mice. The VLP-based vaccine was found to be unstable as only antibodies to the core components, VP3 and VP7, could be detected in the mice serum. The Zera®-VP2ep and Zera®-VP2 vaccine candidates both elicited significant anti-VP2 immune responses in the vaccinated mice without the use of adjuvant. These results demonstrate that Zera®-VP2ep and Zera®-VP2 has potential as BTV vaccines and their development should be further investigated.

Nomenclature

Abbreviations

bp	base pairs
BSA	bovine serum albumin
BT	Bluetongue
BTV	Bluetongue virus
C	cytosine
CAMV	Cauliflower mosaic virus
cELISA	competitive enzyme linked immunosorbent assay
CLP(s)	core-like particles
CP	coat protein
CPMV	Cowpea mosaic virus
DIVA	**d**ifferentiate **i**nfected from **v**accinated **a**nimals
DNA	deoxyribonucleic acid
dNTP	deoxy-ribonucleoside triphosphates (dATP, dCTP, dTTP and dGTP)
dpi	days post infiltration
dsRNA	double stranded ribonucleic acid
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
ER	endoplasmic reticulum
g	gram(s)

G	guanine
H	histidine
hr(s)	hour(s)
IPTG	isopropylthio-β-D-galactoside
kb	kilobase(s)
kDa	kilodalton(s)
L	litre(s) / lysine
LA	Luria agar
LB	Luria broth
M	molar / methionine
mA	milli-amperes
MCS	multiple cloning site
MES	2-morpholinoethanesulfonic acid
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
MLV	modified live virus
mM	millimolar
ng	nanogram(s)
NLS	m-lauroyl-sarcosine
nm	nanometre(s)
NS	non-structural

OIE	Office International des Epizooties
O/N	overnight
OD	optical density
p	plasmid / proline
PAGE	polyacrylamide gel electrophoresis
PB(s)	protein bodies
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PTGS	post-transcriptional gene silencing
Q	glutamine
R	arginine
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-qPCR	real time reverse transcriptase polymerase chain reaction
s	second(s)
S	serine
SDS	sodium dodecyl sulphate
T	threonine
TBE	Tris-borate-EDTA buffer
TBSV	Tomato bushy stunt virus
T-DNA	transfer-DNA
TEM	transmission electron microscopy

TSP	total soluble protein
TSWV	Tomato spotted wilt virus
Tris	Tris(hydroxymethyl)aminomethane
UTR	untranslated region
UV	ultraviolet
V	valine / volts
v	volume
VIB	viral inclusion bodies
VLP(s)	virus-like particle(s)
VP(s)	viral protein (s)
w	weight
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Y	tyrosine

Symbols

α	alpha
β	beta
γ	gamma
µg	microgram(s)
µL	microlitre(s)
µm	micrometre(s)
%	percentage
°C	degrees Celsius

Chapter 1:

Literature Review

1.1. Introduction

Bluetongue (BT) is a non-contagious, arthropod-borne viral disease that affects cattle, sheep, goats and wild ruminants (Roy, 2004). BT was first recognised and described in South Africa after the introduction of fine-wool sheep from Europe over 200 years ago (Maclachlan and Mayo, 2013; Vellema, 2008). The classical form of BT is seen in sheep; however cattle are also a natural reservoir for the virus (Roy, 2004). The causative agent of BT is the Bluetongue virus (BTV), the type species of the genus *Orbivirus* in the family *Reoviridae* (Mertens *et al.*, 2004). BTV is mostly transmitted by adult females of the haematophagous midges that belong to the genus *Culicoides* (du Toit, 1944).

BTV is endemic in tropical and sub-tropical regions and prior to 1998, outbreaks of BT in Southern European countries were rare (Mellor and Boorman, 1995). However, since 1998 BTV has become one of the most widespread animal pathogens as it has spread to areas that were previously free of the virus (Purse *et al.*, 2005). Outbreaks of BT occur when susceptible sheep are introduced into BTV-endemic regions or when the virus spreads to naïve sheep populations at the interface of endemic and non-endemic regions (MacLachlan, 2004). In 2006 BTV serotype 8 (BTV-8) was detected in northern Europe (Netherlands, Belgium, Germany and the north of France); this was the first time that BTV had been detected beyond the latitude of 52 °N. In subsequent outbreaks the northernmost limits of BTV moved beyond 54 °N (Vellema, 2008; Wilson and Mellor, 2009).

In 1996 worldwide economic losses due to BT were estimated to exceed 3 billion US dollars per year (Tabachnick, 1996). These losses are both direct (weight loss, abortions, death, reduction in milk production etc.) and indirect, such as trade restrictions, surveillance, vaccination, vector control and the treatment of affected animals (MacLachlan and Osburn, 2006; Wilson and Mellor, 2008). The BTV-8 outbreak in Europe in 2006 is significant as it is believed to have caused greater economic damage than any other single-serotype outbreak (Wilson and Mellor, 2009). Due to its ability to rapidly spread, the serious economic consequences associated with outbreaks and the impact of the disease on animals (Purse *et al.*, 2005) BT is classified as a List A notifiable disease by the Office International des Epizooties (OIE).

1.2. Bluetongue virus

1.2.1. Classification and variation of BTV

BTV is a virus with a ten-component double stranded ribonucleic acid (dsRNA) genome that causes an insect-borne, infectious non-contagious disease of both domesticated and wild ruminants; it is the type species of the genus *Orbivirus* that is classified into the family *Reoviridae*. *Reoviridae* is one of the largest families of virus that includes major human pathogens, such as rotavirus, as well as pathogens of insects, reptiles, fish, plants and fungi (Mertens *et al.*, 2004). Orbiviruses differ from other members of the *Reoviridae* family in that they can multiply in both arthropod and vertebrate cells, causing severe disease and high mortality (Roy, 2004). BTV is transmitted between its hosts by *Culicoides* spp., causing disease in ruminants worldwide (Mellor *et al.*, 2008).

BTV virions are complex three-layered icosahedral structures that are ~80 nanometres (nm) in diameter. The virions are composed of a core of ten segments of dsRNA encapsulated by seven structural proteins (four major and three minor proteins) that are arranged into three distinct layers as shown in Figure 1.1.

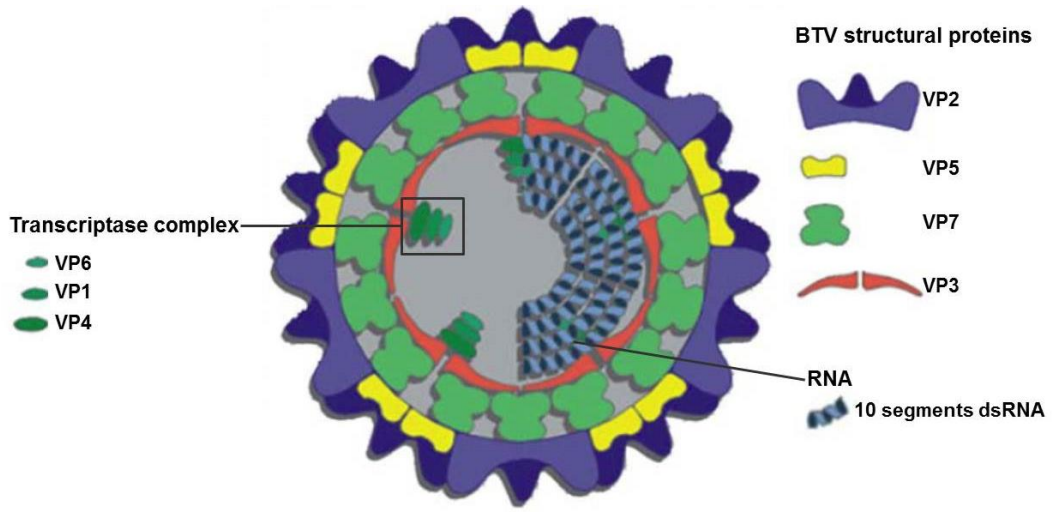

Figure 1.1: A schematic of the BTV particle showing the four structural proteins, the transcriptase complex and the dsRNA genome (Mertens *et al.*, 2004). *Image used with permission from the publisher.*

The three minor proteins (viral protein [VP] 1, VP4 and VP6) are enclosed by the sub-core that is made up of VP3. The core-surface layer consists of VP7, both VP3 and VP7 are major proteins. The outer capsid is composed of major proteins VP2 and VP5 which is laid onto the foundation provided by the core. The minor proteins together with genomic RNA form the virus replication complex, whereas the four major proteins make up the capsid of the virus (Mertens and Diprose, 2004; Mertens *et al.*, 2004; Roy, 1992; Roy, 2004).

In addition to the structural proteins BTV has four non-structural (NS) proteins (NS1, NS2, NS3 and NS3a) which are involved in virus replication and assembly in BTV-infected cells (Roy, 2004).

VP2 is the most variable of the BTV capsid proteins and contains the epitopes involved in virus neutralisation and serotype determination (DeMaula *et al.*, 2000; Huismans and Erasmus, 1981). Twenty six distinct serotypes of BTV have been identified based on neutralisation activity of VP2 as well as with BTV specific real time reverse transcriptase polymerase chain reaction [RT-PCR, (Hofmann *et al.*, 2008; Maan *et al.*, 2011a; Orru *et al.*, 2006)]. Each serotype shows variation that is associated with the geographical origins of the virus from around the world (Darpel *et al.*, 2007). Recent molecular studies on BTV isolates from different geographic regions have further divided BTV into two major topotypes, namely the eastern lineage which includes BTV isolates from South East Asia, India, China and Australia, and the western lineage which includes virus isolates from Africa and North or South America (Maan *et al.*, 2012; Maan *et al.*, 2011a; Maan *et al.*, 2008).

1.2.2. Three-dimensional (3-D) structure of BTV

The 3-D structure of BTV cores and virions has been determined by cryoelectron microscopy (Cryo-EM) and reconstructed using computational methods. Cryo-EM of the BTV core showed that it has a diameter of 69 nm, with the surface exhibiting icosahedral symmetry (Grimes *et al.*, 1997). Figure 1.2 B and C show that the core is divided into two layers (VP3 and VP7) that enclose the ten dsRNA segments and the proteins that are responsible for replication (Figure 1.2 A). A total of 120 molecules of VP3 (Figure 1.2 B) provide a smooth scaffold for the VP7 trimers to associate with (Figure 1.2 C). The surface of the core consists of clusters of VP7 trimers that have knob-like protrusions organised into pentameric and hexameric units with channels between them. In total there are 780 VP7 molecules per particle. The layer formed by VP7 is essential for the deposition of VP2 and VP5, which constitute the outer capsid (Roy, 2004).

Analysis of cryoelectron micrographs of BTV virions show that the two outer capsid proteins, VP2 and VP5 (Figure 1.2 D), attach in an orderly fashion to the foundation provided by the surface (VP7) of the core. (Roy, 2004). The capsid is icosahedral and the two outer capsid proteins have very distinctive morphologies. VP5 is globular and 120 of these proteins sit in the channels that are formed by the VP7 trimers. The sail-shaped VP2 spikes are located above 180 of the VP7 trimers and form triskelion-type motifs that cover most of the VP7 molecules. The VP2 spikes protrude 4 nm above the globular proteins and contain the virus neutralising epitopes. VP2 and VP5 form a continuous layer around the core, except for holes on the five-fold axis (Hewat *et al.*, 1992a; Hewat *et al.*, 1992b; Roy, 2004).

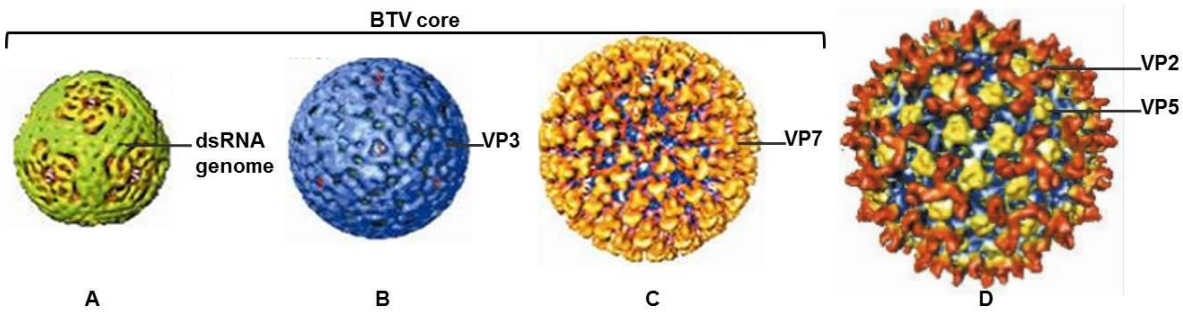

Figure 1.2: A surface representation of a cryoelectron micrograph of BTV, adapted from Roy (2004). (A) The RNA genome and the replication complex that is formed by VP1, VP4 and VP6, (B) the smooth inner layer formed by 120 molecules of VP3 and (C) protrusions of the 260 VP7 trimers in the outer layer. Together A, B and C form the BTV core. (D) The topography of the globular-shaped VP5 and the sail-shaped VP2 displayed on a whole virus particle. *Image used with permission from the publisher.*

1.2.3. Virus Proteins

The BTV core contains the 10 dsRNA segments, each one encoding a single viral protein, except for the smallest RNA segment, S10. The three minor protein components; VP1 (RNA dependent RNA polymerase, 150 kilodalton [kDa]), VP4 (capping enzyme, 76 kDa) and VP6 (helicase, 36 kDa) constitute the transcriptase complex (Mertens *et al.*, 1984; Roy, 1992; Singh *et al.*, 2004) .

The two major proteins that make up the core - VP3 (102 kDa) and VP7 (38 kDa) - are both hydrophobic (Roy, 1992). VP3 is highly conserved among BTV serotypes as well as different genera of the *Reoviridae* family. VP3 has an important functional role in the structural integrity

of the virus core (Huismans and Van Dijk, 1990; Mertens *et al.*, 2004). VP7 is highly conserved among the BTV serotypes and contains the group-specific antigenic determinants. VP7 is often used in current diagnostic serological tests - such as the commercially available competitive enzyme linked immunosorbent assays (cELISA) - as the BTV serogroup antigen for the detection of BTV (MacLachlan, 1994; MacLachlan, 2004; Roy, 1992; Roy, 1996; Vellema, 2008).

The core of BTV is surrounded by a diffuse outer capsid layer which comprises of VP2 (110 kDa) and VP5 (58 kDa). These proteins are the least conserved among virus serotypes (Roy, 1992; Roy, 1996) and together they are involved in cell attachment and viral entry during initiation of infection (Hassan *et al.*, 2001). Specificity of interactions between the proteins of the outer capsid (especially VP2) and neutralising antibodies can be evaluated by serum neutralisation assays, which distinguish between the different virus serotypes (Singh *et al.*, 2004). VP2 is the most variable, serotype-specific protein, the viral haemagglutinin and the cellular receptor binding protein (Hassan and Roy, 1999; Nason *et al.*, 2004; Roy, 1992). Antibodies to VP2 elicit virus neutralisation properties and confer resistance to reinfection with a homologous serotype (MacLachlan, 1994; Roy, 1992; Vellema, 2008). VP5 binds to the surface of cells and plays a role in membrane destabilisation in order for the core to gain access to the cytoplasm (Forzan *et al.*, 2004; Hassan *et al.*, 2001).

In addition to the seven structural proteins there are four non-structural proteins, NS1, NS2, NS3 and NS3a, that can be found in infected cells. In contrast to the structural proteins, the sequences of the four NS proteins are highly conserved among serotypes (Roy *et al.*, 1990a). These proteins are involved in virus replication and assembly (Roy, 1992; Roy, 2004). NS1 and NS2 form two virus-specific structures in the cytoplasm of infected cells - tubules and granular viral inclusion bodies (VIBs), respectively (Cromack *et al.*, 1971; Lecatsas, 1968). The BTV sub-core and core particles are assembled within the VIBs in the cytoplasm of infected cells (Mertens *et al.*, 2004). NS3 and NS3a are both encoded by the same gene segment and are associated with intracellular, smooth-surfaced vesicles and the plasma membrane. These proteins mediate the release of BTV from infected cells (Mertens *et al.*, 2004; Roy, 1992).

The evolutionary rate (genetic drift) of the multi-segmented RNA viruses is very high as they have no proof-reading activity and their genomes are able to reassort, resulting in antigenic shift (Gale *et al.*, 2009). During reassortment, different strains of BTV that infect the same cell can exchange their genome segments. This process is enabled by the segmented nature of

the BTV genome and plays a significant role in the generation of new virus strains that have different serological and biological properties (Roy *et al.*, 2009; Singh *et al.*, 2004).

1.2.4. Replication

BTV can replicate in both wild and domestic ruminants. Replication takes place in both the host and the *Culicoides* insect vector (Roy *et al.*, 2009).

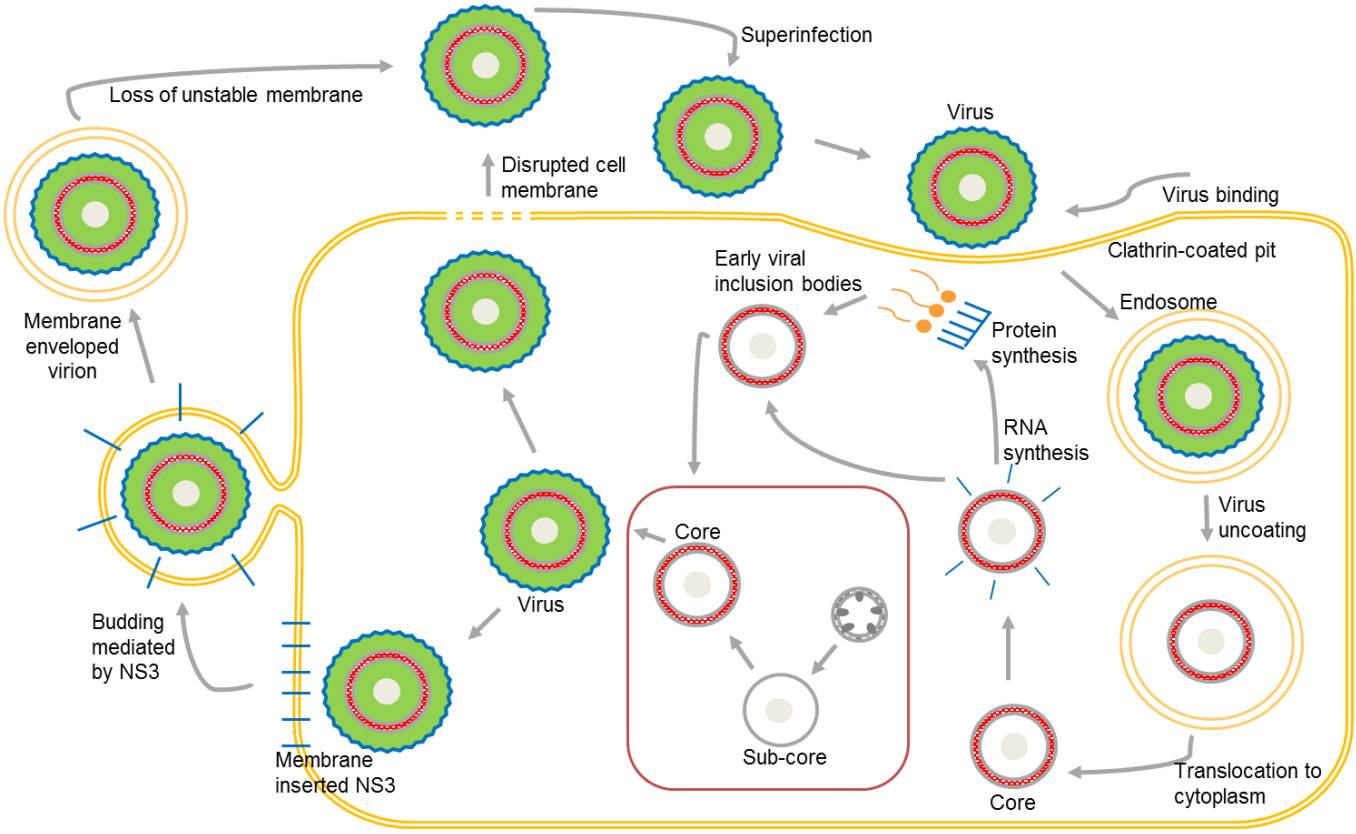

Figure 1.3: A schematic presentation of the lytic replication cycle of BTV. Image adapted from Mertens (2002).

Figure 1.3 shows a schematic representation of the replication cycle of BTV (Mertens, 2002). BTV interacts with the cell surface via the VP2 trimers and enters the cell via a clathrin-dependent endocytosis pathway (Forzan *et al.*, 2004; Hyatt *et al.*, 1993). The reduction of pH in the endosome induces the association of VP5 with the endosomal membrane and the

delivery of the transcriptionally active core into the cytoplasm (Forzan *et al.*, 2004; Mertens *et al.*, 2004). The transcriptase function of the core particle is activated by the removal of VP2 and VP5 during cell entry (Mertens and Diprose, 2004).

Positive sense single stranded (ss) RNA copies that act as messenger RNAs are transcribed by the VP1 molecules in the BTV core from each of the ten genome segments (Boyce *et al.*, 2004). The RNA molecules are capped by the guanylyl-transferase and transmethylase activities of VP4 and exit the particles via channels that are situated at the five-fold axes of the core particle (Mertens and Diprose, 2004). Viral RNA is directed to the VIB, where encapsidation of different segments within the VP3 shell involves interactions with VP6 (helicase), NS2 (ssRNA binding protein), VP1 and VP4. DsRNA is synthesized from the positive strand RNA by VP1 (Boyce *et al.*, 2004); each dsRNA segment associates with a different transcriptase complex (Nason *et al.*, 2004).

The fragile VP3 sub-cores act as a scaffold for the addition of VP7 trimers, thereby forming stable cores (Grimes *et al.*, 1997; Tanaka and Roy, 1994). VP2 and VP5 are added to the core surface at the periphery of the VIB upon entry of the host cytoplasm. The mature particles are transported within the cytoplasm on microtubules that involve the interaction of VP2 with vimentin (Bhattacharya *et al.*, 2007). The virions are released from infected cells when NS3 mediates the destabilisation of the cell membrane; budding and cell death/lysis also play a role in release of the virions (Mertens *et al.*, 2004).

1.3. Bluetongue Disease

1.3.1. Clinical signs

BTV can infect all known species of domestic and wild ruminants. Severe disease usually occurs in the fine-wool and mutton breeds of sheep as well as some species of deer (Mellor and Boorman, 1995). BTV infection of cattle, goats and wild ruminant species is mostly asymptomatic or subclinical. In BTV endemic areas BTV-infected sheep develop only mild or no obvious disease (MacLachlan, 2004). The blue tongue after which the disease is named is seen only in serious clinical cases (Wilson and Mellor, 2009).

Onset of the disease in sheep is typically characterised by high fever lasting 5 - 7 days (Roy, 2004). Clinical signs of disease can include fever, depression, excessive salivation, nasal discharge, facial oedema, hyperaemia and ulceration of the oral mucosa, coronitis, lameness and death (Darpel *et al.*, 2007; Mellor and Boorman, 1995). Abortion can occur in pregnant

animals as well as teratogenic defects in calves (Roy, 2004). The severity of clinical disease and mortality rate is influenced by the breed and age of the animal as well as the virus strain that causes the infection (Mellor and Wittmann, 2002). In acute phases of BT, clinical signs in sheep are mainly associated with damage to microvascular endothelial cells (Singer *et al.*, 2001).

Those animals that do recover from BT may suffer from a number of long-lasting secondary effects, such as reductions in milk production, weight gain, wool break and temporary infertility (Wilson and Mellor, 2009).

1.3.2. Pathogenesis in ruminants

Pathogenesis of BTV infection is similar in sheep and cattle as well as other species of ruminants. After an animal is infected with BTV through the bite of a *Culicoides* vector, the virus travels to the regional lymph node where initial replication takes place (Barratt-Boyes *et al.*, 1995; Maclachlan *et al.*, 2009). The virus then spreads throughout the body to a variety of tissues, where replication occurs mainly in mononuclear phagocytic and endothelial cells (MacLachlan, 2004; Maclachlan *et al.*, 2009).

Viraemia is cell-associated and can be prolonged in domestic ruminants. During viraemia BTV is associated with all blood cells, but late in the course of infection the virus becomes associated with the erythrocytes. The longer lifespan of erythrocytes facilitate prolonged infection of ruminants, as well as the infection of the haematophagous insect vectors that feed on viraemic ruminants (Barratt-Boyes *et al.*, 1995; MacLachlan, 2004; Maclachlan *et al.*, 2009; Singer *et al.*, 2001). Infectious virus can co-circulate with neutralising antibodies for several weeks (Maclachlan *et al.*, 2009). The maximum period of viraemia in sheep is about 50 days and in cattle about 100 days (Mellor and Wittmann, 2002).

1.3.3. BTV insect vector

Arthropods of the genus *Culicoides* classified into the family Ceratopogonidae within the order Diptera are the vectors responsible for transmission of BTV (du Toit, 1944). These small (3 mm) haematophagous midges occur throughout the world (Figure 1.4) on all large land masses, except for Antarctica, New Zealand, Patagonia and the Hawaiian Islands (Mellor, 2000; Purse *et al.*, 2005; Walton, 2004; Wilson and Mellor, 2009). *Culicoides* are spread by

the wind over long distances, and movement of infected midges has been implicated as the most likely source of a number of BT outbreaks (Wilson and Mellor, 2008). *Culicoides* that are of veterinary importance breed in organically rich, damp soil that is found near their hosts (Wilson and Mellor, 2009).

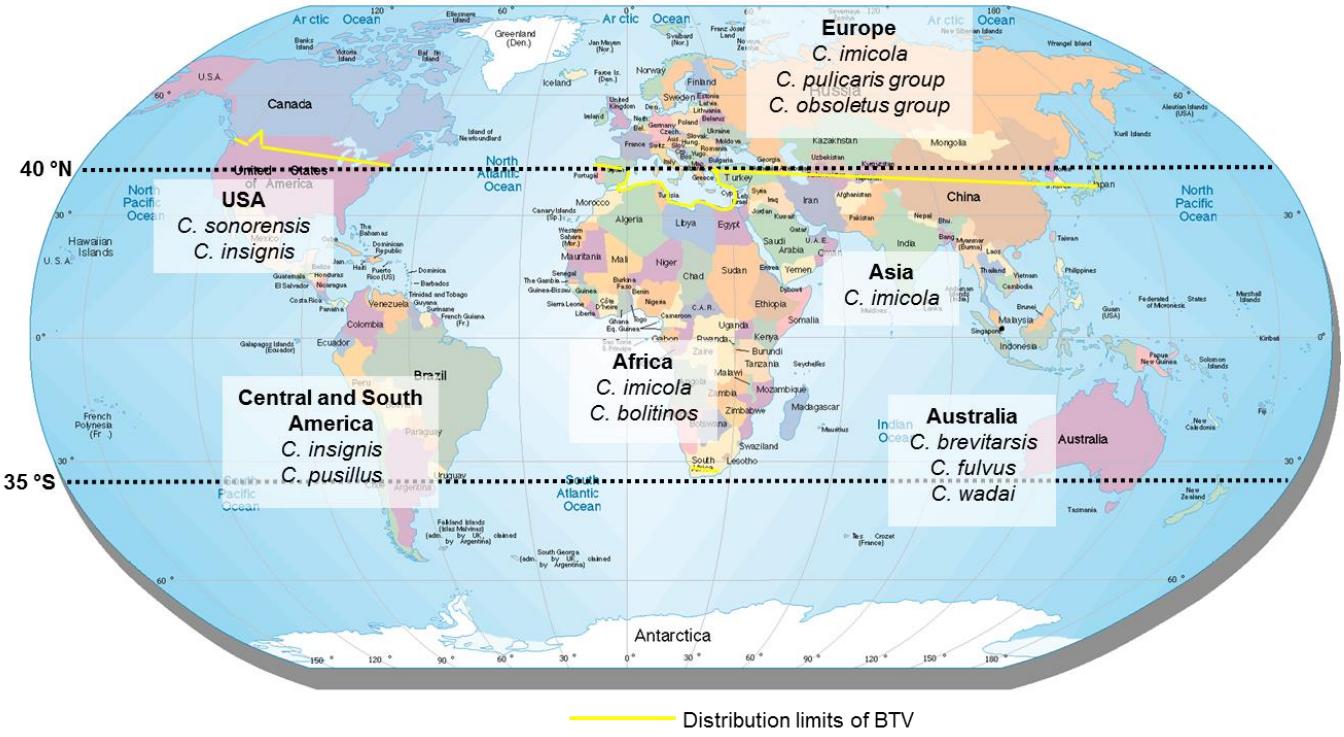

Figure 1.4: Distribution map of BTV and *Culicoides* vectors. Image adapted from Purse *et al.* (2005).

Approximately 50 of the 1500 species of C*ulicoides* play a role in BTV transmission worldwide. For *Culicoides* species to be effective vectors of BTV, they must occur in large numbers and feed almost only on ruminants.

Males do not feed on blood, but adult females undertake flight activity to seek a blood meal. After ingestion, infected blood is directed to the hind part of the mid-gut where BTV replicates in the gut cells, progeny of the virus particles escape into the hemocoel and infect a range of secondary target organs, including salivary glands. After replication in the salivary gland cells, BTV is available for transmission during biting activity (Mellor, 2000). The insects can only transmit BTV after this extrinsic incubation period of 4 - 20 days, depending on the temperature and serotype (MacLachlan, 2004; Mellor *et al.*, 2000; Purse *et al.*, 2005). BTV infection of *Culicoides* persists for the remainder of their lifetime, where adults survive approximately 20

days (MacLachlan, 2004). The extrinsic incubation period can be shorter at higher temperatures indicating an increased rate of virogenesis, therefore compensating for the shorter lifespan of the vector at higher temperatures (Gale *et al.*, 2009).

Peak populations of adult *Culicoides* occur in the late summer and autumn, when BT is usually detected in ruminants (Mellor and Boorman, 1995). Midges of *C. obsoletus* and *C. pulicaris,* which are indigenous to the north Palearctic, have been identified as new vectors of BTV-8 since the outbreaks occurred in northern Europe in 2006. Northern Europe recently experienced higher temperatures which increased the competence of these indigenous midges to serve as vectors for BTV (Gale *et al.*, 2009).

1.3.4. Epidemiology

Prior to 1998 outbreaks of BT in Europe were sporadic and it was considered an exotic disease. However from 1998 to 2005 several countries in the Mediterranean basin were affected by different BTV strains. In 2006 BTV-8 was detected in the Netherlands after which it spread to neighbouring countries, causing a devastating epidemic in 2007. The BTV-8 epidemic was believed to have caused greater economic damage than any other single-serotype outbreak (Wilson and Mellor, 2009).

The distribution of BTV is limited to areas where *Culicoides* vector species are present (Mellor *et al.*, 2000) and transmission occurs during the time of the year when climatic conditions are favourable for adult vector activity (Mellor, 2000). In a phenomenon termed “overwintering” it is possible that BTV can be maintained in the host or vector, and after months of no detection, BT outbreaks can reappear (Takamatsu *et al.*, 2003; Wilson and Mellor, 2008). Adult *Culicoides* can survive for more than three months at 10 °C (Lysyk and Danyk, 2007), while virus replication may effectively stop. Once temperatures are favourable, the latent virus can replicate again (Mullens *et al.*, 1995). It is possible that this phenomenon contributed to the re-emergence of BTV-8 in northern Europe in the spring of 2007 (Wilson *et al.*, 2007).

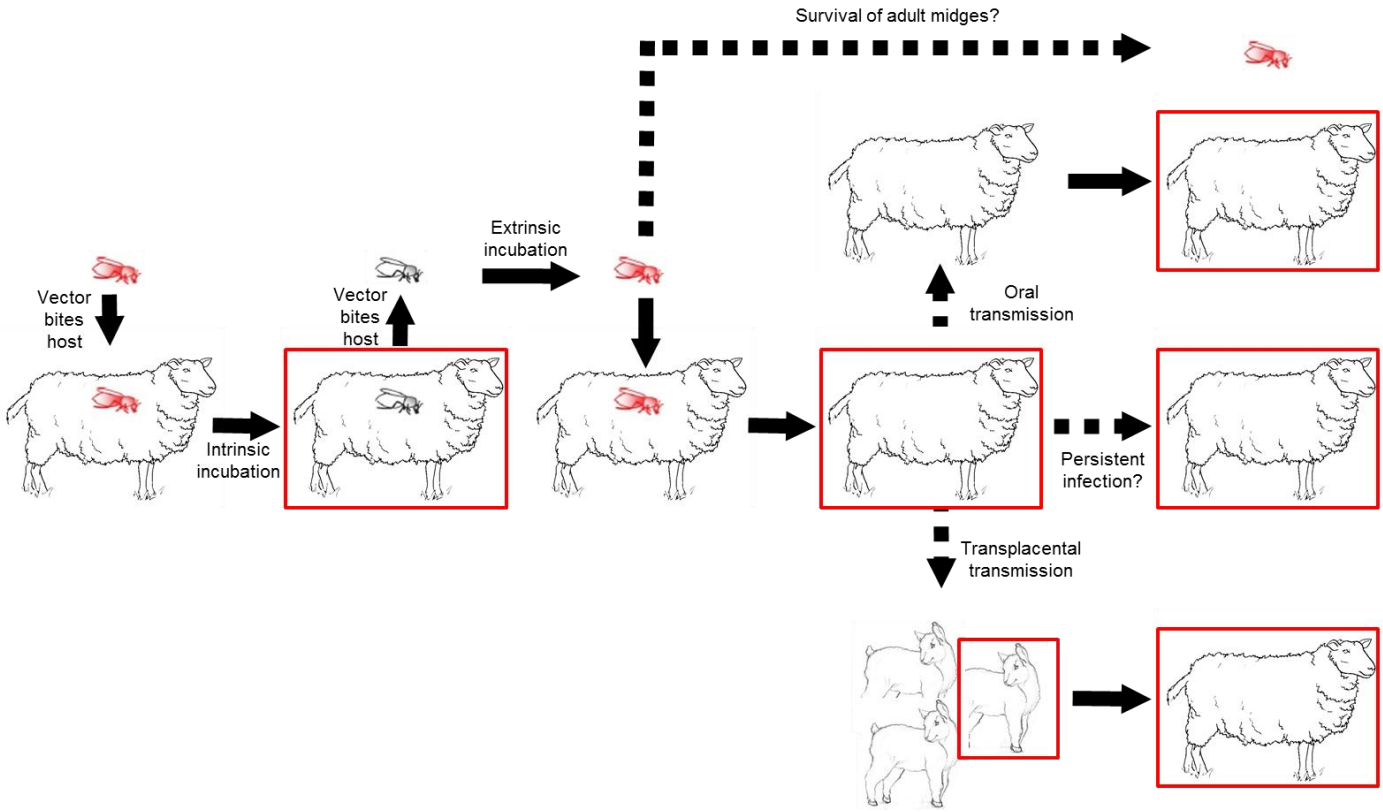

Figure 1.5: Transmission routes of BTV. Possible secondary transmission routes (oral and transplacental) are also indicated. Adapted from Wilson and Mellor (2009).

On rare occurrences some strains of BTV can be transmitted by secondary mechanisms, such as oral or transplacental transmission within ruminant populations (Figure 1.5), however these mechanisms are not important in the long-term survival and spread of the virus (Mellor *et al.*, 2008; Mellor and Wittmann, 2002; Wilson and Mellor, 2009).

In regions of the world where enzootic BTV infection occurs throughout the year, BT is uncommon. This can be attributed to population immunity or it can be a result of co-evolution of the animal host and the virus (Huang *et al.*, 2009).

1.3.5. Global distribution of BTV

BTV occurs world-wide in a broad band, from about 35 °S to 40 °N, that covers much of the Americas, Africa, southern Asia, northern Australia and occasionally the southern fringes of Europe (Figure 1.4). In North America, Asia and most recently in northern Europe, the

occurrence of BTV can extend up to 50 °N (Gibbs and Greiner, 1994; Mellor *et al.*, 2008; Purse *et al.*, 2005). Existence of BTV in these areas is a reflection of *Culicoides* distribution and the temperature required for BTV replication and transmission by these vectors.

In 2006 BTV-8 extended northwards into areas of Europe that has never before been affected by the virus, and persisted in these areas causing the greatest outbreak of BT on record. The reasons for the change in BT epidemiology are linked to extensions in the distribution of its vector species *C. imicola* and the involvement of novel *Culicoides* vectors, likely a result of climate change (Mellor *et al.*, 2008; Purse *et al.*, 2005).

In Europe prior to 1998, only transient and limited incursion of single BTV serotypes into the Mediterranean were documented (Maclachlan and Mayo, 2013). However, since 1998 multiple BTV serotypes (1, 2, 4, 6, 8, 9, 11 and 16) have been detected in Europe (Mellor *et al.*, 2008). BTV-8 which was first identified in northern Europe in 2006 has spread upwards throughout the Mediterranean basin above 50 °N right up to regions of Scandinavia (Saegerman *et al.*, 2008; Wilson and Mellor, 2008). All the BTV serotypes that have invaded the Mediterranean basin after 1998, except for BTV-8, were shown to have originated from the adjacent regions of Africa or Asia (Purse *et al.*, 2005).

BTV serotypes 10, 11, 13 and 17 are endemic to extensive areas of North America. BTV-2 was documented in America 30 years ago and in recent years an additional 10 serotypes have been identified in the south eastern United States (MacLachlan, 2010). It is assumed that these viruses originated in the adjacent Caribbean ecosystem after which they spread to North America (Maclachlan, 2011).

In Australia and Israel novel serotypes have been identified in recent years. Historically BTV serotypes 2, 4, 6, 10 and 16 were present in Israel, but recently BTV serotypes 8, 15 and 24 emerged in this country (Shimshony, 2004). In Australia eight serotypes (1, 3, 9, 15, 16, 20, 21 and 23) were identified previously, with only serotypes 1 and 21 spreading into eastern Australia annually. However, in 2007/2008 BTV serotypes 2 and 7 were detected for the first time in the Northern Territory of Australia (MacLachlan, 2010).

New BTV serotypes (BTV-25 and 26) were recently identified with RT-PCR in Switzerland and the Middle East (Hofmann *et al.*, 2008; Maan *et al.*, 2011a). It is difficult to interpret the BTV status of other regions in the world, especially the Middle East and Asia, due to the lack of adequate surveillance (Maclachlan, 2011).

1.3.6. Disease surveillance and diagnosis

Control of endemic or emerging BT in certain regions relies on the rapid detection of either the virus (virological surveillance) or the disease (clinical surveillance). This is best accomplished by on-going field surveillance for infection and not only disease surveillance, as infections can be mild or subclinical. Surveillance should include evaluation of livestock as well as that of potential vector species present in the region. Sensitive and specific diagnostic assays should be used for virus detection (Maclachlan and Mayo, 2013; Souza Monteiro *et al.*, 2012).

Traditional detection and typing methods such as virus isolation and serum/virus neutralisation assays can take weeks and depend on the availability of reference virus-strains and antisera (Jeggo, 1986). Serum neutralisation tests can show a heterotypic response to multiple BTV serotypes, especially if the animal was previously infected or vaccinated with more than one type of BTV, thereby obtaining inconclusive results (Maan *et al.*, 2012).

The most conserved protein among the BTV serotypes, VP7, contains the serogroup-specific antigenic determinants and is often used in current diagnostic serological tests, such as cELISAs, as the antigen for the detection of BTV (MacLachlan, 1994; Vellema, 2008). However, since these cELISAs rely on the detection of anti-VP7 antibodies, they cannot be used to determine virus serotype.

An indirect ELISA was developed by Barros *et al.* (2009) to facilitate differentiation between vaccinated and infected animals. This assay is based on a differential antibody response to NS3 observed between naturally infected and vaccinated animals. The theory is that inactivated vaccines contain mainly capsid proteins, therefore they are less likely to elicit antibody responses against non-structural proteins. In the study it was shown that animals infected with BTV had greater levels of NS3 antibodies than animals that were immunised with inactivated BTV vaccines (Barros *et al.*, 2009). However, this diagnostic method is not commercially used, possibly since the presence of non-structural proteins within vaccine preparations could vary from batch to batch, thereby skewing the results obtained.

Nucleic-acid based typing assays are available for the detection of BTV, such as the group-reactive quantitative RT-qPCR assays and sequencing that can reliably detect all BTV serotypes with sensitivity and specificity (Hofmann *et al.*, 2008; Mayo *et al.*, 2010). These methods are advantageous as they are fast, sensitive and more specific than the established serological assays. Individual virus lineages can be identified by comparing the sequence data of strains that had previously been analysed. Full-length sequence data of VP2 (segment 2) from multiple isolates of individual types of BTV, including eastern and western strains, has

become available and a complete set of “serotype-specific” primers for all 26 BTV serotypes have been designed and evaluated. The assays using these primers have been shown to provide rapid and reliable BTV serotype and strain identification in less than 24 hours (Maan *et al.*, 2012).

1.3.7. Prevention and control

Hypothetically, BTV infection of wild and domestic animals can be controlled either by elimination of the midge vector or by protecting the animal hosts from infection and disease (Maclachlan and Mayo, 2013).

Vector-based control strategies to limit the spread of BTV are aimed at either reducing or eradicating populations of either *Culicoides* adults or larvae. These strategies are challenging considering the enormous midge populations in many regions and their extensive breeding sites (Maclachlan and Mayo, 2013). Control of the immature life-stages (larvae) by removing breeding sites and using pesticides (mature midges) can be employed to reduce populations of adult *Culicoides* at a local level (Carpenter *et al.*, 2008; Holbrook, 1986; Mintiens *et al.*, 2008). The efficacy of this strategy is limited in areas that require broad application and there are concerns with regards to the effects of the insecticides on the environment. Decoys such as repellents and attractants have been evaluated to lure midges and reduce the biting rate among animals; however their use is complicated and requires a daily application regimen (Maclachlan and Mayo, 2013).

Animal-based control strategies include stabling of livestock, movement restrictions, culling of infected animals and vaccination. The use of protective housing is mostly impractical considering extensive livestock production systems and the sheer number of livestock that need to be stabled (Huang *et al.*, 2009; Napp *et al.*, 2011). The implementation of movement restrictions can be logistically challenging and economically devastating (Papadopoulas *et al.*, 2009). Arboviral diseases are not easily managed by culling of animals as infections can be subclinical and the vector midges harbouring the virus can spread widely. Furthermore, there is also societal resistance to the large-scale slaughter of healthy animals (Maclachlan and Mayo, 2013).

Vaccination is central to the response of BT outbreaks. However, vaccination is difficult given that there are 26 serotypes of BTV; this, together with serotype-specific immunity in livestock can make vaccination problematic (Noad and Roy, 2009b; Zientara *et al.*, 2010).

1.4. Vaccines against BTV

The most effective means to protect animals against disease and to reduce spread of the virus after infection is vaccination. To combat the direct losses incurred with BTV outbreaks and to minimize the circulation of BTV, vaccination of livestock was implemented by various countries according to their individual national policies, the serotype of virus and the availability of vaccines (Caporale and Giovannini, 2010; Savini *et al.*, 2008).

A number of vaccines have been developed against BT. These include inactivated whole virus vaccines, live attenuated virus vaccines (modified live virus vaccines), recombinant vaccines and virus-like particle (VLP) vaccines. While these vaccines have various advantages and disadvantages, only attenuated virus vaccines and some inactivated vaccines are presently commercially available (Niedbalski, 2011; Noad and Roy, 2009b; Osburn *et al.*, 1996; Savini *et al.*, 2008). The most important reasons for BT vaccination are to prevent clinical disease, to reduce environmental spread of BTV, and to protect susceptible animals during movement between affected and BTV-free zones (Niedbalski, 2011). Ideally a vaccine for BT should protect against more than one serotype, should not revert to virulence or recombine with circulating strains of the virus, and should not be able to replicate in the field (Roy *et al.*, 2009). It is also important that vaccines against BT are safe to use and allow for **d**ifferentiation between naturally **i**nfected and **v**accinated **a**nimals [DIVA, (Niedbalski, 2011)].

1.4.1. Inactivated vaccines

Inactivated vaccines are prepared by treating cell-cultured BTV with beta-propiolactone, gamma radiation or binary ethylenimine. Vaccines prepared in this way are commercially available and have shown good immunogenicity and safety. The risks of reassortment of genes with field strains, reversion to virulence and teratogenicity are avoided with the use of inactivated vaccines (Di Emidio *et al.*, 2004; Roy *et al.*, 2009).

The first inactivated vaccine to be developed and used in the field after an outbreak of BT in Europe was the vaccine against BTV-2, which was used to vaccinate sheep in 2005 in Corsica (France). In 2005-2006 monovalent BTV-4 and bivalent BTV-2 and BTV-4 inactivated vaccines were developed and used in Corsica, Spain, Portugal and Italy (Rodriguez-Sanchez *et al.*, 2008; Savini *et al.*, 2008).

The disadvantages of using inactivated vaccines is that a single dose produces only transient neutralising antibodies and does not provide sufficient long-lasting immunity, therefore booster

immunisations are required, which increases costs. These vaccines are also expensive to produce and require that large amounts of infectious virus be grown before inactivation. In addition, there are no strategies available at present for regular diagnostic purposes that can differentiate infected animals from vaccinated animals (Savini *et al.*, 2008).

1.4.2. Modified live virus (MLV) vaccines

MLVs are produced by adapting BTV field isolates to *in vitro* growth through serial passaging in tissue culture or embryonated chicken eggs. A strong antibody response is directly correlated with the MLVs' ability to replicate in a vaccinated host and can last up to one year. These vaccines are more affordable to produce in large quantities than inactivated vaccines, protective immunity is stimulated after a single inoculation and they have proven effective in the prevention of clinical BT in the areas where they are used (Dungu *et al.*, 2004a; Savini *et al.*, 2008).

The first egg-adapted attenuated BTV vaccine was developed in South Africa in the early 1950's (Alexander and Haig, 1951) and since then MLV vaccines for many serotypes of BTV have been developed and are available outside of Europe. These countries include the United States of America, Turkey, South Africa and India (Savini *et al.*, 2008).

Despite the apparent success of MLVs for controlling BTV, a number of documented or potential drawbacks have been reported. These include depressed milk production in lactating sheep as well as abortion/embryonic death and teratological effects after the vaccination of pregnant ewes with MLV vaccines (Monaco *et al.*, 2004; Osburn *et al.*, 1971; Schultz and Delay, 1955; Young and Cordy, 1964).

The vaccine virus can cause viraemia for over two weeks in vaccinated sheep and the viraemia has been reported to be sufficient for transmission of the vaccine strain to the insect vector (Schwartz-Cornil *et al.*, 2008; Veronesi *et al.*, 2010; Veronesi *et al.*, 2005). In both North America and Europe studies have shown that live, attenuated BTV vaccine viruses (or its individual gene segments) used to vaccinate animals, can be acquired and transmitted by *Culicoides* in the field thereby contributing to the genetic diversity of circulating viruses (Batten *et al.*, 2008a; Osburn *et al.*, 1996). Of particular concern is the reassortment of MLV genes with those of wild-type virus strains that co-infect the same animal. In Italy, 2002, a circulating BTV-16 strain was found to be generated by reassortment between BTV-2 and BTV-16 MLV

strains (Batten *et al.*, 2008b). As with inactivated vaccines, there is the inability to serologically distinguish naturally infected from MLV vaccinated animals (Savini *et al.*, 2008).

1.4.3. Recombinant vaccines

A number of experimental recombinant vaccines have been investigated which aim to meet the requirements for an effective BT vaccine. These requirements include low cost of production, DIVA compliance, protective immunity against multiple serotypes, no risk for reversion/reassortment and long term protective immunity after a single dose (Roy *et al.*, 2009; Schwartz-Cornil *et al.*, 2008).

1.4.3.1. Recombinant subunit protein vaccines

It has been demonstrated that VP2 purified from BTV was able to elicit protective immune responses in sheep (Huismans *et al.*, 1987). VP2 alone and in combination with other viral proteins have been tested in small-scale animal experiments to analyse the protective efficacy using different approaches. These include purified recombinant VP2 and VP5 which were successful in eliciting neutralising antibodies in sheep when used together (Roy *et al.*, 1990b), a recombinant vaccinia BTV vaccine expressing VP2 and VP5 which afforded protection against homologous challenge (Lobato *et al.*, 1997), as well as other recombinant poxvirus-based systems where the poxvirus is used as a vector for the transfer of BTV genes into host cells, resulting in the expression of BTV proteins (Boone *et al.*, 2007). A capripox vector expressing VP2, VP7, NS1 and NS2 provided only partial protection against heterotypic BTV challenge (Perrin *et al.*, 2007; Wade-Evans *et al.*, 1996). A recombinant canarypox virus vaccine co-expressing VP2 and VP5 was described that elicited neutralising antibodies and protected sheep against challenge with BTV (Boone *et al.*, 2007). A major advantage of this vaccine in terms of BTV is that the existing VP7 competitive diagnostic ELISA assay would distinguish vaccinated from naturally infected animals. This vaccine is still in the developmental stages (Savini *et al.*, 2008).

1.4.3.2. Virus-like particles (VLPs) as vaccines

VLPs are structures that resemble the authentic conformation of native viruses and are formed when multiple subunits of protein complexes interact with each other in such a way that it results in self-assembly (Grgacic and Anderson, 2006). BTV VLPs contain only the four structural proteins - VP2, VP3, VP5 and VP7 - of BTV and mimic the structure of authentic virus particles. They are readily recognised by the immune system and present viral antigens in a conformation that is more authentic than other subunit vaccines, making them excellent immunogens (Noad and Roy, 2003). VLPs have been shown to be non-infectious and unable to replicate as they contain no viral genetic material (Rodriguez-Limas *et al.*, 2013). Since BTV VLPs do not contain the non-structural proteins or virus RNA, it is possible to distinguish between vaccinated and infected animals using molecular diagnostic techniques (Niedbalski, 2011). VLPs are excellent vaccine candidates as there is no risk of reversion to virulence or reassortment with wild virus strains as they contain only structural proteins (Roy *et al.*, 2009).

The repetitive, high density display of epitopes on VLPs render them more immunogenic than subunit vaccines or chemically inactivated vaccines. The particulate nature of VLPs combined with epitope display enhance the humoral, cell-mediated and mucosal immune responses (Grgacic and Anderson, 2006; Noad and Roy, 2003; Roy and Noad, 2008). VLPs are excellent candidates for BTV vaccines, since both humoral and cellular immunity is important for protection against BTV (Calvo-Pinilla *et al.*, 2012). In addition to the above-mentioned advantages of using VLPs as a vaccine, they are also safe to produce and handle and the host cells for their production do not necessarily come from mammalian sources, and therefore would not contain mammalian derived pathogens (Roy, 1996).

VLPs for BTV serotype 10 were first produced in insect cells using a baculovirus-based protein expression system; this system was shown to efficiently produce large amounts of proteins with the correct folding and assembly of large complexes (French *et al.*, 1990). BTV VLPs are formed by the co-expression of the four major structural proteins of the virus (VP2, VP3, VP5 and VP7); these proteins self-assemble into triple-layered VLPs. Sheep vaccinated with the insect cell expressed BTV-10 VLPs developed neutralising antibodies and were protected against challenge with a homologous serotype, these studies showed that VLPs are both safe and effective to use as potential vaccines for BTV (Roy *et al.*, 1990b; Stewart *et al.*, 2012). In other experiments it was shown that low doses of VLPs were sufficient to give complete protection from virus challenge and none of the vaccinated animals developed clinical signs or viraemia. Furthermore, sheep vaccinated with VLPs that were made separately from two different serotypes were shown to have complete protection against the vaccine serotypes as

well as partial protection against related non-vaccine serotypes. It was also demonstrated that vaccination with VLPs provided long lasting protective efficacy over 14 months (Noad and Roy, 2003; Roy *et al.*, 1994; Roy *et al.*, 2009). Despite their efficacy, however, the cost of producing VLPs in insect cells is very high, which has hindered further development and commercialisation of these vaccine candidates, alternative methods of production would be desirable to facilitate large-scale development of these vaccines.

Recently BTV-8 VLPs were successfully expressed and assembled in *Nicotiana benthamiana* (*N. benthamiana*) plants using a transient plant expression vector. The plant-expressed VLPs elicited an immune response in sheep providing protective immunity against challenge with a BTV-8 field isolate (Thuenemann *et al.*, 2013).

1.5. Plant expression systems

Over the last 20 years, plant-based expression of heterologous proteins has attracted much interest as it has many advantages over other eukaryotic expression systems for the expression of proteins. These include high biomass, ease of scale up, cost effectiveness and a low risk of contamination (Lai and Chen, 2012; Sainsbury and Lomonossoff, 2008). Plant-based expression now represents a viable platform for the production of medical and industrial proteins. Furthermore, plant-based expression systems are safe to use as they do not contain the human and animal pathogens that are potentially an issue when using mammalian cell-based production systems (Fischer *et al.*, 2012; Pogue *et al.*, 2010; Rybicki, 2010; Xu *et al.*, 2012).

Several classes of recombinant proteins have been produced in plants. These include monoclonal antibodies, vaccine antigens, therapeutic enzymes, growth hormones, industrial enzymes (hydrolases and proteases) and biopolymers such as spider silk proteins, elastin-like polypeptides and collagen (Tiwari *et al.*, 2009; Xu *et al.*, 2012). Recently, good manufacturing practice (GMP) procedures have been established for the production of recombinant proteins in whole transgenic plants and transient expression systems; this should lead to an increase in the number of plant-derived pharmaceutical proteins and rapid-response vaccines that enter clinical development stages (Fischer *et al.*, 2012).

A wide variety of plants has been utilised for the expression of human and animal vaccines, with the hepatitis B surface antigen expressed in transgenic tobacco being one of the first VLP candidate vaccines (Mason *et al.*, 1992; Rybicki, 2010). Ever since 1992 well-characterised viruses such as human immunodeficiency virus, human papilloma virus, influenza virus, foot

and mouth disease virus and rotavirus, to name but a few, have been successfully investigated for the formation of VLPs in plants (Scotti and Rybicki, 2013).

Nicotiana spp. is one of the most popular candidates for the commercial production of recombinant proteins (Fischer *et al.*, 2004; Twyman *et al.*, 2003). The continual use of tobacco makes it a well-established expression host, and furthermore the high biomass yields, rapid scalability and the fact that it is a non-food crop make it suitable for commercial molecular farming (Fischer *et al.*, 2004; Stoger *et al.*, 2002).

Limitations associated with plant expression systems include low yields of recombinant protein, which is most likely a result of protein instability, low levels of expression and lack of post-translational modifications (Fischer *et al.*, 2004). Several advances for yield increase have been made toward addressing these shortcomings. These include maximising the rate of transcription and translation by using strong plant promoters (Twyman *et al.*, 2003), codon-optimisation of the gene of interest, co-expression with viral silencing suppressors (Takeda *et al.*, 2002; Voinnet *et al.*, 2003), targeting of the recombinant proteins to different subcellular compartments within the plant cell (Fischer *et al.*, 2012; Maclean *et al.*, 2007; Twyman *et al.*, 2003) and fusion of the protein of interest to signal sequences that drive sequestration of the recombinant protein into large protein bodies (PBs) that are protected from degradation by host cell enzymes (Torrent *et al.*, 1994).

PBs are organelles derived from the endoplasmic reticulum (ER) that are able to store stable amounts of zeins - prolamin-type storage proteins that are used as a source of nitrogen during early germination of maize seeds (Müntz, 1998). These zein polypeptides can oligomerise into larger complexes that ultimately self-associate into PBs (Galili, 2004). γ-Zein, one of the maize prolamin storage proteins, has been shown to be naturally accumulated at high levels in PBs in the ER. Heterologous plant-based expression of the Zera® sequence (ERA Biotech, Spain), generated from the N-terminal proline-rich domain and Pro-X sequence of γ-zein results in ER retention and formation of membrane-delineated PBs in non-seed tissues (Geli *et al.*, 1994; Torrent *et al.*, 2009a; Torrent *et al.*, 2009b). Expression of the Zera® domain fused to target proteins has been shown to result in the high-level accumulation of recombinant proteins in large, stable PBs called StorPro® (ERA Biotech, Spain) organelles that are protected from degradation and are easy to purify (Geli *et al.*, 1994; Torrent *et al.*, 2009a).

Currently, most of the vaccines that are available on the market are manufactured using strategies such as chemical inactivation and attenuation of pathogens, or subunit vaccines that are produced using cell culture techniques. Plant-produced vaccines provide relatively inexpensive and safe alternatives to the abovementioned methods (Santi, 2009). There are

three ways in which industrial-scale recombinant vaccines can be produced in plants: (i) stable transgenic or transplastomic expression, (ii) transient expression and (iii) *in vitro* culture systems where plant biomass is cultured in bioreactors under sterile conditions (Xu *et al.*, 2012).

Extensive research has been carried out using stably transformed plants; however the reduced development and production times associated with transient expression (3-9 months for transgenic versus a few days for transient expression) make it a more viable option for the production of proteins of commercial and academic interest (Sainsbury and Lomonossoff, 2008). Transient expression is mediated by either binary plant vectors or recombinant viral vectors based on RNA plant viruses that are hosted in *Agrobacterium tumefaciens* [*A. tumefaciens*, (Sainsbury and Lomonossoff, 2008; Xu *et al.*, 2012)].

Agrobacterium is a gram-negative plant pathogen that is known for its natural ability of trans-kingdom DNA transfer. Although *Agrobacterium* is used mainly for plant genetic engineering it can transfect virtually any living cell, from prokaryotes, yeast and fungi to human cells (Tzfira *et al.*, 2004). During the process of agroinfiltration foreign genes are introduced into the abaxial air spaces of intact plants by vacuum or syringe infiltration of recombinant *A. tumefaciens*, which contains the gene of interest within its transfer DNA (T-DNA) region. The production of recombinant proteins within the leaves starts within 24 hours and can continue for several days (Xu *et al.*, 2012).

Transient expression mediated by recombinant viral vectors is one of the most effective means of achieving high-level transient expression of foreign proteins in plants (Giritch *et al.*, 2006; Lindbo, 2007), as these systems make use of the ability of RNA viruses to replicate to high titres within infected cells. There are a number of drawbacks using virus-directed replication of RNA, which include restrictions on the insert size, genetic stability of the constructs during viral replication and vectors that are based on full-length replicons can move from cell to cell through a plant, resulting in bio-containment issues. As alternatives, non-replicating viral vectors have been developed that allow for the high-level and rapid transient expression of proteins, these include potato virus X and the Cowpea mosaic virus (CPMV)-based expression system as well as deconstructed viral vectors based on bean yellow dwarf virus and the tobacco mosaic virus based magnICON® expression system (Huang *et al.*, 2009; Regnard *et al.*, 2010; Sainsbury and Lomonossoff, 2008; Santi *et al.*, 2006). Transient expression using binary plant and non-replicating virus based vectors is limited to the tissue which has been infiltrated with a recombinant *Agrobacterium* suspension (Sainsbury *et al.*, 2009), thereby circumventing the bio-containment problem.

Previously, *Agrobacterium*-mediated transient expression was most commonly used as a method to assess gene expression and to test expression cassette arrangements before continuing with the stable expression of proteins (Fischer *et al.*, 2004; Sainsbury *et al.*, 2009). However, in recent years transient expression in *N. benthamiana* has been shown to be a highly efficient process for both developmental research and the large-scale production of recombinant proteins (D'Aoust *et al.*, 2010; D'Aoust *et al.*, 2008).

A few plant-derived pharmaceutical products have been produced via transient expression using GMP-approved processes. These products include the rapid response influenza VLP vaccine produced by Medicago Inc. (D'Aoust *et al.*, 2010; D'Aoust *et al.*, 2008; Vicente *et al.*, 2011), the influenza virus haemagglutinin subunit vaccine produced by the Fraunhofer Institute (Shoji *et al.*, 2009; Shoji *et al.*, 2011) and the MAPP66 antibody produced by Bayer/ICON that can be used as a combination Herpes simplex virus (HSV) and human immunodeficiency virus (HIV) microbicide. All of these vaccines have either completed, or are in phase I clinical trials (Fischer *et al.*, 2012).

1.6. Concluding remarks

BTV is an economically significant disease that affects ruminants; the most recent outbreak (BTV-8) occurred in northern Europe in 2006 where high mortality rates of livestock were reported (Toussaint *et al.*, 2006; van Wuijckhuise *et al.*, 2006). The most effective means of controlling or protecting susceptible animals against circulating serotypes of BTV is vaccination (Coetzee *et al.*, 2012).

Vaccines against animal viral diseases, such as BTV, are usually either chemically inactivated or "killed" viruses, or attenuated "live" viruses. These vaccines are, however, associated with significant risks, such as incomplete inactivation of killed virus, or the ability of the attenuated virus strain to revert to a more virulent strain capable of causing disease in the vaccinated animals. In the case of BTV with its segmented genome, there is the added risk of genetic exchange of segments (reassortment) between the vaccine strain and field strains, which can result in the production of new strains of the virus (Noad and Roy, 2003; Savini *et al.*, 2008). Moreover, attenuated vaccines have been associated with significant side effects, including abortion and teratological effects (Osburn *et al.*, 1971; Schultz and Delay, 1955; Young and Cordy, 1964). Another problem associated with attenuated vaccines is the requirement for strict biosafety measures in their production and also the inability to differentiate between vaccinated and naturally infected animals, as all of the gene products are made in both cases (Stewart *et al.*, 2010).

It is therefore desirable to develop vaccines for BTV that are DIVA-compliant and safe to use. Furthermore, producing these vaccines in a system that does not require strict biosafety measures will be advantageous. Transient protein expression in plants is an effective method for rapid small- and large-scale production of proteins, providing a cost-effective alternative for the production of subunit and/or VLP BTV vaccines.

1.7. Aims and objectives of the study

The broader objective of this study was to develop plant-based vaccine candidates for BTV. In order to achieve this, the specific aims of the study were four-fold:

(i) Transient expression of recombinant proteins in plants can be enhanced by using expression vectors that target the recombinant protein to different organelles within the plant cells (Maclean *et al.*, 2007). Furthermore, transient expression allows for rapid evaluation of the expression of numerous constructs. Therefore, transient co-expression of the four plant codon optimised capsid genes (VP2, VP3, VP5 and VP7) of BTV-8 was carried out using the pTRA vector suite that targets heterologous protein expression to different subcellular compartments. Expression levels obtained with the pTRA vector suite were compared to expression of the capsid genes obtained with the Cowpea mosaic virus-based *HyperTrans* (CPMV-*HT*) pEAQ vector system in *N. benthamiana*. The pEAQ-*HT* expression system was used for comparison with our in-house pTRA vectors as it was a stipulated objective of the EU FP7 grant that funded this project.

(ii) The second objective was to further optimise co-expression and subsequent purification of BTV-8 VLPs with the plant expression vector that allowed for the most efficient expression of all four capsid proteins. The production of VLP vaccines against BTV *in planta* abrogates the need for high biosafety levels required with insect cell produced VLPs, thereby reducing the costs involved in vaccine production. Recently BTV VLPs were successfully produced in *N. benthamiana* using a transient expression system. Sheep vaccinated with the plant-expressed VLPs were protected against challenge with a field strain of BTV-8 (Thuenemann *et al.*, 2013).

(iii) The third objective of this study was to develop two second generation plant-produced BTV PB vaccines by fusing a predicted synthetic VP2 multi-epitope and the full-length

codon optimised BTV-8 VP2 to Zera® (ERA Biotech, Spain), which drives PB formation. The epitope-based candidate vaccine is proposed to be multivalent, thereby obviating the need for multiple serotype-specific vaccination. The particulate vaccine consisting of the full-length VP2 could be used in animals to protect against a single serotype of BTV; this vaccine could also be produced as a rapid-response vaccine to circulating serotypes of BTV. Both PB vaccine candidates are aimed at being DIVA compliant.

(iv) The final objective of this study was to analyse the immunogenicity of the plant-produced candidate vaccines in BALB/c mice.

Chapter 2:

Agrobacterium-mediated transient expression of BTV-8 plant codon optimised capsid genes in *N. benthamiana* using different expression vectors

2.1. Introduction

BTV virions are architecturally complex, multi-layered particles that consist of seven structural proteins. Figure 1.1 (Chapter 1) shows that the four major structural proteins are organised into three layers. The outer shell of the virion contains two major proteins, VP2 and VP5 that are laid onto the foundation provided by the assembly of VP3 and VP7 (French *et al.*, 1990; Roy, 1996).

When VP3 and VP7 are co-expressed in an insect cell/baculovirus expression system, they form core-like particles (CLPs). Using the same expression system, the co-expression of VP2 and VP5 together with VP3 and VP7 leads to the assembly of VLPs that are devoid of nucleic acid (French *et al.*, 1990; Roy, 1996). However, the cost associated with the production of VLPs in insect cells is very high and alternative methods of production would be desirable (Thuenemann *et al.*, 2013). Despite the proven efficacy of the attenuated vaccine in South Africa (Coetzee *et al.*, 2012), it is desirable to replace these attenuated vaccines with recombinant VLP vaccines, as this would negate the possibility of the emergence of new reassortant viruses of unknown virulence due to co-circulation of vaccine and wild-type viruses in the same herds.

In recent years the heterologous expression of complex high-value proteins in plants has attracted much interest. There are many advantages of using plants over other eukaryotic expression hosts, including their high yield of biomass, ease and affordability of scale-up and lack of the risk of contamination by human pathogens (Rybicki, 2010; Tzfira and Citovsky, 2006). Transient expression systems are flexible and allow for rapid expression of high concentrations of recombinant protein in a matter of days (Fischer *et al.*, 1999). Transient expression can be achieved by the use of infective plant virus-based vectors where the gene of interest is cloned into the genome of a viral plant pathogen which is used to infect the plant, or by infiltration of plants with recombinant *Agrobacterium* containing the transgenes of interest inserted into the T-DNA region of the bacterial tumour-inducing plasmid [pTi, (Fischer *et al.*, 1999; Tzfira *et al.*, 2004)]. The *Agrobacterium vir* (virulence)-system processes and transfers the T-DNA from the bacterium to the plant cell nucleus where it is transcribed and translated

using host cell enzymes, resulting in transient expression of recombinant protein (Kapila *et al.*, 1997; Tzfira *et al.*, 2004; Zupan *et al.*, 2000).

As a defence mechanism against virus infection plants have an RNA silencing system known as post-transcriptional gene silencing (PTGS), which can suppress the transient expression of foreign proteins. Several plant viruses encode silencing suppressors that counteract PTGS (Takeda *et al.*, 2002; Voinnet *et al.*, 2003). The NSs protein of tomato spotted wilt virus (TSWV) and P19 of tomato bushy stunt virus (TBSV) are examples of such proteins (Takeda *et al.*, 2002; Voinnet *et al.*, 2003). *A. tumefaciens*-mediated transient expression of recombinant proteins can be enhanced and extended by co-infiltrating plants with recombinant *A. tumefaciens* cells that encode the silencing suppressor gene (Maclean *et al.*, 2007).

Expression levels of heterologous proteins in plants can be increased by using signal peptides that target the expressed protein to different organelles within the plant cell. It was shown that the Human papilloma virus (HPV) major capsid protein, L1, was expressed at higher levels when recombinant protein was targeted to the chloroplast compared to cytoplasmic localisation or retention in the endoplasmic reticulum [ER, (Maclean *et al.*, 2007)]. Moreover, targeting of the recombinant proteins to different subcellular compartments may facilitate easier purification when production is scaled up.

BTV-8 VLPs have been successfully produced in *N. benthamiana* using a Cowpea mosaic virus-based *HyperTrans* (CPMV-*HT*) and associated pEAQ transient expression system which targets expression to the cytoplasm (Thuenemann *et al.*, 2013). In this chapter transient co-expression of the four BTV-8 VPs in *N. benthamiana* was compared by using the binary *A. tumefaciens* pTRA suite of vectors pTRAc-HT, pTRAkc-AH and pTRAkc-rbcs1-cTP, which target recombinant protein to the cytosol, apoplast and chloroplast respectively, in order to determine whether the expression yields could be improved by targeting the recombinant proteins to different subcellular compartments (Maclean *et al.*, 2007). The pTRA vectors used in this study were available in-house at UCT. In addition to the pTRA vectors, the VPs were also co-expressed using pEAQ-*HT* which has the P19 silencing suppressor sequence of TBSV incorporated into the T-DNA, enabling expression of the gene of interest and the silencing suppressor from a single plasmid (Sainsbury *et al.*, 2009), to compare which system best co-expressed the four VPs.

2.2. Materials and Methods

2.2.1. PCR amplification of the BTV-8 capsid (VP) genes

The BTV-8 *VP2*, *VP3*, *VP5* and *VP7* capsid gene sequences (Genbank accession numbers: AM498052, AM498053, AM498056 and AM498057, respectively) were codon optimised for *Nicotiana spp.* translation and synthesized by GeneArt (Germany).

The four codon optimised genes were modified by Eva Thuenemann (John Innes Centre, UK) using the primers listed in Table 2.1 to facilitate directional cloning of the genes into the *Age*I and *Xho*I sites of the pEAQ-*HT* vector (Figure 2.1 A, obtained from G. Lomonossoff, John Innes Centre, UK) to yield four different constructs. The four pEAQ-*HT* plasmids (pEAQ-*HT*VP2co, pEAQ-*HT*VP3co, pEAQ-*HT*VP5co and pEAQ-*HT*VP7co) were used as template DNA in subsequent PCR DNA amplifications to modify the 5' and 3' termini of the four BTV-8 capsid genes to facilitate cloning into the pTRA vector suite. The primers used for the various PCR reactions are listed in Table 2.1.

PCR amplification of the capsid genes was performed with the primers listed in Table 2.1. The PCR reactions consisted of 50 ng of the appropriate template DNA, 200 µM dNTPs, 1 µM of each primer, 1x Buffer A and 1 unit (U) KAPA Taq DNA Polymerase (KAPA Biosystems). For modification of *VP2* and *VP3* the reactions were amplified with an initial denaturation step at 94 °C for 5 min. Twenty seven cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 60 s and elongation at 72 °C for 90 s were carried out followed by a final elongation step at 72 °C for 5 min. For amplification of *VP5* and *VP7* the cycling conditions were modified by annealing at 55 °C for 60 s and elongation at 72 °C for 45 s. The amplified products were separated on 0.8% w/v TBE (89 mM Tris base, 89 mM boric acid and 2 mM EDTA [pH 8]) agarose gels containing 2.5 mg/mL ethidium bromide and visualised under short wavelength ultraviolet (UV) illumination.

O'GeneRuler™1kb DNA ladder (Fermentas) was used as molecular weight marker on all the agarose gels.

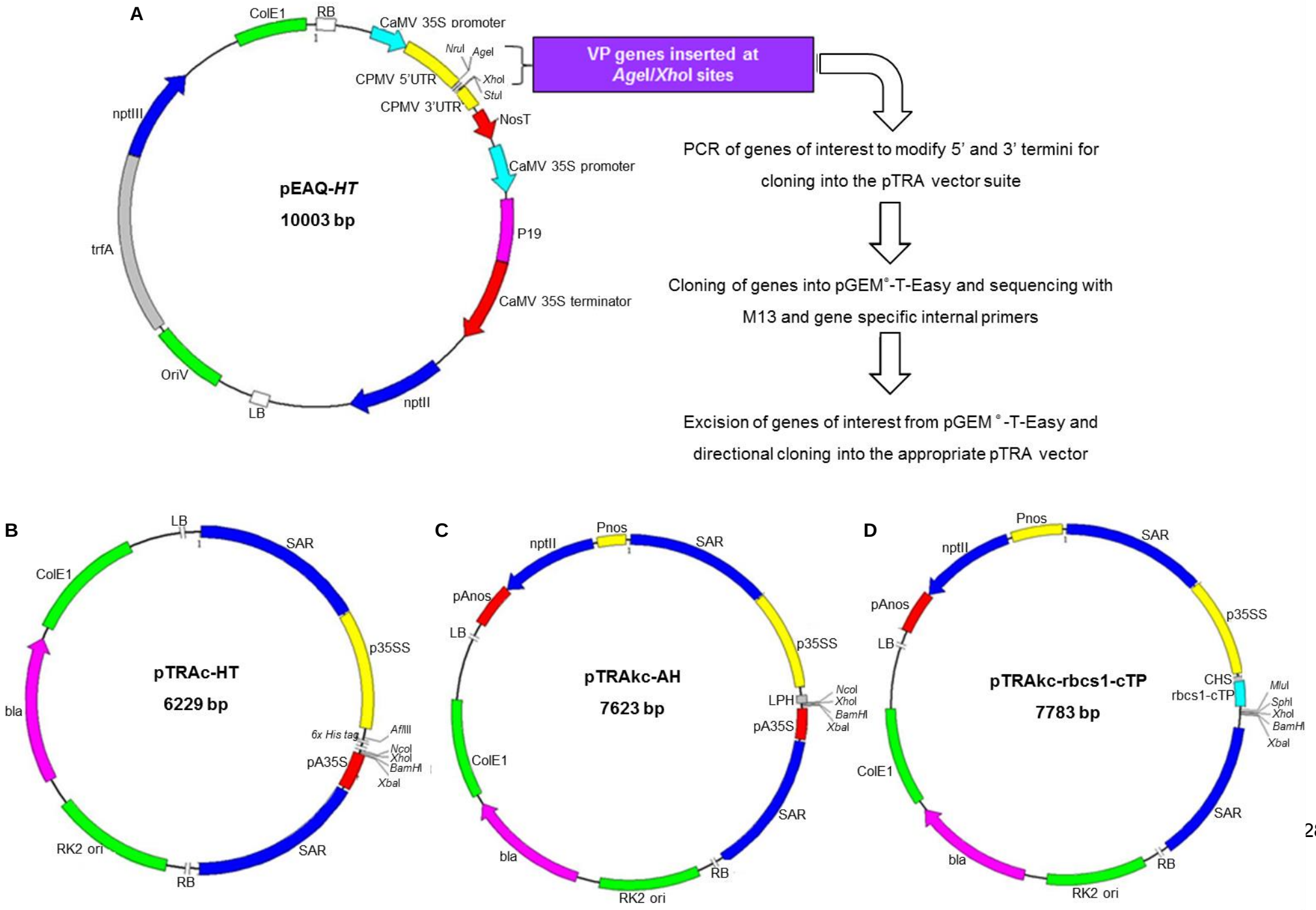
A
ColE1
RB
CaMV 35S promoter
NruI
AgeI
XhoI
StuI
CPMV 5'UTR
CPMV 3'UTR
NosT
CaMV 35S promoter
P19
CaMV 35S terminator
nptII
LB
OriV
trfA
nptIII
pEAQ-HT
10003 bp
VP genes inserted at AgeI/XhoI sites
PCR of genes of interest to modify 5' and 3' termini for cloning into the pTRA vector suite
Cloning of genes into pGEM®-T-Easy and sequencing with M13 and gene specific internal primers
Excision of genes of interest from pGEM®-T-Easy and directional cloning into the appropriate pTRA vector
B
LB
SAR
ColE1
p35SS
pTRAc-HT
6229 bp
bla
6x His tag
AflIII
NcoI
XhoI
BamHI
XbaI
pA35S
RK2 ori
SAR
RB
C
nptII
Pnos
SAR
pAnos
LB
p35SS
pTRAkc-AH
7623 bp
LPH
NcoI
XhoI
BamHI
XbaI
pA35S
ColE1
SAR
bla
RB
RK2 ori
D
Pnos
nptII
SAR
pAnos
p35SS
LB
CHS
rbcs1-cTP
MluI
SphI
XhoI
BamHI
XbaI
pTRAkc-rbcs1-cTP
7783 bp
ColE1
SAR
bla
RB
RK2 ori

Figure 2.1: *From the previous page.* A flow diagram displaying the strategy that was followed to modify the BTV-8 capsid genes and their subsequent cloning into the pTRA vector suite. The capsid genes were directionally cloned and subcloned into the plant expression vectors: (A) pEAQ-*HT*, (B) pTRAc-HT, (C) pTRAkc-AH and (D) pTRAkc-rbcs1-cTP. The vector elements that are necessary to achieve expression using the pEAQ-*HT* and pTRA vectors *in planta* are shown in the figure. **(A) pEAQ-*HT*:** RB and LB: right and left borders for T-DNA integration, 35S promoter from Cauliflower mosaic virus (CaMV), 5'UTR: modified 5' UTR from CPMV RNA-2, 3'UTR from CPMV RNA-2, NosT: nopaline synthase terminator, P19: suppressor of gene silencing from TBSV, 35S terminator from CaMV, *npt*II: kanamycin resistance gene, OriV: pRK2 origin of replication, TrfA: replication essential locus and ColEI; the pBR322 *E. coli* origin of replication (Sainsbury *et al.*, 2009). **(B-D) pTRA vectors:** P35SS: CaMV 35S promoter with a duplicated transcriptional enhancer, CHS: chalcone synthase 5' UTR, pA35S: CaMV 35S polyadenylation signal that enables foreign gene expression, SAR: the expression cassettes are flanked by two copies of the Rb7 scaffold attachment region, ColEI ori: origin of replication for *E. coli,* RK2 ori: *Agrobacterium* origin of replication, *bla*: gene for ampicillin / carbenicillin resistance. pTRAc-HT (B) contains a 6x histidine tag at the N-terminal, pTRAkc-rbcs1-cTP (C) contains chloroplast-transit peptide sequence of the potato *rbcS*1 gene (rbcs1-cTP) and pTRAkc-AH (D) contains a plant codon optimised signal peptide sequence from the murine mAB24 heavy chain gene (LPH). In addition, both (C) and (D) contain *npt*II,the gene for kanamycin resistance and pNos/pAnos the promoter/polyadenylation signal of the nopaline synthase gene (Maclean *et al.*, 2007).

Table 2.1: Sequences of the primers used to modify the BTV-8 capsid genes for subsequent cloning into the different plant expression vectors that were tested.

Expression vector	Insert	Size (kb)	Restriction sites (5' / 3')	Primer	5' – 3' Sequence[1]	Tm (°C)	Subcellular target	Construct
pTRAc-HT	*VP2*	~2.8	*Nco*I *Xho*I	cVP2coF	GCGAATTC**CCATGG**AAGAACTCGCTATCCCAA	56	Cytosol	pTRAc-HTVP2co
				cVP2coR	GC**CTCGAG**TCAAACGTTGAGGAGCTTAGTAAG	54		
	VP3	~2.7		cVP3coF	GCGAATTC**CCATGG**CTGCTCAAAATGAGCAAAG	59		pTRAc-HTVP3co
				cVP3coR	GC**CTCGAG**TTAAACAGTTGGAGCAGCAAGC	58		
	VP5	~1.5		cVP5coF	GCGAATTC**CCATGG**GAAAGATTATTAAGTCCCTCTC	55		pTRAc-HTVP5co
				cVP5coR	GC**CTCGAG**TCAAGCGTTCCTAAGGAAGAGT	54		
	VP7	~1		cVP7coF	GCGAATTC**CCATGG**ATACAATTGCTGCTAGGG	54		pTRAc-HTVP7co
				cVP7coR	GC**CTCGAG**TCACACATAAGCAGCCCTAG	57		
pTRAkc-AH[2]	*VP2*	~2.8	*Nco*I *Xho*I	See note 2 below			Apoplast	pTRAkc-AHVP2co
	VP3	~2.7						pTRAkc-AHVP3co
	VP5	~1.5						pTRAkc-AHVP5co
	VP7	~1						pTRAkc-AHVP7co
pTRAkc-rbcS1-cTP[3]	*VP2*	~2.8	*Mlu*I *Xho*I	cTPVP2coF	GTGG**ACGCGT**TAGGTGC**ATG**GAAGAACTCGCTATCCCAA	56	Chloroplast	pTRAkc-rbcs1-cTPVP2co
	VP3	~2.7		cTPVP3coF	GTGG**ACGCGT**TAGGTGC**ATG**GCTGCTCAAAATGAGCAAAG	59		pTRAkc-rbcs1-cTPVP3co
	VP5	~1.5		cTPVP5coF	GTGG**ACGCGT**TAGGTGC**ATG**GGAAAGATTATTAAGTCCCTCTC	55		pTRAkc-rbcs1-cTPVP5co
	VP7	~1		cTPVP7coF	GTGG**ACGCGT**TAGGTGC**ATG**GATACAATTGCTGCTAGGG	54		pTRAkc-rbcs1-cTPVP7co
pEAQ-*HT*	*VP2*	~2.8	*Age*I *Xho*I	pEAQ-HTVP2F	GC**ACCGGT**ATGGAAGAACTCGCTATCCCAA	56	Cytoplasm	pEAQ-*HT*VP2co
	VP3	~2.7		pEAQ-HTVP3F	GC**ACCGGT**ATGGCTGCTCAAAATGAGCAAAG	59		pEAQ-*HT*VP3co
	VP5	~1.5		pEAQ-HTVP5F	GC**ACCGGT**ATGGGAAAGATTATTAAGTCCCTCTC	55		pEAQ-*HT*VP5co
	VP7	~1		pEAQ-HTVP7F	GC**ACCGGT**ATGGATACAATTGCTGCTAGGG	54		pEAQ-*HT*VP7co

[1] The restriction sites are underlined and in bold.

[2] The 5' and 3' termini were modified using the same primers that were used to modify the genes for cloning into pTRAc-HT.

[3] The 3' termini were modified with the pTRAc-HT reverse primers to add *Xho*I restriction sites to the capsid genes.

2.2.2. Cloning of amplified genes into pGEM®-T-Easy for sequence analysis

DNA was purified from the agarose gels using the QIAquick® Gel Extraction kit (Qiagen) following the manufacturer's instructions. The purified DNA was ligated into the pGEM®-T-Easy Vector system (Promega) according to the manufacturer's instructions and incubated overnight (O/N) at 4 °C.

The ligated plasmid constructs were used to transform DH5-α chemically competent *E. coli* cells (E. cloni™, Lucigen) according to the method described by Sambrook *et al.* (1989). The transformed cells were selected on Luria Bertani (LB) media plates supplemented with ampicillin (100 µg/mL), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, [80 µg/mL]) and IPTG (Isopropylthio-β-D-galactoside, [0.5 mM]) that were incubated for 16 hrs at 37 °C.

White colonies were inoculated into 5 mL LB broth supplemented with 50 µg/mL ampicillin and incubated with agitation for 16 hrs at 37 °C. Plasmid isolations were performed using the QIAprep® Spin Miniprep kit (Qiagen) according to instructions provided by the manufacturer. Recombinant pGEM®-T-Easy clones were verified by restriction enzyme digestion. Recombinant DNA (~500 ng) was digested for 1 hr at 37 °C using 1 U X*ho*I (Fermentas) per reaction according to the manufacturer's instructions. Linearized DNA was separated on 0.8% w/v TBE agarose gels containing 2.5 mg/mL ethidium bromide.

After verification of the clones with restriction digests the plasmid DNA was sequenced (Macrogen Inc., Netherlands) using the M13 forward and reverse primers. Gene-specific internal primers (Table 2.2) were designed for sequencing in order to obtain sequence data for the entire length of the sequence. Sequence data were analysed using DNAMAN (Lynnon BioSoft).

Table 2.2: The gene-specific internal primers used to sequence the entire length of the genes of interest.

Gene	Primer	5' – 3' sequence
VP2	coVP2-IF	GTACAACCTTCTCCGTAATTGGT
	coVP2-IR	CCTTAGCAGATCTTCCAACAGCT
VP3	coVP3-IF	AGGCTTCAGGGATATATTGAG
	coVP3-IR	TCACAGCCTTAACAGCCTCTG
VP5	coVP5-IF	CTATTGAGGTTGAAAGGGATGGA
	coVP5-IR	TGATCTCTTTGTGGTTCTCTTC
VP7	coVP7-IF	CTAGAGGTGATGTTCAGCAGAT
	coVP7-IR	CTGGAAAATCTGCTGAACATCA

2.2.3. Sub-cloning of the BTV-8 capsid genes into the pTRA vector suite[1]

The capsid genes were sub-cloned into the three different pTRA binary vectors (Figure 2.1): pTRAc-HT, pTRAkc-AH and pTRAkc-rbcs1-cTP (kindly provided by Dr Rainer Fischer, Fraunhofer Institute, Aachen, Germany). These vectors are designed to target recombinant proteins to the cytosol, apoplast and chloroplast, respectively (Maclean *et al.*, 2007). The *VP2* and *VP3* genes modified for cloning into pTRAc-HT, pTRAkc-AH and pTRAkc-rbcs1-cTP were excised from the pGEM®-T-Easy backbone by first digesting the plasmid DNA with either *Nco*I or *Mlu*I and *Sca*I for 1 hr at 37 °C after which the DNA was gel-purified with the QIAquick® Gel Extraction kit (Qiagen). The entire volume of purified DNA was subsequently digested with *Xho*I for 1 hr at 37 °C and gel-purified. This two-step digestion from pGEM®-T-Easy yielded *VP2* and *VP3* genes with 5' *Nco*I or *Mlu*I sites and 3' *Xho*I sites. *VP5* and *VP7* were excised from pGEM®-T-Easy by double digesting with either *Nco*I or *Mlu*I and *Xho*I, yielding genes flanked by 5' *Nco*I or *Mlu*I sites and 3' *Xho*I sites.

The pTRAc-HT and pTRAkc-AH (Figure 2.1 B and C) expression vectors were linearized by restriction digestion with *Nco*I and *Xho*I and pTRAkc-rbcs1-cTP (Figure 2.1 D) was linearized with *Mlu*I and *Xho*I. The vector DNA was dephosphorylated using 1 U rapid alkaline phosphatase (Roche) according to the manufacturer's instructions. The BTV-8 genes were

[1] All restriction enzymes used in the section were obtained from Fermentas and digests were carried out according to the manufacturer's instructions.

directionally sub-cloned into the pTRA plant expression vectors yielding the constructs listed in Table 2.1.

DH5-α chemically competent *E. coli* cells were transformed (Sambrook *et al.*, 1989) with the plasmid constructs and recombinant clones were selected on LB media plates supplemented with ampicillin (100 µg/mL) O/N at 37 °C. Colonies were inoculated into 5 mL LB broth supplemented with 50 µg/mL ampicillin and incubated with aeration for 16 hrs at 37 °C. Small scale plasmid isolations were performed on the cultures using the QIAprep® Spin Miniprep kit (Qiagen).

For confirmation of the recombinant pTRA constructs 500 ng - 1 µg plasmid DNA was digested for 1 hr at 37 °C with 1 U each of the restriction enzymes (5' *Nco*I/*Mlu*I and 3' *Xho*I) that flank the genes of interest (Table 2.1). Digested DNA was separated on 0.8% w/v TBE agarose gels containing 2.5 mg/mL ethidium bromide.

2.2.4. Transformation of *A. tumefaciens*

A. tumefaciens LBA4404 and *A. tumefaciens* GV3101::pMP90RK cells were made electrocompetent using the method described by Shen and Forde (1989). Fifty to 100 ng of the pEAQ-*HT* and pTRA recombinant plasmids were electroporated into *A. tumefaciens* LBA4404 and *A. tumefaciens* GV3101::pMP90RK, respectively. Transformation of the *A. tumefaciens* strains was carried out by electroporation according to the method described by Maclean *et al.* (2007). Recombinant pEAQ-*HT* clones were selected on LB media plates at 27 °C containing 30 µg/mL kanamycin and 50 µg/mL rifampicin. The pTRA clones were selected under the same conditions except that the plates were additionally supplemented with 50 µg/mL carbenicillin.

Successful transformation was confirmed by colony PCR (as in Section 2.2.1) using the gene-specific primers listed in Table 2.1, as well extracting plasmid DNA (QIAprep® Spin Miniprep kit, Qiagen) from O/N liquid cultures of putative recombinant clones and back-transformation of the DNA into DH5-α chemically competent *E. coli* cells. Transformed *E. coli* was plated onto LB plates supplemented with antibiotics to select for the recombinant plasmids.

2.2.5. *A. tumefaciens*-mediated transient expression

Starter cultures of recombinant pEAQ-*HT* and pTRA *A. tumefaciens* cells, including *A. tumefaciens* LBA4404 containing pBIN-NSs, the plasmid carrying the TSWV *NSs* silencing suppressor gene (Prins *et al.*, 1996), were supplemented with the relevant antibiotics and grown in LB broth as described by Maclean *et al.* (2007). To prevent clumping of the recombinant LBA4404 *A. tumefaciens* cells, the medium was supplemented with 2 mM magnesium sulphate ($MgSO_4$).

The starter cultures were used to inoculate induction medium (Maclean *et al.*, 2007) with the addition of the appropriate antibiotics, including 20 µM acetosyringone and the cultures were incubated O/N at 27 °C with agitation. The cells were harvested from the O/N cultures by centrifugation at 1 000 *x g* for 5 min and resuspended in infiltration medium (10 mM magnesium chloride [$MgCl_2$], 10 mM 2-morpholinoethanesulfonic acid [MES], 3% sucrose and 150 µM acetosyringone in water, pH 5.6). The cell suspensions were incubated at 22 °C for 2 hrs to allow for expression of the *vir* genes prior to infiltration. After incubation the cultures were diluted to the required optical density (OD_{600}) in infiltration medium. The cell densities (OD_{600}) used for co-infiltration of the constructs were varied in order to find the optimal cell concentration where all four capsid proteins were successfully co-expressed.

For each one of the expression vectors used, the relevant recombinant *A. tumefaciens* constructs were co-infiltrated into six-week-old *N. benthamiana* leaves by injecting the suspension into the abaxial spaces using a blunt-ended syringe (Maclean *et al.*, 2007). Recombinant *A. tumefaciens* pTRA constructs were co-infiltrated with pBIN-*NSs*, the pEAQ-*HT* recombinant strains did not require co-infiltration with the silencing suppressor as it already has the P19 silencing suppressor integrated on the T-DNA of the plasmid under control of the CaMV 35S promoter (Sainsbury *et al.*, 2009). As a negative control the plants were infiltrated with infiltration medium. Time-trials were conducted to evaluate and compare co-expression of VP2, VP3, VP5 and VP7 with the different vectors. The plants were grown at 22 °C under 16hrs/8hrs light/dark cycles until harvested.

2.2.6. Protein extraction and western blot analysis

Three leaf discs (cut by using the cap of a microcentrifuge tube) were harvested for each vector co-expressing recombinant proteins at 1, 3, 5 and 7 days post infiltration (dpi) and ground up in liquid nitrogen. The leaf material was resuspended in 70 µL per disc of bicine

buffer (50 mM bicine [pH 8.4], 20 mM sodium chloride [NaCl], 0.1% N-lauroyl-sarcosine [NLS], 1 mM DL-dithiothreitol [DTT] and 1x Complete Mini EDTA-free protease inhibitor cocktail [Roche]) by vortexing. The crude leaf extracts were clarified by centrifugation at 15 000 *x g* for 5 min in a bench top centrifuge.

For western blot analysis, the crude plant extracts were incubated at 90 °C for 10 min in sample application buffer (Sambrook *et al.*, 1989). The proteins were separated on 8% SDS polyacrylamide gels where equal amounts of total protein were loaded in each lane. For all the western blots PageRuler™ Prestained Protein Ladder (Thermo Scientific) was used as molecular weight marker. After electrophoresis the proteins were transferred onto nitrocellulose membranes at 15 volts (V) for 1 hr using a Trans-blot®SD semi-dry transfer cell (Bio-Rad). After electroblotting the membranes were blocked in blocking buffer (5% non-fat dairy milk [NFDM] and 1x PBST [137 mM NaCl, 10 mM disodium hydrogen phosphate (Na_2HPO_4), 2.7 mM potassium chloride (KCl), 2 mM potassium dihydrogen phosphate (KH_2PO_4) at pH 7.4 and 0.1% Tween-20]). The membranes were probed O/N at 4 °C with 1: 2000 sheep serum containing antibodies that reacted equally with all four VPs (Thuenemann *et al.*, 2013) diluted in blocking buffer. The membranes were washed four times with blocking buffer for 15 min each and afterward incubated in a 1: 10 000 dilution of anti-goat/sheep alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich) in blocking buffer for 1 hr at 37 °C. After incubation in secondary antibody the membranes were washed four times with 1x PBST, with 15 min for each wash. Detection was performed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) phosphatase substrate (BCIP/NBT 1-component, KPL).

Protein extraction and western blot analysis was repeated at least three times to confirm co-expression of the BTV-8 VPs.

2.3. Results

2.3.1. PCR amplification of the BTV-8 codon optimised capsid genes

The BTV-8 capsid genes, *VP2*, *VP3*, *VP5* and *VP7* were successfully amplified with the primers listed in Table 2.1 to modify the 5' and 3' termini for cloning into the pTRA vectors. PCR amplification of the genes with the different primer sets were expected to yield the following fragments: *VP2:* 2.8 kb, *VP3:* 2.7 kb, *VP5:* 1.5 kb and *VP7:* 1 kb.

Figure 2.2 shows the results of PCR amplification, indicating that all four genes were successfully amplified. Figure 2.2 A shows bands at the expected sizes for all four capsid genes that were amplified with the pTRAc-HT primer sets. These genes were modified to contain 5' *Nco*I and 3' *Xho*I restriction enzyme sites to facilitate cloning into the pTRAc-HT and pTRAkc-AH expression vectors.

Figure 2.2 B shows the four genes at the expected sizes that were modified with the gene-specific pTRAkc-rbcs1-cTP forward and pTRAc-HT reverse primers to contain 5' *Mlu*I and 3' *Xho*I restriction enzyme sites.

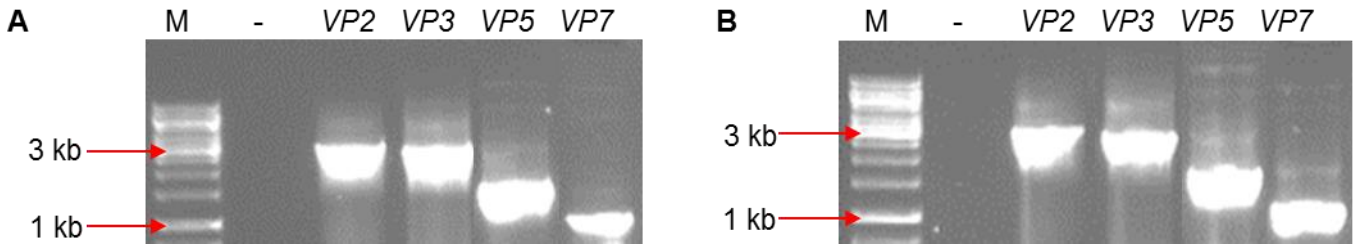

Figure 2.2: PCR amplification of the BTV-8 capsid genes using (A) the pTRAc-HT gene-specific primer sets containing the 5' *Nco*I and 3' *Xho*I restriction enzyme sites and (B) the pTRAkc-rbcs1-cTP gene-specific forward primers containing the 5' *Mlu*I enzyme site and the pTRAc-HT gene-specific reverse primers with the 3' *Xho*I enzyme sites. The negative controls (-) containing water instead of DNA template were amplified with the appropriate primer sets. Lanes M represents the molecular weight marker, O'GeneRuler™1kb DNA ladder (Fermentas).

The relevant bands on the gel were purified using the QIAquick® Gel Extraction kit (Qiagen) and subsequently cloned into the pGEM®-T-Easy vector.

2.3.2. Verification of pGEM®-T-Easy clones

The PCR-modified genes were successfully cloned into the pGEM®-T-Easy vector and transformed into *E. coli,* yielding the constructs listed in Table 2.3.

Table 2.3: Table showing the constructs created by cloning of the PCR-modified genes into pGEM®-T-Easy.

Gene	Construct	Restriction sites (5' / 3')
VP2	cVP2GEM®-T-Easy	*Nco*I / *Xho*I
VP3	cVP3GEM®-T-Easy	*Nco*I / *Xho*I
VP5	cVP5GEM®-T-Easy	*Nco*I / *Xho*I
VP7	cVP7GEM®-T-Easy	*Nco*I / *Xho*I
VP2	cTPVP2GEM®-T-Easy	*Mlu*I / *Xho*I
VP3	cTPVP3GEM®-T-Easy	*Mlu*I / *Xho*I
VP5	cTPVP5GEM®-T-Easy	*Mlu*I / *Xho*I
VP7	cTPVP7GEM®-T-Easy	*Mlu*I / *Xho*I

The pGEM®-T-Easy recombinant clones were verified by restriction enzyme digestion using the *Xho*I sites which flank the 3' ends of all the genes of interest. Figure 2.3 A and B show that both the cVP- and cTPVPGEM®-T-Easy clones contained the genes of interest as linearized fragments were obtained after restriction digestion of the recombinant DNA at ~5.8 kb, ~5.7 kb, ~4.6 kb and ~4 kb for modified *VP2*, *VP3*, *VP5* and *VP7*, respectively.

The recombinant pGEM®-T-Easy clones were sequenced with M13 forward and reverse primers as well as gene-specific internal primers (Table 2.2) to obtain sequence data for the entire sequence of each individual gene. The sequences were analysed in DNAMAN (Lynnon Biosoft) by multiple alignment of the sequence data with theoretical sequences of the different plasmids. Sequence analysis confirmed that the genes were intact (data not shown).

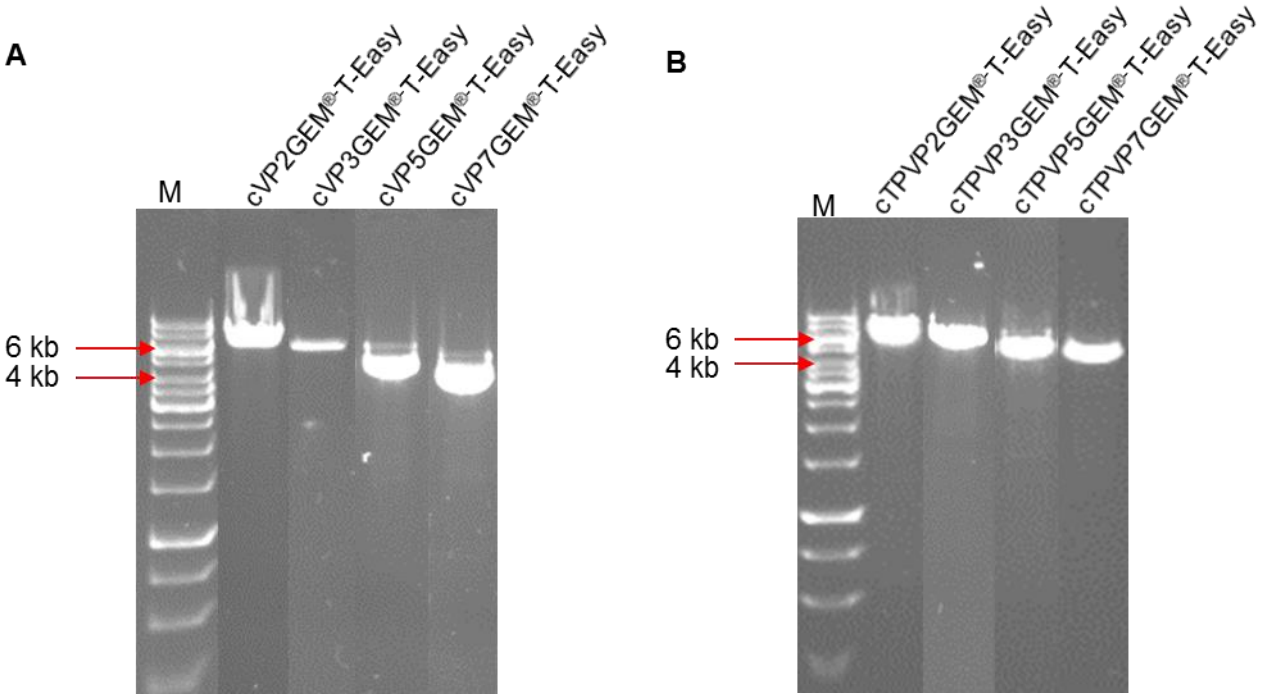

Figure 2.3: Recombinant pGEM®-T-Easy clones were verified by linearizing the plasmid DNA with *Xho*I restriction enzyme digestion. (A) Shows the pGEM®-T-Easy clones with the genes modified to contain 5' *Nco*I and 3' *Xho*I restricting sites and (B) shows the clones containing 5' *Mlu*I and 3' *Xho*I restriction sites. Clones containing the genes of interest yielded bands at ~5.8 kb, ~5.7 kb, ~4.6 kb and ~4 kb for modified *VP2*, *VP3*, *VP5* and *VP7*, respectively. Lanes M represent the molecular weight marker.

2.3.3. Sub-cloning into the pTRA vector suite

2.3.3.1. Excision of the capsid genes from the pGEM®-T-Easy vector backbone

Digestion of cVP2- and cVP3GEM®-T-Easy with *Nco*I and *Xho*I resulted in fragments at ~3 kb consisting of either VP2 and VP3 and the pGEM®-T-Easy backbone. As these fragments were indistinguishable on agarose gels, the genes could not be excised using this strategy. Therefore, the cVP2- and cVP3GEM®-T-Easy plasmid DNA was first restriction digested using *Nco*I and *Sca*I (which cuts the pGEM®-T-Easy backbone); this yielded fragments at ~4.8 kb and ~1.1 kb for cVP2 and ~4.6 kb and ~1.1 kb for cVP3. In both instances the larger fragment was purified from the gel and subsequently digested with *Xho*I, yielding cVP2 and cVP3 with *Nco*I and *Xho*I 5' and 3' restriction sites. In the case of cVP5- and cVP7GEM®-T-Easy, the genes were excised from the pGEM®-T-Easy backbone by double digesting with *Nco*I and *Xho*I. Bands at ~1.5 kb for cVP5 and ~1 kb for cVP7 were gel-purified yielding genes flanked with 5' *Nco*I and 3' *Xho*I restriction sites (results not shown).

The same strategy was followed to excise the genes from cTPVP2-, VP3-, VP5- and VP7 GEM®-T-Easy, however *Mlu*I was used instead of *Nco*I yielding genes flanked by 5' *Mlu*I and 3' *Xho*I restriction sites (results not shown).

2.2.3.2. Verification of recombinant pTRA clones

The capsid genes were successfully cloned into the pTRAc-HT, pTRAkc-AH and pTRAkc-rbcs1-cTP plant expression vectors and transformed into *E. coli*.

Recombinant pTRA clones were screened by small scale plasmid isolations on O/N cultures and the subsequent restriction enzyme digestion of the plasmid DNA with *Nco*I and *Xho*I for the pTRAc-HT and pTRAkc-AH clones and *Mlu*I and *Xho*I for pTRAkc-rbcs1-cTP clones (Figure 2.4). Restriction enzyme digestion resulted in ~6.2 kb, ~7.6 kb and ~7.7 kb bands that constitute linearized pTRAc-HT, pTRAkc-AH and pTRAkc-rbcs1-cTP vector backbones, respectively (Figure 2.4, black arrows). In all cases the insert VP DNA resulted in bands at ~2.8 kb, ~2.7 kb, ~1.5 kb and ~1 kb for *VP2*, *VP3*, *VP5* and *VP7* respectively (Figure 2.4, white arrows). These restriction profiles confirmed that the VP genes were successfully sub-cloned into the pTRA vectors.

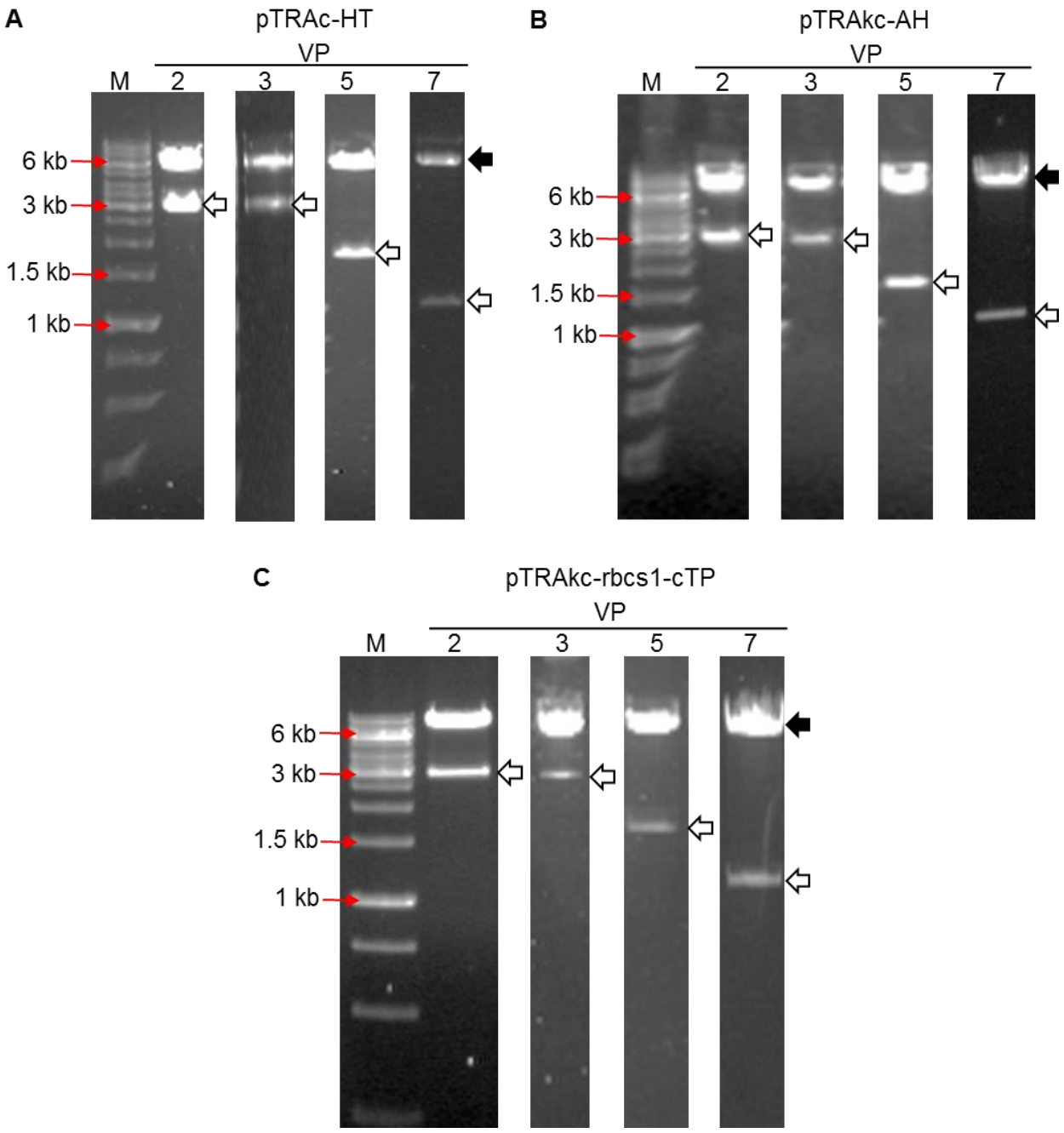

Figure 2.4: pTRAc-HT (A) and pTRAkc-AH (B) recombinant clones were verified by *Nco*I / *Xho*I restriction enzyme digestion, whereas pTRAkc-rbcs1-cTP (C) recombinant clones were verified by restriction enzyme digestion with *Mlu*I and *Xho*I. Black arrows indicate the linearized vector backbone at ~6.2 kb, ~7.6 kb and ~7.7 kb for pTRAc-HT, pTRAkc-AH and pTRAkc-rbcs1-cTP respectively. White arrows indicate the insert DNA at ~2.8 kb, ~2.7 kb, ~1.5 kb and ~1 kb for *VP2*, *VP3*, *VP5* and *VP7* respectively. The molecular marker is represented by lanes M.

2.3.4. Confirmation of successful *A. tumefaciens* transformation

The recombinant pTRA constructs were transformed into *A. tumefaciens* GV3101::pMP90RK and recombinant pEAQ-*HT* constructs were transformed into *A. tumefaciens* LBA4404. Successful transformation was confirmed by colony PCR and back-transformation of the plasmid DNA extracted from the transformed *A. tumefaciens* into *E. coli*.

2.3.5. Transient expression of BTV-8 VPs in *N. benthamiana*

To evaluate whether recombinant pTRA and pEAQ-*HT A. tumefaciens* constructs expressed the BTV-8 VPs in plants, individual recombinant constructs were infiltrated into *N. benthamiana* at an OD_{600} value of 0.25 per construct. All the vectors successfully expressed the individual capsid proteins; VP2, VP3, VP5 and VP7 (results not shown) and subsequent experiments were carried out to optimise co-expression of the four VPs with each vector set, as the aim was to eventually produce assembled BTV-8 VLPs.

Recombinant *A. tumefaciens* constructs harbouring the pEAQ-*HT* plasmids was initially co-infiltrated into *N. benthamiana* leaves using a blunt-ended syringe with a relative vector concentration ratio of 1:1:1:1 (VP2:VP3:VP5:VP7), at an OD of 0.25 for each construct. Preliminary screening at 1, 3, 5 and 7 dpi of the pEAQ-*HT* infiltrated leaf tissue using western blot analysis showed that although VP2, VP3 and VP7 were present in the crude extracts, VP5 was not co-expressed (results not shown). The lack of expression of VP5 could possibly have been a result of the low *A. tumefaciens* cell concentration used for infiltration. It has been shown by Wroblewski *et al.* (2005) that an increase in the cell concentration used for infiltration can lead to an increase of transgene expression, therefore the concentration of the recombinant *A. tumefaciens* constructs encoding VP5 was subsequently doubled (for both the pTRA and pEAQ-*HT* constructs) and infiltration was carried out at a relative vector concentration ratio of 1:1:2:1 (VP2:VP3:VP5:VP7).

Figure 2.5 shows the expression results after each set of four recombinant constructs were co-infiltrated into *N. benthamiana* using a relative vector concentration of 1:1:2:1 (VP2, VP3, VP5:VP7), at an OD_{600} of 0.25 for each construct and an OD_{600} of 0.5 for the construct carrying VP5.

Co-expression of the VPs cloned into the pTRAc-HT plant expression vector (Figure 2.5 A), resulted in the detection of expected bands from 3 dpi onwards for VP3 (~102 kDa) and VP7

(~38 kDa), respectively. A very faint ~59 kDa band (red arrow) constituting VP5 was barely detected at 1 dpi, however detection of this protein was not very convincing as non-specific protein bands were also detected on this day and the band at ~59 kDa could possibly have been due to non-specific binding of the antibody. The expected band at ~111 kDa for VP2 was not detected over the course of the 7 day time-trial.

Co-infiltration with the pTRAkc-AH set of constructs (Figure 2.5 B) resulted in very low-level accumulation of VP5 at 1, 3 and 5 dpi (red arrow). VP7 was detected at ~38 kDa from 3 dpi, onwards. No VP2 or VP3 was detected with western blot analysis.

When *N. benthamiana* was co-infiltrated with the pTRAkc-rbcs1-cTP set of constructs (Figure 2.5 C), accumulation of VP7 (~38 kDa) was detected at 3 and 5 dpi, with VP3 (~102 kDa) and VP5 (~59 kDa) barely visible at 5 dpi (green arrow) and 1 dpi (red arrow), respectively.

Co-infiltration with the pEAQ-*HT* set of constructs resulted in the successful co-expression of all four VPs at 5 and 7 dpi (Figure 2.5 D). Expected band sizes of 111 kDa, 102 kDa, 59 kDa and 38 kDa corresponding to VP2, 3, 5 and 7 respectively, were visualized with the most protein detected at 7 dpi.

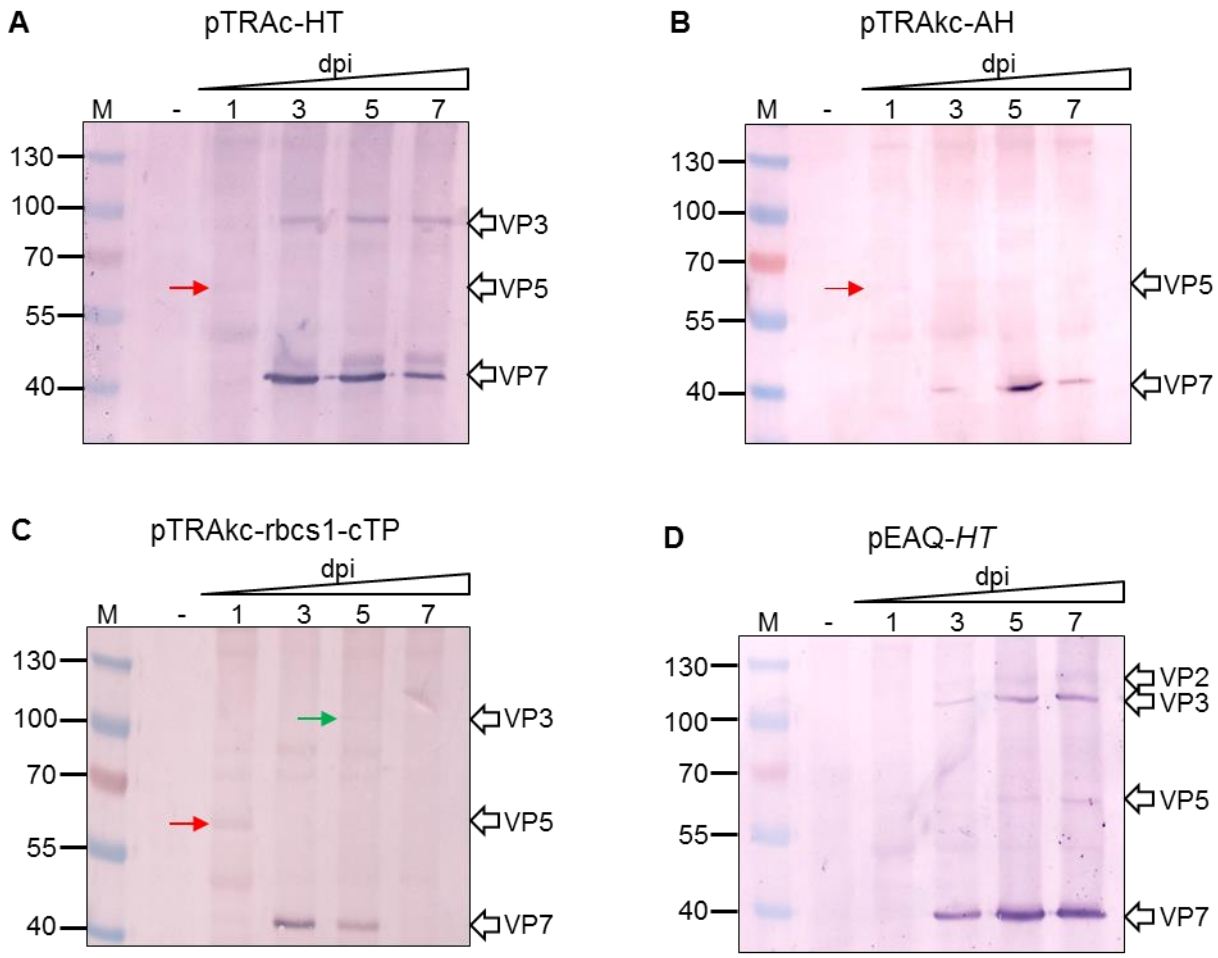

Figure 2.5: Co-expression of the BTV-8 VPs (VP2, VP3, VP5 and VP7) over 7 days using four different plant expression vectors: (A) pTRAc-HT, (B) pTRAkc-AH, (C) pTRAkc-rbcs1-cTP and (D) pEAQ-*HT*. Equal amounts of total protein were loaded in each lane. Each set of recombinant constructs was co-infiltrated at OD_{600} of 0.25 for VP2,VP3, VP7 and 0.5 for the VP5-carrying construct, with a relative vector concentration of 1:1:2:1 (VP2:VP3:VP5:VP7). VPs that were expressed are indicated by the white arrows. Red and green arrows indicate faint bands obtained for VP5 and VP3 expression, respectively (A-C). The negative controls are crude leaf extracts of plants infiltrated with infiltration medium. Lanes M indicate the molecular weight size marker.

Wroblewski *et al.* (2005) showed that an increase in the concentration of *A. tumefaciens* cells used for infiltration could increase transgene expression and subsequent levels of expressed protein. These findings were found to be applicable to heterologous expression in *N. benthamiana* as it was shown above (Figure 2.5) that expression of VP5 was achieved after doubling the *A. tumefaciens* cell concentration used for infiltration. Therefore, to determine whether an increase in cell density would affect co-expression of the BTV-8 VPs with the pTRA

and pEAQ-*HT* sets of constructs, infiltration of *N. benthamiana* using increased concentrations of all the recombinant *A. tumefaciens* constructs were tested.

Infiltration of *N. benthamiana* with the pTRAc-HT constructs at an OD_{600} of 0.5 for each construct carrying VP2, VP3 and VP7 and OD_{600} of 1.0 for VP5, resulted in the co-expression of VP2, VP3 and VP7 (Figure 2.6 A). Expression of VP7 (~38 kDa,) was detected throughout the time-trial, with VP3 (~102 kDa) detected from 3 dpi onward. VP2 (~111 kDa) was detected on the western blot at 3 and 5 dpi, after which expression ceased. With pTRAkc-AH (Figure 2.6 B) the increased cell concentration used for co-infiltration resulted in the high level accumulation of VP7 from 1 dpi onward, with VP2 and VP3 detected from 3 dpi until the end of the time-trial. VP5 was not expressed using either pTRAc-HT or pTRAkc-AH.

The increase in cell density used for co-infiltration of the pTRAkc-rbcs1-cTP constructs resulted in expression of all four VPs (Figure 2.6 C). Bands representing VP2, VP3 and VP7 was detected at ~111 kDa, 102 kDa and 38 kDa, respectively from 1 dpi onward, whereas VP5 (~59 kDa) was barely detected at 1 dpi (red arrow). Even though an increase in the *A. tumefaciens* cell concentration used for infiltration of the pTRA constructs enhanced expression of the VPs, all four proteins were rarely co-expressed on the same day. Western blot analysis of the pTRAkc-AH and pTRAkc-rbcs1-cTP constructs infiltrated at higher cell concentrations (Figure 2.6 B and C, grey arrows) showed the presence of an unknown protein at ~80 kDa that was co-expressed with the VPs.

On the other hand, co-expression of the VPs with the pEAQ-*HT* vector was enhanced with the increase in the cell concentration used for infiltration (Figure 2.6 D). The presence of all four VPs was shown by western blotting from 1 dpi onward, although bands representing VP2 and VP5 were very faint at 1 and 3 dpi (blue and red arrows, respectively). The accumulation of these proteins increased over the course of the time-trial with the most intense bands for all four proteins visible at 7 dpi.

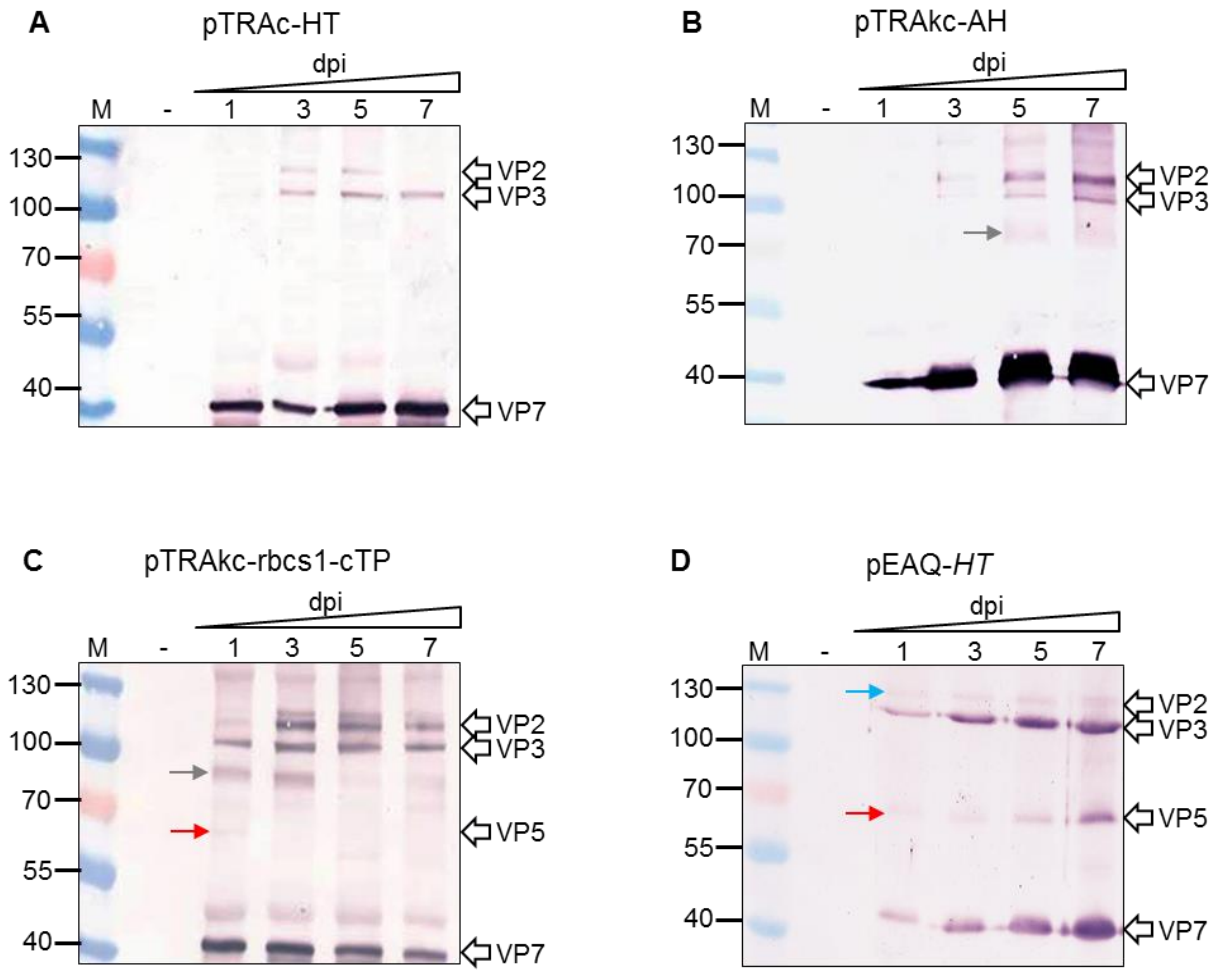

Figure 2.6: Co-expression of the BTV-8 VPs (VP2, VP3, VP5 and VP7) over 7 days using: (A) pTRAc-HT, (B) pTRAkc-AH, (C) pTRAkc-rbcs1-cTP and (D) pEAQ-*HT*. Each set of recombinant constructs was co-infiltrated at OD_{600} of 0.5 for each individual construct (OD_{600}=1.0 for VP5), with a relative vector concentration of 1:1:2:1 (VP2:VP3:VP5:VP7). Expressed proteins are indicated by the white arrows. Red and blue arrows indicate bands representing VP5 and VP2, respectively (C and D). Grey arrows (B and C) indicate an unknown protein at ~80 kDa. The negative controls are crude leaf extracts of plants infiltrated with infiltration medium. Lanes M represent the molecular weight size marker.

Increasing the cell density to OD_{600} of 1.0 for each construct did not enhance co-expression of the capsid proteins using the pTRA vectors, as a comparison of corresponding bands on the western blots were found to be similar to those found on blots developed using samples from expression using an OD_{600} of 0.5 for each construct (results not shown).

The increase in cell density to OD_{600} of 1.0 for infiltrating the pEAQ-*HT* constructs resulted in similar accumulation levels to those obtained when infiltrating at OD_{600} of 0.5 for each *A. tumefaciens* construct (results not shown).

Infiltrated leaves were examined over the course of 7 days for the visible effects caused by increasing the cell density used for infiltration (Figure 2.7). *N. benthamiana* infiltrated with infiltration medium served as a control for evaluating the effects caused by infiltration with the different recombinant constructs. Leaves infiltrated with the pTRA constructs at OD_{600} of 0.5 and 1.0 developed slight chlorosis from 3 dpi onward. The chlorotic symptoms did not worsen as the time-trial progressed. Plants infiltrated with the pTRAkc-AH and pTRAkc-rbcs1-cTP sets of constructs developed chlorosis at 3 dpi using an infiltration OD_{600} = 0.5, the leaves became more chlorotic over the last days of the time-trial, showing signs of necrosis at the injection sites (7 dpi). At 3 dpi chlorosis was observed in leaves infiltrated with the pTRAkc-AH and pTRAkc-rbcs1-cTP sets of constructs at OD_{600} = 1.0. The symptoms worsened progressively over the course of the time-trial, resulting in severely necrotic leaves at 7 dpi (Figure 2.7). Leaves infiltrated with the pEAQ-*HT* set of constructs at both OD_{600} = 0.5 and 1.0 were the healthiest with signs of chlorosis observed from 3 – 7 dpi, but no necrosis occurred.

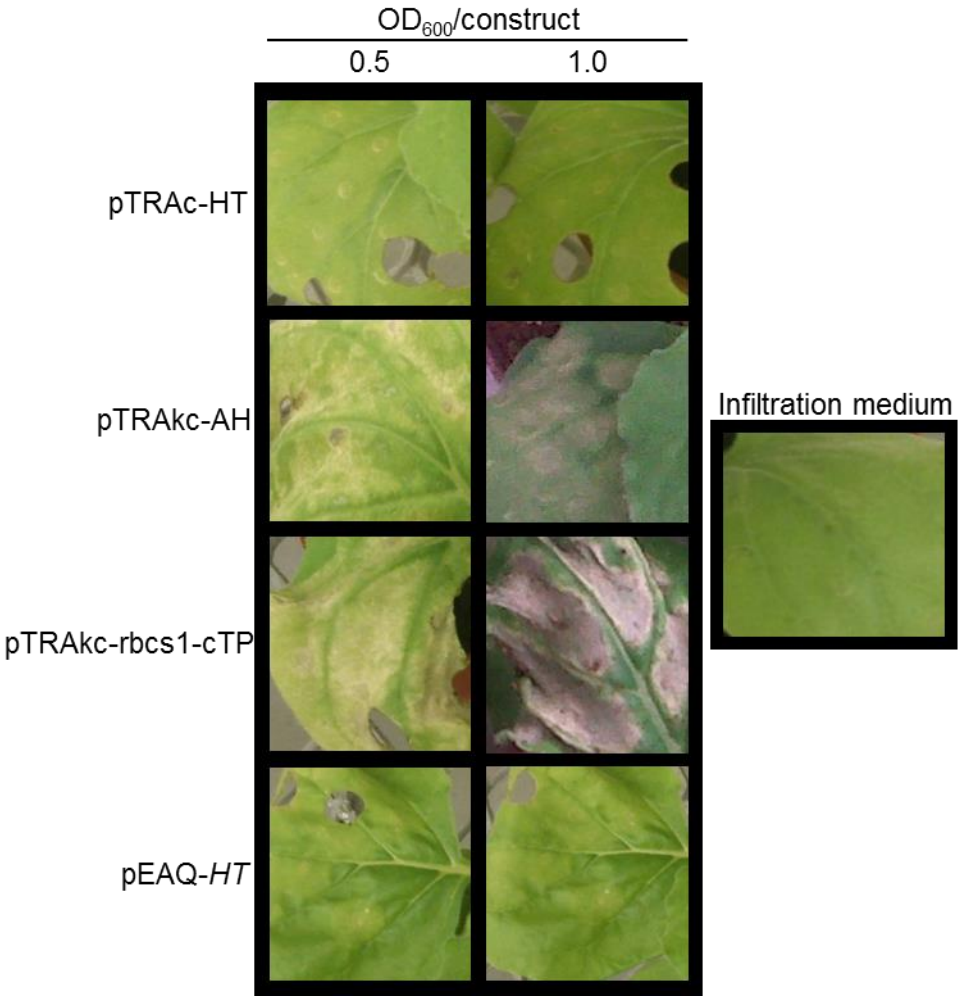

Figure 2.7: Photographs of infiltrated leaves prior to protein extraction at 7 dpi. Four plant expression vectors were used to co-express the BTV-8 VPs: pTRAc-HT, pTRAkc-AH, pTRAkc-rbcs1-cTP and pEAQ-*HT*. Each set of recombinant constructs was co-infiltrated at either OD_{600} of 0.5 or 1.0 for each individual construct, with a relative vector concentration of 1:1:2:1 (VP2:VP3:VP5:VP7). Plants infiltrated with infiltration medium served as an indicator of the effects caused by infiltrating with different cell concentrations with the expression vectors.

2.4. Discussion

In recent years, plants have become increasingly popular for the expression of recombinant proteins (Rybicki, 2009a; Rybicki, 2010). Transient expression by means of agro-infiltration is a fast and convenient method for evaluating expression of heterologous proteins in plants (Fischer *et al.*, 1999). While the successful production of BTV-8 VLPs in *N. benthamiana* has been reported elsewhere (Thuenemann *et al.*, 2013), this study – performed simultaneously – was aimed at comparing the ability of several different plant expression vectors to transiently co-express the four capsid proteins of BTV-8 (VP2, VP3, VP5 and VP7) in *N. benthamiana* leaves to determine whether expression levels could be increased by targeting the recombinant proteins to different subcellular compartments.

Expression of the BTV-8 VPs with the pTRA plant expression vectors using a relative vector concentration ratio of 1:1:2:1 at an infiltration OD_{600} of 0.25 resulted in poor co-expression of the four VPs. Only VP7 was expressed using an infiltration OD_{600} of 0.25 for all the pTRA expression vectors; little to no expression of VP2, VP3 and VP5 was achieved using these infiltration conditions (Figure 2.5 A, B and C).

Expression was optimised by increasing the *A. tumefaciens* cell concentration used for co-infiltrating, which resulted in the successful co-expression of three out of the four VPs. VP2, VP3 and VP7 were co-expressed using the pTRAc-HT (Figure 2.6 A) vector which retains recombinant protein in the cytosol of plant host cells, these three proteins were also expressed using pTRAkc-AH and pTRAkc-rbcs1-cTP (Figures 2.6 B and C) which target recombinant proteins to the apoplast and chloroplast, respectively. VP5 was only detected at 1 dpi using the pTRAkc-rbcs1-cTP expression vector (Figure 2.6 C, red arrow). It has been found that VP5 is cytotoxic and degrades easily when expressed without being fused to another protein (Hassan *et al.*, 2001). Furthermore, research has shown that VP5 interacts strongly with VP7 and assembly of VP5 precedes that of VP2 in the making of VLPs (Nason *et al.*, 2004). It is possible that VP5 was expressed during the early stages of the time-trials using all the pTRA vectors; however it was degraded due to the absence of a CLP scaffold for it to interact with to form VLPs. The presence of the 6x histidine tag on all the pTRAc-HT constructs may have further prevented proper folding of the VPs to form CLPs.

BTV core and sub-core particles assemble within viral inclusion bodies in the cytoplasm of infected cells (Boyce *et al.*, 2004; Mertens *et al.*, 2004); it is possible that assembly of recombinant CLPs and VLPs also takes place in the cytoplasm of the plant cell. Furthermore the cytoplasm of both animal and plant cells have a pH of 7.0 - 7.5, whereas the apoplast and chloroplast have more acidic pH values of 5.8 and 4, respectively. These lower pH values

could have resulted in failure of the BTV proteins to accumulate when expressed with the pTRAkc-AH and pTRAkc-rbcs1-cTP vectors, which target expressed proteins to the chloroplast and apoplast, respectively. Thus, while the pTRA vectors were shown to be very useful in producing HPV VLPs at high yield (Maclean *et al.*, 2007), they were not effective in the case of BTV VLP production.

Further increasing the cell density of the recombinant pTRA constructs to an OD_{600} of 1.0 did not have any effect on increasing the VP co-expression. In the case of pTRAkc-AH and pTRAkc-rbcs1-cTP it did, however, cause necrosis of the plant leaves which lower cell densities did not (Figure 2.7). The necrosis seemed to correlate with the expression of an unknown protein at ~80 kDa (Figure 2.6 B and C, grey arrows) that was detected on the western blots. It is possible that the higher concentration of *A. tumefaciens* cells may have played a detrimental part in normal plant regulatory functions (Wroblewski *et al.*, 2005), causing the plant cells to die off and the subsequent expression of proteins involved in the process.

In contrast to the pTRA vectors, all four capsid proteins of BTV-8 were successfully co-expressed using the pEAQ-*HT* vector (Figures 2.5 and 2.6 D). This difference in expression observed between the pTRA vector suite and the pEAQ-*HT* vector could be attributed to the presence of the P19 silencing suppressor which is integrated into the T-DNA of the pEAQ-*HT* vector. This ensures that both the gene of interest and the silencing suppressor are transferred to the same plant host cells, thereby ensuring concomitant post transcriptional gene silencing activity and expression of recombinant protein (Sainsbury *et al.*, 2009). In the case of the pTRA vectors, *A. tumefaciens* cells hosting the silencing suppressor plasmid (pBIN-*NSs*) were co-infiltrated with the plant expression vectors containing the genes of interest. This does not necessarily guarantee the successful delivery and transfer of silencing suppressor T-DNA to the same host plant cells as the *A. tumefaciens* constructs containing the recombinant plasmids with the genes of interest (Sainsbury *et al.*, 2009). Successful co-expression of the four BTV-8 VPs and the silencing suppressor from five separate *A. tumefaciens* constructs within the same plant cell, require five separate transfer events with the pTRA vectors – these factors taken together with the size of the larger VPs (VP2: ~111 kDa and VP3: ~102 kDa) could possibly constitute a substantial bottleneck in co-expression of the four VPs.

In conclusion, these results show that the pTRA vector suite only co-expressed some of the VPs, therefore these vectors are not suitable for the co-expression of the four BTV-8 VPs. It was concluded from this work that pEAQ-*HT* is the most effective of all the vectors tested for

co-expression of the four BTV-8 VPs and it was decided to conduct further optimisation and purification experiments with this set of constructs.

Chapter 3:

Transient expression, characterisation and purification of BTV-8 VLPs

3.1. Introduction

Virus-like particles (VLPs) resembling mature viral particles in size and shape are formed by the self-assembly of viral structural proteins. It is advantageous to use VLPs as vaccines as they have been shown to stimulate both humoral and cellular immune responses (Grgacic and Anderson, 2006). VLPs have the ability to stimulate a strong humoral immune response as B cells recognise the specific repetitive epitopes exposed on the surfaces of these particles (Jegerlehner *et al.*, 2002; Mason *et al.*, 1992; Whitehead *et al.*, 2014). Since VLPs are similar in size to native virions, they are able to be taken up by the dendritic cells which presents them to the major histocompatibility complex, resulting in the stimulation of a cellular immune response (Chackerian, 2007).

BTV has a complex structure where the four major structural proteins – VP2, VP3, VP5 and VP7 – are concentrically arranged around the segmented genome and other minor structural and non-structural proteins. In an effort towards making a safer vaccine for BTV, VLPs that lack the viral genome and that are identical to native BTV virions have been produced in insect cells using only the four outer capsid proteins of BTV (French *et al.*, 1990; Roy, 1992). However, high production costs and contamination with host-associated products or viruses are just some of the drawbacks associated with the production of VLPs in insect cells (Kushnir *et al.*, 2012).

Agrobacterium-mediated transient expression in plants provides a more cost-effective, convenient and fast alternative for the production of vaccine candidates, including VLPs (Chen and Lai, 2013; Rybicki, 2010; Scotti and Rybicki, 2013). Several reasons make plants a viable and appealing vehicle for the production of VLP vaccines; these include the cost-effectiveness and scalability of the production system as well as the possibility of producing rapid response plant-produced vaccines in response to pandemics. For the production of influenza VLPs, Medicago Inc. (Canada) and the Fraunhofer Institute (USA) proved that it was possible to proceed from the acquisition of nucleic acid sequence data through to the purification of grams of protein within a month using an *Agrobacterium*-mediated transient expression system in tobacco (D'Aoust *et al.*, 2010; D'Aoust *et al.*, 2008; Rybicki, 2009b; Shoji *et al.*, 2009; Shoji *et al.*, 2011).

The plant expression platform has the possibility of providing a fast and scalable method for the production of BTV VLPs. Recently, BTV-8 VLPs were successfully produced in *N. benthamiana* by using the pEAQexpress vector, where either VP2 and VP5 or VP3 and VP7 were incorporated on the same T-DNA. These VLPs were shown to protect sheep against lethal challenge with the virus (Thuenemann *et al.*, 2013).

In this study transient expression of BTV-8 VLPs using separate pEAQ-*HT* plasmids carrying the capsid genes to be expressed was further optimised and VLP assembly from the four separately expressed capsid proteins was analysed. *In situ* localisation of the particles was investigated using transmission electron microscopy. VLP purification using ultracentrifugation was optimised to achieve concentration of the VLPs, after which structural characterisation of the purified particles was carried out. Purified VLPs were used for subsequent downstream immunogenicity studies in mice (Chapter 5).

3.2. Materials and Methods

3.2.1. Optimisation of BTV-8 co-expression using pEAQ-*HT*

Since it was shown in Chapter 2 that the pEAQ-*HT* plant expression vector was the most successful at co-expressing the BTV-8 structural proteins, additional optimisation experiments using this set of constructs consisting of pEAQ-*HT*VP2co, pEAQ-*HT*VP3co, pEAQ-*HT*VP5co and pEAQ-*HT*VP7co were conducted.

A. tumefaciens LBA4404 harbouring the abovementioned pEAQ-*HT* recombinant plasmids was cultured and prepared for syringe-infiltration as described in section 2.2.5. Maintaining a cell concentration of OD_{600} of 0.5 per recombinant *A. tumefaciens* construct, the effects of different infiltration ratios of the four recombinant pEAQ-*HT* plasmids were tested. In addition to the infiltration ratio of 1:1:2:1 (VP2:VP3:VP5:VP7) used in Chapter 2, the relative vector concentration infiltration ratios of 2:1:1:1 and 1.5:1:1:6.5 were also tested. The 2:1:1:1 ratio was chosen at random and the ratio of 1.5:1:1:6.5 correlated to the ratio of the number of copies of each of the VPs on the BTV virion as determined by cryo-electron microscopy (Hassan and Roy, 1999; Roy, 1992; Roy, 2004).

Three leaf discs were harvested for each infiltration ratio from 6 dpi to 9 dpi and protein was extracted and prepared for western blot analysis as described in section 2.2.6.

3.2.2. Transmission electron microscopy (TEM) of crude plant extracts

TEM was carried out on crude plant extracts to determine at which ratio and dpi co-expression of the four BTV-8 VPs resulted in the formation of VLPs. Copper grids (mesh size 200) were made hydrophilic by glow discharging at 25 mA for 30 s using a Model 900 SmartSet Cold Stage Controller (Electron Microscopy Sciences). The grids were floated on a 1:200 dilution of BTV-8 sheep serum made against plant-produced VLPs (Thuenemann *et al.*, 2013) for 2 min and washed twice with sterile water. Thereafter the grids were floated on a 1:10 dilution of crude plant extract for 5 min and washed three times with sterile water. The samples were negatively stained for 1 min with 2% w/v uranyl acetate and viewed using a Technai G^2 transmission electron microscope (FEI). Particles were measured using the Ruler tool in Adobe® Photoshop® CS6.

3.2.3. Embedding of infiltrated leaf sections for *in situ* TEM

The BTV-8 pEAQ-*HT*VP2co, pEAQ-*HT*VP3co, pEAQ-*HT*VP5co and pEAQ-*HT*VP7co constructs were cultured and combined (as described above) in a ratio of 1:1:2:1 (VP2:VP3:VP5:VP7) and syringe-infiltrated into the abaxial surfaces of six-week-old *N. benthamiana* plants.

At 9 dpi a whole leaf was picked from the infiltrated plant and a 3 cm x 3 cm piece was cut out with a scalpel blade in the presence of 2.5% gluteraldehyde (25% gluteraldehyde diluted in 0.1 M phosphate buffer [20 mM sodium dihydrogen (ortho) phosphate (NaH_2PO_4) and 80 mM disodium hydrogen (ortho) phosphate (Na_2HPO_4), pH 7.4]). The leaf sample was soaked in 2.5% gluteraldehyde for 6 hrs after which it was cut into 1 mm x 3 mm fragments, also in the presence of 2.5% gluteraldehyde. The leaf fragments were soaked in 2.5% gluteraldehyde O/N at 4 °C. The following day the leaf fragments were washed 3 times, 5 minutes for each wash, in 0.1 M phosphate buffer (pH 7.4). The leaf fragments were fixed for 1 hr in one part 2% osmium tetroxide and one part 0.2 M phosphate buffer (40 mM NaH_2PO_4 and 160 mM Na_2HPO_4, pH 7.4), after which it was washed twice for 5 min each with 0.1 M phosphate buffer followed by two washes of 5 min each with sterile water.

After washing, the leaf fragments were sequentially dehydrated. The leaf fragments were incubated for 5 min each in 30%, 50%, 70%, 80%, 90% and 95% ethanol. The fragments were incubated for 10 min in 100% ethanol; this step was repeated twice. After the ethanol series the leaf fragments were further dehydrated by 10 min incubation in 100% acetone, repeated twice. The leaf fragments were mixed O/N in 1:1 acetone: Spurr's (Agar Scientific) resin.

The following day half of the 1:1 acetone: Spurr's resin mixture was removed and replaced with 100% Spurr's resin to yield a 1:3 acetone: Spurr's resin mixture. The samples were mixed for 4 hrs at room temperature, after which the acetone/resin mixture was removed and replaced with 100% Spurr's resin. The leaf fragments were incubated in 100% Spurr's resin for three days at 4 °C. The 100% Spurr's resin was replaced with fresh resin and incubated for 4 hrs at room temperature after which the resin was replaced again and incubated overnight at room temperature. The following day the specimens were embedded in 100% Spurr's resin and incubated for 24 hours at 60 °C.

The embedded leaf specimens were cut into ultrathin sections with a diamond knife and collected onto copper grids (mesh size 200). The copper grids were stained with uranyl acetate for 10 min after which they were washed five times, 15 seconds each, with water. The

grids were blotted dry and transferred to lead citrate for 10 min after which the grids were washed with water and blotted dry.

Grids were viewed and the particles measured as described above (Section 3.2.2).

3.2.4. Protein extraction and density gradient purification of BTV-8 VLPs

Thirty six-week-old *N. benthamiana* plants were syringe infiltrated using a ratio of 1:1:2:1 of the recombinant *A. tumefaciens* strains containing the pEAQ-*HT* plasmids expressing VPs 2, 3, 5 and 7. The plants were grown for 8-9 days at 22 °C under 16hrs/8hrs light/dark cycle.

Infiltrated leaves were harvested and immediately cut into fine pieces. The plant material was thoroughly homogenised with a Waring-type blender in three volumes of ice cold bicine buffer (50 mM bicine [pH8.4], 20 mM sodium chloride [NaCl], and 1 x Complete Mini, EDTA-free protease inhibitor cocktail [Roche]) lacking NLS and DTT. The homogenate was incubated at 4 °C for 30 min with gentle agitation after which it was filtered through four layers of Miracloth™ (Merck) and further clarified by centrifugation at 1 000 *x g* for 10 min at 4 °C.

The crude plant sap was overlaid onto 5 mL of a 30% iodixanol (Optiprep™, Sigma-Aldrich) cushion prepared in 50 mM Tris-HCl, pH 8.4 and 20 mM NaCl after which it was centrifuged for 2 hrs at 79 000 *x g* in a SW 32 Ti rotor (Beckman). The 30% iodixanol cushion was collected after centrifugation from the bottom of the tube and diluted to 15% with 50 mM Tris-HCl, pH 8.4 and 20 mM NaCl, after which it was overlaid onto a discontinous iodixanol gradient diluted in 50 mM Tris-HCl, pH 8.4 and 20 mM NaCl. The discontinous gradient was centrifuged as described above and fractions were collected from the bottom of the tubes and analysed by western blotting (as described in Chapter 2).

3.2.5. TEM of purified BTV-8 VLPs

Copper grids were glow discharged as described in section 3.2.2. The grids were floated on the fractionated samples from the density gradients for 5 min, after which they were washed five times with sterile water. The samples were negatively stained for 1 min with 2% w/v uranyl acetate and viewed as described in section 3.2.2.

3.2.6. Quantification of purified VLPs

Purified fractions were resolved on SDS-Page gels and treated with Coomassie-blue stain to confirm that no contaminating plant proteins were co-purified on the iodixanol gradients (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid). Thereafter, the total soluble protein (TSP) present in the iodixanol fractions containing VLPs was determined using the Bio-Rad DC Protein Assay according to the manufacturer's instructions. Bovine serum albumin (BSA, Sigma-Aldrich) was used as protein standard and the absorbance was read at 750 nm using a Bio-Tek Powerwave XS spectrophotometer.

3.3. Results

3.3.1. Co-expression of the BTV-8 capsid proteins with pEAQ-*HT*

Time-trials of the co-expression of the BTV-8 capsid proteins were extended to 9 dpi to determine if longer expression would lead to increased levels of co-expressed proteins. Co-infiltration of the recombinant *A. tumefaciens* harbouring the pEAQ-*HT* plasmids in a ratio of 1:1:2:1 (Figure 3.1 A) resulted in the co-expression of VP2 (~111 kDa), VP3 (~102 kDa), VP5 (~59 kDa) and VP7 (~38 kDa) from 6 dpi onward. The most intense bands for all four proteins were observed at 7 and 8 dpi, after which the recombinant protein levels appeared to be slightly less.

Co-infiltration of the recombinant pEAQ-*HT* constructs in a ratio of 2:1:1:1 (Figure 3.1 B) resulted in the presence of VP2, VP3 and VP7 on the western blot at 6 dpi with no VP5 visible. At 7 and 8 dpi, all four VPs constituting the VLPs were observed, with VP5 becoming less visible after 9 dpi. In comparison, infiltration at a cell density ratio of 1.5:1:1:6.5 (Figure 3.1 C) resulted in co-expression of all four capsid proteins at 6 dpi, with VP5 at very low levels (red arrow). From 7 to 9 dpi only VP2, VP3 and VP7 could be detected on the western blot.

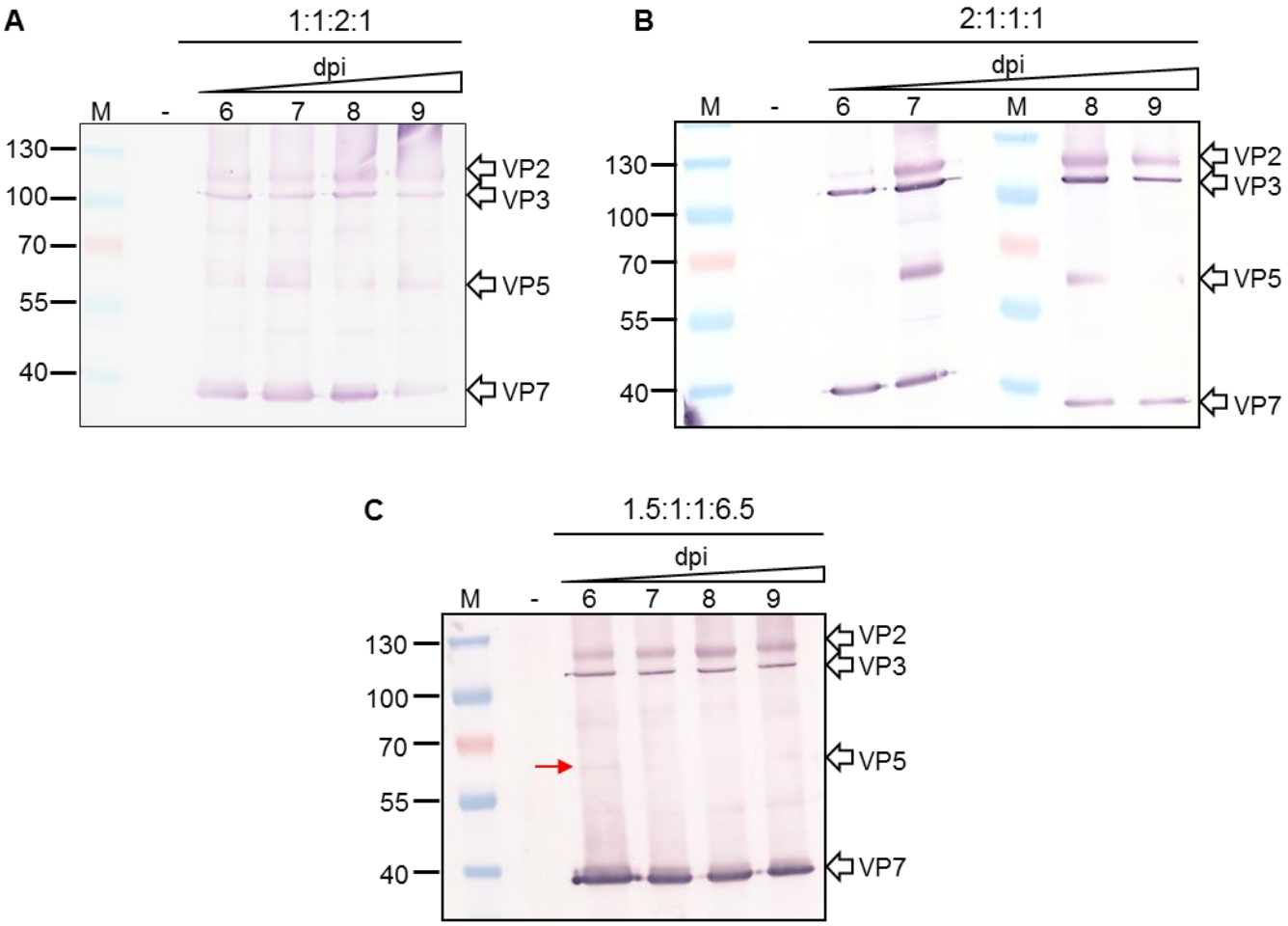

Figure 3.1: Western blot analysis of the BTV-8 capsid protein co-expression with different infiltration ratios of recombinant pEAQ-*HT* plasmids. (A) Infiltration using a relative vector concentration ratio of 1:1:2:1 (VP2:VP3:VP5:VP7), (B) 2:1:1:1 and (C) 1.5:1:1:6.5, the red arrow indicates expression of VP5 at 6 dpi. The negative control was crude leaf extract of a plant infiltrated with infiltration medium and lanes M represent the molecular weight marker.

3.3.2. TEM of the crude plant extracts infiltrated with different ratios of recombinant *A. tumefaciens* constructs

TEM was carried out on the crude leaf extracts of the 6 – 9 dpi pEAQ-*HT* samples that were infiltrated using the relative vector concentration ratios of 1:1:2:1 (VP2:VP3:VP5:VP7), 2:1:1:1 and 1.5:1:1:6.5 to assess whether co-infiltration resulted in the assembly of intact CLPs or VLPs. It was expected that the particles could be distinguished from each other by a difference

in size with CLPs ranging in diameter from 60 - 69 nm and VLPs having diameters ranging in size from 72 – 80 nm.

A mixed population of both putative CLPs and VLPs was confirmed from 6 – 9 dpi using an infiltration ratio of 1:1:2:1 (Figure 3.2 A[i] and B[i]). Sub-core (VP3 only) particles and CLPs were observed in crude *N. benthamiana* extracts infiltrated with all three ratios (results not shown for 7 and 8 dpi), however there were no putative VLPs visible in the leaves which were co-infiltrated using the ratios of both 2:1:1:1 (Figure 3.2 A[ii] and B[ii]) and 1.5:1:1:6.5 (Figure 3.2 A[iii] and B[iii]) throughout the time-trial. With the 2:1:1:1 and 1.5:1:1:6.5 infiltration ratios the amount of particles that were visible decreased after 6 dpi, with mostly protein aggregates visible at 9 dpi (results not shown for 7 and 8 dpi). No CLP or VLP-like structures were observed in the negative control sample that was infiltrated with infiltration medium only (Figure 3.2 C).

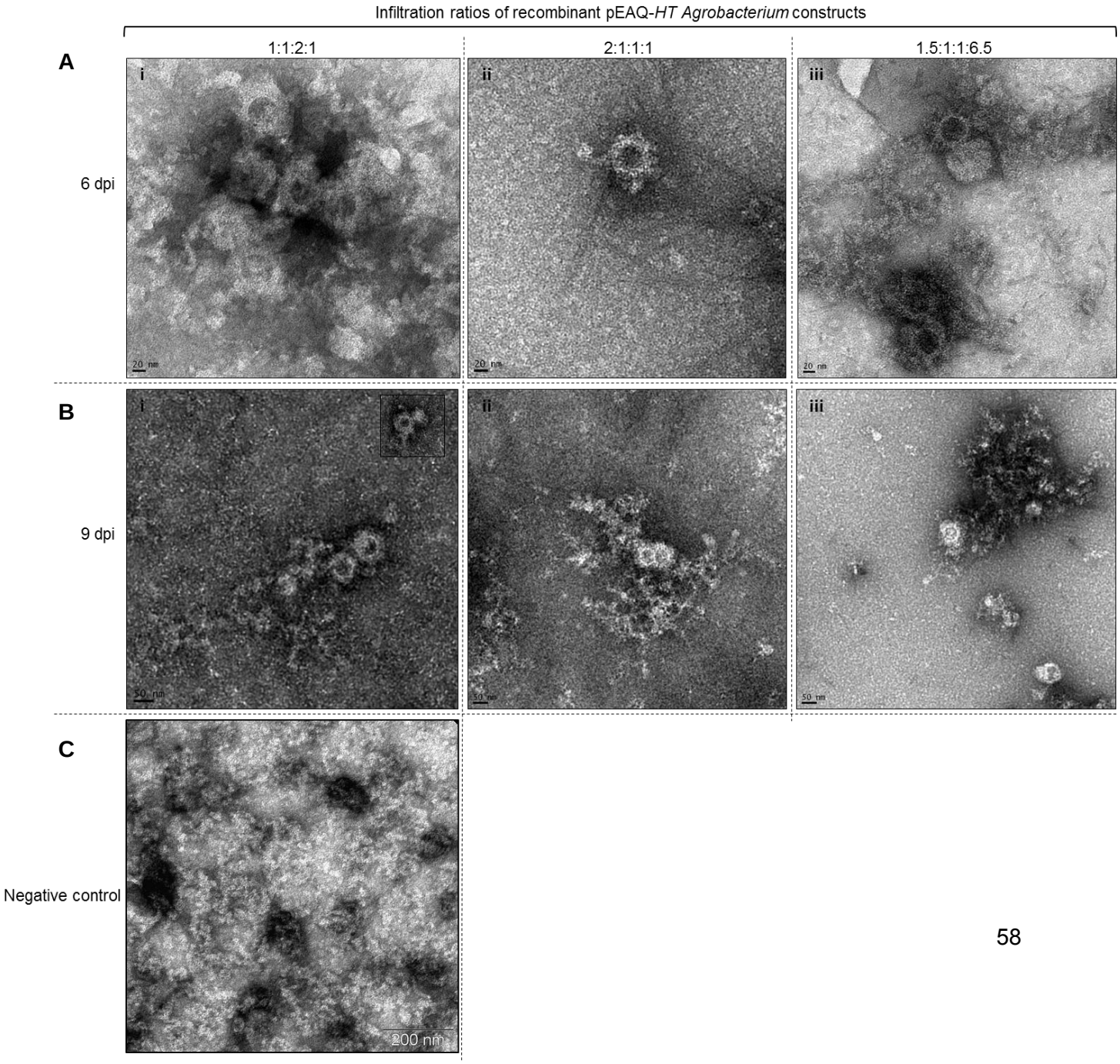

Figure 3.2: *From the previous page*. Transmission electron micrographs of leaves infiltrated with the three different ratios - (i) 1:1:2:1, (ii) 2:1:1:1, (iii) 1.5:1:1:6.5 - tested of *A. tumefaciens* harbouring the recombinant pEAQ-*HT* plasmids at 6 dpi (A) and 9 dpi (B). (C) A negative control crude extract of a plant infiltrated with infiltration medium only was also viewed. The crude extracts were captured onto copper grids with sheep serum and viewed using a Technai G^2 transmission electron microscope (FEI). Scale bars: (A) 20 nm and (B) 50 nm (C) 200 nm.

3.3.3. *In situ* TEM of BTV-8 capsid proteins co-expressed in *N. benthamiana*

Leaves infiltrated with recombinant *A. tumefaciens* constructs using a relative vector concentration ratio of 1:1:2:1 (VP2:VP3:VP5:VP7) were harvested at 9 dpi, embedded in Spurr's resin and subsequently viewed using TEM.

Western blot analysis of the leaves harvested for *in situ* TEM showed that all four capsid proteins were successfully expressed at 9 dpi (results not shown). Aggregates of spherical particles arranged in linear arrays were observed in the cytoplasm of the plant cells expressing VLPs (Figure 3.3). The particles ranged from 61 – 83 nm in diameter, indicating the existence of a mixed population of particles consisting of both CLPs and VLPs. Figure 3.3 (A[i] and B[i]) shows two fields of view at a magnification of 5700 x and 8000 x, respectively. Figure 3.3 (A[ii] and B[ii]) show the particles in more detail (17 000 x magnification). TEM showed that CLPs and VLPs were not expressed in every plant cell (results not shown), but when a cell did produce the particles they were present in large numbers. Negative control samples infiltrated with infiltration media (Figure 3.3 C) were also evaluated using TEM. No similar structures were observed in these control samples.

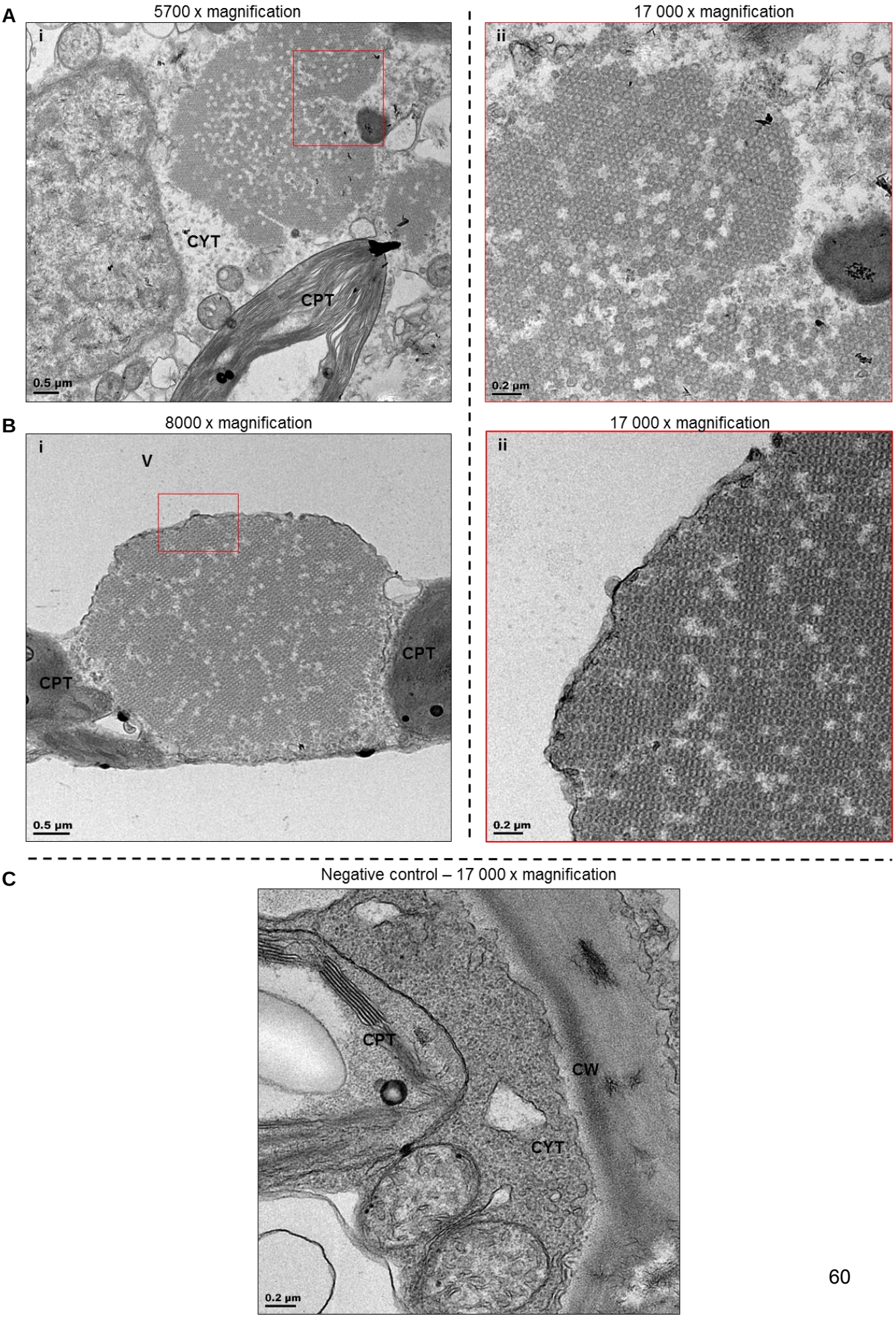
A
5700 x magnification
i
CYT
CPT
0.5 µm
17 000 x magnification
ii
0.2 µm
B
8000 x magnification
i
V
CPT
CPT
0.5 µm
17 000 x magnification
ii
0.2 µm
C
Negative control – 17 000 x magnification
CPT
CW
CYT
0.2 µm

Figure 3.3: *From the previous page*. (A) and (B): Transmission electron micrographs of leaf sections that were infiltrated with recombinant pEAQ-*HT* plasmids using an infiltration ratio of 1:1:2:1 (VP2:VP3:VP5:VP7) and harvested at 9 dpi. A(i) and B(i) represent two fields of view at 5700 x and 8000 x magnification, respectively. A(ii) and B(ii) show the particles in more detail at 17 000 x magnification. Particles were present in paracrystalline arrays. Scale bars left panel: 0.5 µm, right panel: 0.2 µm (C) Negative control leaf infiltrated with infiltration medium at 17 000 x magnification (Scale bars: 0.2 µm). CW: cell wall, CPT: chloroplast, CYT: cytoplasm, V: vacuole.

3.3.4. Density gradient purification of putative BTV-8 VLPs

Since VLPs were observed in both the crude extracts and embedded sections of leaves infiltrated with pEAQ-*HT* constructs in the ratio of 1:1:2:1 (VP2:VP3:VP5:VP7), the number of plants infiltrated was scaled up to produce larger amounts of VLPs for purification with density gradient centrifugation.

3.3.4.1. Initial VLP purification

Infiltrated leaf tissue was harvested at 6 and 9 dpi after which VLPs were immediately extracted by grinding leaves in liquid nitrogen and resuspending the leaf material in bicine buffer containing NLS and DTT. The crude extracts were overlaid onto discontinuous iodixanol gradients consisting of 10 – 60% iodixanol (in 10% increments), followed by ultracentrifugation.

Western blot analysis of undiluted iodixanol fractions collected from the bottom of the tubes showed smears in lane 4 (Figure 3.4 A) and lanes 4 and 5 (Figure 3.4 B) – representing 30 – 40% iodixanol - for VLPs purified at 6 and 9 dpi, respectively. At 6 dpi (Figure 3.4 A) only VP7 (orange arrow) was detected in the pellet (P), with no protein present in fractions 1 – 3, representing 50 – 60% iodixanol. Three distinct bands representing either VP2 or VP3 (purple arrow), VP5 (red arrow) and VP7 (orange arrow) was detected in fraction 4. Analysis of fractions 1 – 3 at 9 dpi (Figure 3.4 B) showed no bands representing the VPs, however fraction 3 showed a band at ~50 kDa (black arrow), which could possibly constitute degradation products. No protein was detected in the pellet (P) collected after gradient centrifugation at 9 dpi.

Two-fold serial dilutions using bicine buffer were made of fraction 4 and fractions 4 and 5, collected at 6 and 9 dpi respectively and analysed on western blots. Western blot analysis (Figure 3.4 C) of the diluted 30 – 40% iodixanol fractions showed the presence of VP2, VP3, VP5 and VP7 (blue, green, red and orange arrows respectively) in the ½ dilution of fraction 4 collected at 6 dpi, with no VP5 detected at the ¼ and $^{1}/_{8}$ dilutions. Serial dilutions of both fractions 4 and 5 collected at 9 dpi, show the presence of VP3, VP5 and VP7 (green, red and orange arrows respectively), but no VP2 was detected. Dilutions of fraction 5 (9 dpi) show the presence of additional bands on the western blot. These bands possibly constitute degradation products of the VPs, indicating their instability during the purification process.

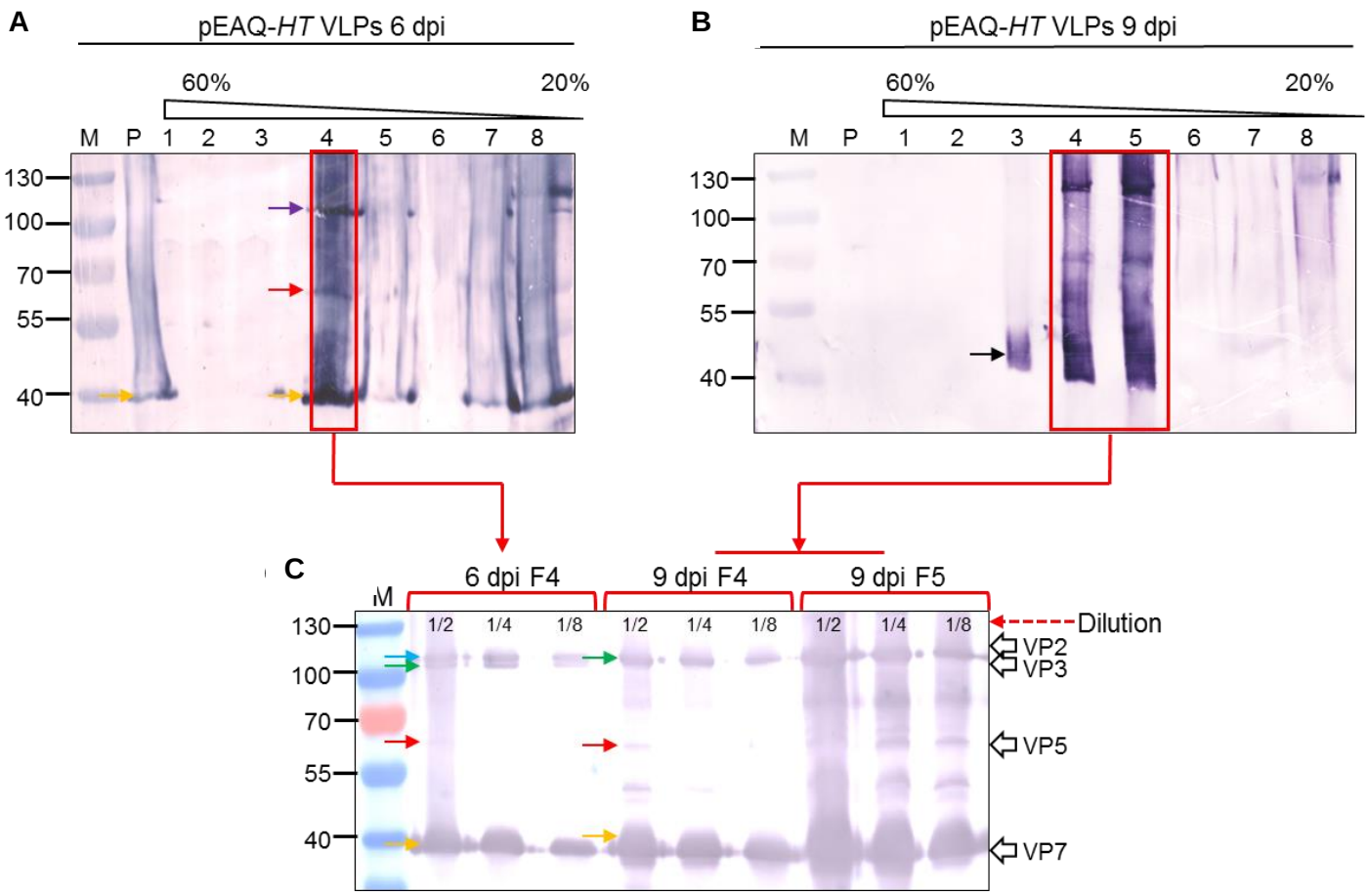

Figure 3.4: Western blot analysis of fractions collected after density gradient purification of putative BTV-8 VLPs. Iodixanol fractions ranging from 20 – 60% of VLPs purified at 6 and 9 dpi are shown in A and B, respectively. Lanes P represent the pellets collected after gradient centrifugation and numbers 1 – 8 indicate the fraction numbers collected from the bottom of the tubes. (C) Shows a dilution series of fraction 4 from 6 dpi and fractions 4 and 5 from 9 dpi. Lanes M represent the molecular weight marker. VP2: blue arrow, VP3: green arrow, VP5: red arrow and VP7: orange arrow. Band representing VP2 and/or VP3 (A): purple arrow.

Fraction 4 collected at 6 dpi and fractions 4 and 5 collected at 9 dpi were fixed to copper grids and visualised using TEM.

Transmission electron micrographs of fraction 4 (6 dpi) showed particles ranging in size from 51 nm to 70 nm (Figure 3.5 A[ii]). The smaller particles (~51 nm, black arrows) represent putative sub-core particles which consist of only VP3, these particles had a smooth surface and appeared to be either hexagonal, angular or round, depending on their orientation on the grid. Larger particles representing putative CLPs (red arrows) closely resembled CLPs

produced in insect cells, the particles had a spiky appearance due to the layer of VP7 trimers arranged around the sub-core particles.

TEM analysis of fractions 4 and 5 collected at 9 dpi (Figure 3.5 B[ii] and [iii]) showed similar results to those found at 6 dpi, with mostly putative sub-core particles and CLPs present in the iodixanol fractions. Control samples Figure 3.5 A[i] and B[i]) collected from the top of the tubes after gradient ultracentrifugation were also analysed with TEM; no structures resembling sub-core particles or CLPs were observed on these grids.

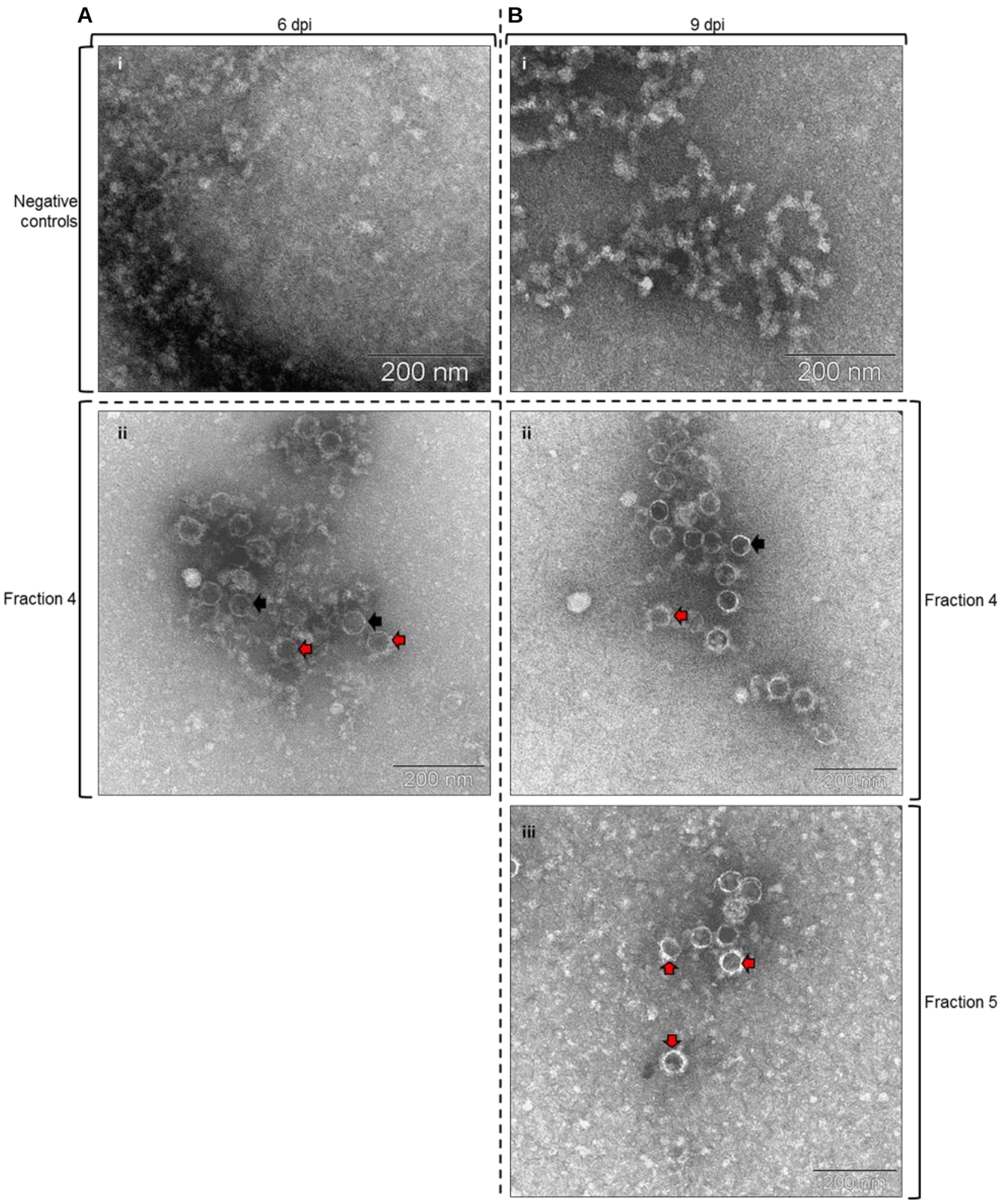

Figure 3.5: TEM analysis of iodixanol fractions 4 (A[ii]) collected at 6 dpi and fractions 4 and 5 (B[ii] and [iii]) collected at 9 dpi. A (i) and B (i) are the negative controls collected from the top of the tube after ultracentrifugation. Fractions from 6 and 9 dpi consisted of particles from 51 – 70 nm in size, representing putative sub-core particles (black arrows) and CLPs (red arrows). Scale bars: 200 nm.

3.3.4.2. Optimisation of VLP purification

Since the purification process appeared to be detrimental to the stability of the particles as indicated by western blot and TEM analysis of the iodixanol purified fractions (Figures 3.4 and 3.5), several small modifications were made to the extraction method. Plant tissue was not ground up in liquid nitrogen, but thoroughly homogenised instead in ice cold bicine buffer lacking DTT and NLS. DTT is a redox reagent that reduces disulphide bridges of proteins and prevents intra-molecular disulphide bonds from forming between cysteine residues, while NLS is a detergent that denatures proteins by reducing solvent-inaccessible disulphide bonds.

Proteins were co-expressed for 9 days - since VLPs were detected in both the crude extract (Figure 3.2 B[i]) and *in situ* (Figure 3.3 A and B) - after which recombinant protein was immediately extracted from the leaf material. The clarified crude plant extract was overlaid onto a 30% iodixanol cushion and centrifuged as described in section 3.2.4. Ultracentrifugation through the iodixanol cushion further clarified the crude plant extract by removing particulate matter and contaminating plant proteins that were co-extracted with the VLPs. The 30% iodixanol cushion containing the VLPs could be stored O/N at 4 °C without adverse effects on the particles.

The 30% iodixanol cushion was diluted to 15% in Tris buffer (pH 8.4) and overlaid onto either a discontinous gradient containing 20 – 60% iodixanol in 10% increments (1 mL of each step, total volume of 10 mL) or a continuous 20 – 60% gradient (total volume of 24 mL). After centrifugation 1 mL fractions were collected from the bottom of the tubes containing both the discontinous gradient and the continuous gradient.

Western blot analysis of the fractions collected from the discontinous iodixanol gradient (Figure 3.6 A) showed that no protein was detected in fractions 1 and 2, which represent ~50% and ~60% iodixanol respectively. Bands corresponding to the expected sizes of VP3 and VP7 were detected in fraction 3 and all four VPs were detected from fraction 4 onward. Fractions 4 - 6 corresponds to approximately 30 - 40% iodixanol, where the VLPs were expected to be found.

Transmission electron micrographs of fraction 4 (Figure 3.6 B [i] and [ii]) show that intact VLPs (green arrows) ranging from 73 – 80 nm in size were purified on the discontinous gradient. The fuzzy appearance of the outer layer of the particles is due to the assembly of VP2 and VP5 with the CLPs. Less structured uniform particles (blue arrows) possibly representing assembly intermediates between CLPs and VLPs were co-purified with the putative VLPs.

The plant produced VLPs were similar to VLPs (Figure 3.6 B[iii]) produced using the baculovirus expression system (Roy, 2004).

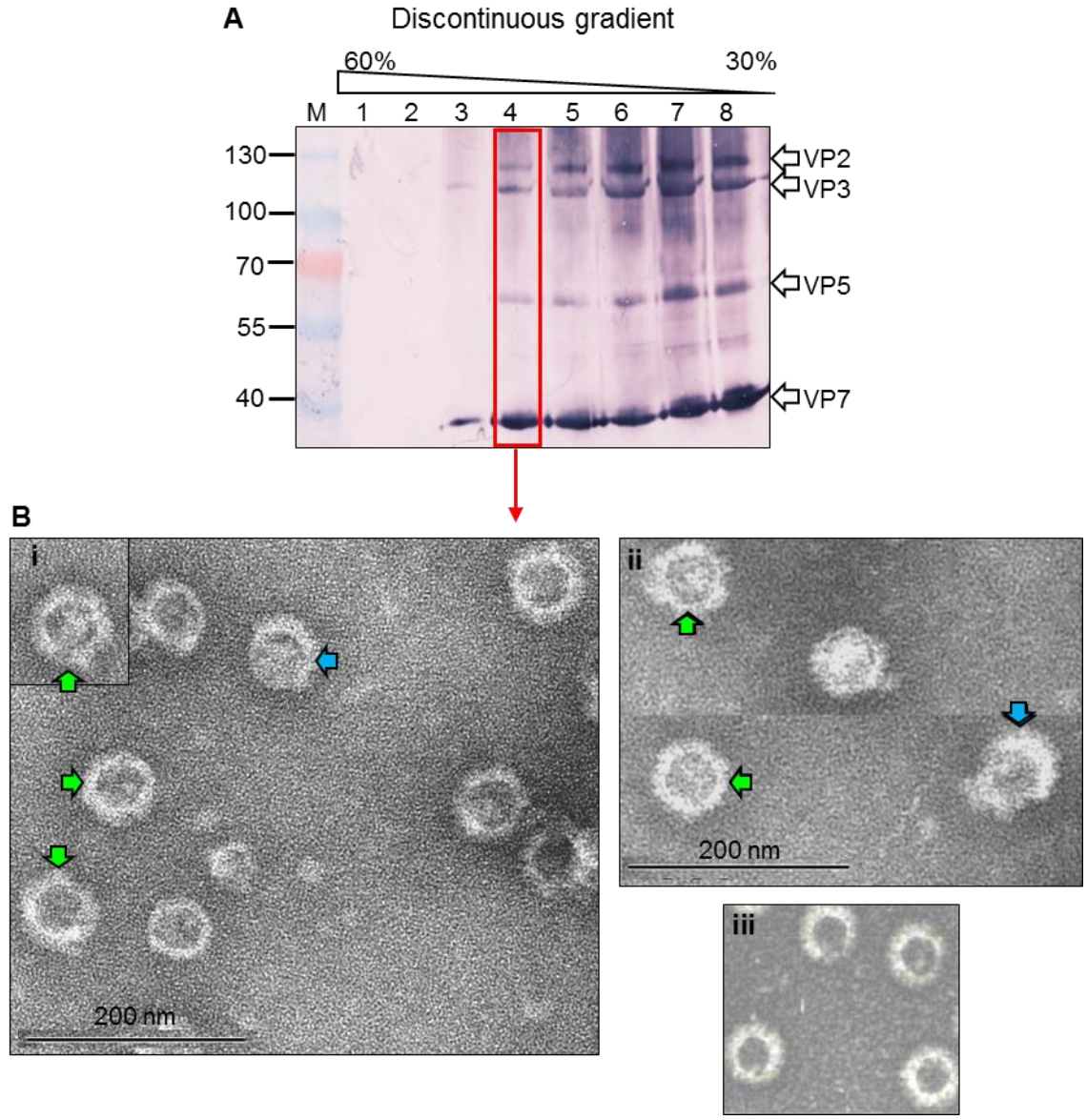

Figure 3.6: (A) Western blot analysis of fractions collected from a discontinuous iodixanol gradient. Lane numbers indicate the fractions collected from the bottom of the tubes. Bands the size of VP2, VP3, VP5 and VP7 were seen from fraction 4 onward. Lane M indicates the molecular weight marker. B (i) and (ii) TEM of fraction 4 show both intermediate structures (blue arrows) and VLPs (green arrows). Scale bars are 200 nm. B (iii) TEM of baculovirus-expressed VLPs (Roy, 2004).

Western blot analysis of the fractions collected of the continuous iodixanol gradient showed that no protein was present in the first 11 fractions (fractions 1-10: results not shown). In Figure 3.7 A bands representing VP7 (orange arrow) were observed in fractions 13 – 14, with

VP3 (green arrow) also present in fractions 15 and 16. VP3, VP5 and VP7 was detected in fraction 17 and all four VPs were detected from fraction 18 onward. Even though all four VPs were detected on the western blot, TEM analysis of fraction 19 showed that only CLPs ranging from 60 – 69 nm in diameter were purified; no VLPs were observed (Figure 3.7 B). The results indicate that even though VP2 and VP5 were co-purified with the CLPs, they were not assembled into VLPs, possibly due to instability of the multi-layered particles when using this purification method.

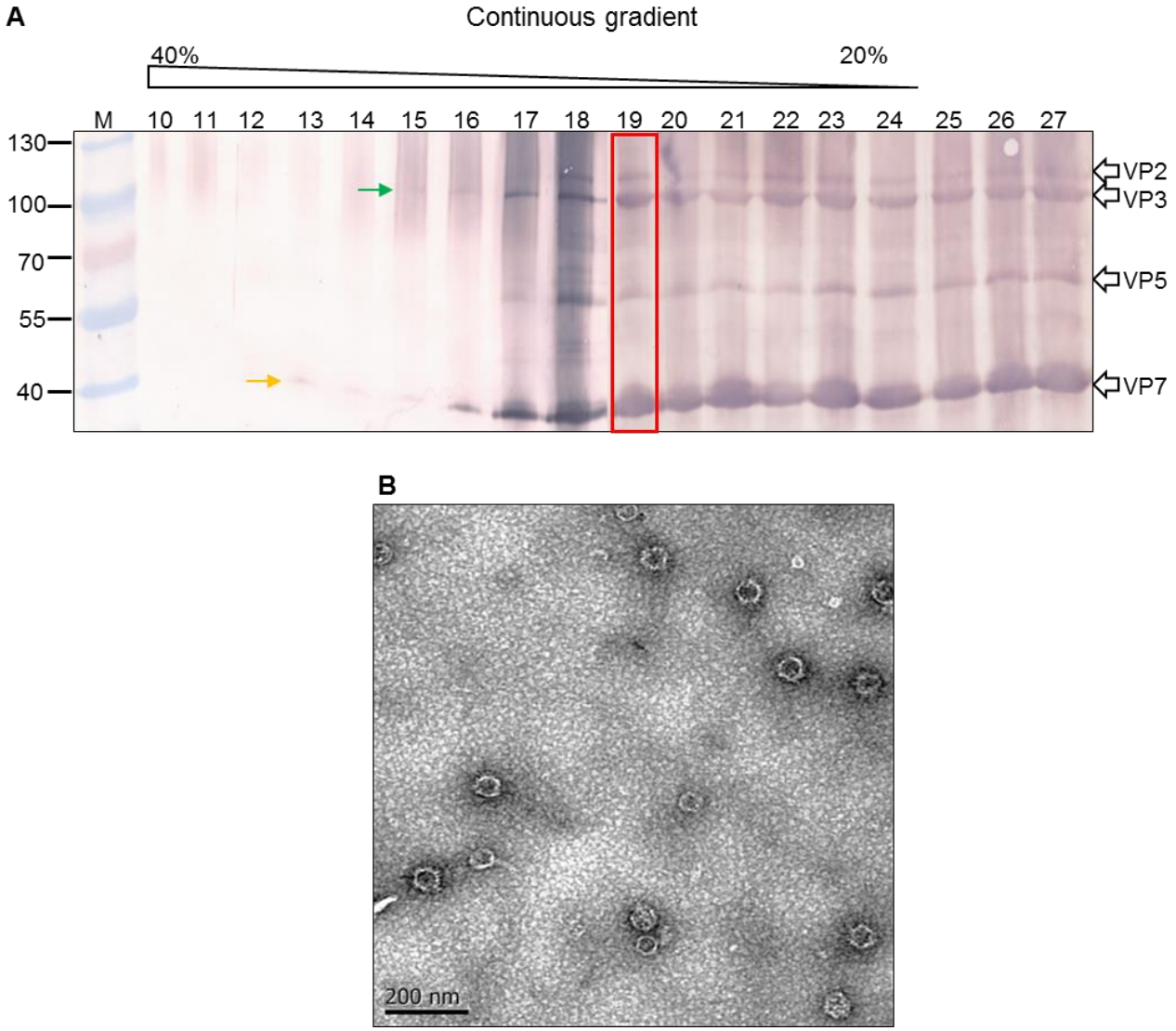

Figure 3.7: (A) Western blot analysis (two blots joined) of particles purified using a continuous iodixanol gradient. Lanes 10 – 27 show the fractions collected from the bottom of the tubes. Bands representing the four VPs can be seen from lane 17 onward. Lane M represents the molecular weight marker. VP3: green arrow and VP7: orange arrow. (B) TEM of fraction 19 shows that CLPs were purified on the continuous gradient. Scale bar: 200 nm.

Particles produced for animal experiments (Chapter 5) were extracted as above and subsequently purified on an iodixanol cushion followed by ultracentrifugation through a discontinuous iodixanol gradient. The discontinuous gradient was slightly modified to consist of 1 mL 60%, 2 mL 50%, 2 mL 40 %, 3 mL 30% and 2 mL 20% iodixanol. The modifications were carried out to prevent overloading of the gradient with the sample and to allow for selection of VLPs in the ~30% iodixanol fraction. The best fractions from the first round of centrifugation were pooled and applied to a second discontinous gradient consisting of half the volumes of appropriately diluted iodixanol and centrifuged as described above. Three fractions of 500 µL each that contained VLPs were pooled (results not shown) and quantified.

3.3.5. Total soluble protein (TSP) quantification of VLPs

Coomassie-stained SDS-Page gels (results not shown) showed that now contaminating plant proteins were co-purified with the VLPs on the gradient, however bands representing the four VPs could not be visualised on the gel due to the low levels of purified protein. Therefore, the TSP of the pooled iodixanol fractions containing VLPs was determined using the Bio-Rad DC Protein Assay kit. A standard curve was plotted using the average absorbance values obtained for the BSA standards (Figure 3.8). Using the equation on the chart, the TSP was calculated at ~1.75 mg total protein in a volume of 1.5 mL. The final yield of VLPs was determined to be ~27 mg TSP per kilogram of fresh leaf material.

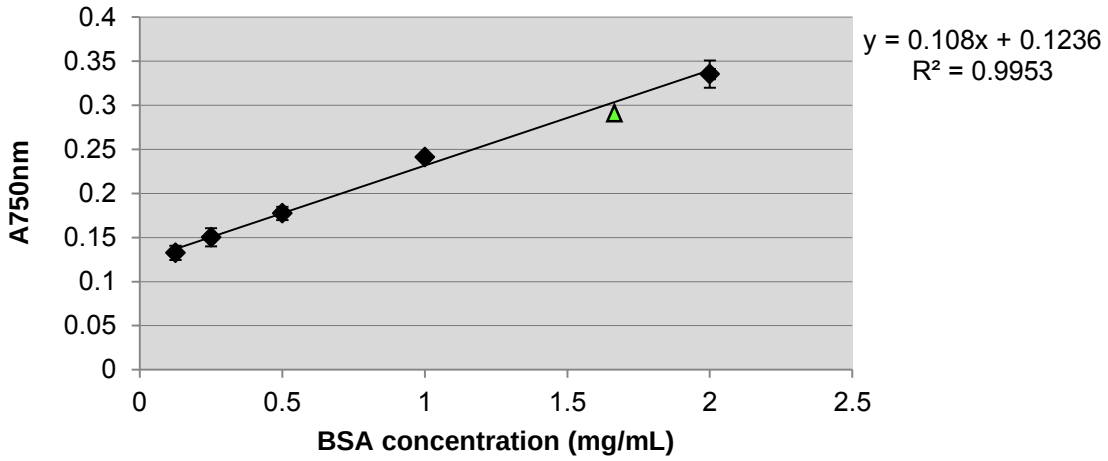

Figure 3.8: TSP quantification of the pooled iodixanol fractions containing VLPs using BSA as a standard. The green triangle indicates the average absorbance obtained for the VLPs. The error bars indicate the standard deviation.

3.4. Discussion

VLPs are excellent vaccine candidates as they combine the advantages of using whole-virus vaccines and subunit vaccines. These advantages include: a well-defined structure and uniformity with repetitive structures, the preservation of antigenic conformation, safety for use as they are non-infectious and non-replicating and the fact that they can be DIVA compliant when using diagnostic tests that are based on the detection of non-structural BTV proteins or antibodies (Barros *et al.*, 2009; Crisci *et al.*, 2012). BTV is a structurally complex virus with a capsid made up of concentric layers of the four capsid proteins VP2, VP3, VP5 and VP7. Production of VLPs that contain multiple capsid proteins that interact with each other is technically challenging as all the capsid proteins must be co-expressed by the same cell in order for the VLP to be assembled (Noad and Roy, 2003).

In this study, transient co-expression of the four BTV-8 capsid proteins in *N. benthamiana* was further optimised by varying the infiltration ratios of the recombinant *A. tumefaciens* constructs carrying the pEAQ-*HT* plasmids. The infiltration ratios were varied in order to assess the effect on protein accumulation and the ability of the co-expressed proteins to assemble into VLPs. Expression using the 1:1:2:1 infiltration ratio was continued as it was shown to result in the successful co-expression of VP2, VP3, VP5 and VP7 (Chapter 2).

Co-expression time-trials were extended to 9 days to ascertain if this would lead to increased levels of accumulation and samples were collected from 6 dpi onward. Western blot analysis (Figure 3.1) of the expression time-trials using different ratios showed that all four capsid proteins were successfully expressed using the three infiltration ratios. All four proteins were expressed from 6 – 9 dpi after infiltration using the 1:1:2:1 ratio (Figure 3.1 A). Infiltration using the 2:1:1:1 (Figure 3.1 B) and 1.5:1:1:6.5 (Figure 3.1 C) ratios resulted in the co-expression of the four capsid proteins at 7 – 8 dpi and 6 dpi, respectively.

TEM analysis of the crude plant extracts from the time-trials showed that plants infiltrated with VP2:VP3:VP5:VP7 in a ratio of 1:1:2:1 resulted in the successful expression of VLPs (76 – 82 nm) from 6 – 9 dpi (Figure 3.2 A[i] and B[i]). TEM analysis further showed that co-infiltration with the four separate *A. tumefaciens* constructs resulted in the “spontaneous” assembly of separately expressed VPs into CLPs and VLPs.

In contrast, TEM analysis of expression using the 2:1:1:1 and 1.5:1:1:6.5 ratios showed that sub-core like particles and CLPs were expressed at 6 dpi (Figure 3.2 A[ii-iii]) in the plants, however no VLPs were detected in the crude extracts from 6 – 9 dpi. At 9 dpi (Figure 3.2 B[ii-iii]) mostly protein aggregates and sub-core like particles were observed using TEM, indicating degradation of the particles over extended periods of expression. The microscopy results

correlated with the expression profiles seen on western blots, where only VP2, VP3 and VP7 were detected after 9 dpi (Figure 3.1 A and B). The EM results indicated the importance of VP5 in the assembly of VLPs; it appeared that VP5 was not as readily expressed as the other VPs. Therefore, increasing the infiltration volume of the recombinant *A. tumefaciens* harbouring VP5, increased the likelihood of VP5 being successfully co-expressed with the other VPs.

Based on these results further expression studies were conducted by co-infiltrating plants with a 1:1:2:1 ratio of the recombinant *A. tumefaciens* constructs.

Leaves co-expressing the capsid proteins were harvested at 9 dpi and embedded in Spurr's resin to ascertain where in the plant cell the particles were assembled and also to determine the size of the particles *in situ*. Electron micrographs of leaf sections co-expressing the four capsid proteins showed the presence of mixed populations of both CLPs and VLPs (measuring 61 – 83 nm in size) present in paracrystalline arrays within the cytoplasm of plant cells (Figure 3.3 A and B), indicating that particles were assembled by interactions of the expressed VPs. Similarly, work done by French and Roy (1990) showed that CLPs also aggregated as paracrystalline arrays within the cytoplasm of insect cells co-expressing VP3 and VP7. BTV virions have been shown to assemble within the cytoplasm of infected host cells, where after virions are released from the cells in a process mediated by the non-structural protein, NS3 (Boyce *et al.*, 2004; Mertens *et al.*, 2004). The *in situ* results from this study and work done by French and Roy (1990) suggest that recombinantly-produced CLPs and VLPs assemble within the cytoplasm, similar to native BTV virions. The absence of NS3 from recombinantly-produced particles possibly keep them localised within the cytoplasm, resulting in the formation of paracrystalline arrays that consist of a multitude of CLPs and/or VLPs. TEM analysis showed that not every plant cell expressed CLPs and/or VLPs. These results were expected because for VLPs to be produced in a host cell, four transfer events – from each one of the four recombinant *A. tumefaciens* constructs – into the cell is required for co-expression of the four capsid proteins and subsequent VLP formation.

In order to determine when VLPs could be effectively purified from crude plant extract, leaves co-expressing the four VPs were harvested at 6 and 9 dpi, and protein was extracted using bicine buffer containing NLS and DTT (as in Chapter 2). After purifying these crude extracts on discontinuous iodixanol gradients, western blot analyses (Figure 3.4) and TEM (Figure 3.5) of the purified fractions showed that only sub-core like particles and CLPs were purified using this extraction buffer at both days. For subsequent experiments, proteins were co-expressed

for 9 days as this expression time frame was shown to result in the formation of VLPs within the plant cell using TEM (Figure 3.3 A and B).

Since the presence of NLS and DTT in the buffer used to extract these heteromultimeric complexes appeared to negatively affect the stability of the VLPs (Figures 3.4 and 3.5), these substances were omitted from further extraction processes. In addition, the crude plant extract was centrifuged through a 30% iodixanol cushion which resulted in further clarification of the crude plant sap by removing contaminating plant proteins that were co-extracted with the VLPs, as well as any particulate matter that remained in the extract after low-speed centrifugation.

Purification of VLPs was compared by overlaying the diluted 30% iodixanol cushion containing the particles onto either a discontinous gradient or a continuous gradient. Both western blot analysis and TEM showed that centrifugation through a discontinous gradient resulted in the successful purification of intact VLPs measuring 73 – 80 nm in diameter (Figure 3.6 B[i-ii]). The purified plant produced VLPs were shown to be similar to VLPs produced by Roy (2004) in insect cells (Figure 3.6 B[iii]). In contrast, the continuous gradient resulted in the purification of mostly CLPs (Figure 3.7 B) although all four proteins were detected on a western blot (Figure 3.7 A). From these results it was found that although the four proteins constituting VLPs were still present in the purified iodixanol fractions, purification through the continuous gradient most likely resulted in the dissociation of the two outer capsid proteins, VP2 and VP5, from the CLPs. It was determined that the discontinuous gradient was important for maintaining the structural integrity of VLPs during the purification process. Similarly, it was also shown that the centrifugation of insect cell produced VLPs through a discontinuous sucrose gradient was important for the purification of structurally intact VLPs (Stewart *et al.*, 2010). It is possible that the less steep continuous gradient results in dilution of the VLPs throughout the gradient, causing the outer capsid proteins to dissociate from the more stable CLPs whereas selection for VLPs on the discontinuous gradient is more stringent.

VLPs that were produced for animal experiments (Chapter 5) were purified on a discontinuous iodixanol gradient and the best fractions obtained from the first gradient were subjected to a second round of ultracentrifugation through a smaller gradient to concentrate the particles.

The final yield of VLPs that was purified after 9 dpi from plants co-infiltrated with four different *A. tumefaciens* constructs using a ratio of 1:1:2:1 (VP2:VP3:VP5:VP7) was determined to be ~27 mg TSP per kilogram of fresh leaf tissue (Figure 3.8). It was shown by Thuenemann *et al.* (2013) that increased yields (up to ~70 mg [TSP] VLPs per kilogram of fresh weight plant material) of BTV-8 VLPs were obtained by using the pEAQexpress vectors where VP3 and

VP7 or VP2 and VP5 were expressed from the same T-DNA. In this approach VP3 was under control of the wild type CPMV 5' UTR which resulted in down-regulation of CLP synthesis and a shift towards higher levels of VLP expression. Furthermore, the fact that two proteins were carried on the same T-DNA halved the number of *A. tumefaciens* constructs that needed to be co-infiltrated into plants and also reduced the number of transfer events that were necessary to result in VLP expression from plant cells (Thuenemann *et al.*, 2013).

In conclusion, it has been shown that co-infiltration of the *A. tumefaciens* constructs harbouring the pEAQ-*HT* plasmids using a relative vector concentration ratio of 1:1:2:1 resulted in the expression of intact VLPs in plants. To the best of our knowledge this is the first work demonstrating the presence of assembled BTV-8 VLPs in paracrystalline arrays within the cytoplasm of plant cells. The importance of using a discontinuous gradient for the purification of structurally intact VLPs was also demonstrated. The expression approach used here, is flexible in terms of being able to vary individual *A. tumefaciens* construct densities for "fine-tuning" protein expression. This may be necessary for making vaccines for different BTV serotypes, as there is no guarantee that the individual VPs of these will express in the same way as has been shown for BTV-8. On the other hand, the expression approach used by Thuenemann *et al.* (2013) - where two proteins are expressed from a single T-DNA - could have the potential to be used as a rapid response production system for production of BTV VLPs where VP3, VP5 and VP7 serve as a "universal core" and the serotype specific VP2 is interchanged on the T-DNA, depending on the strain of BTV that will be targeted with the vaccine.

Chapter 4:

Design, expression and purification of BTV protein body vaccines

4.1. Introduction

The most efficient means of controlling Bluetongue disease (BT) in sheep is through vaccination. A vaccine produced by Onderstepoort Biological Products (OBP, Pretoria) that consists of attenuated field strains is widely used in South Africa. The vaccine is sold as three bottles each containing five different BTV serotypes (Bottle A: serotypes 1, 4, 6, 12 and 14; Bottle B: serotypes 3, 8, 9, 10 and 11 and finally Bottle C: serotypes 2, 5, 7, 13 and 19). The most attenuated virus strains are administered first, thereby reducing the possible side effects caused by the less attenuated virus strains in the remainder of the vaccine preparations (Coetzee *et al.*, 2012; Roy *et al.*, 1990b). Several side effects including the development of mild clinical symptoms (Veronesi *et al.*, 2010), decreased milk production (Savini *et al.*, 2008) and transplacental infection (MacLachlan *et al.*, 2000; Osburn *et al.*, 1971) have been documented with the use of these vaccines.

VLP vaccines have been produced using insect cell and plant-based expression systems. The VLP-based vaccines were shown to be safe and effective, with vaccinated sheep protected against virus challenge (Roy *et al.*, 1990a; Stewart *et al.*, 2012; Thuenemann *et al.*, 2013). A disadvantage of these however, is that they are protective against only one of the 26 BTV serotypes, unless they are administered as a combination of VLPs produced against different serotypes. In South Africa 22 of the 26 known BTV serotypes have been detected in the country and it has been found that multiple BTV serotypes co-circulate with each vector season (Coetzee *et al.*, 2012): this demonstrates the necessity for use of a multivalent vaccine for BTV in this region. Unfortunately, it may be distinctly unfeasible to propose using multi-serotype VLP-based vaccines, as there would be problems producing sufficient volumes of so many types, and for a cost that is even remotely comparable to the live attenuated vaccine.

The BTV structural protein VP2 is the major serotype-specific antigen of BTV (Huismans and Erasmus, 1981; Roy *et al.*, 1990b). It has been shown that ≥50 µg doses of both VP2 obtained from isolated and purified BTV and recombinantly-produced VP2 induced neutralising antibodies and protected some, but not all of the sheep that were vaccinated, against viral challenge. Lower doses were shown to provide protection; however no neutralising antibodies were detected after vaccination (Huismans *et al.*, 1987; Roy *et al.*, 1990b). Even though these subunit vaccines have been shown to be safe for use in sheep (Roy *et al.*, 1990b), it is

desirable to enhance the immunogenicity of these vaccine candidates. The immunogenicity of proteins can be increased by fusing them to other immunogenic proteins, by adding adjuvant to the vaccine formulation (Rybicki, 2010), or by fusion to signal sequences that drive assembly and sequestration of the protein into protein bodies (Torrent *et al.*, 1994). Particulate proteins with repeating sequence motifs, such as protein bodies, are favoured for uptake by antigen presenting cells, thereby enhancing the immune response (Whitehead *et al.*, 2014).

Protein bodies (PBs) are endoplasmic reticulum (ER)-derived organelles that are found in maize seeds. These organelles stably store massive amounts of zeins as a source of protein within the ER (Müntz, 1998). Once expressed and targeted to the ER for post translational modification, these zein polypeptides oligomerise in large complexes and eventually self-associate into protein bodies (Galili, 2004; Herman and Larkins, 1999; Larkins and Hurkman, 1978). The proline-rich N-terminal (including a tandem-repeat domain) of one of these zeins - γ-zein - was shown to be important for ER retention and the formation of PBs in both maize seeds and a wide range of eukaryotic cells (Ludevid *et al.*, 2004).

Zera® (ERA Biotech, Spain) is a synthetic peptide generated from the N-terminal proline-rich domain of γ-zein (Ludevid *et al.*, 2004). The Zera® sequence (see Figure 4.1) is made up of 112 amino acids that include the γ-zein signal peptide and the first 93 amino acids of γ-zein. The complete Zera® sequence contains four regions (Figure 4.1): a γ-zein ER-targeting signal peptide (blue box), 11 hydrophobic non-proline amino acids that contain a CGC motif that is important for packing of protein bodies due to the formation of inter-and intra-chain disulphide bonds (green box, N-term), the proline-rich repeat domain containing eight repeats of the hexapeptide PPPVHL (grey box) which is important for the assembly of PBs and finally, a proline-X (Pro-X region) sequence where proline residues alternate with other amino acids (Llop-Tous *et al.*, 2010; Ludevid *et al.*, 2004). The main driving forces behind the self-assembly of PBs are hydrophobic interactions between the $(PPPVHL)_8$ repeat regions of two or more Zera® chains and inter-chain disulphide bond formation that stabilises and strengthens the oligomers (Llop-Tous *et al.*, 2010).

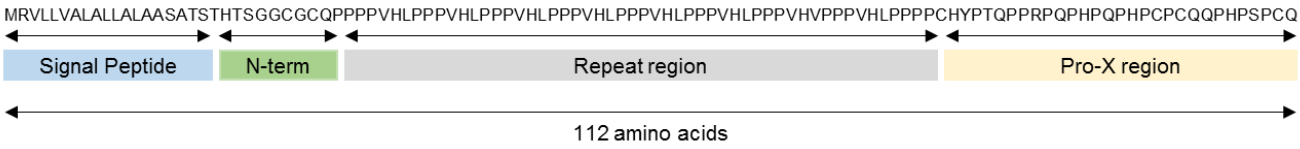

Figure 4.1: A schematic representation of the Zera® regions, including the amino acid sequence. Image adapted from Llop-Tous *et al.* (2010).

It has been demonstrated that fusion of Zera® to different proteins of interest results in the retention of recombinant proteins within PBs called StorPro® organelles (ERA Biotech, Spain). Zera® fused to several different proteins including red fluorescent protein (Joseph *et al.*, 2012), enhanced cyan fluorescent protein (Llop-Tous *et al.*, 2010), human growth hormone (Llompart *et al.*, 2010), calcitonin (Ludevid *et al.*, 2004) and epidermal growth factor (Torrent *et al.*, 2009a) has been shown to result in the formation of PBs in the leaves of both transiently and stably transformed tobacco plants.

Synthetic PBs (StorPro® organelles) retain many beneficial characteristics of natural PBs, including the presence of chaperones that facilitate folding of recombinant proteins, better accumulation within dense membrane-bound structures that allow for easier purification and subcellular sequestration that protects the proteins from intracellular degradation (Torrent *et al.*, 2009a).

Zera®-fused subunit vaccine proteins have been shown to induce cellular and humoral immune responses as well as having intrinsic adjuvancy and immunomodulatory properties (http://www.erabiotech.com/index.php/applications/zera-vaccines/protein-particle-vaccines). It has been shown that fusion of the HPV-16 E7 protein to Zera® enhanced immunogenicity of the protein. The study further demonstrated the adjuvant activity of Zera®, whereby free protein bodies mixed with the HPV-16 E7 protein resulted in an enhanced immune response (Rybicki *et al.*, 2011; Whitehead *et al.*, 2014).

As has been described previously, the antigenic characteristics of VP2 make it an excellent candidate for a BT vaccine. By presenting VP2 to the immune system in particulate form, immunogenicity of the protein will probably be enhanced and less of the antigen may be needed for vaccination. Therefore, the aim of this study was to develop two novel VP2-based BTV vaccine candidates. For the first vaccine candidate, the putative antibody-binding epitopes of multiple serotypes of VP2 were predicted with *in silico* methods and a synthetic

peptide based on the predicted epitopes was constructed for VP2. The second vaccine candidate consisted of the full-length plant codon-optimised BTV-8 VP2. Nucleotide sequences representing both the synthetic epitope sequence and the full-length plant codon-optimised BTV-8 VP2 were fused to the Zera®-encoding sequence to drive PB formation when the fusion protein genes were expressed in *N. benthamiana*. Protein expression and purification of both vaccine candidates was optimised and TEM of infiltrated leaves was carried out to determine if the Zera®-fused proteins accumulated into PBs when expressed in *N. benthamiana*.

4.2. Materials and Methods

4.2.1. *In silico* epitope prediction of VP2

Eight full-length BTV VP2 amino acid sequences (Table 4.1) were selected from Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and the most likely epitopes of each of these sequences was predicted with COBEpro [(Sweredoski and Baldi, 2009), http://scratch.proteomics.ics.uci.edu/], an online program for the prediction of continuous B-cell epitopes.

The VP2 amino acid sequences were aligned using ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/) and two predicted B-cell epitope regions whose amino acid compositions corresponded to most of the aligned VP2 sequences - belonging to the eight different BTV serotypes - were selected for inclusion into the synthetic epitope-based vaccine. A third homologous region was also included in the synthetic epitope-based vaccine sequence.

Table 4.1: The BTV serotypes used in the design of the putative VP2 epitope and their corresponding Genbank accession numbers.

BTV serotype	Genbank accession numbers
1	ADI79209
2	CA079950
6	ADI49552
8	AM498052
10	JN704634
13	AAX48783
17	AAB30550
23	AAA56867

4.2.2. Construction of the fusion product Zera®-VP2ep with assembly PCR

4.2.2.1. PCR amplification of Zera®

The plasmid pZera®1 (Torrent *et al.*, 2009a) obtained from ERA Biotech was used as a template for the PCR amplification of Zera®. Primers were designed to introduce 5' *Age*I and 3' *Nco*I restriction enzyme sites to the gene termini (Table 4.2; Zera®-FP and Zera®-RP). The PCR reaction consisted of 50 ng of the template DNA, 200 µM dNTPs, 1 µM of each primer, 1x Buffer A and 1 unit (U) KAPA Taq DNA polymerase (KAPA Biosystems).

Zera® was amplified by initial denaturation at 94 °C for 5 min. Thereafter, 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s and elongation at 72 °C for 45 s were carried out followed by a final elongation step at 72 °C for 5 min. The amplified product was separated on a 1% w/v TBE (89 mM Tris base, 89 mM boric acid and 2 mM EDTA [pH8]) agarose gel containing 2.5 mg/mL ethidium bromide and visualised under short wavelength ultraviolet (UV) illumination.

The amplified Zera® fragment was purified from the agarose gels using the QIAquick® Gel Extraction kit (Qiagen) following the manufacturer's instructions. The purified DNA was used as a template in the subsequent assembly PCR of the fusion product, Zera®-VP2ep.

Table 4.2: Primers and oligomers used for PCR of individual genes and the assembly PCR of fusion products[1].

Gene / Oligomer	Size (bp)	Primer	Restriction sites (5' / 3')	5' – 3' sequence[2]	Tm (°C)
Zera®	352	Zera®-FP[3, 4]	*Age*I	GC**ACCGGT**ATGAGGGTGTTGCTCGTT	62.7
		Zera®-RP	*Nco*I	GC**CCATGG**CTGGCACGGGCTTGGAT	58.3
VP2ep	70	VP2ep-F	*Mlu*I	GC**ACGCGT**TTCCCAGAGCACCTTCGACACGATGTAACAAAT	76.7
		VP2ep-R	*Xma*I	GC**CCCGGG**TCCACGCAGGCAGGGATACGGATTTGTTACATC	79.8
Linker			*Nco*I/*Mlu*I	**CCATGG**GAAGCGGCGGCGAAA**ACGCGT**	75.9
		Linker-VP2ep-FP[3]		GAAGCGGCGGCGAAAACGCGTTTCCCAGAGCACCTT	77.3
		Linker-Zera®-RP[3]		TTTCGCCGCCGCTTCCCATGGTCTGGCACGGGCTTG	81.4
		VP2ep-RP[3, 4]	*Xma*I	GC**CCCGGG**TCCACGCAGGCAGGG	56.8
Sequencing primers		pEAQ-FP		GACGAACTTGGAGAAAGATTGTTAAGC	61.2
		pEAQ-RP		AACCAGAGTTAAAGGCCTCGAGC	62.3

[1] All primers and oligomers were synthesized in-house at the Department of Molecular and Cell Biology (University of Cape Town).

[2] The restriction enzyme sites are underlined and in bold.

[3, 4] Primers used for first and second stage assembly PCR of Zera®-VP2ep.

4.2.2.2. Assembly of VP2ep

The putative multi-epitope sequence - VP2ep - was designed to contain 5' *Mlu*I and 3' *Xma*I restriction enzyme sites. Two oligomers - VP2ep-F and VP2ep-R (Table 4.2) - consisting of 41 bp each were designed and synthesized with 12 complementary base pairs to facilitate fusion of the fragments during assembly PCR to yield VP2ep. The assembly PCR reaction consisted of 1 µM each of VP2ep-F and VP2ep-R, 200 µM dNTPs, 1x Buffer A and 1 U KAPA Taq DNA polymerase. The two oligomers were assembled by initial denaturation of the DNA at 94 °C for 5 min, followed by 20 cycles of denaturing at 93 °C for 1 min and annealing and elongation at 65 °C for 30 s. A final elongation step was carried out for 5 min at 72 °C. The annealed product was separated on a 2% w/v TBE agarose gel containing 2.5 mg/mL ethidium bromide.

The VP2ep DNA was purified from the PCR reaction using the QIAquick® PCR purification kit (Qiagen) following the manufacturer's instructions and used as template in the assembly PCR of Zera®-VP2ep.

4.2.2.3. Assembly PCR of Zera®-VP2ep

For the fusion of Zera® to VP2ep a nucleotide sequence encoding a short universal peptide linker consisting of glutamate (E), three alanines (A) and lysine (K) - EAAAK (nucleotide sequence in Table 4.2) - was synthesized so as to contain 5' *Nco*I and 3' *Mlu*I restriction sites. Linker primers containing regions that were complementary to both the linker, Zera® and VP2ep were also designed and synthesized (Table 4.2; Linker-VP2ep-FP and Linker-Zera®-RP) to further facilitate assembly of the fusion product.

Assembly PCR of Zera®-VP2ep was carried out in two stages. Figure 4.2 illustrates the fusion product Zera®-VP2ep, including the primers that were used in first and second stage assembly PCR reactions.

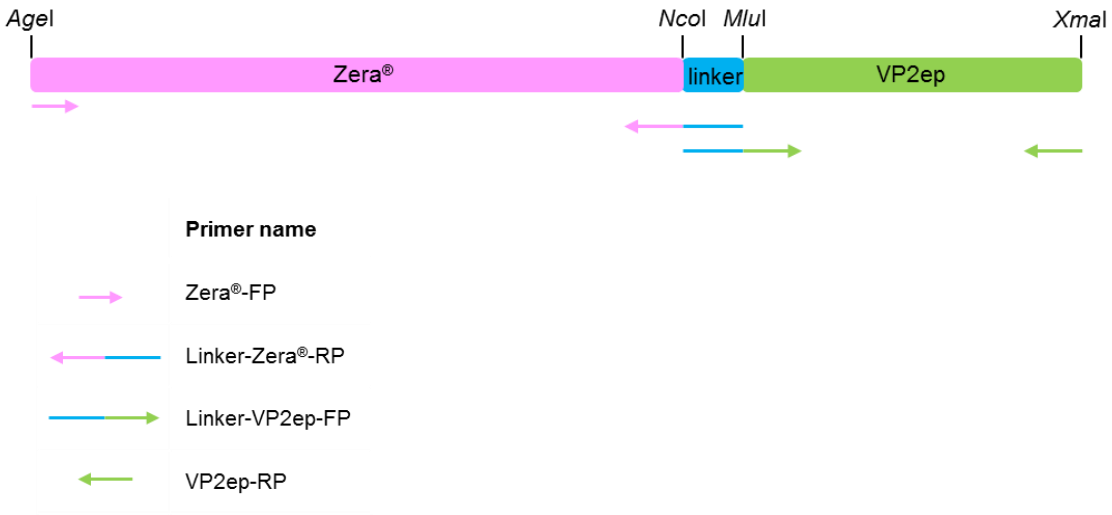

Figure 4.2: Schematic representation of the primers used to create the fusion product Zera®-VP2ep.

The first stage assembly PCR reaction consisted of ~50 ng of each of the following DNA templates: Zera®, VP2ep and the linker. The remainder of the reaction contained 1 µM of each primer (Zera®-FP, Linker-VP2ep-FP, Linker-Zera®-RP, VP2ep-RP), 200 µM dNTPs, 1x Buffer A and 1 U KAPA Taq DNA Polymerase. First stage assembly PCR was carried out with an initial denaturing step at 94 °C for 2 min, followed by 25 cycles of denaturing at 93 °C for 1 min and annealing and elongation at 72 °C for 30 s. A final elongation step was carried out for 5 min at 72 °C.

The assembled PCR product was separated on a 2% w/v TBE agarose gel and purified using the QIAquick® Gel Extraction kit, after which the purified DNA was used as template in second stage PCR of the fusion product.

The second stage PCR reaction contained 50 ng of the template DNA, 1 µM each of Zera®-FP and VP2ep-RP, 200 µM dNTPs, 1x Buffer A and 1 U KAPA Taq DNA Polymerase. Second stage cycling conditions were similar to the first stage assembly PCR conditions, however only 20 cycles of amplification were carried out and the annealing temperature was decreased to 65 °C. The assembled fusion product Zera®-VP2ep was visualised and gel purified as described above.

4.2.3. Cloning of Zera®-VP2ep into pEAQ-*HT*

The entire volume of gel purified Zera®-VP2ep was digested for 1 hr at 37 °C with 1 U each of the restriction enzymes *Age*I and *Xma*I (Fermentas), after which the DNA was gel purified as described above. Similarly, 1 µg of the pEAQ-*HT* vector was linearized and gel purified after which the vector DNA was dephosphorylated using 1 U rapid alkaline phosphatase (Roche) according to the manufacturer's instructions.

Zera®-VP2ep was directionally cloned into the pEAQ-*HT* plant expression vector to yield pEAQ-*HT*Zera®-VP2ep. DH5-α chemically competent *E. coli* cells (E. cloni™, Lucigen) cells were transformed (Sambrook *et al.*, 1989) with the plasmid construct and recombinant clones were selected using kanamycin resistance (50 µg/mL).

pEAQ-*HT*Zera®-VP2ep recombinant clones were screened by colony PCR using the Zera-FP and VP2ep-RP primers (Table 4.2). PCR cycling conditions included an initial denaturation step at 94 °C for 2 min, followed by 20 cycles at 93 °C for 1 min, 65 °C for 30 s and 72 °C for 45 s and a final elongation step at 72 °C for 5 min. PCR products were separated on 2% TBE agarose gel with 2.5 mg/mL ethidium bromide.

Recombinant clones were verified by restriction enzyme digestion of the plasmid DNA. Colonies were inoculated into 5 mL LB media supplemented with 50 µg/mL kanamycin and incubated with aeration for 16 hrs at 37 °C. Small scale plasmid isolations were performed on the cultures using the QIAprep® Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. Recombinant plasmid DNA (~500 ng) was digested for 1 hr at 37 °C using 1 U each of *Age*I and *Xho*I. The digested DNA was separated on a 1% TBE agarose gel stained with 2.5 mg/mL ethidium bromide.

The sequence of the pEAQ-*HT*Zera®-VP2ep construct was verified using the pEAQ-FP and pEAQ-RP sequencing primers (Table 4.2). Sequence data was analysed by alignment with the theoretical sequence in DNAMAN (Lynnon BioSoft).

4.2.4. Construction of pEAQ-*HT*Zera®-VP2

VP2ep was excised from the pEAQ-*HT*Zera®-VP2ep plasmid by restriction enzyme digestion with 1 U each of *Mlu*I and *Xho*I. Similarly, the full-length *N. benthamiana* codon-optimised VP2 was excised from the pTRAkc-rbcs1-cTPVP2co plasmid (Chapter 2). To create the pEAQ-*HT*Zera®-VP2 construct, VP2 was cloned into the *Mlu*I and *Xho*I sites after which it was

used to transform DH5-α chemically competent *E. coli* cells (E. cloni™, Lucigen) as described in Section 4.2.3.

Recombinant pEAQ-*HT*Zera®-VP2 clones were screened by colony PCR with the cTPVP2coF and cVP2coR primers (Chapter 2, Table 2.1) using the cycling conditions described in Section 2.2.1.

The recombinant clones were verified by digesting ~500 ng of the plasmid DNA for 1 hr at 37 °C using 1 U each of *Age*I and *Xho*I. The digested DNA was separated on a 0.8% TBE agarose gel stained with 2.5 mg/mL ethidium bromide.

The pEAQ-*HT*Zera®-VP2 construct was further confirmed by sequencing and sequence analysis as described in Section 4.2.3.

4.2.5. Transformation of *A. tumefaciens* LBA4404

Both expression constructs were transformed into electrocompetent *A. tumefaciens* LBA4404 as described in Section 2.2.4. Recombinant clones were selected on LB media plates at 27 °C containing 30 µg/mL kanamycin and 50 µg/mL rifampicin.

Successful transformation was confirmed by colony PCR as described in Sections 4.2.3 and 2.2.1 for pEAQ-*HT*Zera®-VP2ep and pEAQ-*HT*Zera®-VP2, respectively. Further confirmation was also obtained by back-transformation of the plasmid DNA into DH5-α chemically competent *E. coli* cells as described in Section 4.2.3. Transformed *E. coli* was plated onto LB plates supplemented with the appropriate antibiotics to select for the recombinant plasmids.

4.2.6. *A. tumefaciens*-mediated transient expression of Zera®-VP2ep and Zera®-VP2

Starter cultures of *A. tumefaciens* harbouring the pEAQ-*HT*Zera®-VP2ep and pEAQ-*HT*Zera®-VP2 plasmids were supplemented with 30 µg/mL kanamycin, 50 µg/mL rifampicin and 2 mM $MgSO_4$ and grown in LB media as described by Maclean *et al.* (2007).

The starter cultures were used to inoculate induction medium supplemented with 30 µg/mL kanamycin, 50 µg/mL rifampicin, 20 µM acetosyringone and 2 mM $MgSO_4$. The cultures were propagated O/N at 27 °C with agitation, after which the cells were prepared for syringe

infiltration into six-week-old *N. benthamiana* leaves as described in Section 2.2.5. Time-trials were carried out to evaluate at what OD_{600} and which day the best expression of Zera®-VP2ep and Zera®-VP2 occurred, after which expression was scaled up.

4.2.7. *In situ* TEM of Zera®-VP2ep and Zera®-VP2 PBs

A. tumefaciens cultures harbouring the pEAQ-*HT*Zera®-VP2ep or pEAQ-*HT*Zera®-VP2 plasmids were syringe-infiltrated into the abaxial air spaces of six-week-old *N. benthamiana* plants and protein was expressed for 3 and 7 days, respectively.

Embedding and sectioning of the leaf material was carried out according to the method in Section 3.2.3. Grids were viewed using a Technai G^2 transmission electron microscope (FEI).

4.2.8. Protein extraction

For small-scale expression three leaf discs were harvested for each expression construct at 2, 3, 5 and 7 dpi and ground up in liquid nitrogen. The leaf material was resuspended in 70 µL per disc of buffer PBP3 (100 mM Tris [pH8], 50 mM KCl, 6 mM $MgCl_2$, 10 mM EDTA, 0.4 M NaCl and 1x Complete Mini EDTA-free protease inhibitor cocktail [Roche]) and mixed by vortexing. The leaf extracts were clarified for 5 min at 15 000 *x g* on a bench top centrifuge.

After large scale expression of Zera®-VP2ep and Zera®-VP2, leaves were harvested at 3 and 7 dpi, respectively and were either frozen at -80 °C until further processing or immediately cut up into fine pieces and thoroughly homogenised with a Waring-type blender in five volumes of ice cold PBP3 buffer containing 10% sucrose and 1 x Complete Mini EDTA-free protease inhibitor cocktail. The crude plant extract was incubated at 4 °C with gentle agitation, after which it was filtered through four layers of Miracloth™ (Merck). The homogenate was further clarified by centrifugation at 1 000 *x g* for 10 min at 4 °C.

4.2.9. Sucrose cushion purification of protein bodies

The clarified crude plant extract was overlaid onto 5 mL of a 42% sucrose cushion prepared in buffer PBP3 and ultracentrifuged for 2 hrs at 79 000 *x g* in a SW 32 Ti rotor (Beckman).

After centrifugation the pellet was resuspended in 300 µL buffer PBP3 containing 10% sucrose.

4.2.9.1. Sequential extraction of purified PBs

PBs were sequentially solubilised according to the method described by Joseph *et al.* (2012). The process was carried out by mixing 1 part of the protein body suspension in 1 part buffer SB1 (62.5 mM Tris-Cl [pH 6.8], 25% glycerol and 2% SDS). The mixture was incubated at RT for 1 hr, after which it was centrifuged at 15 000 *x g* for 15 min at 4 °C. The supernatant containing the soluble (S) fraction was aspirated. The pellet was resuspended in 1 part buffer SB2 (buffer SB1 including 350 mM DTT), after which it was incubated and centrifuged as above. The supernatant was aspirated (fraction R) and the pellet resuspended in buffer SB2. The resuspended pellet was incubated at 95 °C for 5 min to solubilise the PB cores and centrifuged as described above, after which the supernatant (H) was collected. The remaining pellet was resuspended in 1 part buffer SB2 (final pellet) and analysed with western and dot blots.

4.2.9.2. "Washing" of PBs

The pellet (obtained after sucrose cushion purification) was resuspended in buffer PBP3 and washed multiple times by centrifugation at 15 000 *x g* for 5 min – each time collecting the supernatant (wash 1, wash 2, etc.) and resuspending the pellet in 500 µL PBP3 buffer containing 10% sucrose. The purified protein bodies were stored at 4 °C until further analysis.

4.2.10. Western- and dot blot analysis of Zera®-VP2ep and Zera®-VP2

4.2.10.1. Production of wild type BTV-8 VP2 antibodies in rabbits for use in western- and dot blot analysis

Since there was no commercially available antibody for BTV VP2, antibodies were raised in rabbits against the wild type (wt) BTV-8 VP2 for the detection of plant-expressed proteins. The wtVP2 protein was expressed in *E. coli* DH5-α using the pCold™TF Cold Shock Expression System (TAKARA). The recombinant *E. coli* culture was incubated for 16 hrs at

37 °C with agitation, after which it was used to inoculate 100 mL LB medium supplemented with 50 µg/mL ampicillin. The culture was incubated with agitation at 37 °C until it reached an OD_{600} of 0.4-0.5. The culture was refrigerated at 15 °C for 30 min after which protein expression was induced by the addition of 0.5 mM isopropylthio-β-D-galactoside (IPTG) and the culture incubated at 15 °C for 24 hrs. After 24 hrs the cells were harvested by centrifugation at 1 000 *x g* for 5 min.

Inclusion bodies were purified from the *E. coli* cell pellet using Bugbuster® (Novagen, USA) according to the manufacturer's instructions. The resuspended wtVP2 inclusion bodies were dialysed O/N at 4 °C using dialysis tubing with a molecular weight cut-off of 10 kDa (Thermo Fischer Scientific, USA) in 2 L sterile Dulbecco's Endo-free PBS (Sigma-Aldrich, USA).

Endotoxin levels were measured using the Limulus Amebocyte Lysate QCL-1000™ kit (Lonza) as per the manufacturer's instructions. Samples were assayed in a 96-well Maxisorp® microtitre plate and the absorbance readings at 405 nm were obtained using a Bio-Tek Powerwave XS spectrophotometer.

The amounts of wtVP2 antigen on a 8% SDS polyacrylamide Coomassie-stained gel was estimated using a bovine serum albumin (BSA, Sigma-Aldrich) standard curve. The bands were quantitated using a Syngene Gene Genius imaging system and GeneTools software (Synoptics Inc., UK).

Deltamune (Pty) Ltd. (South Africa) carried out inoculation and production of the polyclonal antibody in rabbits. For the primary inoculation rabbits were injected subcutaneously with 500 µL of wtVP2 in the presence of Incomplete Freund's Adjuvant (IFA) made according to the manufacturer's instructions (Difco, #263910). This was followed by three subsequent booster vaccinations (in the presence of IFA); the first booster was administered two weeks after the initial injection and thereafter the second and third boosters followed one week after the other. The total amount of antigen used for each dose was ~32 µg. All protocols were approved by the Deltamune Animal Ethics Committee prior to being carried out.

Rabbit serum obtained from Deltamune was used as primary antibody for western– and dot blot analysis of Zera®-VP2ep and Zera®-VP2.

4.2.10.2. Western blot and dot blot analysis of Zera®-VP2ep and Zera®-VP2

For western blot analysis, the plant extracts were incubated at 90 °C for 10 min in 5 x DTT sample application buffer (250 mM Tris-Cl [pH6.8], 500 mM DTT, 10% sodium dodecyl sulphate [SDS], 0.3 mM bromophenol blue and 10% glycerol). Zera®-VP2ep and Zera®-VP2 proteins were separated on 15% and 8% SDS polyacrylamide gels, respectively. Western blot analysis was carried out as described in Section 2.2.6, except that a 1:2000 dilution of the rabbit-raised anti-VP2 polyclonal antibody (α-VP2R) in blocking buffer was used as primary antibody after which the membranes were subsequently probed with a 1:5000 dilution of anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody produced in goats (Sigma-Aldrich).

Dot blots were done by dropping 5 µL of the purified protein wash fractions onto nitrocellulose membranes and letting the membranes dry completely. The membranes were probed O/N at 4 °C with 1:2000 dilutions of α-VP2R, BTV-8 sheep serum (raised against plant-produced VLPs) and BTV-10 guinea pig-produced serum (obtained from Dr Christiaan Potgieter, Deltamune) in blocking buffer. The membranes were washed four times in blocking buffer for 15 min each and afterward incubated for 1 hr at 37 °C in either a 1:5000 dilution of anti-rabbit alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich), 1:10 000 dilution of anti-goat/sheep alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich) or 1:5000 anti-guinea-pig alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich). After secondary antibody incubation the dot blots were washed four times with 1x PBST, with 15 min for each wash. Detection was performed using BCIP/NBT (KPL) substrate.

4.2.10.3. Quantification of purified PBs

PBs were quantified by determining the TSP present in the final pellet obtained after washing the PBs. Quantification was carried out according to the method described in Section 3.2.6, after confirming with Coomassie-stained gels that no contaminating plant-proteins were co-purified with the PBs.

4.3. Results

4.3.1. Prediction of a putative BTV VP2 epitope

In silico prediction (data not shown) of continuous B-cell epitopes was carried out on eight full-length VP2 amino acid sequences that were available on Genbank (http://www.ncbi.nlm.nih.gov/genbank) with COBEpro (Sweredoski and Baldi, 2009) prediction software available from the Scratch protein server (http://scratch.proteomics.ics.uci.edu/). COBEpro lists possible predicted epitopes regions from most likely to least likely. The VP2 amino acid sequences were aligned and two predicted epitope regions that were similar for most of the serotypes was selected for the epitope-based vaccine sequence.

The resulting multi-epitope (VP2ep) consisted of three discontinuous regions that were fused together from the VP2 amino acid sequences. Two predicted epitope regions (Figure 4.3 A, blue and green boxes) that showed consensus among most of the amino acid sequences were selected for the design of the putative epitope-based vaccine and a homologous region across all the serotypes was also included in the sequence (Figure 4.3 A, purple box). In total the putative predicted epitope-based oligomer consisted of 54 bp (codon optimised for *N. benthamiana)* that translated into 18 amino acids (Figure 4.3 B).

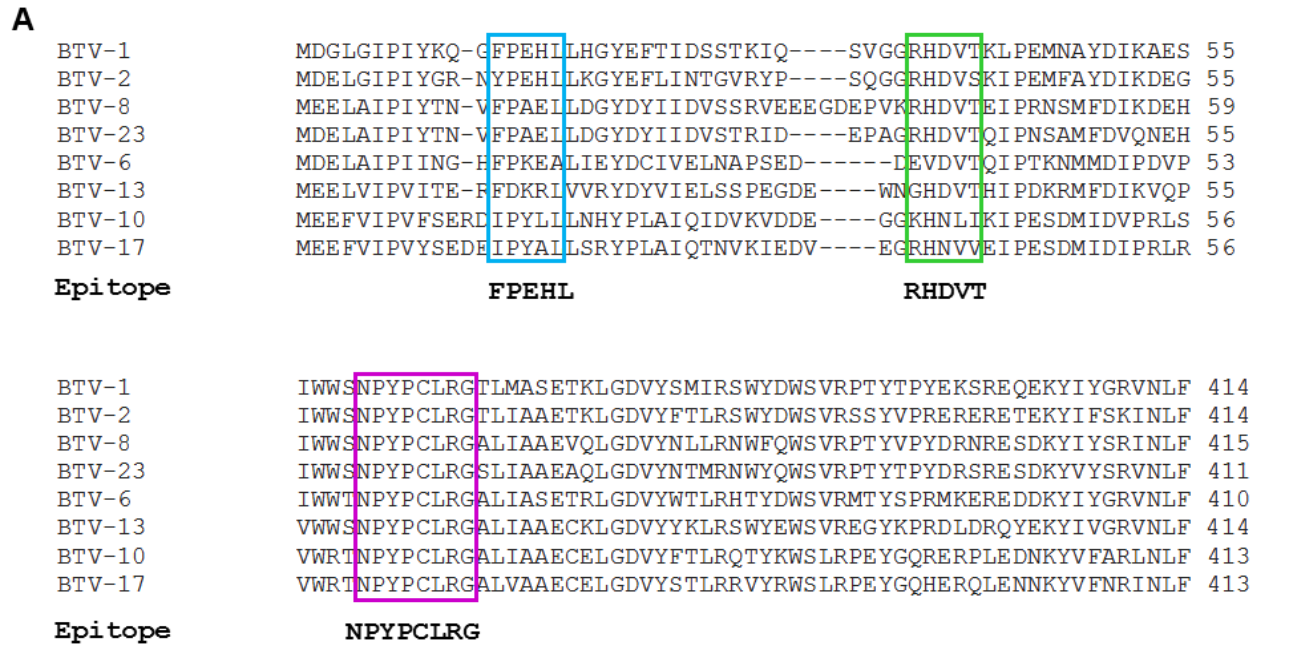

Figure 4.3: (A) Two regions of the ClustalW multiple alignment of the amino acid sequences of eight VP2 serotypes. The predicted epitope regions corresponding to most of the serotypes are shown below the alignment in bold and boxed in blue and green. The homologous region is boxed in purple. (B) The putative amino acid and nucleotide sequences of VP2ep.

4.3.2. Assembly of the fusion product Zera®-VP2ep

4.3.2.1. PCR amplification of Zera®

Zera® was amplified from the pZera™1 plasmid using the primers listed in Table 4.2. The primers were designed to modify the 5' and 3' termini for the addition of *Age*I and *Nco*I restriction enzyme sites, respectively. Amplification resulted in an expected band at 350 bp (Figure 4.4).

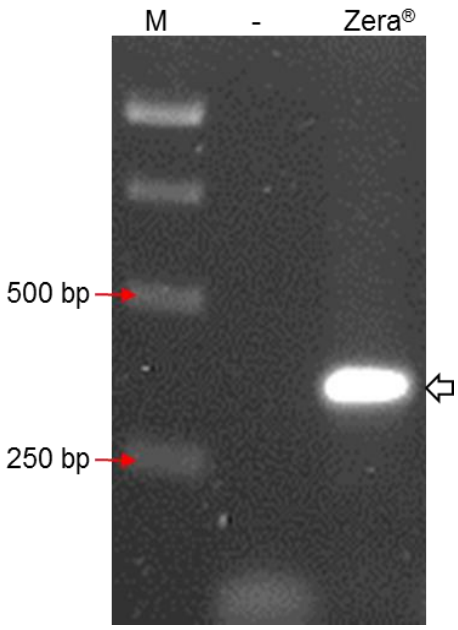

Figure 4.4: PCR amplification of Zera® (white arrow) using Zera®-FP and Zera®-RP. The negative control (-) was amplified with the same primer set, however the reaction contained water instead of template DNA. Lane M represents the molecular weight marker.

The band representing Zera® was excised and gel-purified to use as DNA template in further downstream reactions for assembly PCR of the fusion product.

4.3.2.2. Assembly PCR of VP2ep

Two oligomers (VP2ep-F and VP2ep-R) containing a 12 bp complementary region were designed for the assembly of the synthetic putative epitope by PCR (Figure 4.5 A). VP2ep-F and VP2ep-R also included 5' *Mlu*I and 3' X*ma*I restriction sites respectively, to facilitate cloning into the pEAQ-*HT* expression vector. Addition of the restriction enzyme sites increased the size of the epitope from 54 bp to 70 bp. The two oligomers were successfully annealed and amplified by assembly PCR, resulting in an expected fragment of 70 bp (Figure 4.5 B).

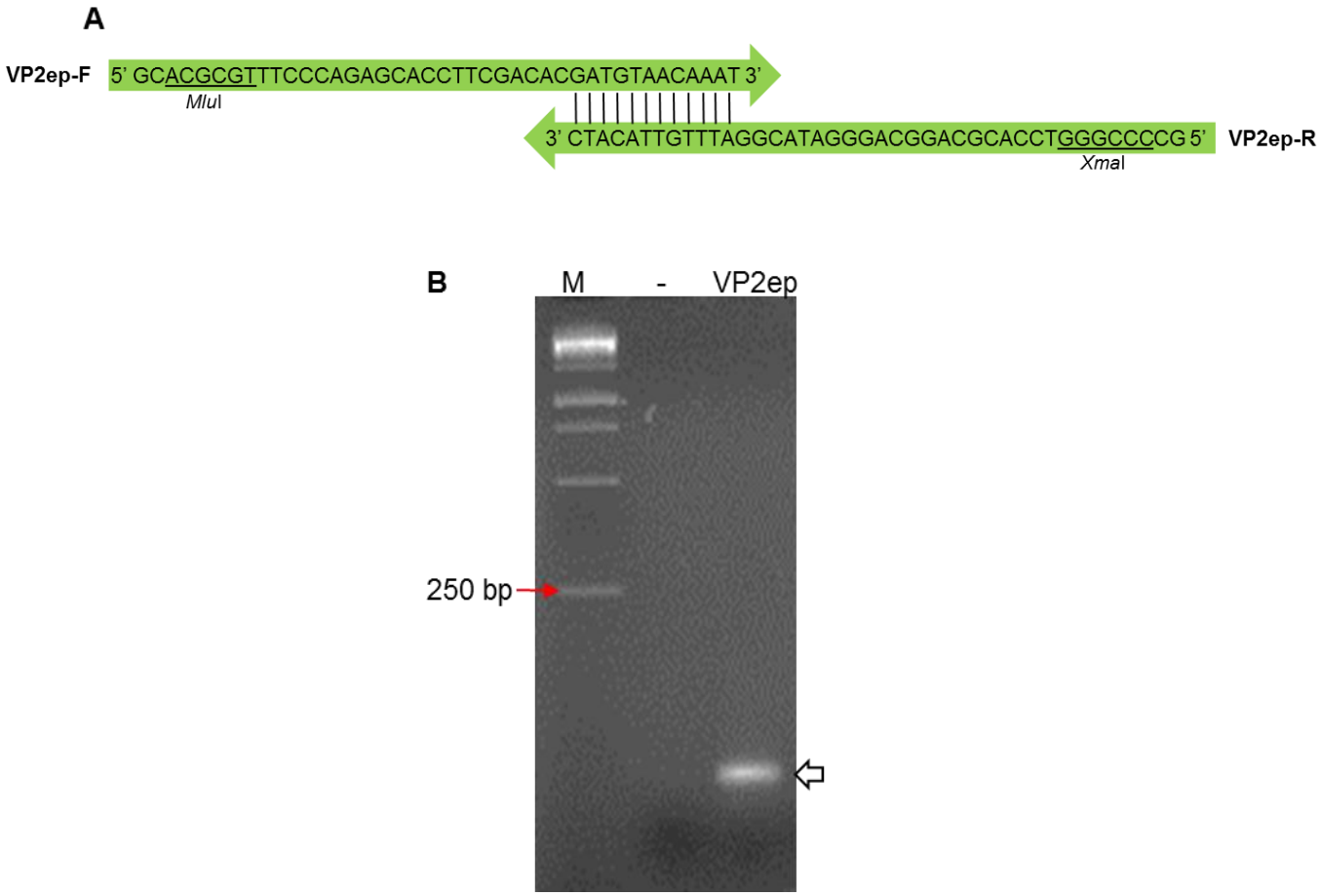

Figure 4.5: (A) Schematic representation illustrating the two oligomers (VP2ep-F and VP2ep-R, with the restriction enzyme sites underlined) and the 12 bp complementary overlap to facilitate assembly of VP2ep. (B) The assembled VP2ep (white arrow) at ~70 bp. The negative control (-) reaction contained no template DNA. Lane M represents the molecular weight marker.

The annealed VP2ep product was purified from the PCR reaction for use as template in subsequent PCR reactions for assembly of the fusion product Zera®-VP2ep.

4.3.2.3. Assembly PCR of Zera®-VP2ep

Zera® was successfully fused to VP2ep with a linker sequence (Table 4.2), as it has been shown that the linking of two proteins or domains with a universal peptide linker can potentially increase the expression of soluble proteins (Arai *et al.*, 2001). The fusion product Zera®-VP2ep was assembled during first stage assembly PCR by using the nucleotide templates Zera®, VP2ep and the linker sequence. These sequences were annealed and amplified with

overlapping primers (a schematic representation of first stage assembly PCR is shown in Figure 4.2).

First stage assembly PCR resulted in a fragment of the expected size at 465 bp (white arrow) which indicated successful assembly of Zera®-VP2ep (Figure 4.6). The smaller bands observed at ~350 bp and ~100 bp could possibly represent Zera® and VP2ep template DNA that did not anneal during assembly PCR.

The Zera®-VP2ep DNA fragment obtained during first stage assembly PCR was gel-purified and used as template in second stage assembly PCR. Second stage PCR was carried out with primers (Table 4.2, Zera®-FP and VP2ep-RP) that flanked the 5' and 3' regions of the complete Zera®-VP2ep fusion; these primers further ensured modification of the termini with *Age*I and *Xma*I restriction enzyme sites. Second stage assembly PCR resulted in an expected fragment of 465 bp (Figure 4.6).

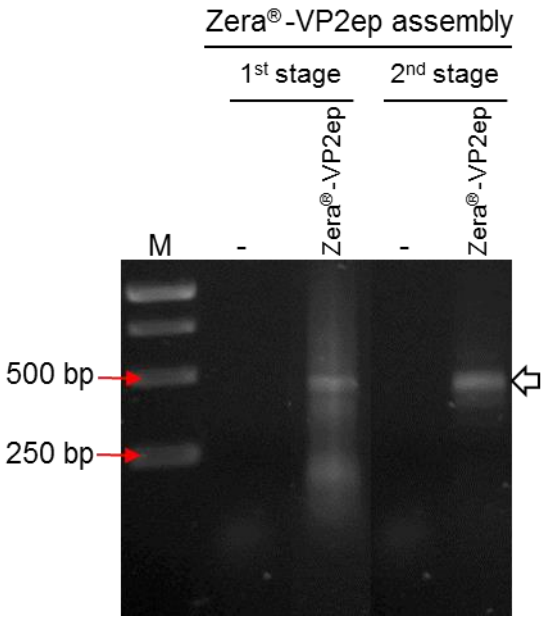

Figure 4.6: First and second stage assembly PCR of Zera®-VP2ep showing a band at 465 bp (white arrow). Both negative controls (-) contained water instead of DNA template. Lane M represents the molecular weight marker.

4.3.3 Verification of pEAQ-*HT*Zera®-VP2ep clones

Zera®-VP2ep was successfully cloned into the pEAQ-*HT* plant expression vector and transformed into both *E. coli* DH5-α competent cells and *A. tumefaciens* LBA4404.

Recombinant clones were screened by colony PCR using Zera®-FP and VP2ep-RP primers that bind the 5' and 3' termini of Zera®-VP2ep. The recombinant clones produced an expected band at ~465 bp. The recombinant pEAQ-*HT*Zera®-VP2ep clone was further verified by restriction enzyme digestion using *Age*I and *Xho*I restriction sites. The *Age*I restriction enzyme site is located at the 5' termini of the gene, whereas the *Xho*I restriction site is located on the vector backbone just downstream of the Zera®-VP2ep *Xma*I 3' site. As expected, restriction enzyme digestion of the recombinant clone showed the presence of a 485 bp insert (results not shown).

The pEAQ-*HT*Zera®-VP2ep plasmid DNA was sequenced and confirmed by aligning the sequence data with the theoretical sequence using DNAMAN multiple alignment software (data not shown).

4.3.4. Construction and verification of pEAQ-*HT*Zera®-VP2 clones

To construct pEAQ-*HT*Zera®-VP2, the pEAQ-*HT*Zera®-VP2ep plasmid (Figure 4.7 A) was digested with the restriction enzymes *Mlu*I and *Xho*I. This facilitated the removal of VP2ep (green box), with Zera® (pink box) and the linker (blue box) remaining on the pEAQ-*HT* backbone (Figure 4.7 B). *VP2* was excised from the pTRAkc-rbcs1-cTPVP2co backbone (Chapter 2) using *Mlu*I and *Xho*I (data not shown), after which it was directionally cloned into pEAQ-*HT*Zera® (*Mlu*I / *Xho*I) resulting in the plasmid pEAQ-*HT*Zera®-VP2 (Figure 4.7 C).

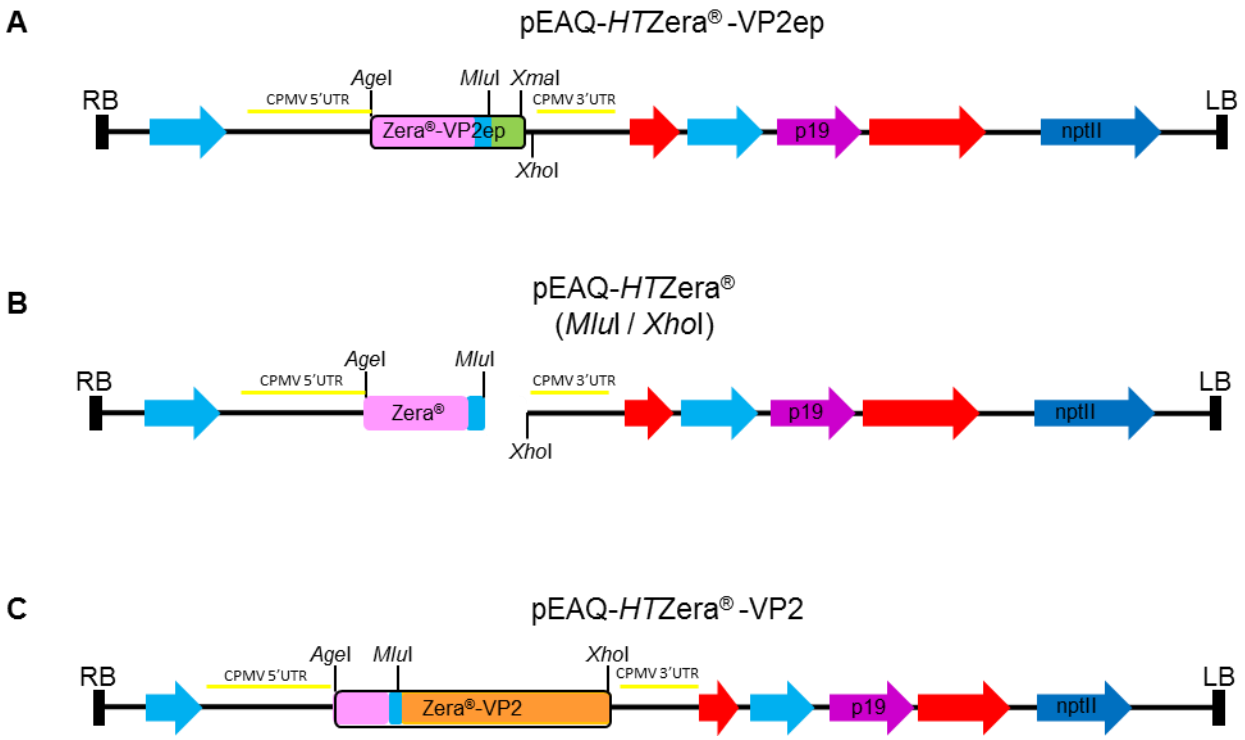

Figure 4.7: A schematic representation of the pEAQ-*HT*Zera®-VP2ep (A) and pEAQ-*HT*Zera®-VP2 (C) plasmids, with the pEAQ-*HT*Zera® (*Mlu*I / *Xho*I) backbone shown in (B). Zera® is represented by the pink box, the linker sequence by the blue box and VP2ep and VP2 is represented by the green and orange boxes, respectively. A detailed map showing all the elements of the pEAQ-*HT* plant expression vector is shown in Figure 2.1 A (Chapter 2).

The pEAQ-*HT*Zera®-VP2 plasmid DNA was successfully transformed into *E. coli* DH5-α competent cells and *A. tumefaciens* LBA4404. The recombinant clones were screened with colony PCR using VP2-specific primers (Table 2.1, cTPVP2coF and cVP2coR). Recombinant colonies resulted in the amplification of expected fragments at 2.8 kb. The recombinant pEAQ-*HT*Zera®-VP2 clone was further verified by restriction enzyme digestion with *Age*I and *Xho*I. Restriction digestion resulted in the expected banding pattern, with the *VP2* insert at 2.8 kb (results not shown).

4.3.5. Optimisation of transient Zera®-VP2ep and Zera®-VP2 expression in *N. benthamiana*

Transient expression of Zera®-VP2ep and Zera®-VP2 in *N. benthamiana* was investigated over a 7 day period, where leaf discs were harvested at 2, 3, 5 and 7 days after infiltration. Plants were infiltrated at an OD_{600} of 0.5, 1.0 and 1.5 with Zera®-VP2ep or Zera®-VP2 to examine the effects of *A. tumefaciens* cell concentration on recombinant protein expression levels. Protein accumulation was analysed with western blotting using rabbit-raised α-VP2R polyclonal serum produced against *E. coli*-expressed wtVP2. Both proteins were detected, with predicted bands of Zera®-VP2ep and Zera®-VP2 at ~16 kDa and ~120 kDa, respectively (Figure 4.8).

Zera®-VP2ep expression was observed on days 2, 3 and 5, with peak expression observed at 3 dpi (Figure 4.8 A) when using an infiltration OD_{600} of 0.5. The infiltrated leaves showed chlorosis with slight necrosis at the infiltration sites at 3 dpi (Figure 4.8 C – top panel), after which the infiltrated leaves became more necrotic. When the cell concentration was increased to an OD_{600} of 1.0 and 1.5, the plants showed severe necrosis with blackening and drying out of the leaves (Figure 4.8 C – top panel). In addition the increased cell concentration resulted in no expression of Zera®-VP2ep at any of the days sampled post infiltration (results not shown).

In contrast, expression of Zera®-VP2 was only detected on western blots by using an infiltration OD_{600} of 1.5 (Figure 4.8 B). Expression was observed from 5 dpi onward, with the highest levels detected at 7 dpi. The high infiltration OD resulted in chlorosis of the leaves at 7 dpi (Figure 4.8 C), however it did not negatively affect expression of Zera®-VP2. Even though the leaves that were infiltrated at lower cell concentrations (Figure 4.8 C) showed similar symptoms of chlorosis, no protein was detected using western blot analysis (results not shown).

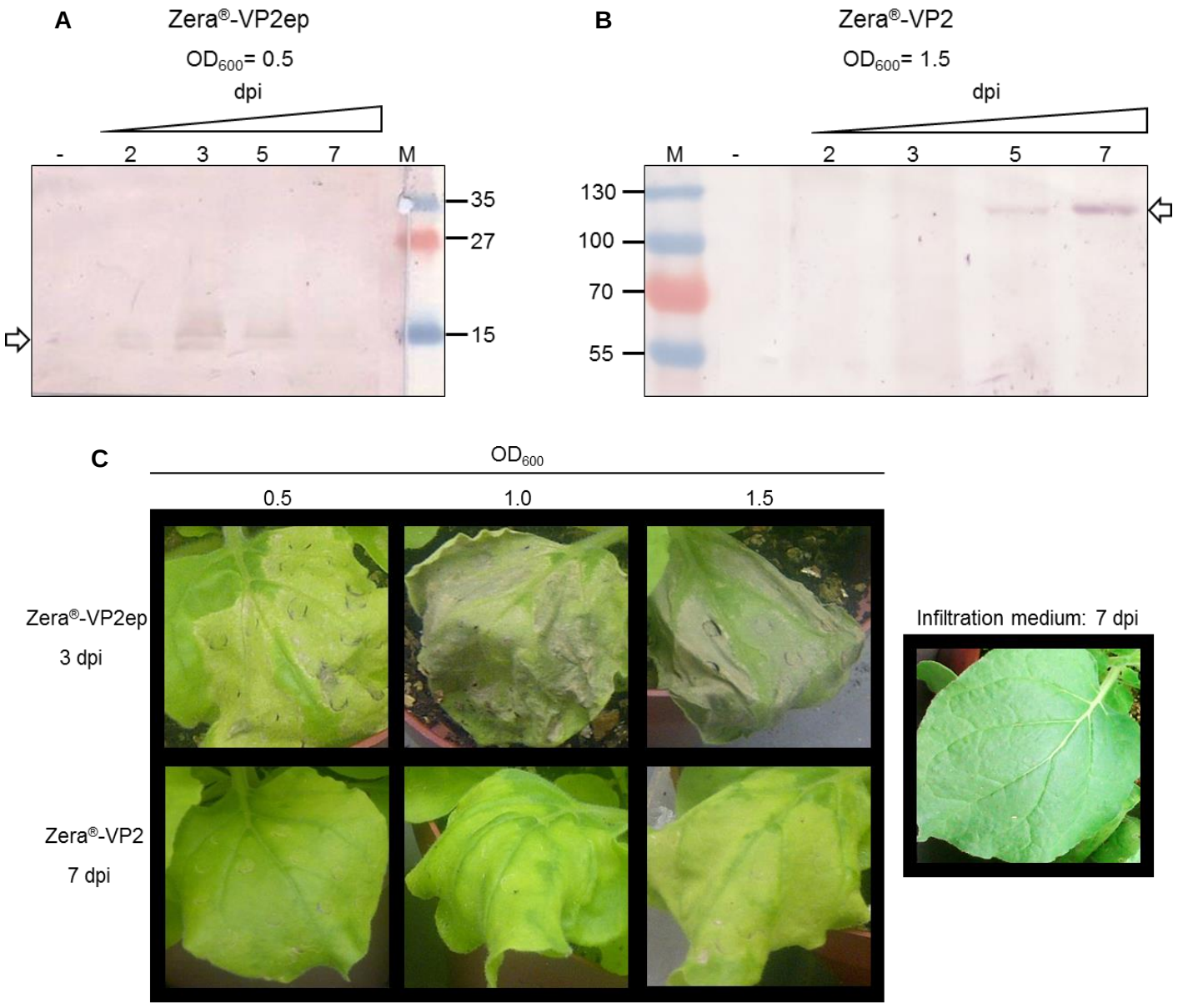

Figure 4.8: Zera®-VP2ep (A) and Zera®-VP2 (B) expression time-trials at infiltration OD_{600} values of 0.5 and 1.5, respectively. Expressed protein in crude leaf extracts were detected by the α-VP2R polyclonal antibody. Negative controls (-) were plants infiltrated with only infiltration medium. White arrows indicate the position of the expressed proteins. Lanes M represent the protein marker with sizes indicated in kDa. (C) *N. benthamiana* leaves infiltrated with different *A. tumefaciens* cell concentrations. Leaves expressing Zera®-VP2ep and Zera®-VP2 were photographed at 3 and 7 dpi, respectively. The negative control shows a leaf infiltrated with only infiltration medium at 7 dpi.

4.3.6. *In situ* characterisation of PBs

Having shown that optimal expression of Zera®-VP2ep and Zera®-VP2 occurred at 3 and 7 dpi respectively, it was undertaken to determine if these polypeptides were sequestered into PBs. Leaves infiltrated with *A. tumefaciens* harbouring the pEAQ-*HT*Zera®-VP2ep and pEAQ-*HT*Zera®-VP2 plasmids were harvested at 3 dpi and 7 dpi respectively, after which the leaf sections were embedded in Spurr's resin and subsequently viewed using TEM (Figure 4.9).

Western blot analysis was carried out on the crude leaf samples with the α-VP2 antibody to confirm that Zera®-VP2ep and Zera®-VP2 were successfully expressed (data not shown).

It has been shown that the Zera® domain containing the $(PPPVHL)_8$ repeats and the Pro-X sequences allow for the accumulation of fusion proteins into membrane-bound protein bodies (Geli *et al.*, 1994; Torrent *et al.*, 2009a). TEM of the embedded leaf material showed the presence of membrane-defined, spherical electron-dense PB-like structures ranging from 0.6 µm to 1 µm in size in the cytoplasm of leaves expressing Zera®-VP2ep and Zera®-VP2 (Figure 4.9 B and C, respectively). These electron-dense structures were embedded in the cytoplasm along with organelles such as mitochondria and chloroplasts. No structures resembling these PBs were observed in any of the negative control samples that were viewed (Figure 4.9 A).

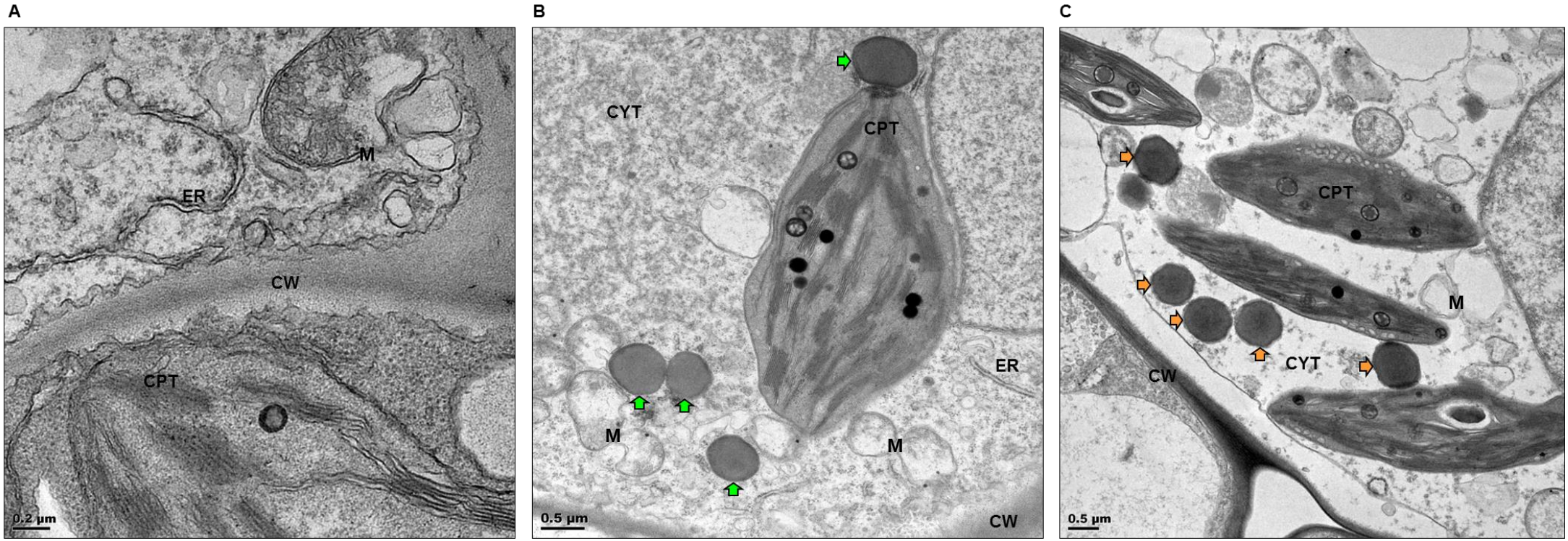

Figure 4.9: Transmission electron micrographs of leaf sections that were infiltrated with (A) infiltration medium, (B) pEAQ-*HT*Zera®-VP2ep (C) and pEAQ-*HT*Zera®-VP2. PBs for both Zera®-VP2ep (B – green arrows) and Zera®-VP2 (C – orange arrows) were present as electron-dense structures within the cytoplasm (CYT) of the infiltrated leaves. No similar structures were present in the negative control samples (A). Scale bars: (A): 0.2 µm; (B) and (C): 0.5 µm. CW: cell wall, CPT: chloroplast, CYT: cytoplasm, ER: endoplasmic reticulum, M: mitochondrion.

4.3.7. Purification of PBs

Since it was shown that Zera®-VP2ep and Zera®-VP2 successfully formed PB-like structures when expressed in *N. benthamiana*, the number of plants infiltrated was scaled up to allow purification of them by density gradient ultracentrifugation.

After homogenisation, the crude plant extract was incubated at 4 °C with gentle agitation to facilitate sufficient extraction of the protein bodies from the plant material. This was followed by clarifying the crude plant sap by filtration and low-speed centrifugation before the supernatant was applied to a sucrose cushion for purification. Plant material infiltrated with infiltration medium only was subjected to the same extraction procedure.

Western blot analysis of the clarified crude protein extracts obtained after low-speed centrifugation showed the presence of bands at ~16 kDa for Zera®-VP2ep (Figure 4.10 A[i]) and ~120 kDa for Zera®-VP2 when using the rabbit-raised α-VP2R polyclonal antibody (Figure 4.10 A[ii]). The detection of an additional band at ~26 kDa on the Zera®-VP2ep western blot (Figure 4.10 A[i], black arrow) could possibly represent oligomerised fusions of Zera®-VP2ep that did not denature completely. No bands were detected in the crude extracts that were obtained from the negative control plants (Figure 4.10 A[i] and [ii]).

Initially it was attempted to purify the PBs using discontinuous sucrose gradients (3 mL each of 19%, 27%, 42% and 56% sucrose in buffer PBP3), however western blot analysis of the fractions collected from the bottom of the tubes after ultracentrifugation showed the presence of Zera®-VP2ep and Zera®-VP2 in all the fractions, including the pellet (results not shown).

Thus, instead of using discontinuous sucrose gradients for purification, the crude negative control, Zera®-VP2ep and Zera®-VP2 plant extracts were overlaid onto 42% sucrose cushions and centrifuged at 79 000 *x g* to facilitate collection of the dense PBs in the pellet. The entire volume of the sucrose cushions was collected from the bottom of the tubes and the pellets resuspended in buffer PBP3. The liquid on top (labelled TOP) of the sucrose cushions was also collected and analysed on dot blots probed with α-VP2R serum.

Dot blots of the fractions collected for the negative control showed no colour development in either the pellet, the 42% sucrose fraction or the top fraction (Figure 4.10 B[i]). These results indicate that the antibody did not react with any of the plant material. Dot blots of Zera®-VP2ep and Zera®-VP2 showed a positive reaction with the pellet sample, indicating successful purification of the recombinant proteins after ultracentrifugation (Figure 4.10 B[ii] and [iii], respectively); no protein was detected in either the top or 42% sucrose fractions.

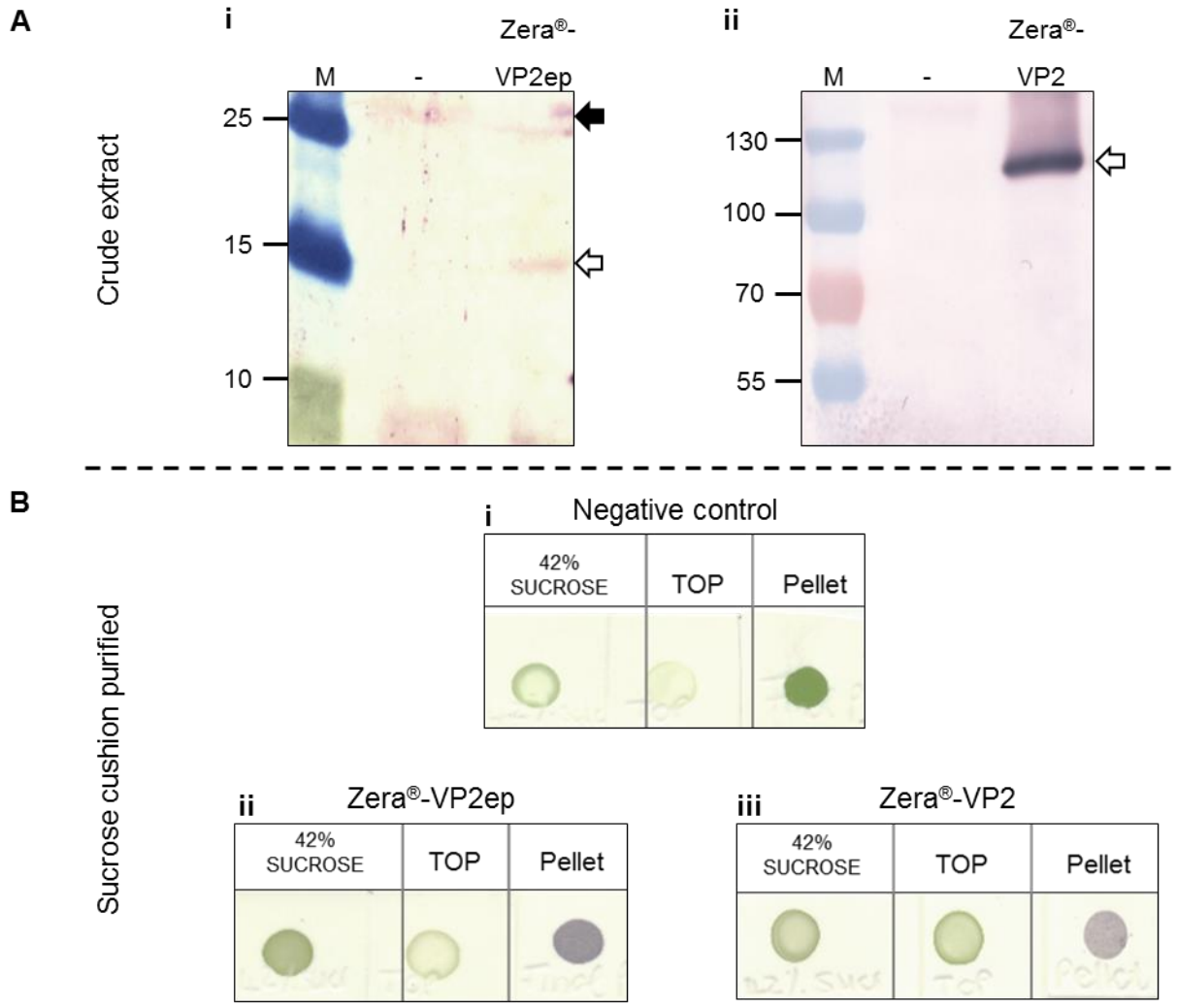

Figure 4.10: (A) Western blot analysis of crude Zera®-VP2ep (i) and Zera®-VP2 (ii) extracted protein using α-VP2R as primary antibody. White arrows indicate the respective proteins with the black arrow showing an oligomerised fusion of VP2ep. In both cases the negative control (-) was plant material infiltrated with infiltration medium only that was extracted using the same method. Lanes M indicate the molecular weight marker. (B) Dot blots of (i) the negative control, (ii) Zera®-VP2ep and (iii) Zera®-VP2 purified using a 42% sucrose cushion.

It was found that the purified PBs were very stable. Western blot analysis of Zera®-VP2ep and Zera®-VP2 purified using 42% sucrose cushions always showed the presence of oligomerised proteins in the denatured samples, even after treating the purified proteins at 90 °C with 5 x sample application buffer containing 500 mM DTT in order to denature them (results not shown).

It was further investigated whether the sequential extraction process described by Joseph *et al.* (2012) would result in the solubilisation of the PBs. The first solubilisation step facilitates the extraction of soluble and membrane-bound proteins, after which the second step extracts proteins that are linked by disulphide bridges. The final heating step should theoretically result in solubilisation of the PB cores (Joseph *et al.*, 2012). After treatment with SDS and DTT, the PBs were mostly present in the final insoluble pellet as seen on the dot blot in Figure 4.11. For both Zera®-VP2ep and Zera®-VP2 protein was detected in the soluble fraction (S), however western blot analysis of these fractions still showed the presence of multiple bands on the blots representing oligomerised fusions of the recombinant proteins that could not be completely denatured (results not shown). After the final solubilisation/centrifugation step the negative control contained no pellet, indicating that whatever protein was present in that sample had been completely solubilised.

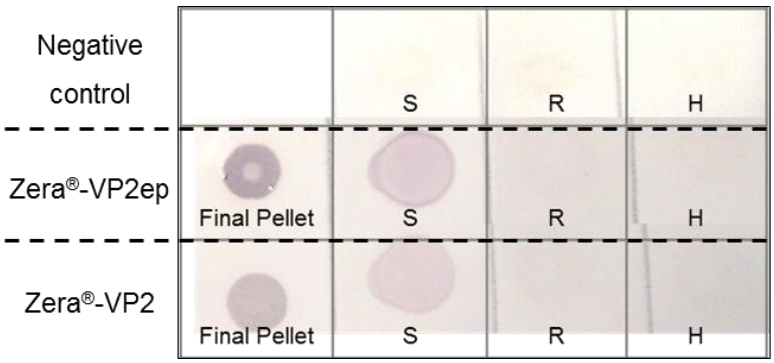

Figure 4.11: A dot blot probed with α-VP2R showing the sequential solubilisation of PBs. The negative control was plant material infiltrated with infiltration medium. S: soluble fraction; R: fraction containing disulphide-linked proteins and H: solubilised protein body cores.

Since the PBs were intended for immunogenic studies in mice, it was not desirable for the extraction buffers to contain reagents such as DTT or SDS, as these might be detrimental to the health of the animals. Therefore, the pellet obtained after ultracentrifugation was washed with buffer PBP3 containing 10% sucrose to stabilise the PBs.

The Zera®-VP2ep pellet was washed six times, and Zera®-VP2 and the negative control samples 10 times each with buffer PBP3 until the final pellet was white. The final pellet obtained after washing was resuspended in 300 µL buffer PBP3 containing 10% sucrose and stored O/N at 4 °C in 1.5 mL microcentrifuge tubes. For both the negative control and Zera®-

VP2ep a white precipitate was present at the bottom of the tubes with green liquid (SNT) containing chlorophyll visible above the precipitate. A sample of the chlorophyll-containing liquid was aspirated for analysis after which the white precipitate was mixed with the liquid resulting in a homogenous solution. In contrast, the resuspended Zera®-VP2 final pellet remained in solution with no precipitate present in the tubes.

The wash samples were analysed with dot blots that were probed with α-VP2R (Figure 4.12 A), BTV-8 sheep serum raised against VLPs (Figure 4.12 B) and BTV-10 guinea pig serum (Figure 4.12 C). Both Zera®-VP2ep and Zera®-VP2 were detected by all the sera used for dot blot analysis (Figure 4.12). The green liquid (SNT) that was collected above the precipitated material in the negative control and Zera®-VP2ep did not react with any of the sera tested, suggesting that the PBs precipitated out of solution after standing O/N.

When probing with α-VP2R (Figure 4.12 A), PBs were only detected in the final pellet for both Zera®-VP2ep and Zera®-VP2. A slight colour reaction was observed for Zera®-VP2ep wash 1, indicating that some of the PBs were solubilised after the first wash. The negative control did not react with the rabbit-raised serum at all.

The BTV-8 sheep serum (Figure 4.12 B) raised against plant-produced VLPs appeared to be more sensitive than the rabbit-raised serum as Zera®-VP2ep was detected in wash 1 and 2, as well as in the final pellet. Zera®-VP2 protein was present in wash 1 – 7, including the final pellet. The detection of the proteins in the wash fractions was possibly the result of gradual solubilisation of the PBs during the wash steps.

The BTV-10 guinea pig serum (Figure 4.12 C) appeared to bind non-specifically to plant proteins, as it showed the presence of protein in wash 1 of the negative control sample. However the results obtained when probing Zera®-VP2ep and Zera®-VP2 were similar to that obtained with the rabbit and sheep serum, showing that the highest concentration of PBs were present in the final pellets.

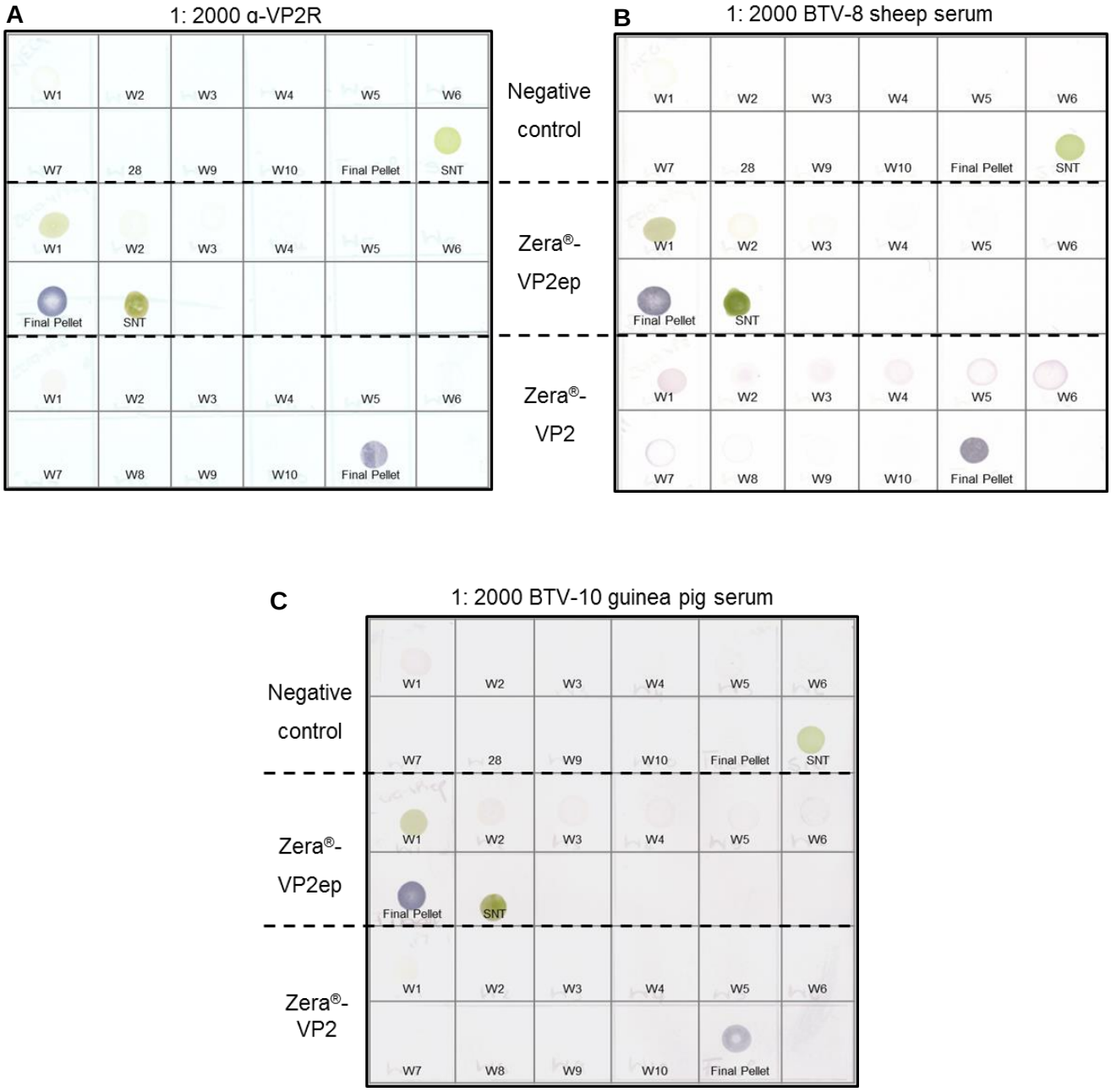

Figure 4.12: Dot blots of the wash fractions of the negative control, Zera®-VP2ep and Zera®-VP2 that were probed with 1: 2000 dilutions of α-VP2R (A), BTV-8 sheep serum raised against plant-produced VLPs (B) and BTV-10 guinea pig serum (C). SNT: green chlorophyll-containing liquid above the white precipitates that were present in tubes containing the negative control and Zera®-VP2ep. W1-W10: wash fractions.

4.3.8. PB quantification

The final resuspended pellets obtained for Zera®-VP2ep and Zera®-VP2 (Figure 4.12) were quantified for testing in subsequent animal experiments (Chapter 5).

Even though bands representing the PBs could not be visualised on Coomassie-stained gels (data not shown), the gels did confirm the absence of contaminating plant proteins in the PB-containing pellets.

Zera®-VP2ep and Zera®-VP2 PBs were quantified by using the Bio-Rad DC Protein Assay kit. The TSP was determined from a standard curve of the average absorbance values obtained for the BSA standards (Figure 4.13).

The TSP of Zera®-VP2ep was calculated to be ~1.39 mg/mL by using both the average absorbance values obtained for Zera®-VP2ep (Figure 4.13, green triangle) and the equation on the chart. The total amount of Zera®-VP2ep PBs that were produced in plants was determined to be ~69.5 mg TSP per kilogram of fresh leaf material.

Similarly, the TSP of Zera®-VP2 was calculated to be ~1.34 mg/mL (Figure 4.13, orange triangle). The total yield of Zera®-VP2 PBs produced in plants was ~35 mg TSP per kilogram of fresh leaf material.

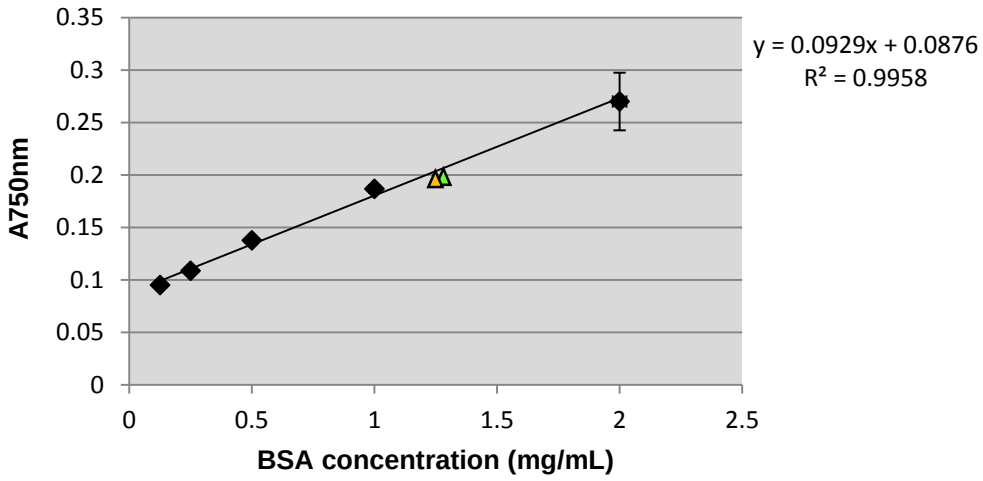

Figure 4.13: TSP quantification of the Zera®-VP2ep and Zera®-VP2 PBs using BSA as a standard. The green and orange triangles indicate the average absorbance values obtained for Zera®-VP2ep and Zera®-VP2, respectively. The error bars indicate the standard deviation.

4.4. Discussion

VP2 is the outermost exposed protein of the BTV virion and it is the most variable protein across the BTV serotypes (DeMaula *et al.*, 2000). Immunologically, VP2 is the most important BTV protein (Cowley and Gorman, 1987; Hassan and Roy, 1999; Marshall and Roy, 1990; Pierce *et al.*, 1995; Urakawa *et al.*, 1994), and VP2 on its own or together with VP5 have been tested as possible vaccine candidates with variable results (Noad and Roy, 2009a; Roy *et al.*, 2009). The aims of this study was to investigate the production of two novel VP2-based vaccine candidates for BTV in *N. benthamiana* that would be cost-effective to produce, easy to purify and DIVA-compliant.

The vaccine candidates designed in this study consisted of a synthetic peptide - VP2ep - constructed from *in silico* epitope predictions of VP2 and the full-length BTV-8 plant codon-optimised VP2. Both the VP2ep and BTV-8 VP2 sequences were fused to Zera® to direct the formation of PBs as both vaccine candidates were aimed at presenting the antigens to the immune system in a particulate form, thereby enhancing the extent of the immune response. In addition, these particular Zera®-fused PB vaccine candidates will allow for DIVA as the commercially available BTV diagnostic tests are mostly based on the detection of the group-specific antigen VP7 (MacLachlan, 1994; Vellema, 2008).

Epitopes are localised regions on the surfaces of antigens that are involved in its recognition by antibodies. These peptide regions also have the ability to elicit an immune response and represent the smallest subunits that can be used therapeutically (Santi *et al.*, 2006; Toussaint *et al.*, 2008). Many advantages such as safety, ease of production and analytical control are associated with the use of epitope-based vaccines: with the presentation of specific epitopes a precise immune response can be directed at conserved and highly immunogenic regions of antigens of interest (Toussaint *et al.*, 2008). B-cell epitopes are parts of antigens that are recognised by the variable regions of antibodies (Sweredoski and Baldi, 2009). Several epitope-based vaccines have been developed for the treatment of various cancers and the prevention of infectious diseases. Epitope-based vaccines for the treatment of ovarian carcinoma, end-stage cervical cancer and melanoma have been successful and have entered or completed phase I and II clinical trials.(Chianese-Bullock *et al.*, 2008; Kenter *et al.*, 2008; Slingluff *et al.*, 2003) Furthermore, an epitope-based vaccine derived from the Epstein-Barr virus latency antigens has been shown to be immunogenic in pre-clinical trials in mice (Depil *et al.*, 2007).

For the Zera®-VP2ep vaccine candidate, the putative B-cell epitopes of VP2 from eight BTV serotypes was predicted using a computational system. Two predicted epitope regions that

were similar for the eight VP2 amino acid sequences were included in design of the epitope-based peptide. Furthermore, to enable detection of the recombinantly expressed fusion protein with the antibodies used in this study, a homologous region consisting of eight conserved amino acids was also included in the peptide sequence. The complete synthetic multi-epitope-based peptide - VP2ep - consisted of a total of 18 amino acids (Figure 4.3).

The VP2ep sequence was successfully synthesized with assembly PCR yielding a final product of ~70 bp (Figure 4.5). VP2ep was fused downstream of the Zera® sequence using assembly PCR, yielding Zera®-VP2ep (Figure 4.6), after which it was successfully cloned into the pEAQ-*HT* plant expression vector (Figure 4.7).

Initial attempts to create a fusion product of Zera® with the full-length plant codon-optimised BTV-8 *VP2* gene by assembly PCR was not successful (results not shown). This was possibly due to the size of the gene (2.8 kb) and the extreme difference in annealing temperatures of the primers used for assembly PCR. Therefore, to make the second vaccine candidate - Zera®-VP2 - VP2ep was excised from the recombinant pEAQ-*HT*Zera®-VP2ep plasmid (Figure 4.7 A) and replaced with the full-length BTV-8 codon-optimised VP2, yielding the construct pEAQ-*HT*Zera®-VP2 (Figure 4.7 C).

Agrobacterium-mediated expression time-trials – where the cell concentration used for infiltration was also analysed - were carried out in *N. benthamiana* for both Zera®-VP2ep and Zera®-VP2. From the time-trials it was determined that the highest levels of Zera®-VP2ep accumulation was achieved at 3 dpi using an infiltration OD_{600} of 0.5 (Figure 4.8 A). Infiltration using an OD_{600} of 0.5 resulted in chlorosis of the infiltrated leaves (Figure 4.8 C – top panel). In contrast, higher cell concentrations (OD_{600} of 1.0 and 1.5) resulted in severe necrosis of the infiltrated leaves at 3 dpi. Moreover, no expression of Zera®-VP2ep was detected after infiltration with the increased cell densities. The fact that the polyclonal rabbit anti-VP2 serum (α-VP2R) that was made against full-length wtVP2 successfully detected the Zera®-VP2ep (Figure 4.8 A) fusion product was very promising. These results confirmed that the epitopes selected for construction of the synthetic epitope-based vaccine were recognised by the antibodies that were present in the serum raised against the “natural” protein.

In contrast to the expression of Zera®-VP2ep, the highest levels of Zera®-VP2 accumulation were observed at 7 dpi (Figure 4.8 B) after using an infiltration OD_{600} of 1.5. The high cell density resulted in chlorosis of the infiltrated leaves (Figure 4.8 C – bottom panel), however it did not negatively affect protein expression. The leaves infiltrated with lower cell densities (OD_{600} of 0.5 and 1.0) developed chlorosis, suggesting expression of proteins, however no

protein could be detected on western blots. It is possible that protein was indeed expressed; however, the levels were too low to be detected using western blots.

When comparing the visible effects of the two fusion products in plants (Figure 4.8 C), it was shown that expression of the epitope-based construct was more detrimental to the plants than expression of the full-length Zera®-VP2 fusion protein. It has been shown that VP2 contains cytotoxic T-lymphocyte epitopes (Andrew *et al.*, 1995): it is possible that some of these cytotoxic epitopes were selected in the design of the epitope-based vaccine and that over-expression of these resulted in necrosis of the plants. Furthermore, it has been postulated that recombinant fusion proteins are accumulated and concentrated to high levels within PBs (Llop-Tous *et al.*, 2010); therefore it is possible that the concentration of the Zera®-VP2ep fusion protein within PBs resulted in necrosis when plants were infiltrated with higher *A. tumefaciens* cell concentrations (OD_{600} of 1.0 and 1.5).

It was shown that transient expression of Zera®-VP2ep and Zera®-VP2 resulted in the formation of intact PBs that were present within the cytoplasm of plant cells as membrane-defined electron-dense vesicles of different sizes (Figure 4.9). These structures were similar in size and morphology to epidermal growth factor PBs produced in SF9 insect cells (Torrent *et al.*, 2009a). Two steps have been described for the formation of PBs: (i) early synthesized Zera® fusion polypeptide chains promote self-assembly of Zera®, resulting in the origin of PBs and thereafter (ii) the continued synthesis of the fusion protein at later stages results in the PB growing by incorporating Zera® fusions into the PBs that were formed during the early stages of expression (Llop-Tous *et al.*, 2010). The different sizes that were observed for the Zera®-VP2ep and Zera®-VP2 PBs (Figure 4.9 B and C, respectively) could be attributed to the occurrence of the two-step process described above.

It was further undertaken to purify Zera®-VP2ep and Zera®-VP2 PBs on a discontinuous sucrose gradient. Analysis of the fractions collected after ultracentrifugation showed that the proteins of interest were present in all the fractions, including the pellet (data not shown). These results could be attributed to the different sizes and therefore densities of early and mature protein bodies. Similar results were obtained by Geli *et al.* (1994) after subcellular fractionation of transgenic plants expressing γ-zein. Therefore, instead of purifying the fusion proteins on a discontinuous gradient, a 42% sucrose cushion was used to separate out contaminating plant proteins form the PBs in the plant extract.

The crude Zera®-VP2ep and Zera®-VP2 plant extracts were analysed with western blotting before purification on the sucrose cushion (Figure 4.10 A). A band of ~16 kDa (Figure 4.10 A[i] – white arrow) confirmed the presence of Zera®-VP2ep in the crude plant sap, an additional

band (almost double the size of Zera®-VP2ep) representing oligomerised fusions of Zera®-VP2ep was detected at ~26 kDa (Figure 4.10 A[i] – black arrow). Western blot analysis of the Zera®-VP2 crude extract showed a single band at ~120 kDa (Figure 4.10 A[ii] – white arrow). Oligomerised fusions were not observed for Zera®-VP2 as an oligomer approximately double the size (~ 240 kDa) of Zera®-VP2 was not visualised on an 8% acrylamide gel. Purification of Zera®-VP2ep and Zera®-VP2 PBs on a sucrose cushion resulted in the sedimentation of the PBs in the pellet (Figure 4.10 B), with no fusion proteins detected in the sucrose cushion or the top fractions. The PBs in the pellet were shown to be very stable (Figure 4.11), as they remained insoluble and oligomerised even after subjection to the sequential extraction process using SDS, DTT and heating as described by Joseph *et al.* (2012).

Recombinantly expressed VP2 is a complex protein that forms multimers stabilised by disulphide bonds (Hassan and Roy, 1999), resulting in the protein being insoluble when expressed in mammalian cells (Capocefalo *et al.*, 2010) and *E. coli* (results not shown). It is possible that the insoluble nature of VP2 combined with the stabilisation afforded by fusion of the protein to Zera®, rendered the fusion proteins completely insoluble and protected them against degradation.

After sucrose cushion purification the pellet containing the PBs was washed multiple times with buffer PBP3 containing 10% sucrose until the resulting pellet was white and free of chlorophyll. The addition of the sucrose further aided in stabilising the PBs during the wash steps. For both the Zera®-VP2ep and Zera®-VP2 wash fractions, dot blots (Figure 4.12) were carried out using α-VP2R (rabbit raised serum against wtVP2), BTV-8 sheep serum (obtained from sheep vaccinated with BTV-8 VLPs) and BTV-10 guinea pig serum. All the antibodies mainly detected the fusion proteins within the final pellet obtained after numerous wash steps. Even though washing of the PB pellets resulted in removal of the chlorophyll present after sucrose cushion purification, it did not result in the solubilisation of either Zera®-VP2ep or Zera®-VP2.

Using TSP quantification, it was shown that ~69.5 mg of Zera®-VP2ep and 35 mg of Zera®-VP2 per kilogram of fresh leaf material was purified from plant material after transient expression in *N. benthamiana*. It is possible that the lower yields obtained for Zera®-VP2 could be attributed to the larger size of VP2 (111 kDa) compared to the small 2 kDa size of the synthetic epitope; furthermore the extremely insoluble nature of Zera®-VP2 possibly resulted in an under-estimation of the total TSP present in the sample.

In conclusion, two novel particulate VP2-based vaccine candidates were successfully expressed in *N. benthamiana* and purified using ultracentrifugation. A novel synthetic epitope

based on the amino acid sequences of eight BTV serotypes was synthesized from predictions obtained using computational software. The Zera®-VP2ep and Zera®-VP2 fusion proteins were successfully detected with rabbit raised serum against the full-length wtVP2, BTV-8 sheep serum raised against BTV-8 VLPS and BTV-10 guinea pig serum. These results showed that antibodies in the sera were able to recognise and bind to the synthetic epitope, suggesting that regions encoding immunogenic epitopes were accurately selected for design of this vaccine candidate. These results are promising, as success of the epitope-based vaccine could obviate the need for vaccination with a mixture of multiple serotypes since it contains putative epitopes for at least eight BTV serotypes. Furthermore, it was shown that the fusion of both VP2ep and BTV8-VP2 to the Zera® sequence resulted in the formation of electron-dense PBs, or StorPro® organelles, when expressed in *N. benthamiana*. The particulate nature of both Zera®-VP2ep and Zera®-VP2 makes these fusion proteins easy to purify and could possibly enhance their immunogenicity (Torrent *et al.*, 1994).

Since both vaccine candidates were shown to be particulate and both antigens could be detected with antisera raised against full-length wtVP2, BTV-8 VLPs and BTV-10, it was undertaken to preliminarily investigate the immunogenicity of these fusion proteins in mice (reported in Chapter 5).

Chapter 5:

Preliminary investigation of the immunogenicity of novel plant-produced candidate vaccines

5.1. Introduction

The existence of 26 serotypes of Bluetongue virus (Maan *et al.*, 2011a; Maan *et al.*, 2011b) complicates control of the disease as animals need to be vaccinated against whichever strains are co-circulating in affected areas. Even though VLP-based vaccines are excellent vaccine candidates, they are not DIVA vaccines, ie. naturally infected animals cannot be distinguished from vaccinated animals with the commercially available competitive enzyme linked immunosorbent assays (cELISA) that detects the presence of VP7 group-specific antibodies in sera (MacLachlan, 1994; Vellema, 2008).

Vaccine antigens are selected by their ability to be immunogenic. VP2 is the major serotype-specific antigen of BTV and a very important component of the vaccines against BTV. Studies have shown that a subunit VP2 vaccine was able to induce neutralising antibodies in sheep (Huismans *et al.*, 1987; Roy *et al.*, 1990b). Chapter 4 of this thesis describes the development of a multivalent DIVA vaccine based on the fusion of three putative B-cell VP2 epitopes predicted for eight BTV serotypes using *in silico* methods. The putative predicted epitopes (VP2ep) and full length VP2 sequences were successfully fused to Zera® to direct protein body formation and these PBs could potentially enhance the immune response.

According to ERA Biotech (http://www.erabiotech.com/index.php/applications/zera-vaccines/protein-particle-vaccines) vaccine antigens fused to Zera® have the ability to induce humoral immunity. Moreover, these antigens have been shown to have intrinsic adjuvant properties, suggesting that the administration of adjuvant with the vaccine formulation is not needed (Whitehead *et al.*, 2014). It was shown in Chapter 4 that the epitope-based vaccine antigen (Zera®-VP2ep) could be detected using rabbit-raised serum obtained from animals inoculated with full-length BTV-8 wtVP2 as well as BTV-8 VLP-vaccinated sheep serum.

BTV is an interferon alpha (IFN-α) inducer in adult mice, which plays an essential role in the antiviral innate immune response. A BTV infection model was established in mice that are deficient in the alpha/beta interferon receptor (IFNAR $^{(-/-)}$) making the mice unable to establish an antiviral state, therefore rendering them susceptible to BTV infection (Calvo-Pinilla *et al.*, 2009). This is a very useful system, especially when performing challenge experiments which

involve live BTV. However, in the present study BALB/c mice were used as test animals since this preliminary study was aimed at evaluating the ability of the plant-produced VLPs, Zera®-VP2ep and Zera®-VP2 candidate vaccines to stimulate humoral anti-VP2 immune responses. The immune responses were evaluated by indirect ELISA.

5.2. Materials and Methods

5.2.1. Immunisation of mice

Female BALB/c mice obtained from South African Vaccine Producers (SAVP, Johannesburg, South Africa) were housed in filter top micro-isolator cages under Biosafety Level 2 (BSL-2) conditions in the Animal Unit at the Health Science Faculty, University of Cape Town. Approval for this study was granted by the Animal Research Ethics Committee at the University of Cape Town (AEC# 011-016).

Female mice (6-8 weeks old) were immunised with the vaccine candidates prepared as described in Chapters 3 and 4 to evaluate the immunogenicity of the plant-produced BTV-8 VLPs and the particulate Zera®-VP2ep and Zera®-VP2 candidate vaccines. Dulbecco's Phosphate Buffered Saline (DPBS, Sigma) was used as a negative control. Vaccination details are shown in Table 5.1 below.

Table 5.1: Plant-produced vaccine candidates used in the immunogenicity study.

Vaccine	Group number [1](n=5)	Adjuvant	Antigen dose (µg)
BTV-8 VLPs	G1	N/A	10
BTV-8 VLPs	G2	IFA	10
Zera®-VP2ep	G3	N/A	10
Zera®-VP2	G4	N/A	10
DPBS	G5	N/A	N/A

1. n = number of mice

The immunogenicity study was carried out in duplicate (Experiment 1 and 2), with each experiment containing a total of 25 mice, divided into 5 groups (G1-G5) of 5 mice each. The TSP of the vaccine candidates (VLPs: Chapter 3, Section 3.3.5; Zera®-VP2ep and Zera®-VP2: Chapter 4, Section 4.3.8) was determined using the Bio-Rad DC Protein Assay kit. The vaccine candidates (G1, G3 and G4) were prepared so as to contain 10 µg antigen in 100 µL DPBS. Only one vaccine candidate, BTV-VLPs (G2), was administered in the presence of Incomplete Freund's Adjuvant (IFA, Sigma-Aldrich). This vaccine was adjusted to contain 10

µg antigen in 50 µL DPBS, after which IFA in a 1:1 ratio was added and the emulsion homogenised by vortexing.

Pre-bleeds were collected from each mouse via the saphenous vein three days prior (Day -3) to vaccination. The vaccine candidates were administered by subcutaneous injection into both the right and left flanks (50 µL each) on Day 0. The mice were boosted on Day 28 with doses containing 10 µg of the appropriate antigens. Final bleeds were obtained via cardiac puncture at Day 56. Serum was isolated from the blood and stored at -80 °C.

5.2.2. ELISA detection of antibodies in mouse sera

5.2.2.1. Indirect ELISA for detection of anti-VP2 antibodies

The anti-VP2 response was determined by indirect ELISA. 96-well Maxisorp® microtitre plates (Nunc) were coated with 100 µL/well (1 µg) of *E. coli*-produced BTV-8 wtVP2 (Chapter 4, Section 4.2.10.1) diluted in coating buffer (10 mM Tris, pH 8.5) and incubated O/N at 4 °C. The plates were blocked with TBS blocking buffer (5% non-fat dry milk in 1x TBS [50 mM Tris, 150 mM NaCl, pH 7.5]) for 2 hrs at RT after which it was washed four times with 1x TST buffer (1x TBS [pH 7.5], 0.05% Tween®20).

To evaluate the anti-VP2 immune response elicited by each mouse, the pre- and final bleed sera were diluted 1:50 in 100 µL TBS blocking buffer and added to each well, after which the plates were incubated for 2 hrs at RT. Blank wells containing no antibody were included as background control. The plates were washed four times with 1x TST buffer and 100 µL of goat anti-mouse IgG alkaline phosphatase conjugate (1:10 000, Sigma) diluted in blocking buffer was added per well and incubated for 1 hr at 37 °C. After incubation the plates were washed four times with 1x TBS (pH 9) buffer and 200 µL SIGMAFAST™ p-Nitrophenyl phosphate (pNPP, Sigma) was added per well. The plates were developed in the dark for 30 min after which the absorbance was detected at 405 nm on a BIO-TEK® Powerwave XS microtitre plate reader.

To determine the anti-VP2 binding titres, mouse sera from each group were pooled into vaccine groups (5 mice/vaccine) for analysis. Final bleed mouse sera were diluted in TBS blocking buffer in a 4-fold series in triplicate ranging from a 1:50 dilution to 1:51 200. Mouse sera from the mice vaccinated with DPBS served as a negative control. Positive control wells to confirm that the ELISA was successful contained sheep serum that was isolated from sheep

previously inoculated with plant-produced BTV-8 VLPs and blank wells with no antibody were included for background control in all the ELISAs. The indirect ELISA was carried out as described above and the anti-VP2 binding titres were expressed as a reciprocal of the maximum serum dilution which produced absorbance values that were three times greater than the corresponding pre-bleed serum diluted at 1:50.

5.2.2.2. Statistical analysis

To calculate the statistical significance of the final bleed anti-VP2 responses between the vaccines and the negative control, a Kruskal-Wallis (KW) test was performed (due non-normal and heteroscedastic data) to compare medians among groups. Statistical analysis was carried out using STATISTICA 64 (StatSoft, Inc.).

5.2.2.3. cELISA for detection of VP7 serogroup-specific antibodies

Final bleed sera for all the vaccine candidates were analysed with the commercially available competitive ELISA (cELISA) BTV Antibody Test Kit (VMRD, Inc.). For this assay plates are coated with VP7, the serogroup-specific BTV antigen. In the cELISA the VP7 serogroup-specific antibodies present in the test sera compete with the horseradish peroxidase (HRP)-conjugated monoclonal VP7 antibody for binding to the antigen (www.cabi.org/ahpc. www.vmrd.com).

The cELISA was carried out according to the manufacturer's instructions, each final bleed serum sample was analysed in triplicate. Positive and negative control samples were included in the test kit. Sera obtained from the Zera®-VP2ep, Zera®-VP2 and DPBS vaccinated mice served as additional negative controls for the cELISA, since the inocula did not contain VP7. The absorbances were determined at 620 nm on a BIO-TEK® Powerwave XS microtitre plate reader.

5.2.3. Western blot detection of anti-VP2 antibodies

E. coli-produced BTV-8 wtVP2 antigen (Chapter 4, Section 4.2.10.1) or plant-produced BTV-8 VP3 (Chapter 2, Section 2.3.5) was incubated at 90 °C for 10 min in sample application

buffer (Sambrook *et al.*, 1989). The proteins (~3 µg TSP each of the *E. coli*-expressed wtVP2 and plant-expressed wtVP3) were separated on 8% SDS polyacrylamide gels, after which they were transferred onto nitrocellulose membranes as described in Chapter 2, Section 2.2.6.

After transferring the proteins onto nitrocellulose membranes, the sections incorporating the 100 kDa to 170 kDa proteins (where VP2 and VP3 were expected to be visualised) were incubated in blocking buffer for 4 hrs at RT. Individual pre- and final bleed sera from both experiments were pooled into vaccine groups (10 mice per vaccine) and diluted 1:100 in blocking buffer. The membrane strips were probed O/N at 4 °C with 1:100 dilution of BTV-8 VLP sheep serum as positive control and the pooled mouse sera.

Following incubation in the primary antibody the strips were washed four times, 15 min each, in blocking buffer. The strips were probed for 1 hr at 37 °C with either anti-goat/sheep alkaline phosphatase-conjugated secondary antibody (1:10 000, Sigma-Aldrich) or anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (1:10 000, Sigma) for the positive control or mouse sera, respectively. The strips were washed four times, 15 min per wash, with 1x PBST and developed using BCIP/NBT (KPL).

5.3. Results

5.3.1. Humoral immune response against BTV-8 VP2

5.3.1.1. Indirect ELISA detection of anti-VP2 antibodies

The humoral anti-VP2 immune responses elicited in the mice were analysed by indirect ELISA, using *E. coli*-produced BTV-8 wtVP2 as coating antigen. Initial analysis of the pre-and final bleed sera of the individual mice showed the presence of anti-VP2 antibodies in only the final bleed sera of mice vaccinated with the Zera®-VP2ep and Zera®-VP2 candidate vaccines, therefore mouse sera from each group were pooled (5 mice/vaccine) for analysis of the anti-VP2 binding titres. In contrast, mice vaccinated with the VLP-based candidate vaccines showed no anti-VP2 response since the absorbance values obtained for the final bleed sera were similar to those obtained for the pre-bleed sera as well as the values obtained for the DPBS vaccinated control mice (Figure 5.1).

The anti-VP2 titres of the mouse sera were determined for the VLP (with and without IFA), Zera®-VP2ep and Zera®-VP2 vaccinated mice. The antibody binding titres were expressed as the reciprocal of the maximum serum dilution containing absorbance values that were three times greater than the corresponding pre-bleed serum at 1:50.

No anti-VP2 response was detected for the vaccine pre-bleeds (Figure 5.1 A) and the negative control vaccine (DPBS, Figure 5.1 C). The positive control indirect ELISA using sheep serum (from sheep vaccinated with plant-produced VLPs) showed a good anti-VP2 response (Figure 5.1 B), thereby validating the indirect ELISA. The VLP vaccine candidates administered in the absence and presence of IFA (Figure 5.1 C; yellow and green lines, respectively) elicited no anti-VP2 immune response with titres similar to the values obtained for the pre-bleed sera and DPBS negative control (Figures 5.1 A and C). However, the Zera®-VP2ep vaccine elicited anti-VP2 titres of 800, with an OD_{405} of ~0.177 (Figure 5.1 C, blue line) compared to an OD_{405} of ~0.0039 obtained for the corresponding pre-bleed serum (Figure 5.1 A, blue marker). In addition, the Zera®-VP2 vaccine candidate elicited anti-VP2 binding titres of 3200 at an OD_{405} of ~0.0097 (Figure 5.1 C, pink line), compared to an OD_{405} value of ~0.0016 obtained for the corresponding pre-bleed serum (Figure 5.1 A, pink marker).

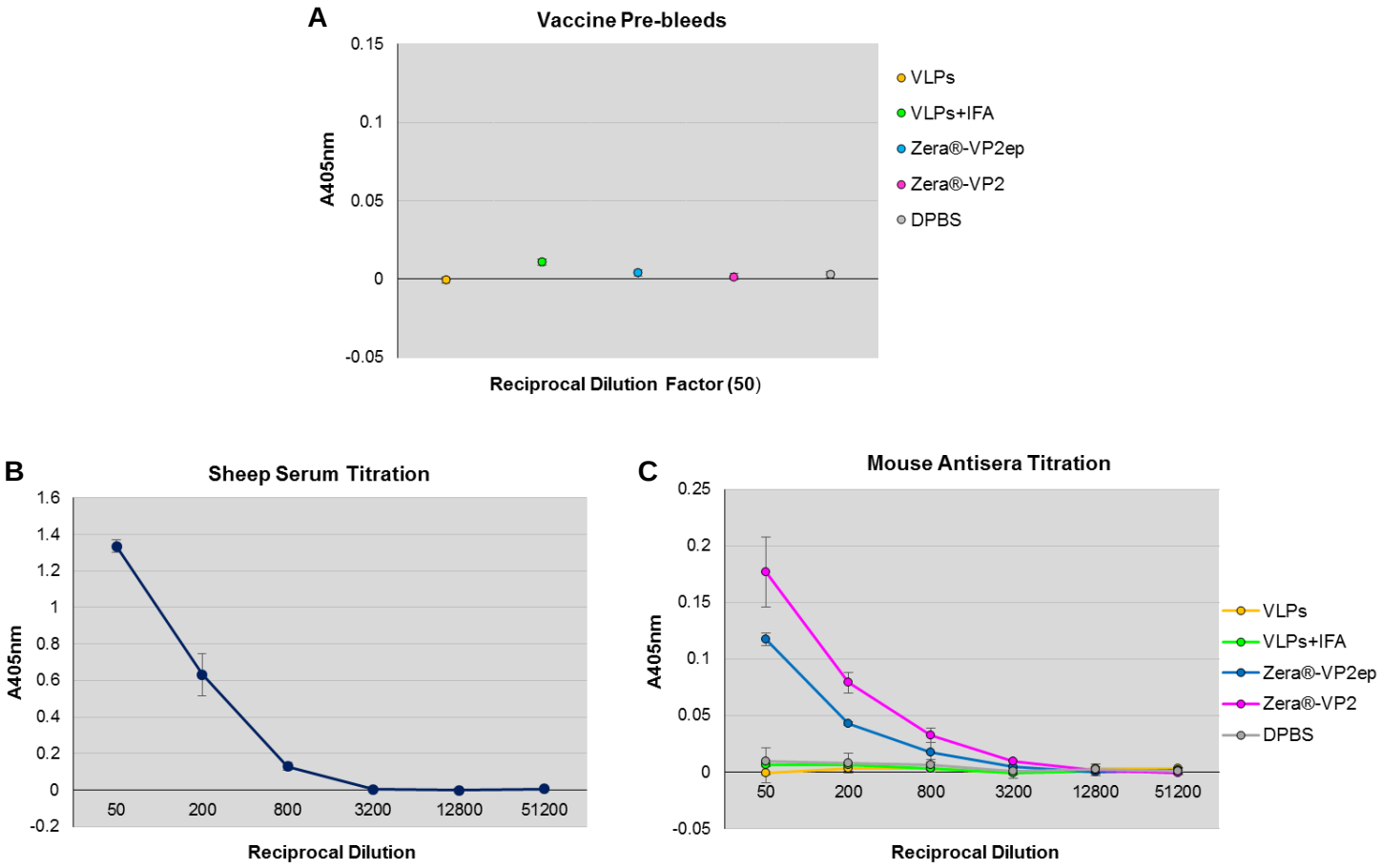

Figure 5.1. Indirect ELISA of mouse sera using *E. coli*-produced wtVP2 as antigen. (A) Absorbance values of the vaccine pre-bleeds at 1:50 dilution. (B) Titration of the ELISA positive control sheep serum produced against BTV-8 VLPs to validate the indirect ELISA. (C) Titration of the mouse antisera produced against the VLPs (yellow line), VLPs+IFA (green line), Zera®-VP2ep (blue line), Zera®-VP2 (pink line) and DPBS (negative control, grey line) vaccine candidates. The markers indicate the mean value of triplicate samples from both experiment 1 and 2, and error bars indicate the standard deviation.

A box whisker plot statistical analysis (Figure 5.2) of the anti-VP2 responses elicited by the Zera®-VP2ep and Zera®-VP2 vaccine candidates, showed that the antibody levels were significantly higher for both vaccines compared to the DPBS negative control (Figure 5.2: KW; H = 22.002, N = 30, $p < 0.0001$). Since no anti-VP2 immune response was detected for VLP vaccinated mice, therefore these sera were not included in statistical analysis.

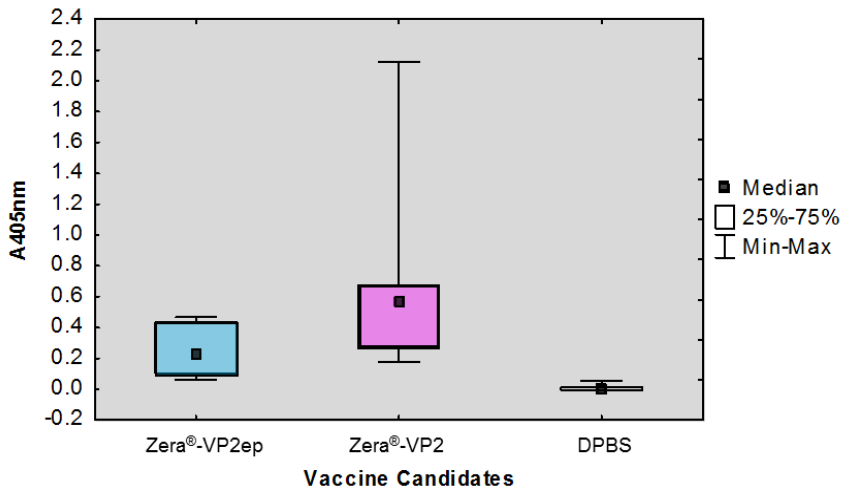

Figure 5.2: A box whisker plot comparing the results of the Zera®-VP2ep (blue box) and Zera®-VP2 (pink box) vaccines with the DPBS (grey box) vaccinated mice to determine the statistical significance of the immune responses. The boxes (blue, pink and grey) indicate the values within which 25-75% of the data are confined. The black boxes indicate the median values and the minimum and maximum values are displayed by the whiskers (vertical bars).

5.3.1.2. Western blot detection of *E. coli*-expressed wtVP2

The ability of the antibodies produced against the two significant test vaccines (Zera®-VP2ep and Zera®-VP2) to recognise BTV-8 wtVP2 was further analysed by evaluating the anti-VP2 humoral immune response of the Zera®-VP2ep and Zera®-VP2 vaccine candidates on western blots using *E. coli* produced BTV-8 wtVP2 as antigen. This BTV-8 wtVP2 protein (~163kDa, Section 4.2.10.1) was expressed as a fusion product with a trigger factor chaperone and a translation enhancing element to achieve high level, soluble expression in *E. coli* (http://www.takara-bio.com).

Individual pre-and final bleed mice sera from both experiments were pooled for each vaccine candidate and analysed for anti-VP2 responses (Figure 5.3).

In Figure 5.3 *E. coli*-expressed wtVP2 (~163kDa, white arrow) was detected with the positive control sheep serum raised against plant-produced BTV-8 VLPs and the final bleed sera obtained from Zera®-VP2ep and Zera®-VP2 vaccinated mice. Non-specific protein bands smaller than the ~163 kDa wtVP2 band were detected with all the serum samples, including the positive control. These bands possibly represent truncated versions of the *E. coli*-expressed wtVP2. It has been shown that heterologous expression in *E. coli* often results in

the expression of truncated versions of the expressed proteins due to abundant codons found in heterologous genes which cause translational stalling and termination of protein expression (Sørensen *et al.*, 2003). No non-specific protein bands were detected with the pre-bleed sera.

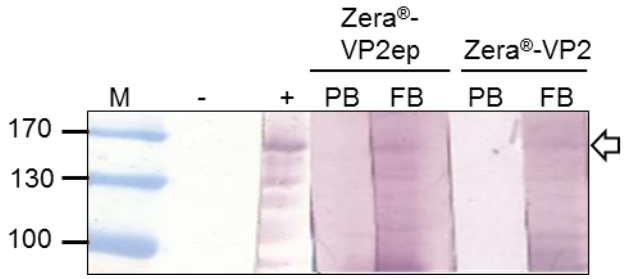

Figure 5.3: Western blot detection of *E. coli*-expressed wtVP2 with 1:100 dilution of pooled mice sera from animals vaccinated with the Zera®-VP2ep and Zera®-VP2 vaccines. Lane M represents the molecular weight marker. The negative control (-) was performed with no primary antibody and sheep serum obtained from BTV-8 VLP vaccinated sheep was used as the positive control (+). Lanes PB and FB represent the pre-and final bleed sera respectively. The white arrow indicates *E. coli*-expressed wtVP2 at ~163 kDa.

5.3.2. Analysis of the humoral immune response elicited by the VLP-based vaccine candidates

5.3.2.1. cELISA detection of anti-VP7 antibodies

Since no anti-VP2 response was detected for the VLP-based vaccines (in the absence and presence of IFA), it was proposed that this was a result of instability of the VLPs and that the outer capsid proteins, VP2 and VP5, had degraded or dissociated from the CLPs by the time the mice were vaccinated. To determine if CLPs, consisting of VP3 and VP7, were present in the vaccine dose at the time of vaccination a cELISA (VMRD, Inc) was carried out on the pooled final bleed sera of all the vaccine candidates using the commercially available cELISA kit (Figure 5.4).

This cELISA is based on antibodies in the test sera competing with the HRP-conjugated monoclonal VP7 antibody for binding to the antigen. Therefore, sera containing anti-VP7 antibodies produces lower OD_{620} values than 50% of the mean negative as the antibodies in the sera outcompete the HRP-labelled antibody for binding to the antigen.

cELISA analysis of the test sera indicated that mice vaccinated with VLPs (Figure 5.4, without [yellow bar] and with IFA [green bar]) produced antibodies against VP7. Lower absorbance values were obtained with the sera of mice vaccinated with VLPs in the presence of IFA, suggesting that the presence of adjuvant enhanced the immune response. As expected, no anti-VP7 response was measured in the kit negative control (light blue bar) as well as the sera obtained from Zera®-VP2ep (blue bar), Zera®-VP2 (pink bar) and DPBS (grey bar) vaccinated mice.

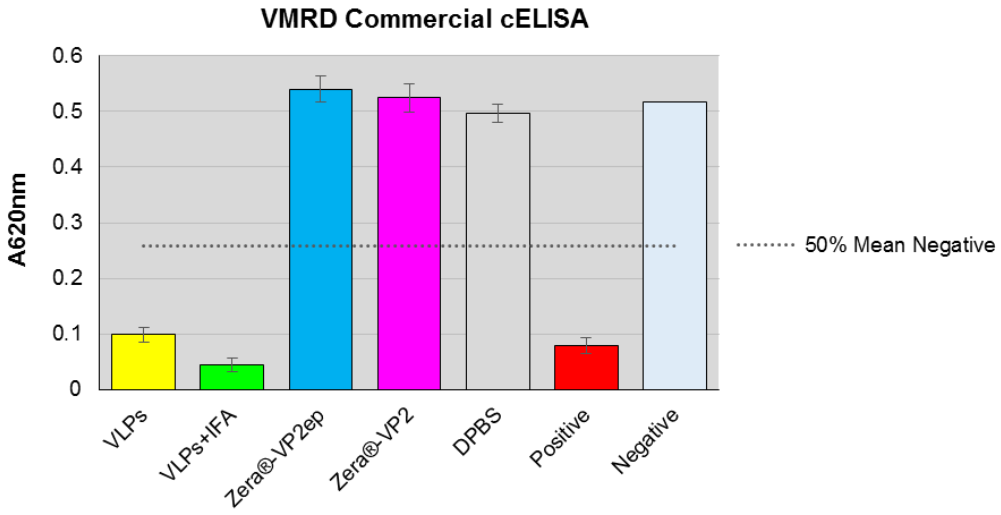

Figure 5.4: Commercial cELISA of mouse sera using VP7 (www.cabi.org/ahpc; www.vmrd.com) as antigen. The dotted line at OD_{620} = ~0.26 represents 50% of the mean values obtained for the negative control. Samples are positive for anti-VP7 antibodies if OD_{620} < 50% of the mean negative control and negative samples have OD_{620} values ≥ 50% of the mean negative control. Positive and negative controls were included in the cELISA kit. Error bars indicate the standard deviation.

5.3.2.2. Western blot detection of plant-expressed BTV-8 VP3

Since it was shown with the commercial cELISA that VP7 was present in the VLP vaccines, it was undertaken to determine if VP3 was still present in the vaccine candidate. These experiments were carried out as there were concerns that the VLPs had degraded after storage at 4 °C. Pooled antisera from mice vaccinated with the VLP candidate vaccine (without and with IFA) was analysed with western blots where plant-expressed BTV-8 VP3 was used as antigen (Figure 5.5)

The VLP-based vaccines - administered without and with IFA - elicited an anti-VP3 immune response, as VP3 bands were detected at ~102 kDa (Figure 5.5, white arrow) using the antisera and positive control sheep serum. The anti-VP3 immune response in mice vaccinated with only VLPs was weaker than that of the mice vaccinated with VLPs in the presence of IFA as seen in Figure 5.5. No bands were detected with the pre-bleed sera.

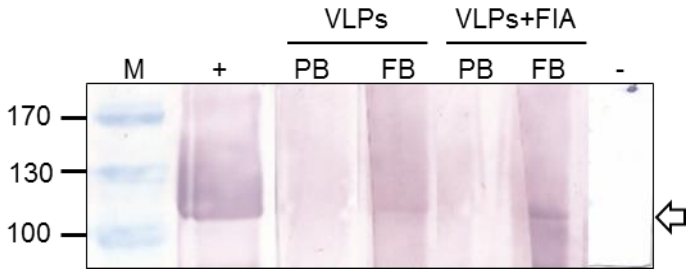

Figure 5.5: Western blot detection of plant-expressed VP3 with 1:100 dilution of pooled mice sera from animals vaccinated with the VLP-based vaccines (without and with IFA). Lane M represents the molecular weight marker. The negative control (-) was performed with no primary antibody and sheep serum obtained from BTV-8 VLP vaccinated sheep was used as the positive control (+). Lanes PB and FB represent the pre-and final bleed sera respectively. The white arrow indicates plant-expressed VP3 at ~102 kDa.

5.4. Discussion

The most effective means of controlling BT is through vaccination and many vaccine candidates have been developed against BT – with varying degrees of success (Stewart *et al.*, 2012). Recombinant vaccines are promising vaccine candidates as they have the potential to be safer than attenuated vaccines with no risk of reassortment and they can be DIVA compliant (Calvo-Pinilla *et al.*, 2012; Noad and Roy, 2003)

In this study, a preliminary investigation of the immunogenicity of three novel plant-produced vaccine candidates was carried out. BALB/c mice were subcutaneously vaccinated with 10 µg each of the VLP vaccine (without and with IFA) and the protein body (Zera®-VP2ep and Zera®-VP2) vaccine candidates. The mice received booster vaccinations containing 10 µg of plant-produced antigen after 28 days.

The detection of anti-VP2 antibodies in mouse antisera was done by indirect ELISA and western blotting using *E. coli*-produced wtVP2 as antigen. The vaccine pre-bleeds and negative control vaccine (DPBS) gave no anti-VP2 responses (Figure 5.1 A and C, respectively). For the VLP vaccine candidate no anti-VP2 immune response was detected in mice vaccinated with either the VLPs on their own or in the presence of IFA (Figure 5.1 C), suggesting that the outer capsid protein – VP2 – was not present at the time of vaccination.

To explain this result, we propose that the lack of anti-VP2 immune responses in VLP-vaccinated mice could have been a result of the vaccine doses not consisting of intact VLPs as the particles were not stable during storage at 4 °C, which resulted in the dissociation and degradation of the outer capsid proteins, VP2 and VP5, from the scaffolding provided by CLPs. For VLPs to be suitable as vaccines, it is vital that their structural integrity is preserved during purification and storage and that conditions for maintaining this need to be resolved. Stability of the BTV VLPs could possibly be enhanced by storage in polyols such as sorbitol and trehalose; which are natural substances found in plants, fungi and algae (Bolen, 2004). These polyols have been shown to be safe for use as stabilisers in Norwalk VLP (Kissmann *et al.*, 2008) and the rotavirus VLP vaccine (Peixoto *et al.*, 2007) preparations. Furthermore, it has been reported by Lynch *et al.* (2012) that the addition of 15% trehalose to human immunodeficiency virus (HIV) VLPs resulted in the particles retaining their original appearance over a period of 12 months. The temperature at which VLPs are stored can also play a role in maintaining their structural integrity and it is important that stability studies are carried out at different temperatures over a period of time.

Further analysis of the serum from mice inoculated with VLPs using the cELISA kit (VMRD, Inc) showed the presence of anti-VP7 antibodies (Figure 5.4). Furthermore antibodies in this

serum was also successful in detecting plant-produced VP3 antigen (~102 kDa) with western blot analysis (Figure 5.5). Antisera from mice vaccinated with VLPs showed that the immune response was enhanced when VLPs were administered in the presence of IFA (Figures 5.4 and 5.5). These results further support the abovementioned proposition that the VLPs used for vaccination of the mice were not stable and that the outer capsid proteins had dissociated and degraded leaving only intact CLPs (VP3 and VP7) in the vaccine preparation.

Both the Zera®-VP2ep and Zera®-VP2 vaccine candidates successfully detected *E. coli*-expressed BTV-8 wtVP2 indicating the stimulation of production of antibodies against this protein. The Zera®-VP2ep and Zera®-VP2 vaccine candidates elicited anti-VP2 immune responses with binding titres of 800 and 3200, respectively (Figure 5.1 C). Furthermore, the anti-VP2 responses measured for antisera from both protein body vaccine candidates were shown to be significant ($p < 0.0001$) in comparison to the negative control (Figure 5.2).

Zera®-VP2ep and Zera®-VP2 antisera successfully detected *E. coli*-expressed BTV-8 wtVP2 (Figure 5.3) on a western blot. The final bleed antisera from both vaccine candidates and the positive control sheep serum detected wtVP2 at ~163 kDa; bands representing truncated versions of the *E. coli*-expressed wtVP2 was also detected. No non-specific bands were detected with the pre-bleed sera.

Unfortunately, the lack of an anti-VP2 response in mice inoculated with VLPs (with and without IFA) precluded the comparison of the immune responses measured in mice vaccinated with Zera®-VP2ep and Zera®-VP2.

In conclusion, Zera®-VP2ep and Zera®-VP2 - the two novel protein body vaccine candidates encoding the predicted putative B-cell epitopes of VP2 and full-length codon-optimised VP2 fused to Zera® to make particulate vaccines – were successful in eliciting anti-VP2 humoral immune responses in mice. Results from the indirect ELISA and western blots showed that antibodies raised against the putative multi-epitope, VP2ep (predicted using *in silico* methods, Chapter 4) were able to bind the full length recombinant wtVP2, further supporting the findings (Chapter 4) that immunogenic-specific epitope sequences were successfully predicted and included in the design of this vaccine. Moreover, the reaction of the antibodies in both ELISA and western blots means the antibodies elicited in vaccinated mice would almost certainly bind native protein as well as denatured protein, meaning they have the potential to protect against infection.

Future research of the Zera®-VP2ep vaccine should include studies to determine if the antiserum produced in this study is successful in detecting VP2 from other BTV serotypes,

thereby confirming the multivalency of the epitope-based vaccine candidate. The Zera®-VP2ep vaccine candidate shows potential for further development as a BTV vaccine that is safe, multivalent and DIVA compliant.

Chapter 6:

General discussion and conclusions

6.1. General discussion

The incursion of BTV-8 into northern Europe in 2006 resulted in significant economic losses due to mortality of affected livestock, but more importantly due to the ban of ruminant trade between affected and non-affected regions (Maan *et al.*, 2010). In an effort to limit the direct losses incurred with BTV infection and to minimize circulation of the virus, vaccination of livestock was undertaken by European countries (Noad and Roy, 2009a; Savini *et al.*, 2008). The types of BTV vaccines that are currently available for use in the European community and in South Africa include inactivated virus vaccines and MLV vaccines (Coetzee *et al.*, 2012; Dungu *et al.*, 2004b; Niedbalski, 2011; Noad and Roy, 2009a; Savini *et al.*, 2008). The disadvantages associated with these vaccines include: high cost of production, the risks of reassortment with wild type field strains or reversion to virulence, and lastly, the inability to distinguish naturally infected from vaccinated animals with current serological methods (Batten *et al.*, 2008b; Di Emidio *et al.*, 2004; Roy *et al.*, 2009; Savini *et al.*, 2008).

The problems associated with the use of MLV and inactivated vaccines have led to the consideration of recombinant vaccines for the control of BTV. Various recombinant vaccines have been developed, with VLP-based vaccines being the most promising candidate (Noad and Roy, 2003; Roy and Noad, 2008; Stewart *et al.*, 2013). Even though the insect cell-produced VLPs are effective they are not commercially available, possibly because their cost of production is very high and production of vaccines for different serotypes requires different clones, therefore this system is not economically feasible for producing vaccines against multiple serotypes. Alternative methods for the large scale production of these vaccines is desirable. Plant-based protein expression provides a cost-effective method for large scale production of vaccine antigens (Fischer *et al.*, 2004; Rybicki, 2010; Sainsbury and Lomonossoff, 2008). Recently BTV-8 VLPs were successfully produced via transient expression in *N. benthamiana*, and were shown to elicit protective immunity in vaccinated sheep (Thuenemann *et al.*, 2013). The promise of plant-produced VLPs, led to the present study which investigated optimisation of transient expression of BTV-8 VLPs, as well as the expression of two novel recombinant BTV vaccine candidates that presents VP2 to the immune system in particulate form.

The aims of this study were to compare transient expression of the BTV-8 plant codon-optimised capsid protein genes using different plant expression vectors with a view to forming VLPs which could be potentially used as a vaccine. The best expression vector from this study was selected for further optimisation to increase VLP expression levels and subsequent purification which would be appropriate for dosing and which would facilitate quantitation and characterisation of the VLPs. This study was taken further to produce two novel second generation plant-produced vaccines that utilise the immunogenic capacity of VP2. These vaccine candidates were based on the fusion of multiple B-cell epitopes predicted for VP2 and the full length BTV-8 VP2 fused to a signal peptide that facilitates protein body (PB) formation. The epitope-based vaccine would be simpler and more cost-effective to produce at a large scale as it requires expression of only one recombinant *A. tumefaciens* construct, compared to two or four *A. tumefaciens* constructs required for heterologous expression of VLPs. Lastly, it was undertaken to analyse the immunogenicity of the VLP and PB vaccine candidates in mice.

Expression levels of proteins can be increased by using vectors that target expressed proteins to different organelles within the plant cell (Maclean *et al.*, 2007). Therefore, *A. tumefaciens*-mediated transient co-expression of the BTV-8 capsid proteins in *N. benthamiana* was compared using several different expression vectors available in-house. Expression with the pTRA vectors, which target heterologous proteins to the cytoplasm, chloroplast and apoplast (Maclean *et al.*, 2007), was compared to expression with the pEAQ-*HT* vector, which targets expressed proteins to the cytoplasm (Sainsbury *et al.*, 2009). This is the first study which directly compares co-expression of the four BTV-8 plant codon-optimised capsid proteins using different plant expression vectors. By adjusting the infiltration ratio of the recombinant *A. tumefaciens* constructs harbouring the four capsid genes to 1:1:2:1 (VP2:VP3:VP5:VP7) and by increasing the cell concentration used for infiltration, it was shown that the pEAQ-*HT* vector was the only vector that could successfully co-express all four capsid proteins at detectable levels. Although the pTRA vectors were able to express the proteins separately, none were able to co-express all four capsid proteins simultaneously. Thus, the pEAQ-*HT* vector was therefore selected as the most suitable vector for co-expression of BTV-8 VLPs.

Agrobacterium-mediated transient expression of BTV-8 VLPs with the pEAQ-*HT* vector construts was further optimised by investigating longer expression times as well as the ratios used for infiltration of the four *A. tumefaciens* constructs harbouring the recombinant pEAQ-*HT* plasmids. The best co-expression and purification of VLPs were found to be after 9 days using a relative vector infiltration ratio of 1:1:2:1 (VP2:VP3:VP5:VP7). Both continuous and discontinuous iodixanol gradients were investigated for the purification of VLPs, and it was

shown with TEM that discontinuous gradients were important for the purification of structurally intact VLPs. These results were similar to sucrose gradient purification experiments performed for insect cell-produced VLPs (Stewart *et al.*, 2010). It was further undertaken to determine where in the plant cell the VLPs were localised. TEM analysis of infiltrated leaf sections showed that co-expression of the four capsid proteins resulted in assembly of higher order structures. Mixed populations consisting of large numbers of CLPs and VLPs were present as paracrystalline arrays within the cytoplasm of plant cells. To the best of our knowledge, this is the first report showing assembled CLPs and VLPs within plant cells. Similarly, French and Roy (1990) showed that CLPs aggregated as paracrystalline arrays within the cytoplasm of insect cells co-expressing VP3 and VP7. The final yield of purified VLPs using the optimised protocol was ~27 mg TSP per kilogram of fresh leaf tissue. This was 2.5 times less than the ~70 mg VLPs per kilogram of fresh weight plant material obtained by expression with the pEAQexpress vector (Thuenemann *et al.*, 2013). However, these yields were sufficient to provide doses for testing the immunogenicity of the VLP vaccine candidate.

Even though recombinant VLPs are excellent vaccine candidates (Crisci *et al.*, 2012), they can only provide protection against one of the 26 serotypes of BTV, unless they are administered as a cocktail containing VLPs representing multiple serotypes of the virus. The possibility of instability of the VLPs could result in the existence of a mixture of CLPs and VLPs within a vaccine preparation which presents a problem when vaccinating animals, as these vaccines cannot be tested by the user for the presence of all four proteins in the field prior to vaccination. Furthermore, even though VLP vaccines are DIVA compliant when using nucleic-acid type assays (Hofmann *et al.*, 2008; Mayo *et al.*, 2010), at present the commercially available serological BTV diagnostic tests are mostly based on the detection of the group-specific antigen, VP7 (MacLachlan, 1994; Vellema, 2008). Thus, when animals are vaccinated with recombinantly produced VLPs, antibodies against VP7 will react with the group-specific antigen in the serological diagnostic tests resulting in the inability to distinguish between vaccinated and naturally infected animals.

Therefore, in an effort to find alternative, possibly multivalent vaccine candidates for BTV it was undertaken to design and express two novel second-generation vaccines in *N. benthamiana*. The first vaccine is based on synthetic B-cell epitopes predicted for multiple serotypes of VP2 by using online computational methods; the second vaccine is based on incorporation of the full length BTV-8 VP2 only. By utilising the immunogenic properties of only VP2, these vaccine candidates were aimed at being DIVA-compliant using current commercially available serological diagnostic tests which are dependent on detecting the

presence of VP7. The putative multi-epitope (VP2ep) and full length BTV-8 VP2 were both fused to a synthetic peptide, Zera®, to induce accumulation of the proteins in dense PBs called StorPro® organelles (ERA Biotech, Spain). PBs have been shown to enhance immunogenicity of the fusion proteins by presenting the recombinant protein to the immune system in particulate form. Both Zera®-VP2ep and Zera®-VP2 proteins were successfully expressed in plants and it was shown with TEM that both these constructs directed the accumulation of proteins in electron-dense granules – StorPro® organelles – that were surrounded by a membrane, within the cytoplasm of plant cells. Antibodies raised against the full length wild type BTV-8 VP2, BTV-8 sheep serum raised against VLPs (Thuenemann *et al.*, 2013) and BTV-10 guinea pig antiserum were able to detect Zera®-VP2ep with western– and dotblot analysis. These results were very promising, as it suggested that the predicted putative VP2 multi-epitope contained regions that were able to bind to the antibodies produced against native VP2 in the antisera and it can potentially be used to stimulate antibodies against BTV VP2 representing other serotypes.

It was further demonstrated that the PBs were easily purified on a sucrose cushion due to their dense nature. The purified PBs were extremely stable and insoluble, even after treatment with SDS, DTT and heat. The sequestration of proteins in PBs that are membrane-delimited has been shown to protect fusion proteins from proteolytic degradation (Torrent *et al.*, 2009a). The stability of the PBs is ideal for vaccines, as it means the vaccine candidates can be stored at 4 °C for extended periods of time without the risk of degradation or proteolysis. Expression and purification of the two PB candidate vaccines, Zera®-VP2ep and Zera®-VP2, resulted in yields of ~69.5 mg and ~35 mg TSP per kilogram of fresh leaf material. Another major advantage of using the Zera®-VP2ep and Zera®-VP2 vaccines is the ability to distinguish vaccinated from infected animals by using the commercially available cELISA.

Preliminary tests to analyse the immunogenicity of the VLPs, Zera®-VP2ep and Zera®-VP2 candidate vaccines were carried out in BALB/c mice. It was shown by Thuenemann *et al.* (2013) that VLPs produced using the pEAQexpress expression vector were stable and they were able to elicit neutralising antibody responses in sheep. However, in our study, analysis of the VLP vaccine candidates with and without adjuvant showed that the particles produced were not stable as they had degraded to their core components, VP3 and VP7, by the time of vaccination. It is possible that the VLPs produced by Thuenemann *et al.* (2013) were more stable than the VLPs produced in this study because both inner and both outer capsid genes were expressed from the same T-DNA, ensuring that stable protein complexes from within the plant cells expressing these genes. In contrast, this proof of concept study showed that both the Zera®-VP2ep and Zera®-VP2 candidate vaccines elicited significant anti-VP2 humoral

immune responses in the vaccinated mice. Both these vaccines were administered without adjuvant as it has been shown that Zera® has adjuvanting properties and that it is able to enhance antibody responses in subunit vaccines (Whitehead *et al.*, 2014). These novel PB vaccine candidates have great potential for the use of BTV vaccines, as they are quick and simple to produce and purify. Furthermore, it was shown that these PB vaccine candidates are extremely stable, making them ideal as rapid response vaccines.

6.2. Future research and conclusions

This study was successful in achieving the primary aims of this study, which included identifying the best plant expression vector available to us for the production of BTV-8 VLPs. A purification method was developed to produce sufficient levels of particles for testing in mice. Moreover, two novel PB vaccine candidates, Zera®-VP2ep and Zera®-VP2, were designed and successfully tested for expression in *N. benthamiana.* Continued research is required to determine the efficacy of these novel PB vaccine candidates. This could include investigating alternative expression vectors, such as the MagnICON® expression system to increase expression levels of the PB vaccine candidates.

VLPs were successfully produced in this study; however they were not stable. It is suggested that the pEAQexpress plant expression system described by Thuenemann *et al.* (2013) - where two proteins are expressed from a single T-DNA - be investigated further as a rapid response system for the production of VLP vaccines for BTV. This could possibly be achieved by always using the more conserved proteins VP3, VP5 and VP7 as a "universal core" and interchanging the variable, serotype specific protein, VP2, with VP2 from whichever strain of BTV is circulating at the time of an outbreak.

It is important to determine if the epitope-based vaccine candidate, Zera®-VP2ep, is able to bind antibodies from different BTV serotypes, this would be done by serum neutralisation assays and ELISAs to determine if the predicted epitope is able to cross-react with other BTV serotypes, including BTV-8. By incorporating a wider range of the predicted VP2 B-cell epitopes within the synthetic peptide, cross-reactivity of the candidate vaccine with more serotypes could be achieved. Should the Zera®-VP2ep vaccine candidate be successful, it could obviate the need for vaccination with a cocktail of BTV serotypes, resulting in "broad-spectrum" protection.

Similar to the VLP-based vaccine, the Zera®-VP2 candidate vaccine should also be investigated as a rapid response vaccine, as it is straightforward to replace the current BTV-8

VP2. It was shown by Roy *et al.* (1990b) that vaccination of sheep with both recombinant VP2 and VP5 resulted in complete protection of vaccinated animals, suggesting it could be possible to further enhance the immune response obtained with Zera®-VP2ep and Zera®-VP2 by creating fusion proteins that include VP5.

In conclusion, to the best of our knowledge, this is the first study that directly compares co-expression of the BTV capsid proteins using different expression vectors in plants. This is also the first report that provides TEM evidence of the accumulation of assembled CLPs and VLPs within the cytoplasm of plant cells co-expressing the four BTV-8 capsid proteins. Additionally, this is the first study to investigate the design, expression and immunogenicity of two novel BTV PB vaccine candidates. Importantly, Zera®-VP2ep is the first multi-epitope-based vaccine to be described for BTV.

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