# Investigation of particulate Bluetongue virus vaccines made in plants

By

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# Declaration

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# Abstract

Bluetongue virus (BTV) infects ruminants but predominantly causes severe and often fatal haemorrhagic fever, known as Bluetongue (BT) disease, in sheep. Increasing global temperatures have contributed to the global dissemination of BTV. This is a result of the *Culicoides* insect vectors which function optimally in warm and wet climates. In South Africa, the eradication and control of BTV is made difficult due to the circulation of 21 of the 28 known serotypes and their limited serological cross-reactivity. Current vaccine strategies include live attenuated and inactivated vaccines. Although they have been successful in protecting animals, there are many limitations and risks associated with these vaccine strategies such as reversion to virulence, re-assortment and short-lasting immunity. There is thus a need for vaccines that are safe, scalable, economically viable and effective against multiple serotypes.

A variety of recombinant BTV vaccine strategies have been developed which address and improve upon some of the limitations of the commercially available vaccine strategies. One of the most promising strategies is that of the virus-like particle (VLP). BTV VLPs comprise four structural proteins, namely VP2, VP3, VP5 and VP7. VP2 is highly immunogenic and is responsible for eliciting neutralising antibodies against the virus. Although a number of different BTV VLPs have been produced in traditional protein expression systems, such as insect cells, these production methods are considered too expensive to compete with those of the commercial vaccines. This has led to the consideration of plants as an alternative vaccine expression system. Plants are easily scaled up, upstream processes are cost effective, they are easy to work with, and do not require sterile environments and expensive infrastructure to maintain. A number of studies have shown that when the four major BTV structural proteins are transiently co-expressed in *Nicotiana benthamiana*, they self-assemble into VLPs which can be used as vaccines to protect animals against homologous serotypes.

The aim of this study was to develop and compare two different particulate BTV candidate vaccines made in plants and determine their ability to elicit specific immunity in guinea pigs. The first vaccine approach was to develop a chimeric BTV VLP vaccine. This was achieved by substituting the immunogenic tip domain of the *VP2* gene of BTV serotype 8 (BTV8) with that of the corresponding domain of BTV serotype 1 (BTV1) generating a chimeric VP2 which, when co-expressed in plants with the remaining BTV8 VP3, VP5 and VP7, resulted in chimeric BTV1/8 VLPs. The second approach involved the display of the immunogenic BTV1 VP2 tip domain on a capsid protein particle. Here, the domain was displayed on the surface of the bacteriophage AP205 particle through the application of the SpyTag (ST)/SpyCatcher (SC) bioconjugation method. It was anticipated that these vaccine candidates would be safe to use and allow for rapid production and scalability. Moreover, only a small fragment of the BTV8 *VP2* gene would need to be modified to allow for a VLP to be made against a new serotype.

Chimeric BTV1/8 VLP protein expression in plants, VLP extraction, and purification protocols were optimised using previously made homologous BTV8 VLPs for comparison. Plants were vacuum co-infiltrated with the chimeric BTV1/8 VP2 protein and the BTV8 VP3, VP5 and VP7 proteins. Protein was extracted from the plants and VLPs were subsequently purified by density gradient ultracentrifugation. The VLP proteins were detected on Western blots and Coomassie-stained gels. These methods were optimised to maximise protein yields by increasing the salt concentration of the extraction and

purification buffer, maintaining an alkaline pH throughout the extraction and maturation process and harvesting at five days post-infiltration. Transmission electron microscopy (TEM) confirmed the presence of a mixture of core-like particles (CLPs), assembly intermediates and fully formed VLPs. These optimised methods were sufficient to produce high enough yields of BTV8 and BTV1/8 VLPs with protein yields of 35mg/kg fresh leaf weight (FLW) and 34mg/kg FLW, respectively, to be used in immunogenicity trials in guinea pigs.

The alternative vaccine strategy involved the display of the BTV1 VP2 tip domain on phage AP205 particles. We utilised the ST/SC antigen display technology for the display of the BTV1 antigenic tip domain on the surface of the AP205 capsid. ST was fused to the N-terminal of the AP205 protein (ST-AP205) while SC was fused to the C-terminal of the BTV1 VP2 tip domain used in the chimeric VLPs (BTV1Tip-SC). Both components were expressed in plants and extracted and purified separately before combining for *in vitro* coupling. The ST-AP205 particles were purified by density gradient ultracentrifugation while the BTV1Tip-SC proteins were purified by nickel affinity chromatography. The purified components were coupled *in vitro* in a molar ratio of 1:3 (ST-AP205:BTV1Tip-SC). The 60kDa coupled complex was detected on Western blots and Coomassie gels with an estimated protein concentration of approximately 0.03ug/uL and a coupling efficiency of 44%.

Finally, since there was insufficient coupled protein product for immunisation doses, only the immunogenicity of the plant produced chimeric BTV1/8 VLPs compared with the BTV8 VLPs were tested. Five guinea pigs per vaccine group (BTV8 VLPs and chimeric BTV1/8 VLPs) were immunised with 15ug of the appropriate vaccine and boosted 13 days later. Serum was collected 41 days post immunisation and used to determine whether there was an immunogenic response to the vaccines by Western blotting and indirect enzyme-linked immunosorbent assays (ELISAs). This preliminary immunogenicity trial found that both VLP candidate vaccines induced an immune response in guinea pigs. While the BTV1 VP2 tip display vaccine strategy still requires further optimisation to generate more dose-appropriate yields, the VLP vaccine strategy tested here shows great potential for further development into a BTV vaccine candidate that is safe, scalable and has potential for multivalency.

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# Abbreviations

| | |
|---|---|
| Ad | adenovirus |
| AEC | animal ethics committee |
| AHSV | african horse sickness virus |
| APCH | antigen presenting cell homing |
| BCIP | 5-bromo-4-chloro-3-indoxyl-phosphate |
| BP | base pairs |
| BRU | biopharming research unit |
| BSA | bovine serum albumin |
| BT | bluetongue disease |
| BTV | bluetongue virus |
| CIDR | cysteine-rich inter-domain region |
| CHO | chinese hamster ovary |
| CLP | core-like particle |
| CP | capsid protein |
| DISA | disabled infectious single animal |
| DISC | disabled infectious single cycle |
| DIVA | differentiate infected from vaccinated animal |
| DPI | days post-infiltration |
| DTT | dithiothreitol |
| EDTA | Ethylenediaminetetraacetic acid |
| EHD | epizootic hemorrhagic disease |
| ELISA | enzyme-linked immunosorbent assay |
| EV | empty vector |
| FDA | food and drug administration |
| FLW | fresh leaf weight |
| GFP | green fluorescent protein |
| h | hour/s |
| HIV | human immunodeficiency virus |
| IFNAR | interferon-alpha/beta receptor |
| IgG | immunoglobulin G |
| KDa | kilodaltons |
| LB | luria bertani |
| LBB | luria bertani-based broth |
| MES | morpholineethanesulforic acid |
| MHC | major histocompatibility complex |
| min | minute/s |

| | |
|---|---|
| MLV | modified live virus |
| MVA | modified vaccinia virus ankara |
| MW | molecular weight |
| NBT | nitroblue tetrazolium |
| NLS | N-lauroyl sarcosine |
| NPT | neomycin phosphotransferase |
| NS | non-structural |
| NTC | no template control |
| OBP | onderstepoort biological products |
| PAGE | polyacrylamide gel electrophoresis |
| PB | protein body |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| RAF | research animal facility |
| RB | right border |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| s | second/s |
| SA | south africa |
| SAB | sample application buffer |
| SAVP | south african vaccine producers |
| SC | spycatcher |
| SCLP | sub-core-like particles |
| SDS | sodium dodecyl sulphate |
| SEM | standard error of the mean |
| SNA | serum neutralisation assay |
| SNT | serum neutralisation test |
| ST | spytag |
| TBS | tris-buffered saline |
| T-DNA | transfer deoxyribonucleic acid |
| TEM | transmission electron microscope |
| UCT | university of cape town |
| UTR | untranslated region |
| UV | ultraviolet |
| VLP | virus-like particle |
| WNV-EDIII | west nile virus envelope protein domain II |

# Chapter 1: Literature Review

## 1.1 Bluetongue Virus and Disease

### 1.1.1 Classification

Bluetongue virus (BTV) is a non-enveloped dsRNA virus that is classified as being part of the *Reoviridae* family and *Orbivirus* genus. BTV is the type species of *Orbiviruses*: it has a dsRNA genome consisting of 10 segments encapsulated by an icosahedral protein capsid (Mertens et al., 2004). It is an arthropod-borne virus which is transmitted by haematophagous *Culicoides* spp. midges. This midge also transmits the similar and economically relevant African horse sickness virus (AHSV) (Mellor, 2000).

### 1.1.2 Diversity of serotypes

The eradication and control of BTV is a challenge due to the existence of at least 28 different serotypes and their limited serological cross-reactivity (Rao et al., 2017, Belbis et al., 2017, Bumbarov et al., 2020). Serotypes are determined by the variability of segment 2 of the genome which translates into the VP2 protein. A virus belongs to a specific serotype if the antibodies induced by said virus are able to neutralise viruses known to belong to the specific serotype (Rao et al., 2017).

South Africa continues to have the greatest diversity of BTV with at least 21 serotypes in circulation and approximately 3-5 serotypes involved in each seasonal outbreak. This is likely a result of changes in herd immunity to the serotypes (Coetzee et al., 2012). Although BTV was first described in South Africa (SA) in 1880 (Verwoerd, 2012) and was endemic there until 1943 when an outbreak was reported in Cyprus (Rao et al., 2017), it is likely that the virus had been circulating in wild ruminants before it was first reported. Since then the virus has spread via the *Culicoides* insect vector, and less often by transported animals and meat, to all continents except Antarctica (Maclachlan, 2010). Dispersal can also occur passively when the midges become aerial plankton in strong winds (Mellor et al., 2000). The same serotype in different parts of the world is not necessarily identical. There are certain geographical regions that are separated enough to allow for nucleotide sequence variation within the genome of a serotype. These strains are called topotypes and are typically regarded as either Western or Eastern (Maan et al., 2010).

### 1.1.3 Transmission

BTV distribution is dependent on that of the female *Culicoides* midge. Although these midges are the smallest hematophagous flies measuring at 1-3mm in length, over 50 arboviruses have been isolated from *Culicoides* species including AHSV, epizootic haemorrhagic disease (EHD) virus and BTV (Mellor et al., 2000). Their activity levels are affected by light intensity, temperature and wind speed (Mellor et al., 2000). Increasing global temperatures due to climate change have contributed to the global dissemination of BTV which is explained by the capacity of the insect vectors to function optimally in warm and wet climates (Dommergues et al., 2019, El Moustaid et al., 2021). Outbreaks of Bluetongue disease (BT) occur seasonally, usually around late summer and autumn, when there are large populations of the vector (Coetzee et al. 2012).

Specific vector-competent species are responsible for the transmission of BTV. The predominant vector species differs according to region. In Africa, *C. imicola* is the major vector for BTV with numerous isolations in SA, Zimbabwe, Kenya and Sudan. In South Africa the predominant vector

species include *C. imicola* and *C. bolitinos* (Coetzee et al., 2012). The presence of *C. imicola* in these regions and surrounds is indicative of the presence of BTV (Mellor, 1990). However, there are regions in Europe, for example, where *C. imicola* is not present and different species are responsible for transmission (Maclachlan, 2011). *C. bolitinos* is a major vector found in colder regions as it more effectively supports BTV replication at low temperatures (Venter et al., 1994).

Midges become carriers of BTV when taking a blood-meal from infected ruminants and ruminants become infected when fed on by midges carrying BTV. When the midge takes a blood-meal, the virus replicates both in the mesenteron cells of the gut and in the salivary glands of the insect (Mellor, 2000). The viral concentration rises until 7-9 days post-infection after which it plateaus and remains for the duration of the insect's life. At this stage, the viral load is approximately $10^3$ to $10^4$-fold greater than at the day of infection. Transmission to ruminants occurs around 10-14 days post infection once the virus has replicated within the midge's salivary glands (Mellor, 2000).

There is evidence, however, that BTV can, under certain circumstances, spread via seminal fluid or across the placenta. Saegerman et al. found that trans-placental infection took place in sheep naturally infected with BTV8. Virus was found in aborted foetuses which had foetal malformations. Antibodies for BTV8 were found in viable lambs before colostrum intake. Trans-placental infection thus could allow for over-wintering in pregnant sheep and their lambs during the colder months when there is low vector transmission (Saegerman et al., 2011). This kind of transmission has also been found to occur in cattle (De Clercq et al., 2008, Menzies et al., 2008)

### 1.1.4 Infection

Sub-clinical infection is common amongst cattle and goats whereas sheep are more likely to develop clinical disease that is severe and often fatal (Schwartz-Cornil et al., 2008). Cattle and several other ruminants are considered to act as natural reservoir hosts as they often do not develop symptoms yet have high levels and longer periods of viremia than sheep (Caporale et al., 2014). It has been found that this is a result of the virus's ability to persist in indentations within the cell membranes of non-replicating lymphocytes and erythrocytes. Here the virus is not accessible to neutralising antibodies and the virus can persist without causing major cell damage (Brewer et al., 1994, MacLachlan, 1994). This makes it difficult to eradicate the virus completely as these animals allow overwintering to occur and are often excluded from vaccine regimes.

Once a ruminant is infected with BTV, the virus is transported from the skin to the draining lymph nodes in migratory dendritic cells (DCs). Here the virus undergoes primary replication. DCs can be infected and support viral replication while simultaneously carrying out their normal function of inducing an immune response. Thus, dissemination can occur via DCs and the virus can spread to secondary sites of infection such as the lungs, liver and spleen (Hemati et al. 2009). In addition to DCs, BTV primarily replicates in mononuclear phagocytes and endothelial cells and is able to persist in erythrocytes (Schwartz-Cornil et al., 2008).

### 1.1.5 Symptoms

Bluetongue disease (BT) is described as a haemorrhagic fever but clinical signs can range from mild to severe depending on the serotype of the virus and on the age and health status of the host (Schwartz-Cornil et al., 2008). Common clinical signs include pyrexia, lethargy, haemorrhage into the lymph nodes, heart and lungs and necrosis of oro-nasal mucosal surfaces and the alimentary organs. A

cyanotic blue tongue, from which the virus gets its name, is occasionally observed in sheep (Schwartz-Cornil et al., 2008, Caporale et al., 2014).

### 1.1.6 Structure

The structure of the virus facilitates the ability to infect and replicate within specific host cells. BTV is an icosahedral virus with three concentric capsid layers which surround the ten dsRNA segments present in the viral core (Figure 1.1) (Nason et al. 2004).

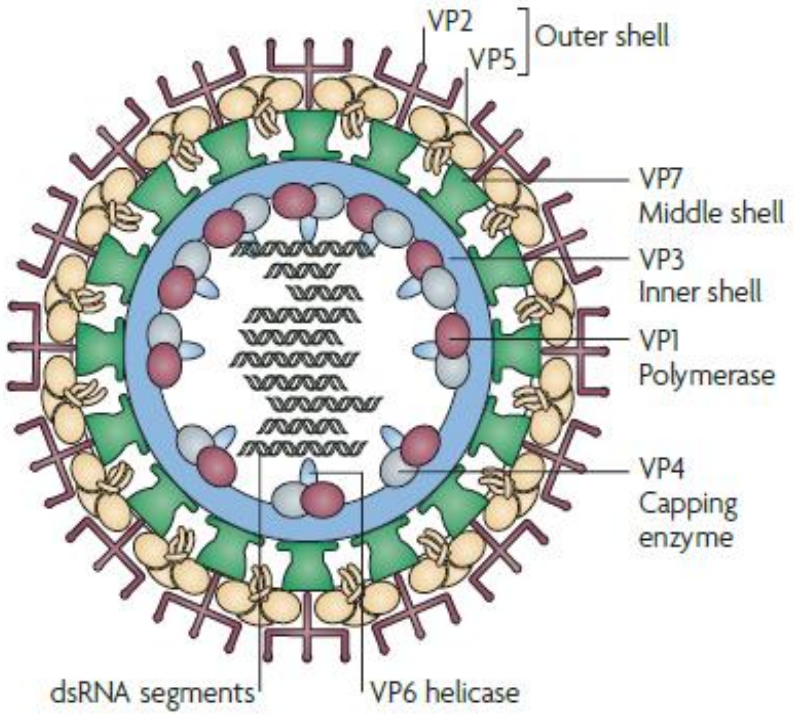



**Figure 1.1: Structure of Bluetongue virus.** The viral capsid surrounds the ten dsRNA segments in the viral core. The inner shell consists of VP3. Associated with the inner shell are the RNA-dependent RNA polymerase VP1, the capping enzyme VP4 and the viral helicase VP6. The VP3 layer is surrounded by the VP7 middle shell. The outer shell consists of VP2 and VP5. Reprinted by permission from Springer, Nature Reviews Microbiology, Prospects for improved bluetongue vaccines (Roy et al., 2009).

The outer capsid (shell) is made up of the major structural proteins VP2 and VP5. The highly variable VP2 protein determines the serotype of the virus and contains the antigenic determinants which induce the neutralising antibody response. Its role in viral entry involves binding to cellular receptors and initiating receptor mediated endocytosis (Forzan et al., 2004). VP5 has similar structural properties to fusion proteins of enveloped viruses. Its major role is in membrane permeabilization, enabling the release of the viral core into the cytoplasm of the host cell (Forzan et al., 2007).

The inner capsid structure comprises more conserved proteins: the major structural protein VP7, forming the middle shell, VP3 forming the inner shell, and the three minor proteins VP1, VP4 and VP6 associated with the inner shell (Figure 1.1). VP3 and VP7 are important structural proteins which aid in the assembly and stability of the viral particle while VP1, VP4 and VP6 are responsible for transcription of the genome (Roy et al., 2009). The viral particle is approximately 73nm in diameter with a 69nm core and the VP2 proteins which add approximately 4nm to its diameter (Hewat et al., 1992, Grimes et al., 1997).

## 1.1.7 Viral entry

It has been shown that viral entry via VP2 receptor-mediated endocytosis involving clathrin-coated pits, facilitates enclosure of the virus within an early endosome (Figure 1.2). The lower pH in the endosome results in degradation of VP2 which exposes VP5. Progression into the late endosome and

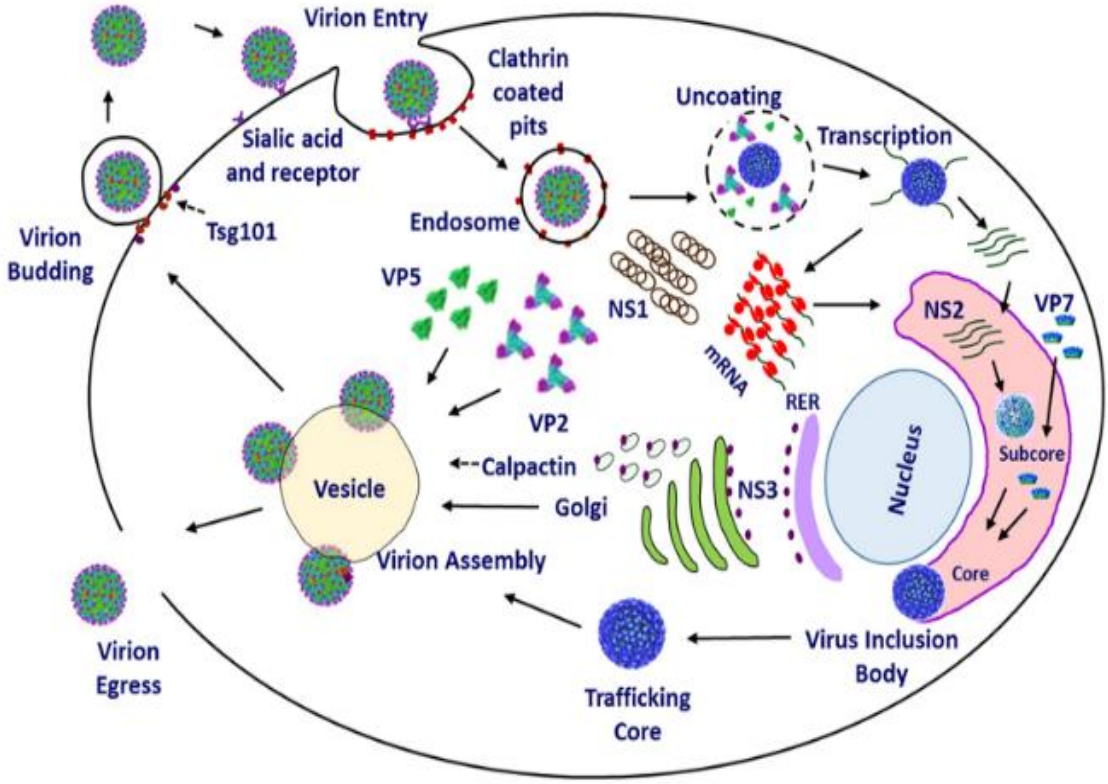



**Figure 1.2: Replication cycle of Bluetongue virus.** VP2 facilitates viral entry via sialic acid attachment followed by clathrin mediated endocytosis. The clathrin coated pits are transported into the endosome where the acidic pH mediates VP5 permeabilisation leading to the core particle entering the host cell cytosol. Transcription and translation take place. NS1 forms tubules and NS2 forms the viral inclusion body containing components for viral core assembly. Complete core particles are trafficked, by interaction with NS3, in exocytotic vesicles during which VP5 and VP2 bind to form complete virions. Particles exit by budding, mediated by Tsg101 and NS3, or by host cell lysis. Reprinted from Virus Research, Volume 182, Patel, A. & Roy, P., The molecular biology of Bluetongue virus replication, pg 5-20, with permission from Elsevier (Patel et al., 2014).

a further drop in pH results in VP5 changing conformation and initiating membrane fusion and the release of the viral core into the cytoplasm so that transcription, translation and assembly of viral particles can occur (Forzan et al., 2007, Zhang et al., 2016). The non-structural (NS) proteins are important for assisting in these processes. NS1 is a tubular protein which enhances the translation of viral proteins. NS2 facilitates the formation of viral inclusion bodies which then recruit the components required for genomic packaging and assembly of the core particle. Once the core is assembled, it is released from the inclusion body and NS3 and NS3A take over. These two isoforms facilitate the final steps of exocytosis and aid in trafficking and release of mature virions from the host cell. The Tsg101 protein interacts with NS3 to mediate the budding of the virions from the cell (Patel et al., 2014).

## 1.2 Global and Local Prevalence of BTV

### 1.2.1 Distribution of serotypes and outbreaks

The *Culicoides* vector has facilitated the global spread of BTV which now exists on all continents except Antarctica (Maclachlan, 2010). In 1998 BTV spread throughout the Mediterranean basin and initiated one of the biggest outbreaks of BTV yet and the first to occur in Europe. Over the course of the outbreak (1998 – 2001) approximately 300 000 sheep died either from disease or because they became infected and subsequently had to be killed to prevent further spread (Mellor et al., 2002). The outbreak affected at least 10 countries in the Mediterranean basin, including Greece, Italy and Tunisia, many of which had not detected BTV ever before. Regions which have not been previously affected by BTV allow for the rapid spread of the virus and are often involved in facilitating outbreaks as they have not implemented the necessary prevention strategies (Carpenter et al., 2009). This is one of the reasons why there are very strict trade restrictions in these areas. Each subsequent year of the outbreak meant further spread of the virus via the *Culicoides* vectors from the Greek islands where it was initially detected in 1998. By 2001 four serotypes had been detected (BTV9, BTV4, BTV16 and BTV2). Monovalent vaccines for each serotype were ordered from South Africa and used on both cattle and sheep (Savini et al., 2008). The outbreak of BTV2 in Italy in 2000 resulted in the death of 90 000 sheep. This was a long and costly outbreak that made Europe more aware of the devastating effects of this virus.

Another devastating outbreak also occurred in Europe but included the northern regions of the continent. This outbreak began in 2006 in the Netherlands with BTV8 which was found to have a sub-Saharan origin (Flannery et al., 2019). This was the first detection of BTV8 in northern Europe (Carpenter et al., 2009). Although there was loss of livestock in 2006, the virus re-emerged in 2007 and resulted in severe loss, including cattle. It was assumed that the virus which had not caused severe damage in 2006 would not be able to overwinter. Only when the damage of the outbreak became apparent in 2007, after considerable economic devastation, were prevention strategies applied. Trade restrictions were implemented and vaccine strategies were investigated to reduce transmission. By 2009 there were only sporadic cases of BTV in Europe (Flannery et al., 2019). Despite the available technology, an inactivated vaccine was only produced late in 2007, almost 2 years into the outbreak. Because the virus spread to areas it had not been found before, it was difficult for authorities to convince veterinarians to look out for early signs of the disease. It resulted in the most severe economic damage caused by a single serotype of the virus (Zientara et al., 2013). During this time new serotypes BTV6 and BTV11 were detected in the Netherlands and Belgium, respectively. It was thought that these strains were derived from illegal modified live virus (MLV) vaccine strains (Zientara et al., 2013).

More recently in 2015 there was an outbreak of BTV8 in France. It progressively spread throughout the country over two years. When compared to the 2007 outbreak, this re-emerging strain did not spread as fast and mainly caused mild symptoms. However, it only differs from the 2007 strain by 11 amino acids. It is postulated that it is a re-emerging strain that had been circulating asymptomatically in wildlife (Flannery et al., 2019). Its attenuation may be a result of selective pressure occurring within the hosts during replication. Presently, BTV8 is still widely prevalent in Europe with 70 outbreaks of BTV8 occurring in 2020 and BTV1, BTV2, BTV4 BTV9 and BTV16 also being detected (Niedbalski, 2022). Additionally, the number of BTV serotypes is continuously expanding as a 29$^{th}$ BTV serotype was recently described in China (Yang et al., 2021).

### 1.2.2 Economic and agricultural impact

The sheep farming industry is economically important in many countries due to the products derived from sheep such as wool, meat, dairy products and leather. BTV causes direct economic loss as a result of mortality and morbidity as well as trade restrictions which may have greater financial implications than direct livestock loss. Indirect economic impact occurs through the cost of surveillance, prevention strategies and vaccines (Caporale et al., 2010, Coetzee et al., 2012, Gethmann et al., 2020).

In order to mitigate the impact of BTV on the economy, surveillance for the disease needs to be consistently carried out. This will allow for quicker epidemiological predictions and accelerate the distribution of relevant serotype-specific vaccines.

## 1.3 Diagnostics

In the early stages of an outbreak there needs to be basic data collected about the virus and its vector so that the epidemiology can be predicted and relevant vaccines can be developed sooner (Carpenter et al., 2009). The diagnosis of BTV in both livestock and wild ruminants plays an important role in the selection of appropriate vaccine strategies during an outbreak as well as determining when an area is virus-free to allow for resumed trade. Diagnostic assays need to have the ability to identify both the BTV serogroup amongst its close relatives as well as the specific serotypes circulating in a given region (Rojas et al., 2019). Because BTV is antigenically related to other *Orbiviruses*, specifically AHSV and EHD, diagnostic methods need to be accurate and reliable in differentiating BTV from these other *Orbiviruses* (Rojas et al., 2019). Serogroup detection usually makes use of the highly conserved BTV VP7 protein while serotype detection makes use of the highly variable VP2 protein which has the greatest sequence variation between serotypes (Rojas et al., 2019). This is also important as most vaccines are serotype specific and thus a diagnosis is required to determine which serotype/s need to be included in a vaccine strategy (Maan et al., 2016). The more efficiently the diagnosis can be made, the more rapidly the vaccine can be produced and distributed. There are a variety of different diagnostic assays that are used commercially that have several limitations often involving expense and accessibility for use in the field (Mohandas et al., 2015). Diagnostic strategies are grouped into two main categories: Antigen detection and antibody detection (Rojas et al., 2019).

### 1.3.1 Virus isolation and characterisation

This involves the isolation of viruses from samples of blood, semen or tissue (Afshar, 1994). Once the virus is isolated, it can then be characterised. Reverse transcription PCR (RT-PCR) is the most common strategy used to detect the virus as well as ELISA assays in some cases.

Immunofluorescent detection is often used to determine the serogroup through the use of monoclonal antibodies which specifically bind VP7 (Pini et al., 1966, Blacksell et al., 1993). Immunoperoxidase strategies are used for detection in fixed cells and tissues (Wechsler et al., 1990). Competitive ELISAs are a similar strategy which make use of multiple antibodies for detection (el Hussein et al., 1989). Although this is highly sensitive there are commonly false negative results and cross reactivity occurs between serotypes (Hamblin, 2004). This can result in an underestimation of the number of infected animals (Anthony et al., 2004).

The early PCR methods made use of reverse transcription PCR. They were sensitive and specific tests that were ideal for detecting the BTV RNA directly without the requirement of viral isolation (Anthony

et al., 2004). However, initially this test could not detect all of the BTV serotypes and had a tendency to give false negatives (Anthony et al., 2004, Vandenbussche et al., 2010). Improvements were made in this diagnostic method with the introduction of a multiplex system which included internal and external control systems - for example the triplex RT-qPCR method (Vandenbussche et al., 2010). In 2014 a quantitative real-time RT-PCR assay was developed which was able to detect 26 BTV serotypes by detection of segment 9 of the genome (Maan et al., 2015). It was shown to be a highly sensitive and specific assay that has become a valuable diagnostic tool (Maan et al., 2015). In 2016, the same group described a type-specific real-time RT-PCR assay which made use of a Taqman fluorescence probe. Serotype-specific primer sets were designed for each known serotype. This method was found to be highly specific but does, in some cases, result in false negatives and would need to be updated regularly to ensure maintenance of sensitivity when sequences change or new isolates are reported (Maan et al., 2016).

### 1.3.2 Antibody detection

Antibody detection relies on the serological response which occurs 7-14 days after infection (Stott et al., 1985). When identifying the serogroup often an ELISA is used. Before the use of the ELISA, agar gel immunodiffusion was used but this test has low sensitivity and specificity and is not the preferred method (Della-porta et al., 1985). Both indirect and competitive ELISAs are used in serogroup identification. The indirect assay is reliable and robust and, in addition to VP7, NS3 can also be used as an antigen (Kramps et al., 2008, Barros et al., 2009a). The competitive ELISA typically makes use of the conserved VP7 antigen to detect antibodies (Afshar et al., 1987). It is often used to determine whether an area is virus-free in order for international trade to resume (Hamblin, 2004). Although different variations of the ELISA have different advantages and are more appropriate under certain conditions, epidemiological scenarios and field conditions can have an effect on the reliability and accuracy of the test (Diaz-Cao et al., 2020). The origin of the sample, for example, can affect accuracy of a test. A sample from a live animal will be a more reliable diagnosis than one from a dead animal that has been through a freeze thaw cycle. When comparing the accuracy of tests these factors need to be considered (Diaz-Cao et al., 2020).

The serum neutralisation test (SNT) is used to detect serotype specific antibodies in order to determine the serotypes present once the serogroup has been identified. This is a highly specific test as it does not cross-react with other *Orbivirus* serogroups and can specifically identify serotypes in a sample. It can also be a good way to declare an area BTV-free or to plan eradication policies however, it is a time-consuming and inefficient method (Anthony et al., 2004, Rojas et al., 2019).

Similar to vaccine strategies there is no one-size-fits-all approach to virus detection and diagnosis. There are many tests that are commercially available and the test that is used in a given scenario needs to be informed by the environment and aim of the diagnosis. Many of the tests can also be used in combination to confirm diagnoses and increase reliability of the results.

Once virus characterisation has been completed, the appropriate control strategy can be put into place. Vaccination is the most effective way to control BTV and prevent the devastating economic losses that can result from a BT outbreak (Erasmus, 1975, Dungu et al., 2004). An important consideration in both diagnostics and vaccine development is that of the ability to differentiate infected from vaccinated animals (DIVA). DIVA compliant vaccines will allow for safe trade to occur between countries regardless of whether BTV has affected them or not. These vaccines can facilitate

surveillance during outbreaks especially in those animals that experience sub-clinical infection (Van Oirschot, 1999). Other important considerations include vaccines that are quick to make, effective against multiple serotypes and affordable (van Rijn, 2019). Although there are commercially available vaccines that have been effective, there are still improvements to be made based on the limitations of these existing strategies.

## 1.4 Current Commercial Vaccine Strategies

### 1.4.1 Modified-live vaccines

Because BT was first described in South Africa, a number of MLV vaccines were developed locally. The global commercially available MLV is produced by Onderstepoort Biological Products (OBP) in Pretoria, South Africa (Feenstra et al., 2017). Attenuation of viruses is achieved by passage of the virus through embryonated chicken eggs and thereafter in tissue culture. This allows for the selection of attenuated or avirulent strains that have a reduced ability to infect and replicate in natural hosts (Dungu et al., 2004).

The OBP MLV strategy includes three separate pentavalent vaccines which cumulatively include attenuated strains of 15 serotypes (Bottle A, B and C). Each bottle contains five serotypes and is administered 3 weeks apart. An annual booster vaccination is also required. The most attenuated strains (Bottle A) are injected first to decrease the risk of the less attenuated strains (Bottle C) causing disease (Feenstra et al., 2017). This vaccine strategy has been successful in South Africa (Dungu et al., 2004) and has also helped in the control of BTV in Europe (Breard et al., 2004, Patta et al., 2004). It is an effective strategy that is cheap, induces both humoral and cellular responses, requires a low dose and prevents BT and circulation of the serotypes included (Feenstra et al., 2017). There are, however, a number of limitations. MLVs have a high potential to revert back to virulence or undergo reassortment with field strains or other vaccine strains (Batten et al., 2008, Nomikou et al., 2015). They cannot be used in pregnant animals as they result in abortions or congenital deformities (De Clercq et al., 2008, Savini et al., 2014). Additionally, it has been shown that the vaccine virus strains can spread via *Culicoides* to unvaccinated animals (Ferrari et al., 2005). Despite the use of MLVs, imposed trade restrictions because of outbreaks still remain, as it is impossible to differentiate between infected and vaccinated animals (it is not DIVA compliant). Because of the numerous drawbacks of the MLV vaccine strategy, inactivated vaccines were developed.

### 1.4.2 Inactivated vaccines

When severe BTV outbreaks began in Europe in 2006, there was a high demand for inactivated (whole-killed) vaccines as MLVs were deemed too risky (Feenstra et al., 2017). Inactivation is achieved through heat, UV radiation and most commonly via chemical methods (Mayo et al., 2017). Inactivated vaccines consist of viral strains that are non-infectious and unable to replicate but are still recognised by the immune system and induce an immune response. The success of these vaccines is evident by the decrease in outbreaks in Europe after mass vaccination (Patta et al., 2004, Zientara et al., 2013). Multiple studies have shown that these vaccines provide protection for at least a year (Wackerlin et al., 2010, Batten et al., 2013) and that they elicit high titres of neutralising antibodies and reduce BTV transmission (McVey et al., 2015). It has also been found that inactivated vaccines are safe for use in gestating animals (van der Sluijs et al., 2012). The disadvantage of inactivated vaccines is that immunogenicity is short-lasting and thus a higher and more frequent dose is required, making vaccination more costly (Hund et al., 2012). In addition, these vaccines are relatively unstable and have

a short half-life (McVey et al., 2015). They have a greater potential for DIVA compliancy than MLVs as there are significantly higher levels of the non-structural protein NS3 in infected, non-vaccinated animals (Barros et al., 2009a). This, however, is not definitely indicative of an animal's infection status.

## 1.5 Recombinant Vaccine Strategies

Although the current vaccine strategies have had success in decreasing circulation of BTV and protecting animals from disease (Table 1.1), the drawbacks that accompany their use has encouraged the development of several recombinant vaccine strategies in an attempt to overcome the limitations associated with current commercial BTV vaccines as well as to ensure DIVA compliance.

**Table 1.1: Criteria fulfilled by each of the currently available BTV vaccine strategies.** '+' indicates degree of fulfillment and '–' means the criterion is not fulfilled. Image under the Creative Commons Attribution (CC-BY) license (Feenstra et al., 2017)**.**

| Vaccine | Onset of Immunity | Duration of Immunity | Animal Safety | Environmental Safety | DIVA | Cost/dose |
|---|---|---|---|---|---|---|
| MLV | +++ | +++ | + | - | - | +++ |
| Inactivated | + | ++ | +++ | +++ | + | + |

### 1.5.1 Serotyped vaccines

The best method for creating vaccines that are easily scalable and quick to adapt to an outbreak of any serotype, has been the use of reverse genetics for the synthesis of serotyped vaccines. Serotyped vaccines include either the backbone of an MLV or an inactivated vaccine virus with an adjuvant. This strategy takes advantage of the already tissue-culture-adapted backbone of a vaccine strain such as BTV1. A study by Nunes et al. investigated the vaccine potential of 16 serotypes using the BTV1 genetic backbone. Not all the serotypes were stable and successful but many did prevent viremia when sheep were challenged with a homologous serotype (Nunes et al., 2014). Even more promising, a chimeric strain of BTV1 was synthesised where part of the BTV1 VP2 sequence was replaced with part of the BTV8 VP2 sequence. This vaccine was cross-neutralised by both BTV1 and BTV8 sheep antisera and thus it can be speculated that there are multiple epitopes present on the VP2 protein (Nunes et al., 2014). The same serotyped vaccine strategy has been applied using already synthesised MLV strains known to be safe for use in vaccines (van Gennip et al., 2012, Feenstra et al., 2015).

### 1.5.2 Viral vector vaccines

A number of strategies involving viral vectors have proven to be promising vaccine candidates. Many of these strategies involve viruses, such as poxviruses or adenoviruses, that do not infect ruminants and have been attenuated and modified to express specific BTV proteins (Boone et al., 2007, Perrin et al., 2007, Calvo-Pinilla et al., 2009, Calvo-Pinilla et al., 2012). For example, chicken embryo fibroblasts are first infected with the wildtype virus (e.g. pox) and subsequently transfected with plasmid vectors containing the BTV genes of interest (Li et al., 2015). The recombinant viruses, selected after plaque assay, are then used to infect the host (Calvo-Pinilla et al., 2012, Li et al., 2015).

It has been found that priming with a DNA vaccine first and then boosting with a recombinant viral vaccine results in good immune responses, especially T-cell mediated immunity (Li et al., 2015). The DNA is able to deliver the antigens directly to the MHC Class I pathway to induce cytotoxic T lymphocytes (CTLs) and the recombinant viruses induce interferon and interleukin expression and a strong humoral response (Calvo-Pinilla et al., 2012). Martin et al. showed that using a recombinant virus vaccine only will provide partial protection but not sterile immunity (Martin et al., 2015). A study by Li et al. found that a DNA prime and a recombinant fowlpox virus (rFPV) boost expressing VP2 and VP5 of BTV1 elicits a stronger immune response than either a DNA prime-boost or a recombinant prime-boost strategy (Li et al., 2015).

Calvo-Pinilla et al. produced a viral vector vaccine from the modified vaccinia virus Ankara (MVA). They used a DNA prime-recombinant MVA boost strategy to compare the immune responses elicited when either VP2 and VP5 or VP2, VP5 and VP7 of BTV4 were included in the vaccines. IFNAR $^{(-/-)}$ (knockout for type 1 interferon receptor) mice were used as a model organism, as challenge with BTV is lethal in these mice. When the DNA and rMVA vaccines only contained VP2 and VP5, there was partial protection against BTV4 challenge. When VP7 was included, there was 100% survival and protection in the mice. Although both methods induced similar levels of neutralising antibodies, when VP7 was incorporated there was a strong humoral and T-cell response (Calvo-Pinilla et al., 2009). These results indicate that inclusion of VP2, VP5 and VP7 in the vaccine combination was necessary to have a complete and sterile protective effect.

In a very similar study, the same strategy was employed but instead of VP5, the non-structural protein NS1 was included with VP2 and VP7. They found that in addition to complete protection against a lethal dose of BTV4, the IFNAR$^{(-/-)}$ mice were also protected against BTV8 and BTV1 challenge (Calvo-Pinilla et al., 2012). The NS1 protein is highly conserved across the BTV serotypes and thus elicits a strong cellular immune response to multiple serotypes. This is a promising result which indicates that it may be possible to have cross-protection against multiple serotypes in a single vaccine. However, the strategy needs to be evaluated in sheep before it can be concluded that it has the same effect in BTV's natural hosts.

Another approach to producing multivalent vaccines is to include antigens from different serotypes in a single dose. Rojas et al. explored this strategy by expressing VP7 of BTV8 and VP2 of BTV1 through a recombinant replication-deficient human adenovirus (serotype 5) (Rojas et al., 2021). Mice immunised with the vaccine were protected against both BTV1 and BTV8 and were partially protected against heterlogous BTV4.

These vaccine strategies are a step forward from the currently available vaccines. They induce both humoral and cellular immune responses and are able to prevent BT but still have DIVA capability as not all proteins, such as NS3 or VP7, are included in the vaccine. There are, however, some drawbacks. The requirement of 2 doses makes the strategy costly and it creates a risk that there is an immune response to the viral vector. The DNA prime-recombinant vector boost method has potential to rectify this problem.

### 1.5.3 DISC and DISA vaccines

Disabled Infectious Single-Cycle (DISC) vaccines are another example of live recombinant vaccines. These include BTV strains that have deletions in key genes. The commonly used DISC vaccine lacks the VP6 coding gene which is essential for viral replication (Matsuo et al., 2011, Celma et al., 2013). This

vaccine can thus infect host cells at the natural sites of infection but cannot replicate and cause viremia. The vaccine still induces an immune response and neutralising antibodies are synthesised (Matsuo et al., 2011, Celma et al., 2013).

DISC vaccines often contain BTV1 mutants which have a BTV1 genetic backbone with a deletion of VP6 but have DNA segments S2 and S6, which code for VP2 and VP5 respectively, from a different serotype. Matsuo et al. recovered reassortant disabled BTV1 viruses in a complimentary cell line that expresses VP6 (BSR9). The reassortant viruses have the BTV1 mutant genetic background but express VP2 and VP5 of BTV8. When transfected into wildtype BSR cells, structural and non-structural proteins were detected and neutralising antibodies were produced with no viral replication or synthesis of infectious particles. When evaluated in sheep, all vaccinated animals had detectable neutralising antibodies seven days post vaccination with no assembly or maturation of viruses. The sheep did not develop any clinical signs when challenged with BTV8. This held true when only one dose was given (Matsuo et al., 2011).

In a similar study, Celma et al. found that after a single dose there was a weak humoral response and only after a second booster vaccine do the animals seroconvert. They also showed that if a multivalent cocktail vaccine is made up of viruses expressing VP2 and VP5 of different serotypes, neutralising antibodies are detected for each of the serotypes included in the vaccine (Celma et al., 2013). This shows promise for developing multivalent vaccines. The DISC vaccine strategy allows for the rapid production of vaccines since the same genetic backbone can be used for all serotypes. The DIVA capability is only possible if the VP6 protein is completely deleted and there is an appropriate detection method available.

A similar vaccine strategy is that of the Disabled Infectious Single-Animal (DISA) vaccine. These are based on MLV strains which have been mutated to have a deletion in the non-structural proteins NS3 and NS3a. This modification means that they are able to replicate locally but cannot be released from the infected cell (van Gennip et al., 2014). For example, a DISA vaccine consisting of a mutated BTV6 strain where the VP2 segment is exchanged for that of the BTV8 serotype is effective. This strategy has been tested successfully on vaccinated sheep challenged with BTV8 (Feenstra et al., 2014). The appeal of this vaccine strategy is the lack of the NS3/NS3a proteins. This omission makes the virus avirulent, have low levels of viremia and an inability to spread through the insect vector (Feenstra et al., 2014, Feenstra et al., 2015). Furthermore, NS3/NS3a proteins typically supress interferon induction and thus their absence may improve the anti-viral response. The use of NS3-based enzyme-linked immunosorbent assays (ELISAs) makes this DIVA competent. Although these ELISAs are not yet commercially available, a proof of concept has been developed (Tacken et al., 2015). A cocktail DISA vaccine consisting of viruses with the same genetic backbone but different serotype specific VP2 proteins would be safe to use as there would not be a risk of reassortment. Additionally, the dose required for protection is slightly lower than that required in MLVs (Feenstra et al., 2017). It has been shown that a 72 amino acid deletion in the NS3 sequence is sufficient for preventing viral replication in the insect vectors. This vacccine is safe and provides long lasting protection in cattle (van Rijn et al., 2021).

Recently, research has focused on the inactive recombinant vaccine strategies. These vaccines include manipulated forms of the virus consisting of specific BTV proteins.

### 1.5.4 Subunit vaccines

Subunit vaccines comprise viral protein components and are a DIVA capable method of vaccination. They only include the parts of the virus that will elicit protective immunity (Feenstra et al., 2017). These vaccines are created by expressing specific BTV genes from plasmid vectors often via a baculovirus expression system in Sf9 cells or via expression in *Escherichia coli* (*E. coli*). The proteins are purified from these cells and, usually with the inclusion of an adjuvant, they are administered as a vaccine (Anderson et al., 2013). The proteins included in the vaccine are not necessarily from the same serotype.

Anderson et al. created a subunit vaccine constituting VP2 of BTV8 and NS1 and NS2 of BTV2 where each protein was expressed and purified separately and added in equal concentrations to make up the final vaccine. This vaccine was compared to a commercially available inactivated vaccine by testing the immune response in sheep. Both vaccines induced similar neutralising antibody reponses but the specific antibody response against each of the subunit components was greater in the subunit vaccine. The NS1 protein elicited a specific T-cell mediated response after both vaccines. Both NS1 and NS2 induced cross-serotype immune responses (Anderson et al., 2013). Subunit vaccines thus have the potential to be developed as multivalent vaccines. The advantage over the inactivated vaccine is the ability to induce a stronger specific antibody response that lasts longer. Furthermore, the lack of the VP7 protein creates DIVA compliancy.

Another subunit vaccine was developed using a similar bacterial expression system. Here, combinations of different proteins, in addition to VP2, were tested to determine the vaccine with the best efficacy. The inclusion of VP7 did not improve the immune response and was excluded to allow for DIVA competancy. The presense or absence of VP5 did have a significant affect on the neutralising antibody reponse. The interaction between VP5 and VP2 seems to enhance this response. Thus the vaccine including VP2 and VP5 was deemed the best vaccine for protection against BTV challenge in mice (Mohd Jaafar et al., 2014).

Legisa et al. used the baculovirus insect cell expression system to create subunit vaccines either comprising VP2 of BTV4 alone or VP2 of BTV4 fused to an antigen presenting cell homing molecule (APCH). These APCH-VP2 subunit vaccines target VP2 to the antigen presenting cells (APCs) to improve the immune response. When IFNAR$^{(-/-)}$ mice were challenged with BTV4, the mice vaccinated with the APCH-VP2 vaccine had a higher titer of neutralising antibodies and a stronger CD4+ and CD8+ specific immune repsonse than the VP2 vaccinated mice (Legisa et al., 2015). This study showed that the attachment of an antigen to APCH can enhance the immune response and thus lower the dose of antigen that is required as it is specifically targeted to immune cells.

A subunit vaccine consisting of a his-tagged VP2 antigen from BTV4 or BTV8 was transiently expressed in *Nicotiana benthamiana* and immunised into IFNAR$^{(-/-)}$ mice (Fay et al., 2019). This induced VP2 serotype specific antibodies and no clinical signs were observed after challenge with the homologous serotype. This is promising for the production of these antigens in plants and their use as a component of a subunit vaccine or use in serological assays.

### 1.5.5 Virus-like particles

Virus-like particles (VLPs) are essentially a protein shell mimicking the virion which lacks viral genetic material. VLPs cannot undergo replication or cause viremia and are thus non-infectious. They can have

self-adjuvanting properties and are recognised readily by APCs (Crisci et al., 2012). Expression of VLPs occurs in similar systems used for subunit vaccines. However, for the assembly of BTV VLPs, expression of four viral proteins are required (VP2, VP3, VP5 and VP7).

Stewart et al. illustrated the success of a baculovirus expressed BTV VLP (Stewart et al., 2010). To increase efficiency of VLP synthesis for any serotype, the highly conserved inner capsid proteins (VP3 and VP7) were pre-integrated into the baculovirus genome. Therefore only two genes need to be manipulated. Here, the core proteins were from BTV10 (VP7) and BTV17 (VP3) and the outer layer proteins from BTV2. Immunogenicity of purified VLPs was evaluated in sheep. Neutralising antibodies were detected and no clinical signs or viremia were observed. The lack of non-structural proteins makes the VLP vaccine DIVA compliant (Stewart et al., 2010). In a later study Stewart et al. showed a similar result using a VLP for BTV1 (Stewart et al., 2012). The potential use of core-like particles (CLPs), made from VP3 and VP7, were also investigated but there was incomplete protection (Stewart et al., 2012).

Pérez de Diego et al. investigated a bivalent VLP vaccine strategy where BTV1 and BTV4 VLPs were included in a single vaccine-boost strategy. Neutralising antibodies against BTV1 were higher than those for BTV4. A BTV1 challenge in sheep vaccinated with the bivalent vaccine did not cause clinical signs or viremia, however, when challenged with BTV4 there was not complete protection in all sheep. It was suggested that this may be due to interference by the BTV1 VLP or individual susceptibility to infection (Perez de Diego et al., 2011).

Despite the immunogenicity and efficacy shown by these BTV VLPs, production in the baculovirus insect cell system is not deemed cost effective enough to compete with the commercial inactivated vaccines (Feenstra et al., 2017). This limitation led to the investigation of BTV VLP production in plants.

## 1.6 Biopharmaceuticals In Plants

### 1.6.1 Limitations of traditional systems

Conventional protein expression systems are well-accepted and favoured by the protein manufacturing industry as they have the ability to produce a high yield of high quality proteins at a low cost (Schillberg et al., 2019). The gold standard systems for industrial protein production are the Chinese hamster ovary (CHO) cells and *E. coli* cells (Nandi et al., 2016, Schillberg et al., 2019). *E. coli,* as well as yeast-based production systems, were the earliest protein production platforms used. These systems are ideal for producing simple proteins such as antibody fragments (Mir-Artigues et al., 2019). They are easy to handle and allow for high production capacity while remaining low-cost (Schillberg et al., 2019). However, the protein modifications required for certain products cannot always be carried out in these systems. Bacteria cannot carry out post-translational modifications (Demain et al., 2009) and although yeasts can modify proteins, these modifications are often not those required for the eukaryotes in question (Guirimand et al., 2021). These systems also require expensive media and infrastructure required for protein production (Nandi et al., 2016, Mir-Artigues et al., 2019). In general, these conventional methods require high upstream operating costs. They require large bioreactors which make it difficult to scale up production and some systems need further processing to achieve the desired fully formed, processed and modified protein. Additionally, there is a risk of contamination by other animal viruses and ensuring a sterile environment adds to upstream processing costs (Ahmad et al., 2012, Moon et al., 2019, Tsekoa et al., 2020).

### 1.6.2 Advantages of plant systems

Although the traditional protein production systems are the gold standard – there are a number of ways in which plant-produced proteins have an advantage over these systems. The appeal of plant-produced proteins is the low complexity of the upstream process and the ability to easily scale-up this process by simply growing more plants (Moon et al., 2019). There is lower upfront investment required, lower operating costs and no need for sterility (Marsian et al., 2016, Rybicki, 2020). Time to market is dramatically reduced compared to the mammalian and bacterial cell systems as transient expression in plants allows for a very short interval between transformation and expression (as little as 3 days) (Merlin et al., 2014, Nandi et al., 2016). Plants also have the ability to carry out most post-translational modifications required for functioning complex eukaryotic proteins (Merlin et al., 2014). Additionally, proteins made in plants retain their ability to self-assemble (Ahmad et al., 2012).

A number of techno-economic analyses have been conducted to determine whether using a transient plant-based platform is in fact economically viable when compared to the established and reliable conventional methods. Most of the published data suggests that the plant-based method does have the potential to compete commercially under certain circumstances (Nandi et al., 2016, Alam et al., 2018, Mir-Artigues et al., 2019). Models predict that there are significant decreases in capital investment and more than 50% reduction in cost of goods compared with published values for traditional biomanufacturing platforms at similar production scales (Nandi et al., 2016). One study found that plant produced proteins have a highly favourable environmental output index as there is low risk to health and safety using this system (Alam et al., 2018). A more recent study created a simplified model for the molecular pharming of antibodies that is universally applicable and allows direct comparison to other models (Mir-Artigues et al., 2019). Here one can compare different expression platforms, modes of expression and different host species. This is a valuable tool that can and should be used when determining whether a given pharmaceutical would be suited to any particular protein expression system or whether plant produced proteins are worth investing in for the long-term production of pharmaceuticals in a developing or developed country.

Currently, plant-produced biopharmaceutical products are mostly used for very specific, niche products which cannot be made using the conventional systems. These include: vaccines and therapeutics intended for oral delivery, when there is a requirement for rapid production of high quantities in the case of emergency vaccines (Tuse et al., 2020) or when there is a need for complex post-translational modifications (Schillberg et al., 2019). Plant derived products can also be more accepted by the consumer as they are considered environmentally friendly with animal-free production methods and human pathogens cannot replicate in plants (Moustafa et al., 2016, Schillberg et al., 2019).

There is still some resistance towards commercialising plant-derived products as there are challenges of low yield, inconsistent product quality and the difficult large scale downstream processing of the proteins (Schillberg et al., 2019). Although scale-up is easier in plants and the upstream cultivation costs are low, the pressure is then added to the downstream extraction and purification steps adding cost to the process.

Despite the few challenges faced when producing plant-derived proteins, it is an ideal system for the local production of protein-based therapies and vaccines in developing countries. In South Africa, and Africa as a whole, there is a trade deficit, supply insecurity and generally poor access to vaccines and

therapeutics required by the population. Plant molecular farming may be a suitable solution to these problems due to the low cost, safety and efficacy of the products both for veterinary and human use (Tsekoa et al., 2020).

### 1.6.3 Transient vs transgenic expression

Plants are able to manufacture recombinant proteins in two different ways. Stable transformation results in transgenic expression in plants where the recombinant genes are integrated into the plant genome. Although this is the cheaper method with the potential for high expression it takes many months for transformation, regeneration and bulk-up and yield can vary (Walwyn et al., 2015). There are also limited plant species in which stable transformation is possible (Moon et al., 2019). Proteins are expressed transiently in leafy plants through the infiltration of transformed *Agrobacteria*. Here, the transgene is transferred into the plants cells where plant machinery is used to translate the recombinant proteins (Mir-Artigues et al., 2019). This method does not have a lag phase and protein is expressed in a short time – just days after infiltration (Wydro et al., 2006, van Zyl et al., 2016, Moon et al., 2019). This is a simpler method that is easy to perform and is more suited to commercialisation (Moon et al., 2019). Although the yield can be affected by a number of factors and RNA silencing suppressors may be necessary to ensure sufficient expression (Wydro et al., 2006), the rapid expression of the protein and the simplicity of this method makes it the preferred method for protein production in plants.

### 1.6.4 *Nicotiana benthamiana*

Although the method of transformation is an important consideration when determining how to manufacture recombinant proteins, the plant itself also needs to be carefully considered. When it comes to transient expression – *Nicotiana benthamiana* is the gold-standard. Previously alfalfa, potato and *Arabidopsis thaliana* have also been used as recombinant protein expression systems but the advantages and feasibility of using *N. benthamiana* has made it the ideal protein-producing plant. These are fast-growing, leafy plants that are easy to infiltrate and which have a large biomass in terms of yield/ha (Shakya et al., 2018). They are favoured for their ability to easily scale-up protein production. They are versatile hosts for many virus-derived expression vectors and have a natural ability to express heterologous gene sequences (Goulet et al., 2019, Tsekoa et al., 2020). Because protein expression in these plants is so well established they are familiar to regulatory agencies such as the FDA and are thus well-accepted as regulation-compliant (Alam et al., 2018). These plants, and related *Nicotiana tabacum,* are accepted hosts for proteins and biopharmaceutical products which have been administered into humans in clinical trials making these commercially relevant plant-based protein expression systems (Tsekoa et al., 2020).

### 1.6.5 BTV plant-based pharmaceuticals

A recent study by Thuenemann et al. illustrated how the use of a cowpea mosaic virus-derived vector can be used to transiently express BTV8 VLPs in *N. benthamiana* via *Agrobacterium tumefaciens*-mediated transient expression (Thuenemann et al., 2013). Mass spectrometry showed that the proteins were expressed in the plant cells and transmission electron microscopy showed that the proteins self-assembled into VLPs. Immunogenicity was evaluated in sheep and serum was found to contain antibodies, mostly against VP2 and the abundant VP7 protein. When sheep were challenged

with BTV8, the response was comparable to that induced when an MLV was used. The sheep were completely protected and an immune response was induced. The sheep vaccinated with the plant-made VLP only produced neutralising antibodies after the booster, confirming the need for a second dose (Thuenemann et al., 2013). This is a highly promising recombinant BTV vaccine. Production and testing is rapid and the method is scalable through the use of vacuum infiltration.

Following the Thuenemann paper, another study looked into the production of a BTV8 VLP through transient expression in *N. benthamiana* (van Zyl et al., 2016). They tested whether the yield of the VLPs can be affected by manipulating the infiltration ratios of the four recombinant *Agrobacterium* cultures containing the recombinant constructs. They also investigated whether targeting of the proteins to particular organelles using the pTRA vector would affect yield. It was found that the pEAQ-*HT* vector was the only vector which expressed sufficient and detectable levels of all four VLP proteins. They showed that differentially controlling the concentration of infiltrated bacteria influenced the yield of VLPs. A ratio of 1VP2:1VP3:2VP5:1VP7 was found to be the ideal ratio as all four VLP proteins were detected by Western blotting and VLPs formed using this ratio were the most fully formed VLPs as observed using electron microscopy. Targeting the proteins to the organelles did not improve yield and it was found that when the pEAQ-*HT* vector is used the VLPs collect in the cytoplasm of the plant cells (van Zyl et al., 2016).

Another plant-based method that has been investigated, is that of the production of protein body (PB) vaccines. The same method involving *A. tumefaciens* and *N. benthamiana* is used. A particular research group (van Zyl et al., 2017) created two PB vaccines; Zera®-VP2ep and Zera®-VP2. Zera® is a synthetic peptide that has the ability to self-assemble into protein bodies through disulphide bonds and hydrophobic interactions between repeat sequences. By fusing VP2 or VP2 epitopes of multiple serotypes to Zera®, the antigen or epitopes can be presented directly to the immune system. The Zera®-VP2ep vaccine had specific epitopes of VP2 from different serotypes fused to Zera®. The antigen or epitopes are sequestrated in the PBs and are thus safe from proteolytic degradation. APCs favour the uptake of PBs thus enhancing the immunogenicity of the vaccine. Zera® is also thought to have adjuvant activity. When used in mice, PBs were successfully formed and anti-VP2 antibodies were detected. Overall, this is a promising vaccine strategy as the PBs are highly stable when stored at 4°C and there may be promise of a vaccine that protects against multiple serotypes. Furthermore, only a single dose is required and it is DIVA compliant.

Chimeric VLPs have the potential to provide protection against multiple serotypes either through cross protection by a single chimeric VLP or through a cocktail of chimeric VLPs targeting different serotypes. This is important for geographical areas which experience multi-serotype outbreaks. A recent study by Mokoena et al. looked at the production of a chimeric VLP vaccine (Mokoena et al., 2019). Because the self-assembly of some monovalent BTV VLPs does not always occur in plants, this group identified the need to explore the use of a core-like particle backbone in combination with capsid proteins of different serotypes in order to develop potential multivalent chimeric vaccines. Here, a BTV8 backbone (VP3 and VP7) was used to assemble single chimeras where BTV8 VP5 is expressed with BTV3 or BTV4 VP2 as well as double chimeras where the VP2 and VP5 proteins are both either from BTV3 or BTV4. They found that the chimeric VLPs were able to self-assemble in plants and elicited seroconversion in sheep. This is a promising study into the use of chimeric BTV VLPs which could be used in combination with homogenous BTV VLPs to produce multivalent vaccines (Mokoena et al., 2019).

## 1.7 Project Rationale And Objectives

Increasing global temperatures have contributed to the global dissemination of BTV (Dommergues et al., 2019). Outbreaks of BTV adversely affect the sheep farming industry and agricultural economy worldwide. Because of the wide distribution of the midges and the ability of some ruminants to act as natural reservoirs of the virus, it is almost impossible to completely eradicate BTV in endemic regions (Caporale et al., 2014). This means that vaccination is the best prevention method and strategy to control the spread of BTV.

Although commercial vaccines have had success in controlling BTV, their limitations leave much to be desired in terms of an ideal BTV vaccine. The MLV risks include reversion to virulence and reassortment with field and vaccine strains. This makes them unsafe and some countries may deem them too risky to be included in vaccine regimes (Batten et al., 2008, Nomikou et al., 2015). The inactivated vaccine has a lower efficacy and requires higher doses more frequently making it expensive (Hund et al., 2012). Many of the recombinant vaccine strategies that have been investigated elicit comparable responses to the current vaccine strategies with additional benefits of safety, multivalent protection and DIVA compliancy. The Biopharming Research Unit (BRU) at the University of Cape Town has previously developed a BTV serotype 8 (BTV8) VLP vaccine produced in *N. benthamiana* which was shown to be immunogenic and efficacious in protecting immunised sheep challenged with BTV8 (Thuenemann et al., 2013, van Zyl et al., 2016). This is a promising step forward for the production of BTV VLPs in plants. However, in South Africa, the eradication and control of BTV is made difficult due to the circulation of 21 of the 28 known serotypes and their limited serological cross-reactivity (Coetzee et al., 2012). Due to the limitations of the currently available BTV vaccines (MLV and inactivated), there is a need for BTV vaccines that are not only safe, scalable, and economically viable but also effective against multiple serotypes.

The aim of this study was to develop two different types of recombinant plant made BTV vaccines and determine their ability to elicit specific immunity in guinea pigs. The first vaccine approach is in the form of a chimeric BTV VLP. This was generated by replacing the immunogenic tip domain of the BTV8 *VP2* gene with that of the corresponding BTV1 *VP2* gene to make chimeric VP2 (while the other VLP proteins are based on BTV8). The second approach involves the display of the BTV1 VP2 immunogenic tip domain on a capsid protein particle. Here, this domain was displayed on the surface of the AP205 particle through the application of the ST/SC bioconjugation method. These vaccine candidates would be completely safe and would allow for rapid production and scalability. Only a portion of the BTV8 VP2 gene would have to be modified to allow for a VLP effective against a new strain to be more easily produced.

Each chapter of this thesis addresses the following objectives:

- To optimise expression, extraction, and purification protocols for chimeric BTV1/8 VLPs with BTV8 VLPs used as a control.
- To investigate the potential for the SpyTag/SpyCatcher technology to be applied for the display of the BTV1 antigenic domain on the surface of the AP205 VLP.
- To determine the immunogenicity of the plant produced vaccines in guinea pigs.

# Chapter 2: Transient expression and purification of chimeric BTV1/8 VLPs in *Nicotiana benthamiana*

## 2.1 Introduction

Conventional protein expression systems which include bacterial, yeast, insect and mammalian cell systems have been tried and tested for many decades. They are often the preferred method of protein expression as they can produce high yields of quality protein (Schillberg et al., 2019). However, these methods can become costly due to the expensive media and infrastructure required to ensure a quality final product. There are strict regulations in place which require expensive equipment and high levels of sterility. Specifically, when working with live virus, high levels of biosafety and containment are required, and only highly skilled and trained personnel can work in these spaces (Laere et al., 2016, Huebbers et al., 2021). Additionally, the protein modifications required for certain products cannot always be carried out in these systems. Bacteria cannot carry out post-translational modifications (Demain et al., 2009) and although yeasts can modify proteins, these modifications are often not those required for the eukaryotes in question (Guirimand et al., 2021). The methods involving fermentation are also considered too slow for the rapid production of vaccines required during an outbreak or epidemic (Tuse et al., 2020, Huebbers et al., 2021).

Plant expression systems offer a number of solutions to the limitations of the systems mentioned above. The upstream processing is low cost and of low complexity, there is no need for sterility and it is simple to scale up (Marsian et al., 2016, Moon et al., 2019, Rybicki, 2020). Plant systems are able to carry out complex post-translational modifications and the proteins retain their ability to self-assemble (Ahmad et al., 2012, Merlin et al., 2014). Transient protein expression in plants results in a faster production time from start to finish (as little as 3 days) (Merlin et al., 2014, Nandi et al., 2016).

Transient expression is achieved through the infiltration of transformed *Agrobacterium tumefaciens* into plants. These are soil bacteria which have the ability to infect plants. They possess a tumour-inducing (Ti) plasmid which contains transfer DNA (T-DNA) that is processed and transferred into the plant cell nucleus through the activity of virulence proteins encoded on the Ti plasmid (*vir* genes). The exported region is then translated into protein using the host plant-cell machinery (Zupan et al., 2000, Gelvin, 2003). These bacteria, in combination with an appropriate plant expression vector, facilitate transient expression in plants.

There have been numerous investigations into the transient expression of BTV VLPs in *N. benthamiana* plants via *A. tumefaciens*. Many of these studies make use of the pEAQ-*HT* vector as the primary vector for recombinant protein expression. This 10003bp cowpea mosaic virus-derived vector has been engineered to express high yields of protein in plants. The vector contains a P19 silencing suppressor as well as a hyper-translatable (*HT*) region which both facilitate the efficient expression and optimal yield of protein when transformed into *A. tumefaciens* and subsequently infiltrated into plants. (Sainsbury et al., 2008, Sainsbury et al., 2009).

The present study makes use of both transient *Agrobacterium*-mediated expression as well as the pEAQ-*HT* vector to express a chimeric BTV1/8 VLP (from heron referred to as 'chimeric VLP'). Chimeric BTV vaccines are of particlular interest as they present potential for making multivalent vaccines. This can be achieved through the production of chimeric particles where protection is provided against

both serotypes included in the particle design (Nunes et al., 2014, Feenstra et al., 2015, Mokoena et al., 2019). Alternatively a chimeric particle, where the chimeric region can be easily exchanged, can be used in a cocktail of particles each inducing a serotype-specific response (Rojas et al., 2021, van Rijn et al., 2021). There are a number of studies which have investigated chimeric BTV vaccines. A chimeric disabled infectious single animal (DISA) vaccine was developed where either the entire VP2 protein was exchanged or only part of it was exchanged with the homologous region of a different serotype while VP3, VP5 and VP7 were based on the same serotype (Feenstra et al., 2015). This study found that it is not always viable to exchange the entire VP2 protein as it is thought that the interaction between VP2 and VP5 is required for, in this case, BTV rescue. The chimeric VP2 constructs that were designed meant that the serotype of the recombinant virus was altered while still allowing the native folding and functionality of VP2 to remain. Similarly another study developed a chimeric inactivated BTV vaccine where a BTV1 backbone with a chimeric BTV8/1 VP2 was produced (Nunes et al., 2014). Both of these studies found that the vaccines with chimeric VP2 proteins were neutralised by antibodies from both serotypes included in the chimeric construct. This suggests that there are neutralising epitopes in multiple places on the VP2 protein and chimeric vaccines could present an opportunity for the development of bivalent or multivalent vaccines. This is of particular relevance in South Africa where multiple serotypes can be in circulation at any given time.

In the present study, the chimeric VLP is based off the fact that the VP2 protein is the outermost protein on the viral particle and induces the neutralising antibody response (White et al., 1990, Hwang et al., 1993). Specifically, the tip domain of VP2 points outwards from the viral particle (Figure 2.1 A and B) and binds to circulating antibodies, thereby inducing a serotype-specific neutralising antibody response during infection (Zhang et al., 2010). We have replaced the approximately 220 amino acid immunogenic tip domain of the BTV8 VP2 capsid protein with that of the corresponding region from the BTV1 serotype (Figure 2.2). This makes a chimeric BTV1/8 VLP where VP3, VP5 and VP7 are based on BTV8 but the VP2 sequence is that of BTV8 VP2 with the tip domain substituted with that of BTV1. If a VLP can assemble when the established backbone of BTV8 VP3, VP5 and VP7 is co-expressed with the BTV1/8 VP2 and induce a BTV1 specific immune response, then the chimeric VLP can potentially be manipulated to create other serotype specific VLPs by replacement of a single domain. This would make the vaccine design more accommodating for outbreaks where the serotypes in circulation cannot be easily predicted before an outbreak.

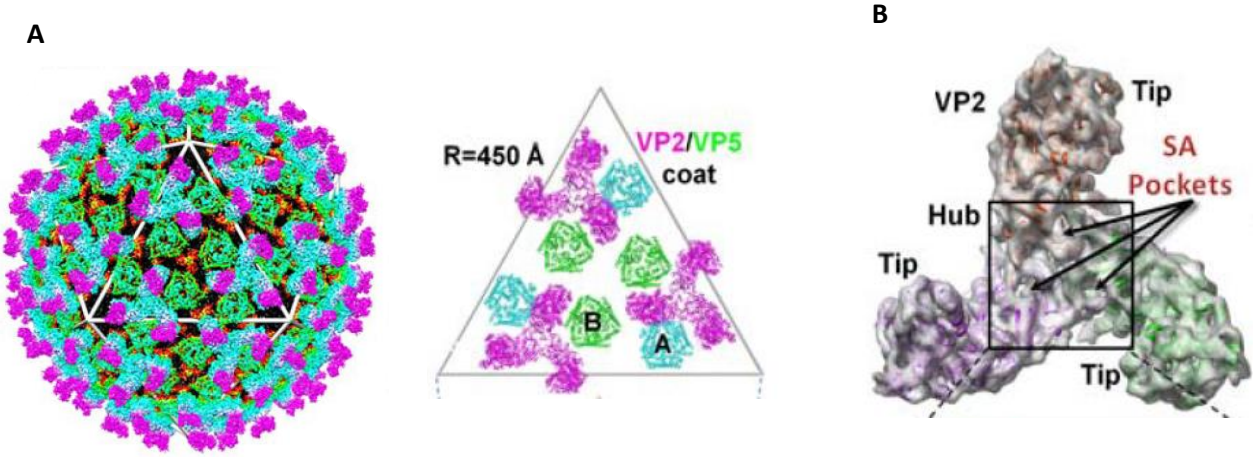


**Figure 2.1: (A)** A 3D model of BTV colour coded by radial position: outer-coat VP2 (magenta and cyan), inner-coat VP5 (green), outer core VP7 (red and black), and inner core VP3 (not visible). **(B)** Top view of the density map of a VP2 triskelion, with three tip domains and a hub which harbours a sialic acid binding pocket. Permission obtained from publisher (Zhang et al., 2010).

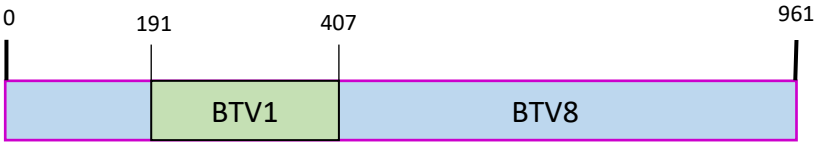



**Figure 2.2: Schematic of chimeric BTV1/8 VP2 protein with amino acid positions indicated.** BTV8 VP2 protein (blue) with antigenic tip domain (green) substituted with that of the corresponding region of BTV1.

This study makes use of existing constructs from the Biopharming Research Unit (BRU). Sequences encoding each BTV serotype 8 VLP structural protein (VP2, VP3, VP5 and VP7) were individually cloned into the pEAQ-*HT* vector. BTV8 VLPs were assembled by co-infiltration of *Agrobacterium* containing the pEAQ-*HT* BTV8 constructs while the chimeric VLPs were assembled through the co-expression of the BTV8 VP3, VP5 and VP7 constructs together with the chimeric BTV1/8 VP2. Preliminary results using these constructs showed that co-infiltration with both the BTV8 VLP constructs as well as the modified chimeric VP2 construct resulted in the assembly of BTV8 and chimeric BTV1/8 VLPs. The VLP proteins (VP2 111kDa, VP3 103kDa, VP5 59kDa and VP7 38kDa) were detected on Western blots and Coomassie blue-stained gels with low intensity. Particles were visualised under the transmission electron microscope (TEM), however many particles were sub-core like particles (SCLP) made up of the VP3 protein alone (approximately 51nm), core-like particles (CLPs) made up of VP3 and VP7 (60-69nm) or degraded VLPs (BRU, unpublished). BTV8 VLPs were used as a control as these particles have been successfully expressed in plants in a previous study (van Zyl et al., 2016) .

In the present study, these pre-existing constructs and preliminary results were used to further optimise the expression and assembly of BTV8 and BTV1/8 VLPs by comparing and improving protein expression and purification processes to maximise VLP production. The optimised methods for production of these VLPs were used to produce vaccine candidates of sufficient quantities for use in animal immunogenicity trials.

A number of factors are known to affect VLP production in plants. For example, co-infiltration with a chaperone can influence how well particles assemble and maintain their structure and stability (Mohl et al., 2019). Additionally, pH and ionic strength of extraction and purification buffers can influence stability of VLPs (Owen, 1964, McCarthy et al., 1998). Taking these factors into account, this chapter explores the transient expression, extraction and purification of both BTV8 and chimeric BTV1/8 VLPs for their optimal production in *Nicotiana benthamiana* plants.

## 2.2 Materials and Methods

### 2.2.1 Recombinant *Agrobacterium* constructs

Previous research conducted in the BRU investigated expression of the BTV8 and BTV1/8 VLPs in *N. benthamiana* where genes encoding the BTV VLP structural proteins were cloned into the pEAQ-*HT* vector, transformed into *A. tumefaciens* and infiltrated into *N. benthamiana* individually or in combination. Preliminary results found that recombinant *Agrobacterium* cultures should be infiltrated at an $OD_{600}$ of 0.5 each and plant material should be harvested on 4 days post-infiltration (4 dpi) for

optimal yields (personal communication). For the present study, these methods were initially followed to confirm previous results obtained before the continuation of optimisation experiments.

The glycerol stocks of recombinant *A. tumefaciens* were obtained from the culture collection of the BRU. These constructs were originally obtained from the Thuenemann research group (John Innes Centre, UK). These included the BTV8 VLP genes (*VP2, VP3, VP5 and VP7*) in pEAQ-*HT* in *A. tumefaciens* LBA4404: pEAQ-HT.BTV8-VP2, pEAQ-HT.BTV8-VP3, pEAQ-HT.BTV8-VP5 and pEAQ-HT.BTV8-VP7. The four genes were modified as described by Thuenemann et al. (Thuenemann et al., 2013) to facilitate directional cloning of the genes into the *Age*I and *Xho*I sites of the pEAQ-*HT* vector to yield four different constructs.

Other constructs used for this work and made previously in the BRU (see corresponding LabCollector numbers) included: pEAQ-HT BTV1/8VP2 (#2156), pEAQ-HT-HSP90 (#2288) and pEAQ-HT (#1348) all hosted in *A. tumefaciens* AGL1 (ATCC BAA-101). The chimeric pEAQ-HT BTV1/8 VP2 construct was designed as discussed in section 2.1. The *HSP90* gene sequence (GenBank accession number AY383484) was codon optimised for equine expression and synthesised by GenScript (USA). The pEAQ-*HT* vector lacking an insert (Sainsbury et al., 2009) in AGL-1 was used as a negative control for expression in plants (Figure 2.3).

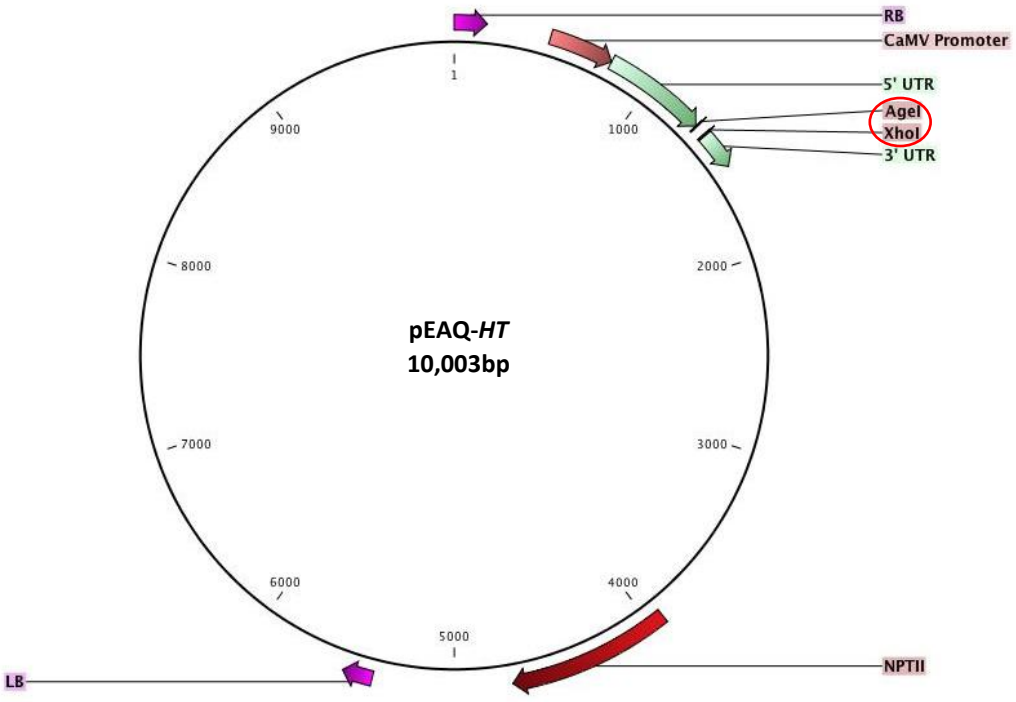



**Figure 2.3: pEAQ-*HT* vector used for cloning.** All genes used in this study were subcloned into the pEAQ-*HT* vector between the AgeI and XhoI restriction enzyme sites (circled in red). RB and LB: right border and left border for T-DNA integration. CaMV Promoter: Cauliflower mosaic virus (CaMV) 35S promoter. 5' UTR and 3'UTR: untranslated regions from Cowpea mosaic virus RNA-2. *NPTII*: kanamycin resistance gene.

### 2.2.2 *Agrobacterium*-mediated infiltration

Glycerol stocks were revived by inoculation into 10mL Luria Bertani (LB) broth (10g/L tryptone, 5g/L yeast, 5g/L NaCl) supplemented with the appropriate antibiotics (50ug/mL kanamycin, 25ug/mL

carbenicillin, 50ug/mL rifampicin). To prevent clumping of the LBA4404 cells, magnesium sulphate ($MgSO_4$) was added to a final concentration of 2mM to the appropriate medium. Cultures were grown at 27°C overnight with agitation. The 10ml cultures were then inoculated into 50mL LBB (2.5g/L tryptone, 12.5g/L yeast, 5g/L NaCl, 1.95g/L 4-morpholineethanesulfonic acid (MES), pH5.6) containing appropriate antibiotics. Incubation was repeated as with the 10mL cultures. The same protocol was followed with the 50mL cultures being inoculated into 500mL LBB but lacking rifampicin and with added acetosyringone (20uM). The 500mL overnight cultures were adjusted to the desired optical density ($OD_{600}$) in resuspension solution (0.975g/L MES, 2.03g/L MgCl2.6H20 pH 5.6). For co-infiltration, each construct was adjusted to an $OD_{600}$ of 0.5 in resuspension solution with the final infiltration suspension consisting of four constructs with a total $OD_{600}$ of 2 (and a final $OD_{600}$ of 2.5 when HSP90 was included). The diluted cultures were supplemented with 200µM acetosyringone and left to stand for 1 h to allow for the *vir* genes of the Ti plasmid to be induced. The diluted bacterial suspensions at the desired optical densities ($OD_{600}$) were vacuum infiltrated into four- to six-week-old *N. benthamiana* plants (grown under 16-hour light/ 8-hour dark cycles at 22˚C). Plants were submerged in the bacterial cultures and infiltrated by applying a vacuum of -100kPa which was released to allow for complete infiltration of the bacterial suspension into the leaves. The negative control culture was prepared for infiltration in the same way ($OD_{600}$ 0.5) and infiltrated into plants in the same manner as the other bacterial cultures.

2.2.3 Protein extraction and clarification

2.2.3.1 Small scale VLP extraction

For the small-scale optimisation experiments, 2g of leaf material was harvested from each group of infiltrated plants at 4 dpi. The plant material was homogenised using an IKA® T25 digital ULTRA-TURRAX in two volumes of bicine buffer (50mM bicine, 50mM NaCl or 100mM NaCl, pH9) containing 1x Roche® EDTA-free complete protease inhibitor. The homogenised material was filtered through 2 layers of Miracloth™ (Merck Millipore) and centrifuged (Beckman Coulter Avanti® J25-I centrifuge) at 15344 x g for 10 min to remove plant debris. The supernatant was incubated at 4°C overnight with agitation. A sample of each fraction was boiled in 1x sample application buffer (SAB) (from 5x SAB – 2% sodium dodecyl sulphate (SDS), 100mM Tris-Cl pH7.5, 2mM EDTA, 52% glycerol, 4.3% mercaptoethanol, bromophenol blue) for 10 min at 95°C before analysis of protein expression by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent detection on Western blots and Coomassie-blue stained gels (section 2.2.5). This method was also followed for the preparation of the negative control samples used in all analyses.

2.2.3.2 Large scale VLP extraction

For the large-scale experiments, approximately 25-30g of leaf material was harvested from each group of infiltrated plants at 4 dpi or 5 dpi. The plant material was homogenised using an IKA® T25 digital ULTRA-TURRAX in 2 volumes of bicine buffer (50mM bicine, 20mM, 100mM or 400mM NaCl, pH9) containing 1X Roche® EDTA-free complete protease inhibitor. The homogenised plant material was incubated at 4°C with gentle agitation for 1 h. The extract was centrifuged (Beckman Coulter Avanti® J25-I centrifuge) at 25931 x g for 30 min, filtered through one layer of Miracloth™ (Merck Millipore) and then centrifuged again at 25931 x g for 20 min. The pH of the clarified extract was corrected to pH8.4 with 1M NaOH and incubated at 4°C with gentle agitation for approximately 48 h.

2.2.4 VLP purification by density gradient ultracentrifugation

Large-scale purification of VLPs was achieved through a discontinuous step-gradient by ultracentrifugation. This had also been optimised previously and the established method was followed without alteration. The clarified plant extract was centrifuged (Beckman Coulter Avanti® J25-I centrifuge) at 25931 x g for 20 min. The supernatant was then loaded onto a discontinuous Optiprep™ (Sigma Aldrich) iodixanol gradient (2mL 50%, 2mL 40%, 2mL 30%, 2mL 20%) diluted in the same buffer in which the material was extracted. The clarified plant extract overlaid on the gradient was centrifuged (Beckman Coulter Optima™ L-100 XP Ultracentrifuge) at 60973 x g (22400rpm) for 3 h at 10°C using a Beckman SW 32 Ti rotor. After centrifugation, the bottoms of the centrifuge tubes were punctured with a syringe needle and 500uL fractions were collected in microcentrifuge tubes. A sample of each fraction was boiled in 1x SAB for 10 min at 95°C before analysis of protein expression by SDS-PAGE and subsequent detection on Western blots and Coomassie-blue stained gels. Clarified extract from the plants infiltrated with the negative control construct were also purified using these methods.

2.2.5 Protein analysis

Equal volumes of samples were resolved through a 10% SDS polyacrylamide gel (Laemmli 1970) at 100 volts (V) for approximately 2 h (Bio-Rad Tetra Cell system). For Western blot analysis, a protein ladder (ThermoScientific™ PageRuler™Plus Prestained Protein Ladder) was used as a molecular weight marker. After gel electrophoresis, the gels were either incubated in Coomassie-blue stain (0.1% (w/v) brilliant blue G-250; 48% v/v methanol, 15% v/v glacial acetic acid) for 1 h at 37°C and then de-stained with de-stain solution (30% v/v methanol,10% v/v glacial acetic acid) overnight at room temperature or transferred to a nitrocellulose membrane for Western blot analysis.

Proteins were transferred from the SDS-PAGE gel to a nitrocellulose membrane (Bio-Rad Trans-Blot® Semi-dry transfer cell) at 15V for 1 h 30 min. Blocking buffer (50 mL/L skim milk, 100mL/L 10 x PBS, 10mL/L 10% Tween-20) was added to the nitrocellulose which was blocked for 30 min with agitation. The membrane was probed with sheep serum (1:2000 or 1:5000 dilution in blocking buffer) containing antibodies raised against plant-produced BTV8 VLPs (Thuenemann et al. 2013) and incubated at 4°C overnight with gentle agitation. Thereafter the membrane was washed for 15 min in blocking buffer four times. Following washing, the membrane was probed with a 1:10000 dilution of monoclonal mouse anti-goat/sheep IgG-alkaline phosphatase antibody (Sigma Aldrich) for 1 h at 37°C. Finally, the membrane was washed as before in blocking buffer lacking milk and the proteins were detected with BCIP/NBT (SeraCare Life Sciences, Inc.).

2.2.6 Transmission electron microscopy

Carbon-coated copper grids (200 mesh size) were glow discharged and prepared with collected fractions from density gradient purifications. Samples were adsorbed onto the grids by floating the grids on 10uL of sample (carbon side down) for 1 min and subsequently washing them three times with 10uL sterile water. Finally, they were negatively stained for 1 min with 2% (w/v) uranyl acetate. Grids were air-dried and samples were visualised with a FEI Tecnai G2 20 Twin transmission electron microscope with bottom-mounted digital camera.

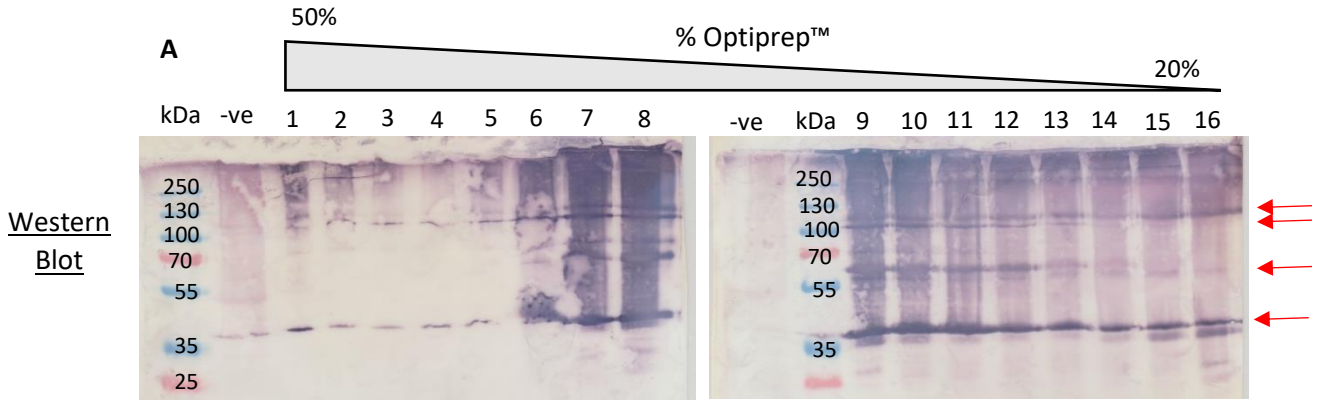
## 2.3 Results

2.3.1 Initial results based on previous work done

To determine the baseline levels of BTV protein expression and VLP formation, the methods previously developed in this research group were followed. BTV8 VLP proteins (BTV8 VP2, VP3, VP5, and VP7) and chimeric BTV1/8 VLP proteins (BTV1/8 VP2, BTV8 VP3, VP5 and VP7) were expressed in *N. benthamiana* plants. Each vector construct encoded the sequence for each one of the VLP proteins cloned into the pEAQ-*HT* vector.

Proteins were detected on the Western blots by serum collected from sheep immunised with BTV8 VLPs. All four BTV8 VLP proteins could be detected on a Western blot (Figure 2.4 A) in the fractions collected from the 40% and 30% Optiprep™ gradients (fractions 7-13) as indicated by the red arrows which from top to bottom represent VP2 at 111kDa, VP3 at 103kDa, VP5 at 59kDa and VP7 at 38kDa. All four of the chimeric VLP proteins were also detected on the Western blot (Figure 2.4 B) in the 30% gradient fractions (9-13) indicated by the red arrows as in Figure 2.4A. The chimeric VP2 protein had a lower band intensity than the BTV8 VP2 protein and was detected in fewer of the fractions. None of the relevant proteins were detected in the negative control. Some bands were detected at approximately 70kDa which do not correspond to any of the VLP proteins. This could be a result of non-specific binding of the polyclonal primary antibody which was taken from sera collected from sheep immunised with BTV8 VLPs.

All four BTV8 VLP proteins were visualised on the Coomassie stained gel in fractions 8 and 9 (Figure 2.4 C) however only VP3 (103kDa) and VP7 (38kDa) were visualised in the chimeric VLP fractions 7-10 (Figure 2.4 D). Red arrows indicate the BTV proteins that were detected. To confirm particle assembly, samples were analysed by transmission electron microscopy (TEM).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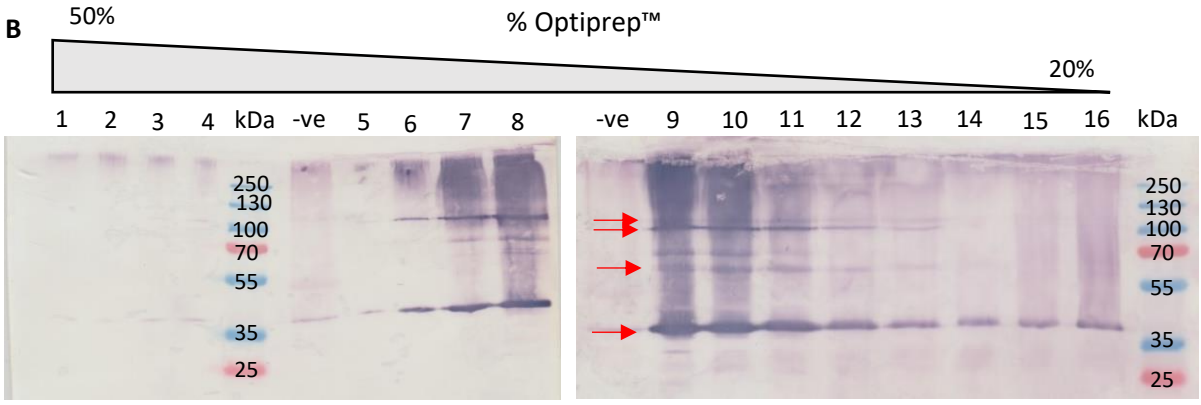
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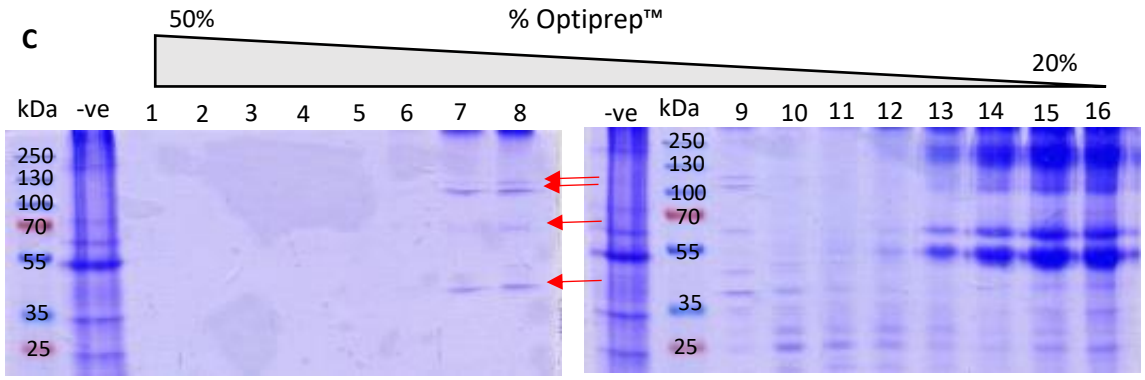
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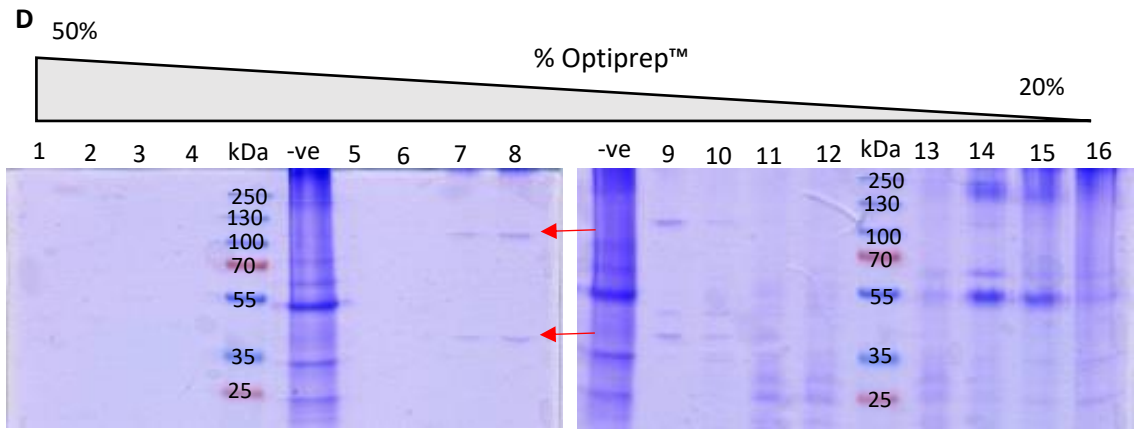
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**Figure 2.4: Detection of purified plant-produced BTV8 and BTV1/8 VLP proteins.** BTV8 or BTV1/8 VLP proteins (VP2, VP3, VP5 and VP7) were co-expressed in plants where each construct was infiltrated at an $OD_{600}$ of 0.5. Plants were harvested at 4 dpi. Fractions of 500uL were collected from the bottom of the tube (Four 2mL gradients: 50%, 40%, 30% and 20% Optiprep™). Western blots and Coomassie-stained SDS-PAGE gels detected proteins from purified fractions from plants expressing **(A and C)** BTV8 VLP proteins or **(B and D)** chimeric BTV1/8 VLP proteins. Proteins were detected on the Western blot with sheep-produced anti-BTV8 VLP anti-serum (1:2000) and anti-goat/sheep alkaline phosphatase-conjugated secondary antibody (1:10000). 1-16: 500uL fractions collected from the bottom of the tube where 1=bottom 50% fraction and 16=top 20% fraction. Red arrows indicate VLP proteins where VP2 is 111kDa, VP3 is 103kDa, VP5 is 59kDa and VP7 is 38kDa. -ve: Negative control pEAQ-*HT* vector. kDa: molecular weight marker in kilodaltons.

Purified fractions collected from the top of the 40% Optiprep™ gradients (fractions 7 and 8) were visualised by TEM with negative staining of carbon-coated copper grids. Some particle-like structures were visualised (Figure 2.5). There were more particles per field of view in the BTV8 fractions (Figure 2.5 A) but most seemed to be partially formed SCLPs or CLPs. CLPs (indicated by yellow arrows) consisting of VP3 and VP7 were identified as having thin light-coloured borders and a round 'spiky' shape. They were smaller than VLPs measuring approximately 60-69nm in diameter. The chimeric fractions (Figure 2.5 B) had fewer particulate structures per frame of view resembling SCLPs and CLPs (yellow arrows).

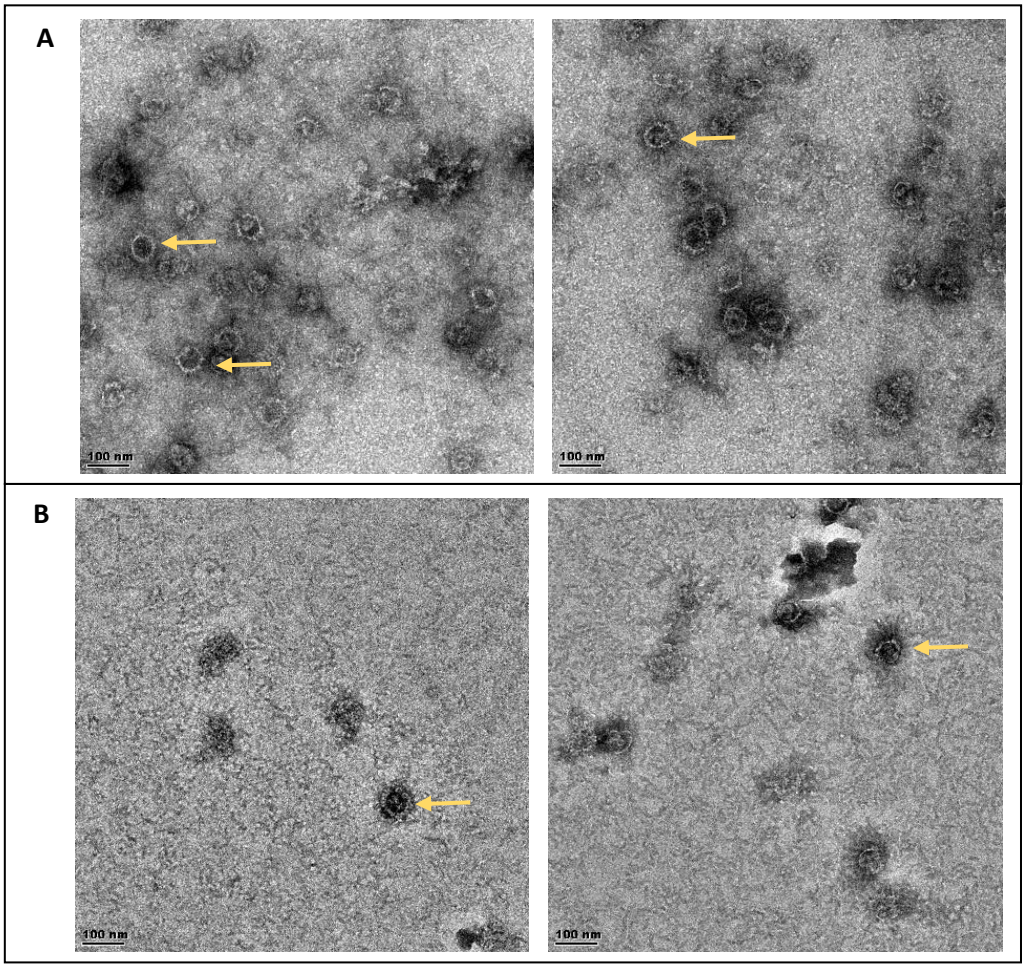


**Figure 2.5: Transmission electron micrographs of plant-purified BTV8 and BTV1/8 particles.** Plant produced particles were purified on an Optiprep™ density gradient and adsorbed to copper grids by negative staining for viewing under the transmission electron microscope. Micrographs of the top 40% purified fractions (fraction 7 and 8) from plants expressing **(A)** BTV8 VLP proteins and **(B)** BTV1/8 VLP proteins are represented. Yellow arrows indicate CLPs. Scale bars represent 100nm.

2.3.2 Small-scale optimisation of VLP production

In order to optimise the expression and extraction of the VLPs based on the previous methods used, a small-scale infiltration was conducted to determine if changes to the methods could improve signal detection of all four VLP proteins and increase the yield of VLPs. This was assessed qualitatively by detection of the VLP proteins on a Western blot (Figure 2.6). We used two different extraction buffers with either 50mM or 100mM NaCl to increase the ionic strength and improve stability of the particles. Some plants were also co-infiltrated with the HSP90 chaperone to determine whether this would enhance particle stability and formation thereby increasing yields. Different dilutions (1:2000, 1:7000 and 1:10000) of primary antibody were tested to see if an increased dilution will prevent the smearing effect seen on the Western blots.

The samples in lanes 4 and 5 (Figure 2.6A) which were extracted in buffer with increased NaCl concentration (100mM) showed similar signal intensity to the samples extracted in 50mM NaCl buffer (Figure 2.6A lane 2 and 3) with a slight increase in the detection of the proteins in the chimeric sample.

Figure 2.6 B shows the comparison of samples from plants infiltrated with or without HSP90 (lanes 2-5). The Western blot (Figure 2.6B) showed that the band intensity for the VLP proteins was similar regardless of whether HSP90 was included or not. There was low VP2 detection in the chimeric samples and low VP5 detection in all samples (Figure 2.6B). The increase in dilution of the primary antibody did not seem to make a difference in the detection of the VLP proteins suggesting the dilution can be increased (Figure 2.6 A and B lanes 7 and 8). The negative control had no bands corresponding to VLP proteins.

Based on the qualitative assessment of band intensity, the increased ionic strength of extraction buffer and co-infiltration with HSP90 were investigated further through large-scale infiltration experiments. An increase in the dilution of the primary antibody was applied to future Western blots as it was clear from these results that an increased dilution of the antiserum did not affect its ability to detect the VLP proteins and could thus help to decrease the smeared staining pattern observed on the Western blots.

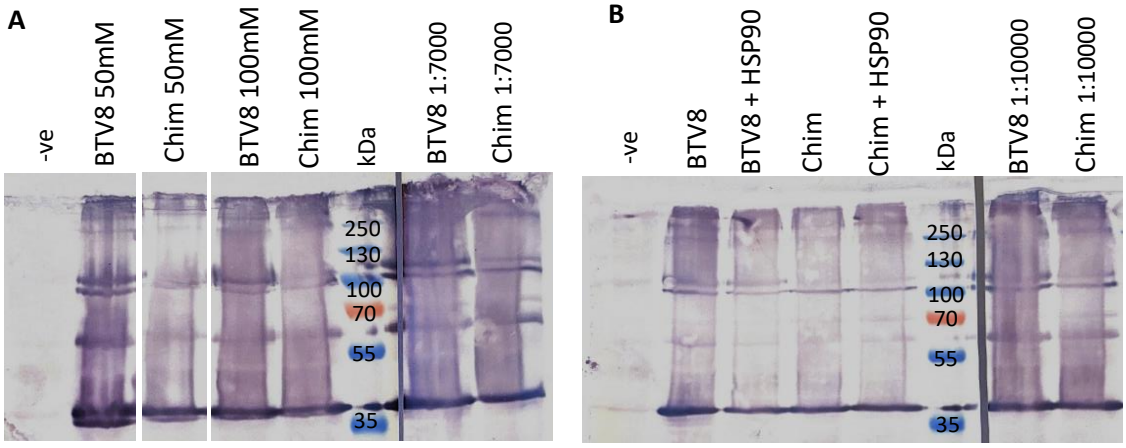


**Figure 2.6: Small-scale expression and crude extract analysis of VLP proteins.** Plants were vacuum infiltrated with BTV8 or BTV1/8 VLP constructs and VLP proteins were detected on a Western blot. Proteins were **(A)** extracted in 50mM NaCl buffer or 100mM NaCl buffer or **(B)** co-infiltrated with without a HSP90 chaperone-encoding vector. Proteins were detected with anti-BTV8 VLP sheep serum (1:2000) and anti-goat/sheep alkaline phosphate-conjugated secondary antibody (1:10000). Blots were probed with greater dilutions of primary antibody (1:7000 and 1:10000). -ve: Negative control pEAQ vector. kDa: molecular weight marker in kilodaltons.

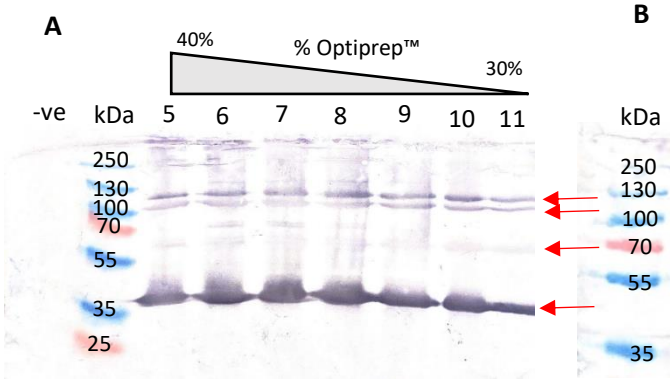
### 2.3.3 Large scale optimisation of extraction buffer with increased salt concentration

To further investigate the effect of an increased concentration of NaCl in the extraction buffer, a large scale infiltration experiment was conducted where plants co-infiltrated with either BTV8 VLP constructs or chimeric BTV1/8 VLP constructs were harvested, extracted and purified in either 100mM NaCl buffer or 400mM NaCl buffer. Having illustrated that a 100mM NaCl buffer improved detection of the VLP proteins in the small-scale experiment, an even greater concentration of 400mM NaCl was investigated based on work previously done in this research group (van Zyl et al., 2017) which suggested this may increase VLP yields. Harvest day was also extended to 5 dpi as plant leaves were not substantially necrotic at 4 dpi and it was reasoned that harvesting one day later could potentially increase protein yields. Previously it had been observed that there was a drop in pH in the clarified plant extract after extraction. Although the pH of the extraction buffer initially used was 9, the pH of the clarified plant extract decreased to 7.2 after clarification. The plant extract was thus adjusted to a pH of 8.4 with 1M NaOH before the maturation period. Samples purified from density gradient ultracentrifugation were collected in 500uL fractions from the bottom of the 50% gradient and analysed on Western blots and Coomassie blue-stained SDS-PAGE gels (Figure 2.7).

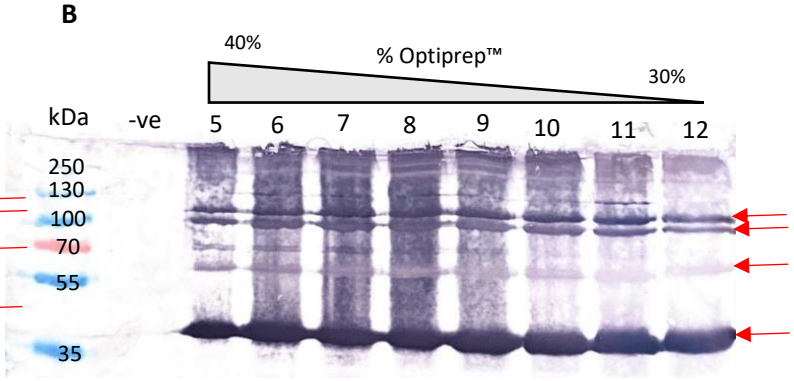
The Western blots with the BTV8 VLP samples (Figure 2.7 A and B) showed detection of all four VLP proteins in both 100mM and 400mM NaCl buffers; however bands had a more intense signal in the samples with the higher salt concentration (Figure 2.7 B). VP5 (59kDa) was barely visible in the 100mM samples (figure 2.7 A). The four proteins were also visualised on the Coomassie gels (Figure 2.7 E and F), specifically in fractions 7-10 (indicated by red circles) of the higher salt fractions which fall at the interface between the 40% and 30% Optiprep™ gradients (Figure 2.7 F).

At the lower salt concentration, all four chimeric VLP proteins were detected on the Western blot in fractions 5 and 6 (Figure 2.7 C) with a low signal whereas at the higher salt concentration all four proteins were detected with high intensity in fractions 7-10 (Figure 2.7 D). At the low salt concentration chimeric VP2 (111kDa) cannot be visualised on a Coomassie gel in the same fractions (Figure 2.7 G) however at the higher salt concentration all four chimeric VLP proteins can be visualised in fractions 7-10 (indicated by red circles) (Figure 2.7 H) illustrating the fact that the higher salt concentration does improve VLP protein signal and detection. There were no bands detected in the negative control lanes. This experiment was repeated three times independently to validate the results.

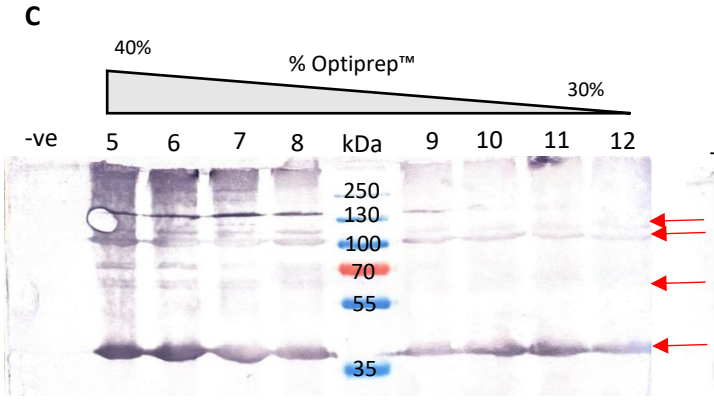
## BTV8 100mM NaCl




## BTV8 400mM NaCl




## Chimeric 100mM NaCl

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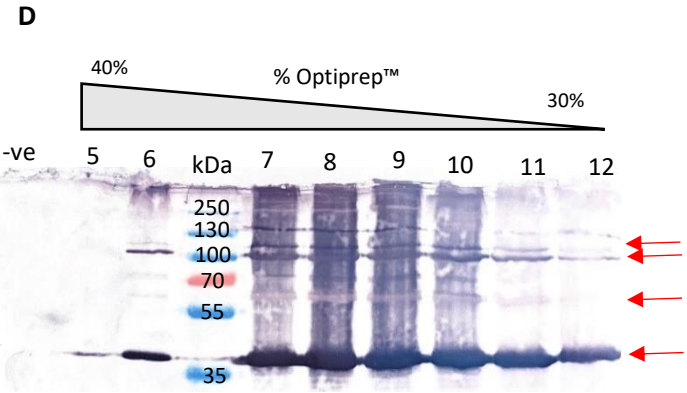
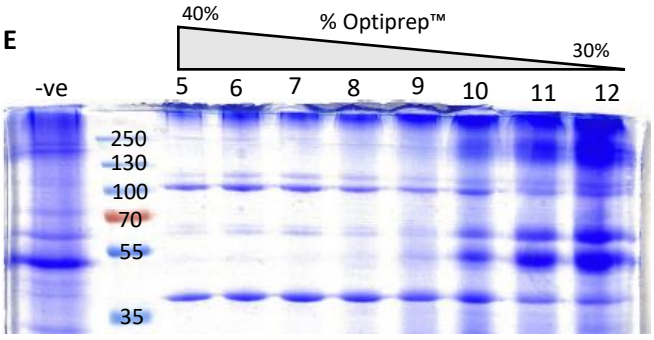
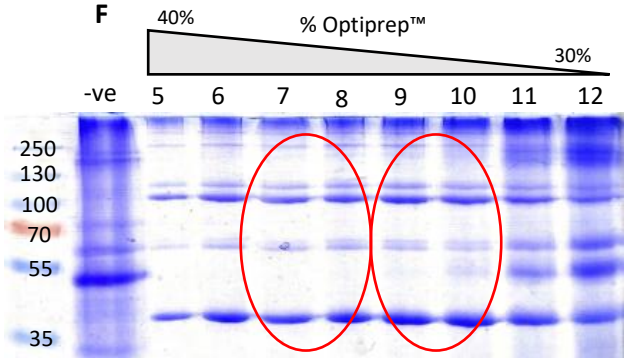


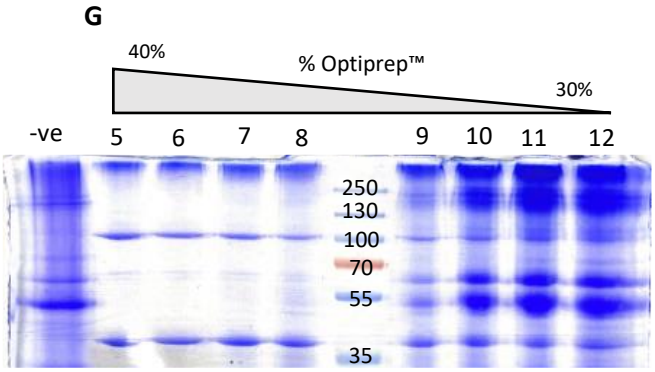

## BTV8 100mM NaCl




## BTV8 400mM NaCl




## Chimeric 100mM NaCl




## Chimeric 400mM NaCl

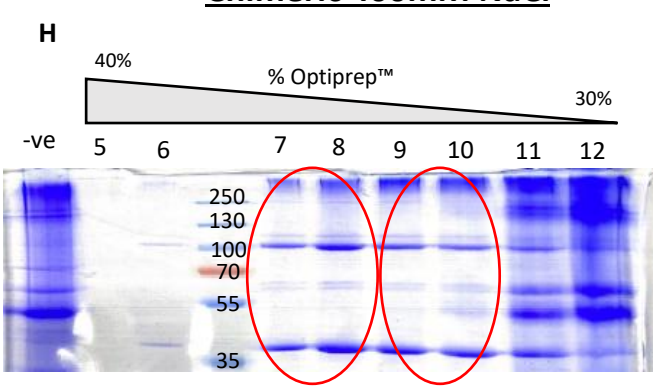
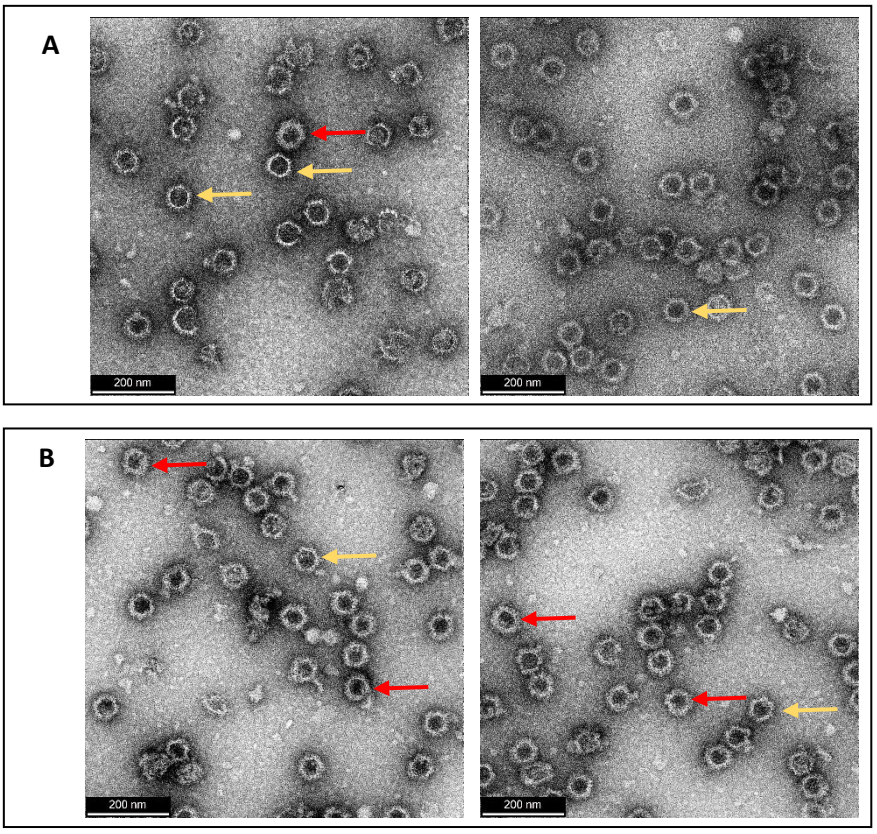


**Figure 2.7: Optimisation of VLP extraction and purification buffer (100mM NaCl vs 400mM NaCl).** BTV8 or BTV1/8 VLP proteins (VP2, VP3, VP5 and VP7) were co-expressed where each construct was infiltrated at an $OD_{600}$ of 0.5 and harvested at 5 days post infiltration. Fresh leaf material was homogenised, clarified and purified in 50mM bicine buffer containing either 100mM NaCl or 400mM NaCl. Purified fractions of 500uL were collected from the bottom of the tube. Western blots and Coomassie blue-stained SDS-PAGE gels detected purified fractions from plants expressing BTV8 VLP proteins in 100mM NaCl buffer **(A+E)** or 400mM NaCl buffer **(B+F)** and purified fractions from plants expressing chimeric BTV1/8 VLP proteins in 100mM NaCl buffer **(C+G)** or 400mM NaCl buffer **(D+H)**. Proteins on Western blots were detected with anti-BTV8 VLP sheep serum (1:5000) and anti-goat/sheep alkaline phosphate-conjugated secondary antibody (1:10000). 5-12: 500uL fractions collected from the bottom of the tube where 5=bottom 40% fraction and 12=top 30% fraction. Red circles indicate fractions where all four VLP proteins were detected on the Coomassie blue-stained gels. Red arrows indicate VLP proteins where VP2 is 111kDa, VP3 is 103kDa, VP5 is 59kDa and VP7 is 38kDa. -ve: Negative control pEAQ vector. kDa: molecular weight marker in kilodaltons.

Samples from the top of the 40% Optiprep™ gradient (fractions 7 and 8) were used to prepare negative-stain carbon-coated copper grids for visualisation under the TEM (Figure 2.8). The BTV8 VLP samples had a similar particle density per field of view despite the variation in NaCl concentration however the lower NaCl concentration resulted in more CLPs (yellow arrows) than VLPs (red arrows) (Figure 2.8 A). The higher salt concentration resulted in the formation of more fully formed BTV8 VLPs (Figure 2.8 B). The chimeric samples had lower particle density at the low salt concentration (Figure 2.8 C) which increased with the increased salt concentration (Figure 2.8 D). Both VLPs and CLPs were observed in both samples.



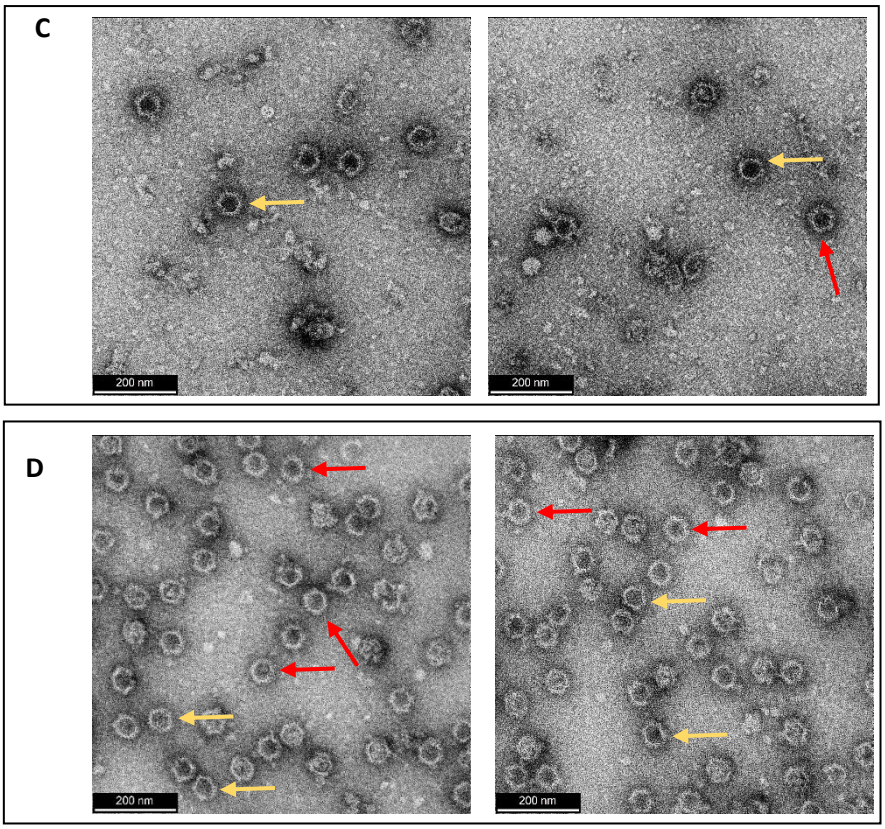
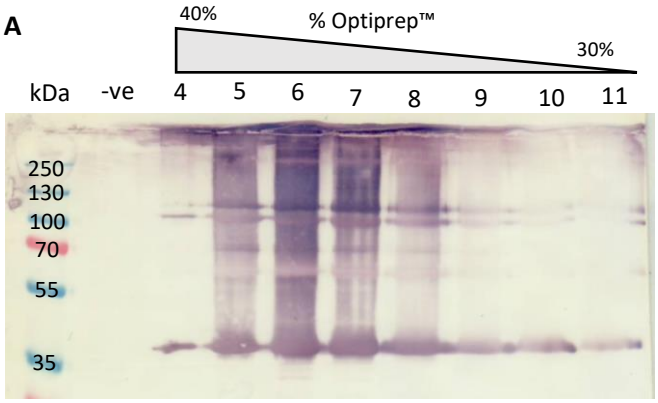



**Figure 2.8: Transmission electron micrographs of plant-expressed BTV8 and BTV1/8 VLPs extracted in either 100mM NaCl or 400mM NaCl bicine buffer.** Plant expressed VLPs were purified on an Optiprep™ density gradient and applied to copper grids by negative staining for viewing under the transmission electron microscope. **A and B**: Micrographs of the top 40% purified fractions (fractions 7 and 8) from plants infiltrated with BTV8 VLP proteins and extracted in (A) 100mM NaCl buffer or (B) 400mM NaCl buffer. **C and D**: Micrographs of the top 40% purified fractions (fractions 7 and 8) from plants infiltrated with Chimeric BTV1/8 VLP proteins and extracted in (C) 100mM NaCl buffer or (D) 400mM NaCl buffer. Yellow arrows indicate CLPs. Red arrows indicate VLPs. Scale bars represent 200nm.

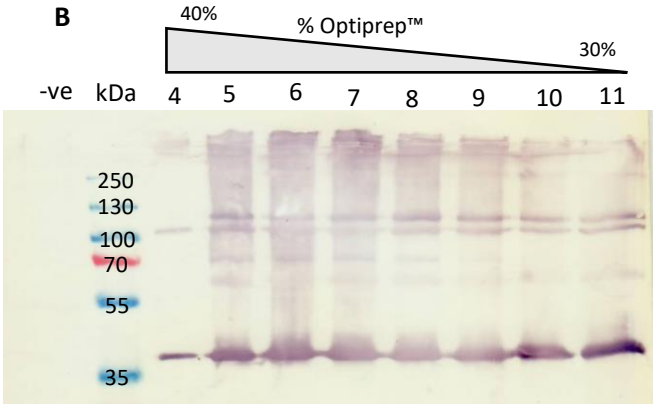
### 2.3.4 Large scale optimisation of VLP production by co-infiltration with HSP90 chaperone

The literature suggests that the HSP90 chaperone can aid in the formation of BTV viral particles and increase stability of the particles (Mohl et al., 2019). Here, a previously-made HSP90-encoding construct transformed into *A. tumefaciens* AGL-1 was co-infiltrated with constructs encoding the four BTV proteins at an $OD_{600}$ of 0.5. Purified samples from plants infiltrated with or without HSP90 were analysed by Western blot and Coomassie blue-stained gels (Figure 2.9). Although the BTV8 VLP proteins were detected on a Western blot regardless of HSP90 (Figure 2.9 A and B), signal intensity was lower in the samples from plants co-infiltrated with HSP90, especially VP5 which was barely visible (Figure 2.9 B). This was also observed on the corresponding Coomassie gels (Figure 2.9 E and F). Similarly the chimeric VLP proteins were also detected regardless of HSP90 inclusion with a slightly lower signal when it was present (Figure 2.9 C and D). Only VP3 and VP7 were clearly visualised on the corresponding Coomasssie-stained gels (Figure 2.9 G and H) with a very faint band corresponding to VP5. These results suggest HSP90 co-infiltration does not improve protein detection or visualisation, and if anything it had a deleterious afffect. There were no bands detected in the negative control lanes. This experiment was independently repeated to validate results.

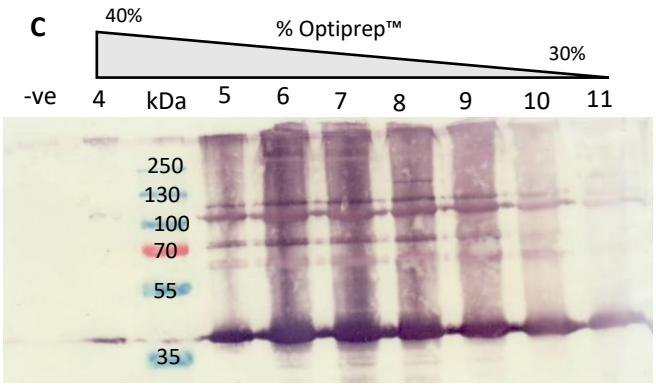
## BTV8



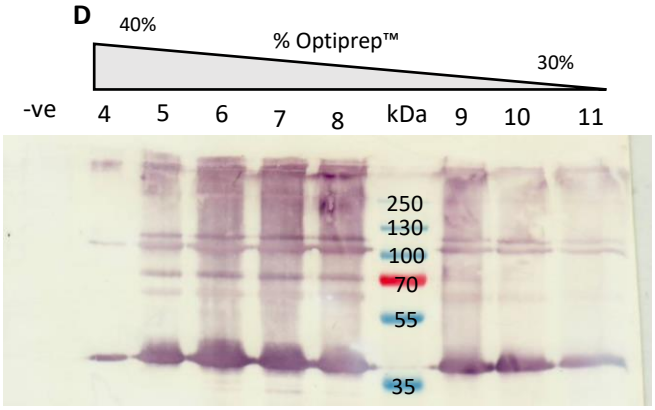

## BTV8 + HSP90



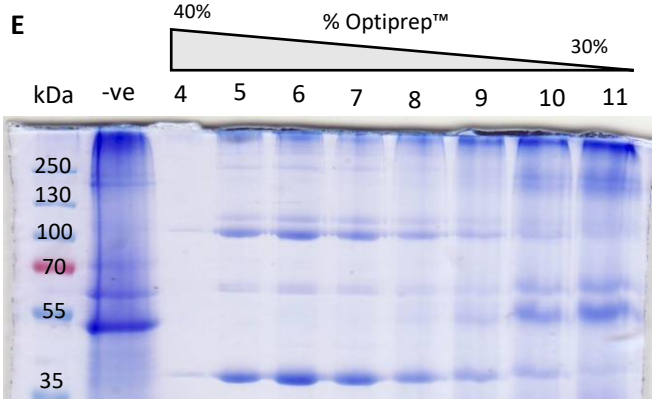

## Chimeric



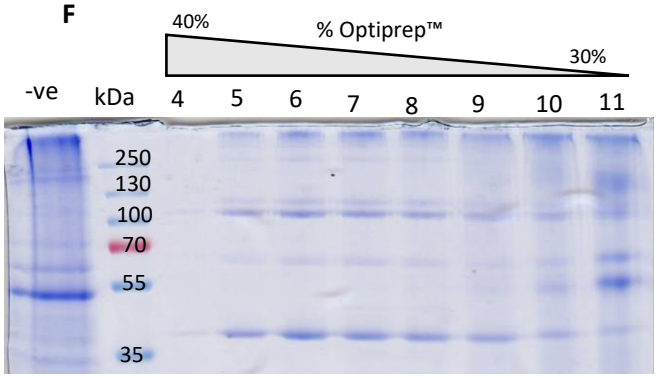

## Chimeric + HSP90



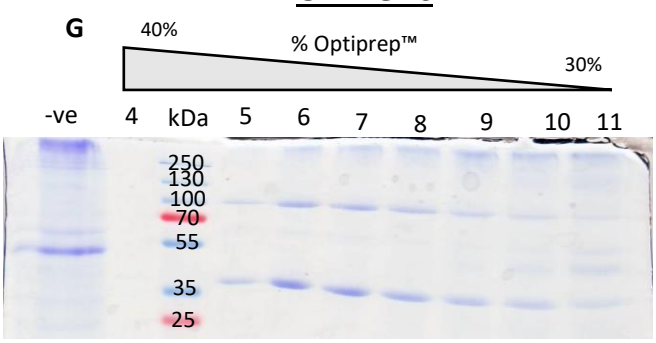

## BTV8




## BTV8 + HSP90




## Chimeric




## Chimeric + HSP90

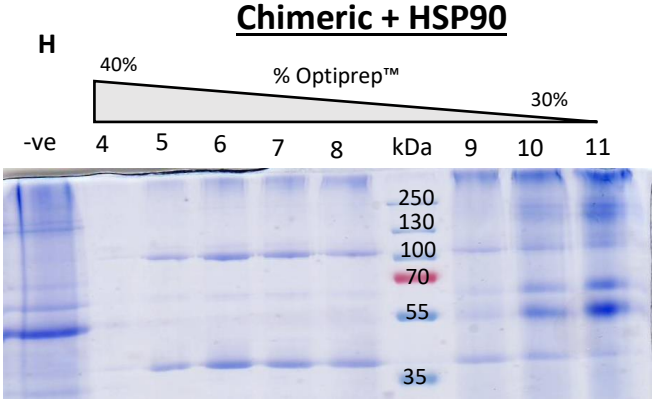


**Figure 2.9: Optimisation of VLP extraction and purification with HSP90 chaperone.** Co-expression of BTV8 or BTV1/8 VLP constructs (VP2, VP3, VP5 and VP7) with or without HSP90 in plants at 5 days post-infiltration where each construct was infiltrated at an $OD_{600}$ of 0.5. Fresh leaf material was homogenised, clarified and purified in 50mM bicine buffer containing 400mM NaCl. Clarified plant extract was purified by density gradient centrifugation (Four 2mL gradients: 50%, 40%, 30% and 20% Optiprep™). 500uL fractions were collected from the bottom of the tube. Western blots and Coomassie blue-stained SDS-PAGE gels detected purified fractions from plants expressing BTV8 VLP proteins without HSP90 **(A+E)** or with HSP90 **(B+F)** and purified fractions from plants expressing chimeric BTV1/8 VLP proteins without HSP90 **(C+G)** or with HSP90 **(D+H)**. Proteins on Western blots detected with anti-BTV8 VLP sheep serum (1:5000) and anti-goat/sheep alkaline phosphate-conjugated secondary antibody (1:10000). 4-11: 500uL fractions collected from the bottom of the tube where 1=bottom 50% fraction and 16=top 20% fraction. -ve: Negative control pEAQ vector. kDa: molecular weight marker in kilodaltons.

Carbon-coated copper grids were prepared with samples from the top of the 40% gradient (fractions 7 and 8) and visualised by TEM (Figure 2.10). The BTV8 samples from leaves not infiltrated with HSP90 showed a low density of particles mostly consisting of VLPs (Figure 2.10 A) whereas in the samples from leaves co-infiltrated with HSP90, there were more CLPs than VLPs per field of view (Figure 2.10 B). A similar phenomenon was observed in the chimeric samples (Figure 2.10 C and D). This was consistent with what was observed on the Western blots and Coomassie-stained gels (Figure 2.9).

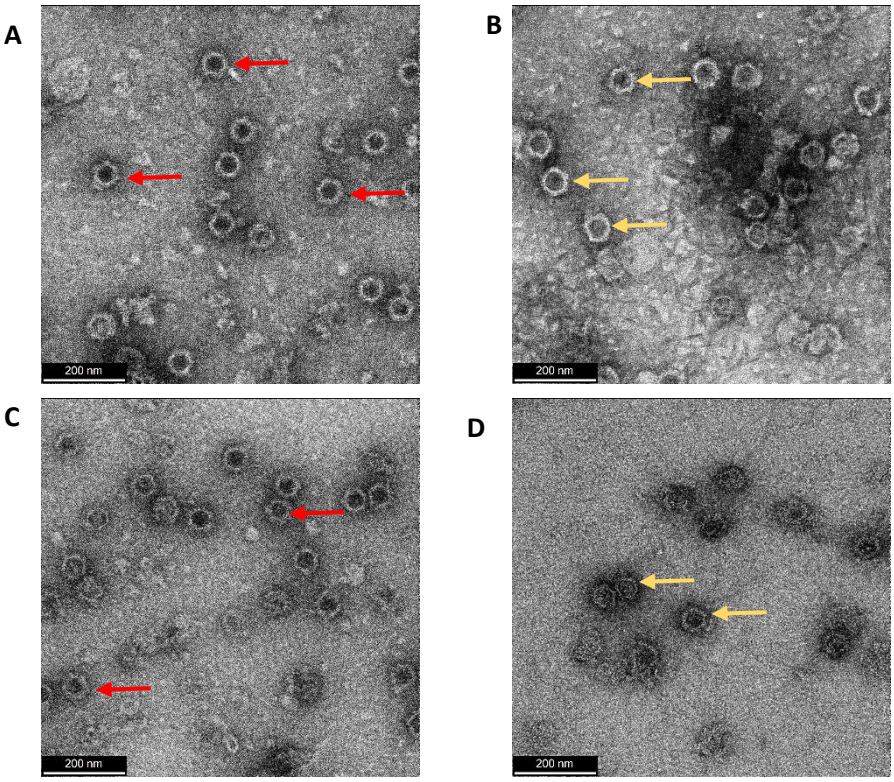



**Figure 2.10: Transmission electron micrographs of plant-expressed BTV8 and BTV1/8 VLPs co-infiltrated with or without HSP90.** Plant expressed VLPs were purified on an Optiprep™ density gradient and applied to copper grids by negative staining for viewing under the transmission electron microscope. **A and B**: Micrographs of the top 40% purified fractions (fraction 7 and 8) from plants infiltrated with (A) BTV8 VLP proteins alone or (B) co-infiltrated with HSP90. **C and D**: Micrographs of the top 40% purified fractions (fractions 7 and 8) from plants infiltrated with (C) chimeric BTV1/8 VLP proteins alone or (D) co-infiltrated with HSP90. Yellow arrows indicate CLPs. Red arrows indicate VLPs. Scale bars represent 200nm.

## 2.4 Discussion

Recently there has been an increased interest in the production of BTV vaccines in plants, specifically BTV VLP vaccines. VLPs are an ideal type of vaccine as they are presented to the immune system similarly to the native virus, however they do not contain any genetic material which could result in virulence. They are safe, immunogenic and antigenically similar to the live virus while having the potential for DIVA compliance as they comprise only four structural proteins (Crisci et al., 2012). The recent studies into BTV vaccines have helped to inform the research done in the present study. These studies include the expression of BTV8 VLPs in *N. benthamiana* (Thuenemann et al., 2013, van Zyl et al., 2016) as well as the development of chimeric BTV VLPs (Mokoena et al., 2019).

The aim of the work described in this chapter was to further optimise the expression, extraction and purification of BTV8 and BTV1/8 VLPs expressed in *N. benthamiana* based on preliminary work conducted in this research laboratory. Here we have optimised these methods through investigating co-infiltration with the HSP90 chaperone, increasing the pH and ionic strength of the extraction buffer and increasing harvest day post-infiltration. As a starting point, the previously established methods were used to conduct a large-scale infiltration experiment to produce the BTV8 and BTV1/8 VLPs. This was done to confirm that the established methods produced the expected results.

On a Western blot, all four BTV8 VLP proteins were detected in fractions corresponding to those within the 30% and 40% Optiprep™ steps (Figure 2.4 A). This was expected based on previous studies purifying BTV VLPs using this method (Thuenemann et al., 2013, van Zyl et al., 2016). All four BTV8 VLP proteins could only be detected in fraction 8 and 9 on a Coomassie blue-stained gel (Figure 2.4 C). These are the interface fractions between the 30% and 40% gradients, confirming they were particulate in nature. VP2 (103kDa) and VP5 (59kDa) were faint but still visible in these fractions. The chimeric BTV1/8 VLP proteins were also detected on a Western blot in fractions 9-13, mostly in the 30% gradient fractions, with less of the chimeric VP2 protein detected compared with BTV8 VP2 (Figure 2.4 B). Only faint bands corresponding to VP3 and VP7 could be detected on a Coomassie gel in fractions 7-10 (Figure 2.4 D). The TEM images show that mostly SCLPs and CLPs were formed in these fractions. There was a slightly greater density of BTV8 particles but overall there were no VLPs present (Figure 2.5). This was not the expected result as the methods that were followed have previously resulted in the production of VLPs, although at low concentrations. The absense of VLPs could be due to a number of reasons. Despite following the established protocol, there may have been unintended changes made to the method by the researcher. The leaves that were harvested and used for extraction and purification of the VLPs may have not been sufficiently infiltrated or those selected for harvesting may have not had the same level of protein expression as previous experiments. Thuenemann et al. found that the co-expression of the BTV8 VLP proteins in *N. benthamiana* results in a mixture of particle types and up to 50% of these particles are assembly intermediates which resemble particles similar to CLPs but not quite whole VLPs (Thuenemann et al., 2013).

Using the previously established methods for the expression and purification of the VLPs we were able to confirm that the protocol does lead to the production of BTV8 and BTV1/8 particles. However, the VLP proteins were not consistently detected in the purified fractions on a Western blot and the particles themselves were not of consistent quality and were low in number per field of view. We could detect all four VLP proteins on Western blots, in certain fractions, but not on the Coomassie-stained gels in some of the corresponding fractions. Because we saw faint bands for BTV8 VP2 and VP5 and no

VP2 or VP5 in the chimeric fractions, it was likely that VLPs would not be forming as a result of insufficient VP2 or VP5. This was confirmed by TEM where only partially formed particles were visualised. CLPs comprising VP3 and VP7 have been found to have a lower immunogenic affect than VLPs (Stewart et al., 2012). CLPs cannot induce a neutralising antibody response and do not confer complete protection to immunised animals (Thuenemann et al., 2013). This is explained by the fact that the neutralising antibody epitopes are found on the VP2 protein which needs to be included for a protective neutralising immune response to be elicited (Erasmus et al., 1981, Appleton et al., 1983, Huismans et al., 1987).

A number of factors involved in the infiltration, harvest and extraction of the proteins could be implicated in the low yield of proteins and VLPs. To address these inconsistencies and to determine whether more optimal expression and extraction conditions could be determined, we made some changes to the established methods. These changes included co-expression with the HSP90 chaperone and increased salt concentration in the extraction buffer. These parameters were applied to a small-scale infiltration experiment where plant crude extract was analysed on a Western blot and Coomassie-stained gel (Figure 2.6). Results from this preliminary experiment would then inform whether it was worth applying the changes to a large scale experiment which consumes more time and resources.

The salt concentration and pH of the extraction buffer are both factors that need to be considered when expressing and extracting VLPs as they can play a role in the stability of the particles. During viral infection, the low pH of the endosome results in the degradation of the viral particles to release the viral core and genetic material (Forzan et al., 2007). It has been found by numerous studies that BTV can be inactivated at a pH of 6.5 and below (Owen, 1964, Verwoerd et al., 1972, Gorman, 1978). The study by Owen found a marked loss of infectivity of BTV between pH 6.1 and pH 6.3 and found that BTV is destroyed in meat at a pH between 5.6 and 6.3. These findings suggest a neutral or higher pH is required for particle assembly and stability. Typically research groups use extraction buffers with a pH of 8.4 which was also used in this study (Thuenemann et al., 2013, van Zyl, 2014, Thuenemann et al., 2018).

Initially bicine buffer containing 50mM bicine and 20mM NaCl was used to extract the proteins as this was commonly used in the extraction of plant VLPs in previous studies (Thuenemann et al., 2013, van Zyl et al., 2016, Mokoena et al., 2019). It has, however, been found that ionic strength can have a marked affect on the stability of protein structures and the assembly of VLPs (McCarthy et al., 1998). Here we compared the VLP yields using extraction buffer containing 50mM NaCl or 100mM NaCl. Detection of BTV8 VLP proteins on the Western blot was similar at both concentrations whereas the chimeric samples showed a greater band intensity in the detection of the VP2 and VP5 proteins at the higher salt concentration (Figure 2.6 A). This was explored further on a large scale (Figure 2.7).

Because the extraction buffer containing 100mM NaCl improved protein detection further (Figure 2.6 A), a large-scale infiltration experiment was conducted where extraction buffers containing either 100mM NaCl or 400mM NaCl were compared. It has been found previously that a greater NaCl concentration (up to 400mM) can improve the stability of particles and thus result in a greater concentration of fully formed VLPs (van Zyl et al., 2017). For example, a 400mM NaCl buffer was used to successfully extract and purify BTV protein bodies from *N. benthamiana* (van Zyl et al., 2017).

The BTV8 VLP proteins were detected on the Western blot and Coomassie gel at both salt concentrations however at the higher salt concentration, band intensity was greater for all VLP proteins. There was a more obvious difference in the band intensity of the chimeric VLP proteins at the different salt concentrations. At the higher salt concentration, VP2 and VP5 were clearly detected on the Western blot and all four VLP proteins were observed on the Coomassie gel which was not the case at the lower NaCl concentration (Figure 2.7). These results give a strong indication that the extraction buffer containing the higher salt concentration, 400mM NaCl, increased the stability of the VLP proteins and thus increased the concentration of purified protein and its detection on a Western blot and Coomassie gel.

The TEM results show correlation between what was detected on the gels and what was visualised under the microscope. There was a greater density of BTV8 particles per field of view (Figure 2.8 A and B) than before (Figure 2.5 A). There was a similar density of VLPs in fractions from both salt concentrations but with more fully formed VLPs present in the fractions from the high salt samples. The chimeric particles were at a lower density at the low salt concentration (Figure 2.8 C) however at the higher salt concentration there was a greater density of particles per field of view and more VLPs than at the lower concentration (Figure 2.8 D). This corresponds to what was seen in the Coomassie-stained gels which had darker bands for the VLP proteins which indicates a greater concentration of protein and thus an increased likelihood of VLP formation.

These results have shown that the ionic strength has an effect on the ability of particles to assemble and remain intact. It has been found that ionic strength and salt concentration do have an effect on electrostatic interactions which influence particle assembly (McCarthy et al., 1998). Protein stability can be increased by improving the electrostatic interactions that occur between charged groups on the surface of the particle (Azia et al., 2009). For example, it was found that the polyomavirus capsid protein, VP1, contains carboxyl groups which are destabilised at a low ionic strength due to electrsotatic repulsion. At a higher salt concentration, this repulsion is shielded and the assembly of the capsid is stabilised in an alkaline solution (Salunke et al., 1989). Although many studies make use of a 20mM NaCl buffer (Thuenemann et al., 2013, van Zyl, 2014, Mokoena et al., 2019), it seems for these particular chimeric BTV1/8 VLPs a higher salt concentration was optimal, similar to results found in the study by Van Zyl et al. where a 400mM salt buffer was used for BTV8 protein body expression (van Zyl et al., 2017). Similar studies often add DL-dithiothreitol (DTT) reducing agent and/or N-lauroyl-sarcosine (NLS) in their extraction buffers which may give additional stability and assembly support by preventing unwanted intra-molecular disulphide bond formation thus only requiring a buffer with a low salt concentration.

In addition to increased ionic strength, the presence of a chaperone has also been found to assist in the correct folding and improved stability of assembled particles (Mohl et al., 2019). Because viral proteins are complex and are produced quickly in large volumes during natural infection, they are susceptible to misfolding and aggregation thus requiring stabilisation (Mohl et al., 2019). Molecular chaperones are able to mediate folding and stabilisation of viral proteins. Mohl and Roy examined the role of the abundant and highly conserved heat-shock chaperone, HSP90, in the viral lifecycle of BTV. Under normal circumstances, this chaperone helps to facilitate protein binding, stabilisation and release and it plays an important role in maintaining protein homeostsis. During BTV infection, they found that HSP90 activity was important in virus replication through its stabilisation of BTV proteins. Without the chaperone, protein levels (specifically VP5 and VP7) and viral titre decreased when

expressed in infected HeLa cells. They also found that HSP90 inhibited the proteosome pathway therefore minimising viral protein degradation. This is a mechanism of action of which BTV has taken advantage to avoid turnover via the proteosome pathway (Mohl et al., 2019). To determine if this principle could be applied to BTV VLPs, HSP90, previously cloned into the pEAQ-*HT* plant expression vector, was co-infiltrated at an $OD_{600}$ of 0.5 with the BTV VLP proteins, thus resulting in a final $OD_{600}$ of 2.5. Again, detection of BTV8 VLP proteins was similar regardless of HSP90 co-infiltration whereas the band intensity of chimeric VP2 and VP5 bands were slightly greater when HSP90 was included (Figure 2.6B). Co-expression with HSP90 was explored further in a large-scale infiltration experiment (Figure 2.9).

The large-scale co-infiltration with HSP90 did not illustrate an obviously notable difference when compared with infiltration without it. The BTV8 VLP proteins were detected regardless with a slightly lower signal intensity in the HSP90 samples (Figure 2.9 A and B). The same result was observed for the chimeric VLP proteins (Figure 2.9 C and D). The Coomassie gels had barely visible bands for VP2 and VP5 in both chimeric VLP samples (Figure 2.9 G and H). The TEM results show a lower density of BTV8 particles and a greater majority of CLPs and SCLPs in the HSP90 samples (Figure 2.10 A and B). The chimeric samples have a similar result where there were VLPs present in the fractions without HSP90 but more CLPs and incomplete particles in the HSP90 samples (Figure 2.10 C and D). These results suggest that the co-infiltration with HSP90 does not greatly improve BTV VLP detection or full particle assembly and thus the inclusion of HSP90 was not carried forward in future experiments. Infiltrating the plants with an additional construct, with a final infiltration $OD_{600}$ of 2.5, may put strain on the plant cell machinery. Additionally, it decreases the chance of all five components being present in each plant cell which is required for the VLPs to assemble. The study which found HSP90 useful in stability and folding (Mohl et al., 2019) was investigating the effect of HSP90 on the native BTV virus which may explain why the chaperone did not have the same effect on VLP assembly in this study. Unpublished work from the same research group found that co-infiltration with HSP90 improved AHSV VLP assembly. This was not observed in the case of BTV VLPs. Further investigation into the co-infiltration conditions is required to confirm whether the inclusion of HSP90 could improve BTV VLP assembly. For example, using differenct infiltration ratios for the VLP constructs as well as the HSP90 construct may result in a diffferent, potentially improved, outcome. Although it has been found that HSP70 and HSP90 are used by viruses to fold proteins and support survival under harsh host conditions, an extensive amount of research still needs to be done to determine the usefulness of these proteins as potential therapeutics and their ability to assist in VLP assembly (Mohl et al., 2019, Lubkowska et al., 2021).

Results from the work carried out in this chapter allowed for the establishment of optimal conditions for infiltration and purification of constructs encoding proteins for BTV8 and chimeric BTV1/8 VLP assembly. The optimal conditions of infiltration of an $OD_{600}$ 0.5, harvesting at 5 dpi and extraction using a 400mM NaCl extraction buffer were incorporated for the further preparation of plant produced BTV8 and BTV1/8 chimeric VLPs for injection into guinea pigs to test for their immunogenicity (chapter 4).

# Chapter 3: Design and transient expression of BTV SpyVLPs in *Nicotiana benthamiana*

## 3.1 Introduction

There has been much research into BTV VLPs which present VP2 on their surface and mimic the conformation of the native virus. In chapter 2, we showed that we could produce chimeric VLPs, presenting a heterologous VP2 on their surface. This vaccine strategy holds great promise for providing protection against multiple serotypes as it is easily manipulated to target any serotype by replacement of the chimeric tip domain region of VP2. A vaccine cocktail of these chimeric particles could therefore be effective at providing protection against multiple BTV serotypes. However, there are other strategies that may be equally immunogenic and effective. Antigen-display technology has taken advantage of the fact that antigens presented to the immune system in multiple, repeating arrays induce a strong and protective immune response (Frietze et al., 2016, Hill et al., 2018).

Some antigen display systems involve the direct linking of the antigen to the VLP. This can be done by genetic fusion into the viral genome, for example the fusion of GFP to the c/e1 epitope of the nucleocapsid of the hepatitis B virus (Kratz et al., 1999), or it can be done at the protein level, post-translationally by chemical conjugation or non-covalent interactions (Hill et al., 2018). These methods have a number of limitations. For example, genetic fusion can affect the folding and assembly of the display particle which may limit the size of the antigen that can be displayed. Post-translational conjugation can become lengthy and complex requiring multiple rounds of purification (Frietze et al., 2016, Hill et al., 2018). An alternative to these methods is the application of a system where two proteins have a natural affinity for one another and can thus facilitate bioconjugation of two protein parts. This is the case for the SpyTag/SpyCatcher (ST/SC) technology which has taken advantage of the rigidity of a bacterial protein's internal isopeptide bond to make bioconjugation a simpler process requiring fewer steps and avoiding the limitations of traditional bioconjugation methods (Zakeri et al., 2012).

The fibronectin binding protein (FbaB) of the gram-positive bacterium *Streptococcus pyogenes* contains an immunoglobulin-like collagen adhesion domain (CnaB2). This domain forms an intramolecular spontaneous isopeptide bond through the nucleophilic attack of the unprotonated amine of a lysine residue and the carbonyl carbon of an aspartate residue. This domain has been split and engineered into two separate parts which, when mixed, will form an irreversible isopeptide bond in minutes (Figure 3.1) (Zakeri et al., 2012). These two parts have been named the 16 amino acid SpyTag (ST) peptide and the 138 amino acid, 15kDa SpyCatcher (SC) peptide. This reaction has been shown to occur rapidly under diverse pH, temperature and buffering conditions with a high yield and is not reversed by boiling or competing peptides (Zakeri et al., 2012).

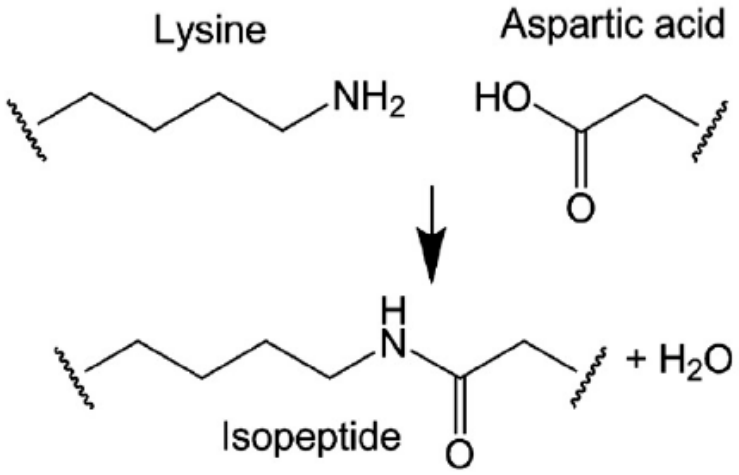



**Figure 3.1:** The formation of the spontaneous intermolecular isopeptide bond by the nucleophilic attack of the unprotonated amine of a lysine residue on SpyCatcher and the carbonyl carbon of an aspartate residue on SpyTag. Permission obtained from publisher (Zakeri et al., 2012).

This ST/SC display technology has been used for a diverse range of bioconjugation applications. Some examples include the 'SpyRing' system used for cyclisation of enzymes (Schoene et al., 2014, Sun et al., 2019), the 'Spy&Go' method for purifying spy-tagged proteins (Khairil Anuar et al., 2019) as well as hydrogel formation (Sun et al., 2014, Gao et al., 2016), characterisation of protein-RNA interactions (Zhao et al., 2019), fluorescent labelling in microscopy (Hinrichsen et al., 2017, Pessino et al., 2017) and phage display applications (Fierle et al., 2019). Most relevant however, the ST/SC system has accelerated vaccine development and optimisation through its application to antigen display on core-like and virus-like particles. 'SpyVLPs' refer to particulate proteins (VLPs) which display an antigen via the ST/SC system. For example, SC can be fused to a capsid protein while ST is fused to an antigen to be displayed. When mixed, the isopeptide bond will form between the ST and SC components and result in a SpyVLP displaying the desired antigen (Figure 3.2).

The RNA *Acinetobacter* phage AP205 has a capsid protein (CP), CP3, which can be expressed in heterologous systems to form VLPs (Brune et al., 2016, Thrane et al., 2016). This coat protein has both the N- and C- termini exposed on the surface (Janitzek et al., 2019). Both termini can undergo genetic fusion with an antigen which will then be evenly distributed on the particle surface (Janitzek et al., 2019). Brune et. al. showed that SC can be fused to the N-terminus of AP205 CP3 and mixed with ST fused to a variety of malarial protein antigens and cancer related peptides to form VLPs displaying the antigen of interest (Figure 3.2) (Brune et al., 2016). Here, the AP205-SC VLPs were easily expressed in *E. coli* and purified before coupling *in vitro*. They also showed that there was robust immunogenicity stimulated in mice which were immunised with the VLPs displaying either CDIR or Pfs25 malarial antigens with no need for a booster or adjuvant (Brune et al., 2016).

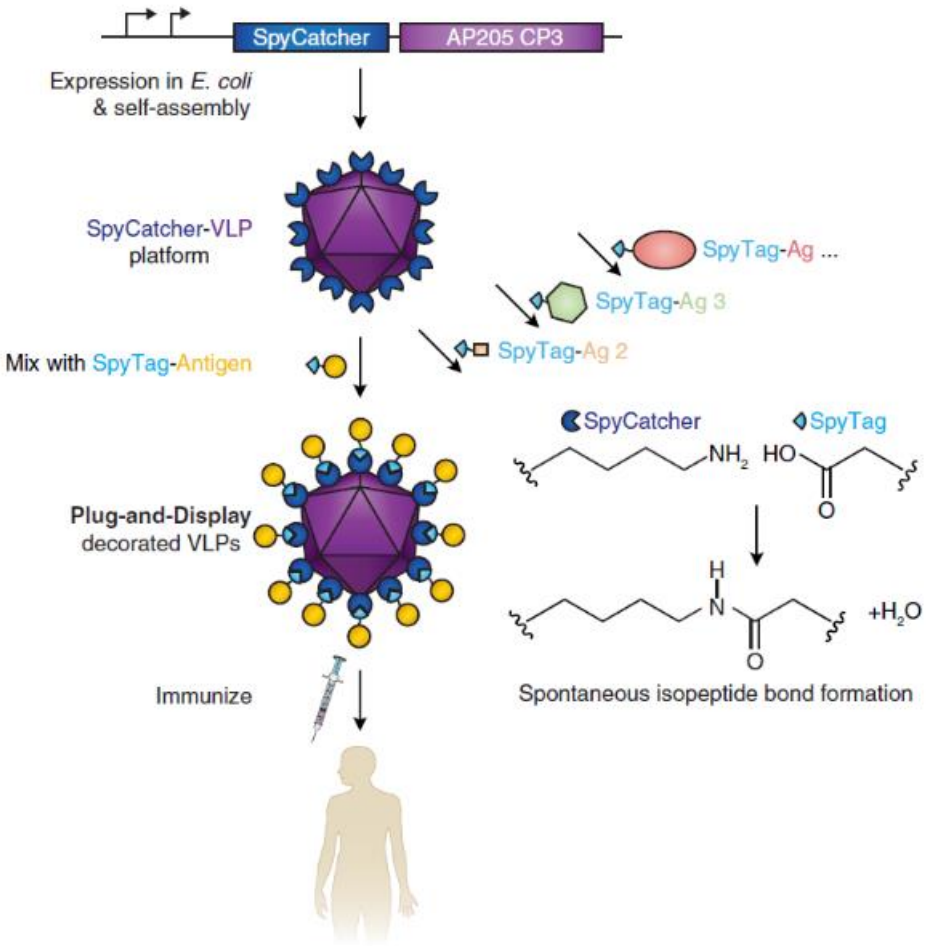



**Figure 3.2: Summary of ST/SC VLP assembly.** SpyCatcher is genetically fused to the N terminus of the AP205 phage coat protein (AP205 CP3). SpyCatcher-VLPs form by spontaneous assembly of monomers. When mixed together with the SpyTag-antigen, the spontaneous isopeptide bond forms with the SpyCatcher-VLPs, resulting in particles displaying the antigen, ready for immunisation. Image under the Creative Commons Attribution (CC-BY) license (Brune et al., 2016).

Other studies have looked at fusing the ST peptide to the AP205 CP3 particle rather than SC. Thrane et al. explored both combinations as well as fusing ST to both the C-and N-termini of AP205 resulting in 360 binding motifs instead of 180 (Thrane et al., 2016). Again, they used ST or SC fused to malarial antigens to determine the ability of these fusion combinations to have an immunogenic effect and prevent parasitic development. They found that immunisation of mice resulted in a higher IgG antibody titre with greater affinity, efficacy and longevity than the monomeric antigenic protein alone. It was found that the VLP with 360 binding motifs is only practical for use with small proteins where steric hindrance is not a concern (Thrane et al., 2016).

The systems discussed above have used bacterial or yeast expression systems for ST- and SC-tagged proteins. Recently, there has been exploration into the use of plants as an expression system for these ST/SC components. For example, the core antigen of hepatitis B (HBcAg) has been designed to display antigens on a core-like particle (CLP) (Peyret et al., 2020). Peyret et al. investigated the expression and conjugation of HbcAg SpyVLPs and GFP model antigen in plants. They were able to express all recombinant proteins in *Nicotiana benthamiana* where conjugation occurred *in vivo*. This method saves time and cost as it avoids additional extraction and purification steps of separate components before *in vitro* coupling (Peyret et al., 2020). Stander et al. expressed ST-AP205 and West Nile Virus

EDIII fused to SC (WNV-EDIII-SC) in *N. benthamiana.* Here they compared *in vivo* conjugation by co-expression, co-extraction and *in vitro* conjugation. It was found that the highest yield of coupled proteins was obtained from the co-extraction method where plants were infiltrated with the separate components, but the leaves were harvested, extracted and purified as a mixture. After immunisation of mice with either the SpyVLP or the antigen alone, the VLP elicited a more significant antibody titre than the soluble antigen. Additionally, the plants were just as effective in their production of the WNV-EDIII protein as *E. coli* as the immune response induced by the plant produced antigens had a similar potency to the immune response elicited in mice immunised with five times the amount of *E. coli* produced WNV-EDIII (He et al., 2014, Stander et al., 2021).

Because it has been demonstrated that the ST/SC system can be employed to assemble antigen-display particles in plants, it was proposed to be a promising strategy to explore for developing a BTV particulate vaccine as an alternative to the chimeric VLPs previously generated (chapter 2). Similar to the chimeric VLPs, this method of antigen display lends itself to the development of vaccines which are safe, easily scalable and rapidly applicable in the case of an outbreak. Both the VLP and ST/SC methods take advantage of the fact that the majority of the vaccine components remain constant regardless of the target serotype or antigen being displayed. The chimeric BTV1/8 VLP comprises the BTV8 backbone where only the tip domain of VP2 needs to be replaced to change serotype targeting and the ST/SC system has already been optimised for display of an antigen on the AP205 particle where only the antigen conjugated to ST needs to be exchanged. These vaccines therefore have the potential to be rapidly produced and distributed when an outbreak occurs.

This chapter investigates the potential for the ST/SC technology to be applied for the display of the BTV1 antigenic domain on the surface of the AP205 VLP. We have described the design, cloning and transient expression of AP205 particles displaying the BTV1 VP2 tip domain using the ST/SC system. As discussed in chapter 2, the tip domain found on the BTV immunodominant VP2 protein is the region known to elicit the predominant neutralising antibody response during BTV infection. Although this is the region which is important for immunogenicity of a vaccine, when VP2 is used as an antigen alone, it is required in much higher concentrations and often with an adjuvant to provide complete protection (Feenstra et al., 2017). It has been found that when expressed in combination with VP5, and even more so when expressed with all four BTV capsid proteins, a lower concentration of VP2 is required to induce a protective neutralising antibody response (Roy et al., 1992). This is thought to be due to the interaction of the proteins, especially VP2 and VP5, which allow for a more desirable conformation and presentation of the neutralising epitopes. Hence, the most effective BTV vaccines display VP2 on the surface of a particle whether that be the attenuated native virus, a VLP or some other particulate structure.

PCR amplification and In-Fusion® cloning were conducted to construct a recombinant pEAQ-*HT* plasmid encoding the BTV1 VP2 tip domain (BTV1Tip) fused to the 5' of the *SC* gene sequence to produce BTV1Tip with *SC* at the C terminal (BTV1Tip-SC). *ST* fused to the N terminal of the AP205 capsid protein (ST-AP205) was expressed from the pEAQ-*HT*-ST-AP205 construct previously designed in the BRU laboratory.

## 3.2 Materials and Methods

### 3.2.1 In-Fusion® cloning of BTV1Tip-SC

In-Fusion® cloning was performed to insert the BTV1Tip-SC fragment into the pEAQ-*HT* vector. First the SC and BTV1Tip fragments were individually amplified from vectors containing these relevant sequences. Subsequently, the two amplified fragments were combined into one fragment (BTV1Tip-SC) by assembly PCR and finally In-Fusion® cloning facilitated the insertion of the BTV1Tip-SC fragment into the linearised pEAQ-*HT* vector (Figure 3.3).

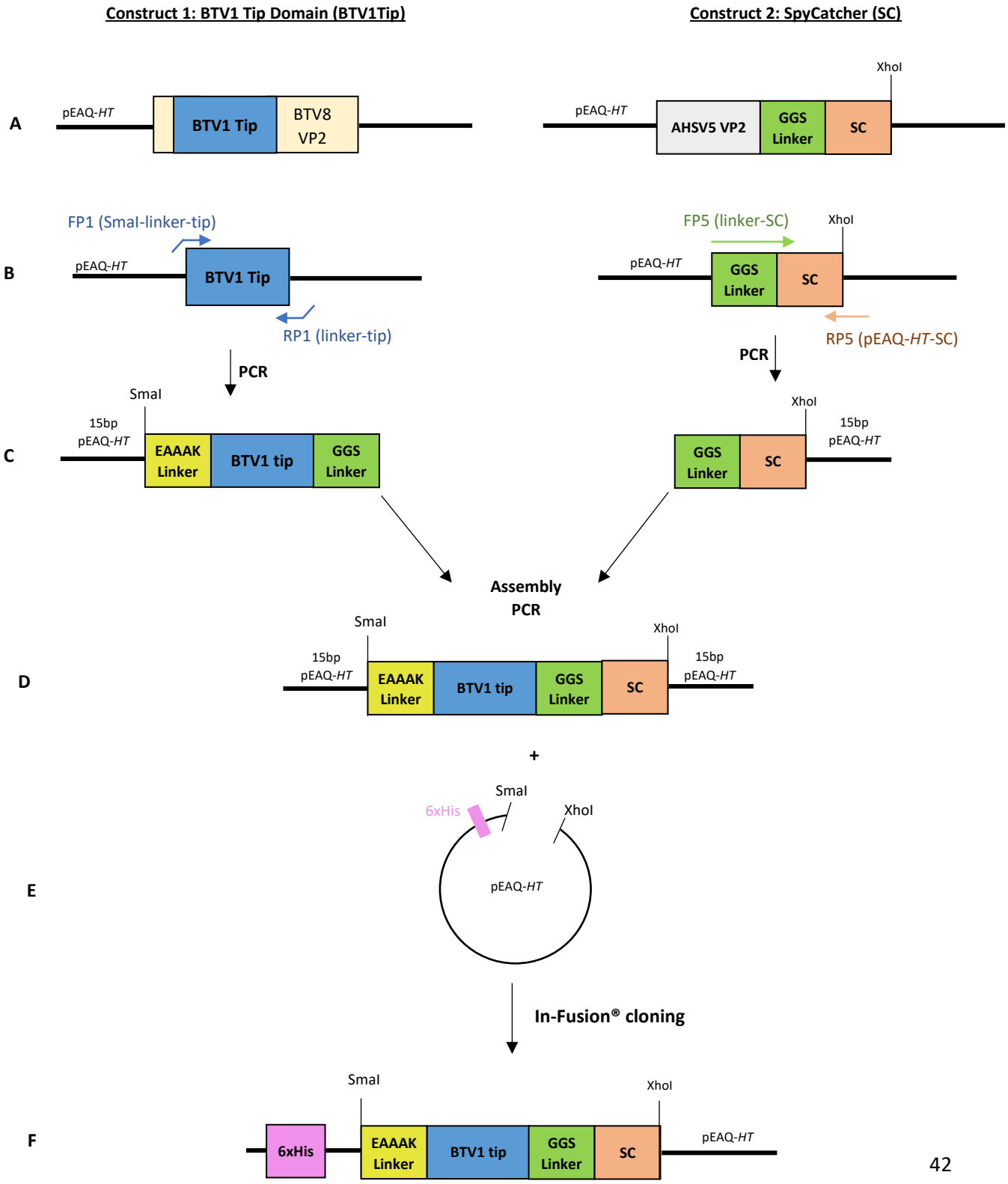


**Figure 3.3: Schematic representation of the steps followed to achieve In-Fusion® cloning. A:** The pEAQ-*HT* vectors containing the relevant sequences, BTV1Tip and SC, were amplified by PCR. **B:** The primers (FP1, RP1, FP5, RP5) were used to modify and amplify the desired fragments by PCR to yield the BTV1Tip and SC fragments with modifications **(C)**. **D:** The final product, BTV1Tip-SC, was generated by assembly PCR using FP1 and RP5 primers. BTV1Tip-SC was cloned into **(E)**, the linearised pEAQ-*HT* vector, by In-Fusion® cloning to form **(F)** the final recombinant pEAQ-*HT* BTV1Tip-SC vector.

3.2.1.1 Isolation of SC and BTV1Tip fragments

*E. coli* glycerol stocks containing each of the relevant constructs were streaked on an LB agar plate supplemented with 30ug/mL kanamycin and incubated at 37°C overnight. A colony from each plate was inoculated into 10mL LB broth supplemented with 30ug/mL kanamycin and incubated at 37°C with agitation overnight. Plasmids were isolated from the cultures using the QIAprep® Spin Miniprep kit (Qiagen) as per manufacturer's instructions.

The SC fragment was amplified from the pEAQ-*HT* AHSV5-VP2domain-SpyCatcher construct which pre-existed in the BRU lab (Lab collector #2194, BRU laboratory). The SC sequence (version 1) is based on that published by Zakeri et al. 2012 (Zakeri et al., 2012). PCR was performed to amplify the *SC* gene which included a flexible GGS glycine linker at the 5' end. The gene was modified by the reverse primer to add a 15bp overlap with the pEAQ-*HT* vector which included an XhoI restriction enzyme site at the 3' end. Primers (FP5 and RP5) designed by a previous BRU student were used to amplify this fragment (Table 3.1).

The BTV1Tip fragment was amplified from the pEAQ-*HT* BTV1/8VP2 construct (see 2.2.1). PCR amplification using the primers detailed in Table 3.1 (FP1 and RP1) allowed for modification of the BTV1Tip sequence to include a 15bp overlap with the pEAQ-*HT* vector, a SmaI restriction enzyme site and a double rigid EAAAK linker at the 5' end. The 3' end was modified to include a 30bp overlap with the flexible GGS linker (present at the 5' end of the SC fragment) to facilitate assembly PCR. The amplification was designed to be in-frame with the histidine sequence (6xHis) of the pEAQ-*HT* vector so that the protein could be purified by nickel affinity chromatography and detected by anti-His antibodies.

Fragments were amplified by PCR of 5ng of the template DNA using Phusion Hot Start II High-Fidelity DNA polymerase (ThermoFisher Scientific) and the primers described in Table 3.1 at a concentration of 0.5uM each. The PCR profile was as follows: one denaturing cycle at 98°C for 30 s, 35 cycles of: 98°C denaturing for 30 s, 65°C annealing for 30 s, 72°C elongation for 30 s and one final elongation step at 72°C for 5 min. Negative controls included an empty vector (pEAQ-*HT*) control, no template control and forward and reverse primer only controls.

The amplified products were visualised on a 0.8% agarose gel stained with ethidium bromide. The O'GeneRuler™ 1kb DNA ladder (ThermoFisher Scientific) was used to confirm the size of the amplified products. The products were purified from the agarose gel using the QIAquick® Gel Extraction kit (Qiagen) as per manufacturer's instructions and stored at -20°C until further use.

3.2.1.2 Assembly PCR amplification of BTV1Tip-SC fragment

Assembly PCR was performed to combine the purified SC and BTV1Tip fragments. The PCR included 10ng of each fragment and the primers described in Table 3.1 (FP1 and RP5) at a concentration of 0.5uM each. Amplification and analysis of products was carried out as in section 3.2.1.1.

3.2.1.3 In-Fusion® reaction

The pEAQ-*HT* vector was linearised with AgeI and XhoI restriction enzymes (ThermoFisher Scientific) as per manufacturer's instructions. The purified BTV1Tip-SC fragment was cloned into the linearised pEAQ-*HT* vector using the In-Fusion® HD cloning kit (Separations) as per manufacturer's instructions. The completed In-Fusion® reaction was transformed into Stellar™ competent *E. coli* cells (Clontech) including the controls stipulated by the manufacturer's instructions.

3.2.1.4 Colony PCR to confirm transformation into *E. coli* and *A. tumefaciens* AGL-1 cells

The transformed *E. coli* colonies were screened for successful transformation of the pEAQ-*HT* BTV1Tip-SC construct by colony PCR using Phusion Hot Start II High-Fidelity DNA polymerase (ThermoFisher Scientific) and the primers described in Table 3.1 (pEAQ-HTf and pEAQ-HTr) at a concentration of 0.25uM each. The PCR profile was as follows: one denaturing cycle at 95°C for 3 min, 30 cycles of: 95°C denaturing for 30 s, 59°C annealing for 30 s, 72°C elongation for 1 min and one final elongation step at 72°C for 5 min. The positive control colony contained the pEAQ-*HT* vector without an insert and the negative control colony included an empty pUC57 vector.

Colonies confirmed to be transformed with the recombinant plasmid were inoculated into 10mL LB broth supplemented with 50ug/mL kanamycin and incubated with gentle agitation at 37°C overnight. The plasmid was isolated using the QIAprep® Spin Miniprep kit (Qiagen) as per manufacturer's instructions. The purified plasmid was verified by sequencing (Macrogen) and stored at 4°C until further use.

3.2.1.5 Electroporation of BTV1Tip-SC into *A. tumefaciens* AGL-1

A 100ng concentration of the purified recombinant pEAQ-*HT* BTV1Tip-SC construct was added to 100µL of *A. tumefaciens* (AGL-1) cells made electrocompetent using the method described by Shen and Forde (Shen et al., 1989) and added to a 0.1cm electroporation cuvette (Molecular BioProducts, Inc.). Following a 5 min incubation on ice, the AGL-1 cells were transformed with pEAQ-*HT* BTV1Tip-SC via electroporation at 1.8kV, 25uF and 200Ω using a Gene Pulser (BioRad). Nine hundred microliters of LB media (lacking antibiotics) were added to the electroporated cells, followed by incubation at 27°C with agitation for 2 h. Thereafter the cells were plated onto LB agar supplemented with carbenicillin (25ug/mL) and kanamycin (30ug/mL) and incubated at 27°C for 2 days. Transformed colonies were screened by PCR as described for *E. coli* colony PCR above (3.2.1.4).

Colonies confirmed to be transformed with the recombinant plasmid were inoculated into 10mL LB broth supplemented with 50ug/mL kanamycin and 25ug/mL carbenicillin and incubated with gentle agitation at 37°C overnight. The culture was stored in 25% glycerol at -80°C until use in infiltration experiments.

**Table 3.1: Primers used in preparation for and confirmation of In-Fusion® cloning.**

| Purpose | Fragment to amplify | Primers* | Ta (°C) | Product size (bp) | 5' addition | 3' addition | Final fragment |
|---|---|---|---|---|---|---|---|
| Fragment generation | SC | FP5 | 65 | 399 | GGS linker | 15bp pEAQ-*HT* overlap | Gly-SC-pEAQ-*HT* |
| | | RP5 | | | | | |
| Fragment generation | BTV1Tip | FP1 | 65 | 786 | 15bp pEAQ-HT, SmaI, EAAAK linker | 30bp complementary to GGS linker | EAAAK-BTV1Tip-Gly |
| | | RP1 | | | | | |
| Assembly PCR | SC and BTV1Tip | FP1 | 65 | 1154 | - | - | BTV1Tip-SC |
| | | RP5 | | | | | |
| Colony PCR | BTV1Tip-SC | pEAQ-HTf | 59 | 1401 | - | - | BTV1Tip-SC |
| | | pEAQ-HTr | | | | | |

* See primer sequences in Appendix (Figure S1)

3.2.2 Small-scale expression of BTV1Tip-SC in *N. benthamiana*

*A. tumefaciens*-mediated infiltration was carried out as described in section 2.2.2. The recombinant AGL-1 culture containing the pEAQ-*HT* BTV1Tip-SC construct (see section 3.2.1.4) was supplemented with 50ug/mL kanamycin and 25ug/mL carbenicillin and the cultures were infiltrated at an $OD_{600}$ of 0.5. For small-scale extraction, 2g of leaf material was harvested from each group of infiltrated plants at 3 and 5 dpi. The plant material was homogenised using an IKA® T25 digital ULTRA-TURRAX® (Sigma Aldrich) in two volumes of buffer. Tris-HCl buffer (50mM Tris-HCl, 20mM NaCl, pH8.4) was used for extraction. Leaf material was extracted in either Tris-HCl buffer without any additives or with added 8M urea or 0.5% Tween® 20. Homogenised leaf material was incubated at 4°C for 1 h with gentle agitation. The extract was then clarified by centrifugation (Beckman Coulter Avanti® J25-I centrifuge) at 15344 x g for 10 min. A sample of the supernatant was boiled in 1x SAB for 10 min at 95°C before analysis of proteins by SDS-PAGE and subsequent detection on Western blots (section 2.2.5). The BTV1Tip-SC protein was detected on a Western blot with a 1:2000 dilution of mouse anti-Histidine IgG primary antibody (BioRad) and 1:10 000 dilution of goat anti-mouse IgG (H/L) (multi-species adsorbed) secondary antibody (BioRad). This method was also followed for the preparation of the negative and positive control samples used in all analyses. The pEAQ-*HT* vector without an insert was the negative control and the positive control was a 48kDa His-tagged NS1 protein from West Nile virus (WNV), pTRAkc-WNV-NS1-HIS-SEKDEL (Lab collector #2387, BRU laboratory), which acted as a positive control for the anti-His antibodies used to detect purified BTV1Tip-SC on a Western blot.

3.2.3 Large-scale expression and purification of BTV1Tip-SC by nickel affinity chromatography

For the large-scale experiments, infiltration and extraction were carried out as in 3.2.2. Here, however, approximately 15g of leaf material was harvested from the plants infiltrated with recombinant AGL-1 at 5 dpi. The plant material was homogenised using an IKA® T25 digital ULTRA-TURRAX in 2 volumes of 0.1M sodium phosphate buffer pH7 (39mL 0.2M $NaH_2PO_4.H_20$, 61mL 0.2M $Na_2HPO_4$, 100mL $dH_20$) containing 8M urea. Sodium phosphate buffer was used as suggested by the manufacturer of the nickel affinity gel (Sigma-Aldrich). The homogenised plant material was incubated at 4°C with gentle agitation for 1 h. The extract was centrifuged (Beckman Coulter Avanti® J25-I centrifuge) at 15344 x g for 20 min, filtered through two layers of Miracloth™ (Merck), centrifuged again at 25931 x g for 15 min and then filtered through four layers of Miracloth™. The resulting supernatant was then centrifuged for a final time at 25931 x g for 10 min. The pH of the clarified extract was adjusted to 7.0. Approximately 21mL of the clarified extract was eluted through a 2mL HIS-Select® Nickel Affinity Gel column (Sigma-Aldrich) and collected in 1mL fractions. The column was equilibrated and washed with 0.1M sodium phosphate buffer containing 8M urea (pH7.0) and eluted with the same buffer containing 250mM imidazole (pH7.0).

Preparation and analysis of samples was carried out as in 3.2.2. The first eight elution fractions were analysed on Western blots and Coomassie-stained SDS polyacrylamide gels.

The fractions with the greatest band density were pooled and dialysed in sterile 0.1M sodium phosphate buffer in 10 000 MW dialysis tubing (ThermoFisher Scientific) overnight at 4°C with stirring. The dialysed sample was then quantified and used for *in vitro* coupling (3.2.5).

The FLW is determined as follows: The concentration of the protein (ug/mL) determined by quantification is converted to mg/ml. This is then converted to mg in the sample by multiplying according to the volume of purified extract used for quantification (volume of extract with given concentration). To determine mg per kg FLW, this mass (mg) is multiplied depending on the mass of leaf material harvested and clarified.

3.2.4 Expression and purification of ST-AP205 from *N. benthamiana*

The ST-AP205 VLPs were expressed and purified as previously optimised by Dr Sue Dennis (BRU, UCT). The ST sequence is based on that published by Zakeri et al. 2012 (Zakeri et al., 2012). The construct was designed so that the ST peptide was fused to the 5' end of the AP205 capsid protein and cloned into the pEAQ-*HT* vector (Dennis, 2019). The recombinant AGL-1 cultures containing the pEAQ-*HT* SpyTag-AP205 construct (Lab collector #1838, BRU laboratory) were infiltrated as described in 2.2.2 at an $OD_{600}$ of 0.25. Leaves were harvested at 4 dpi and homogenised in 2 volumes of 1 x phosphate buffered saline (PBS) (PBS: 137mM NaCl, 2.7mM KCl, 10mM, $Na_2HPO4$, pH7.4) using a Moulinex™ juice extractor. The homogenised leaf material was incubated with gentle agitation at 4°C for 30 min. The crude extract was then filtered through four layers of Miracloth™ and clarified by centrifugation at 25000 x g for 20 min at 4°C. The VLPs were purified through a discontinuous Optiprep™ density gradient by ultracentrifugation. The 7mL step gradient consisted of 2ml 35% Optiprep, 3mL 29% Optiprep and 2mL 23% Optiprep diluted in extraction buffer and layered under approximately 30mL clarified plant extract. The samples were centrifuged (Beckman Coulter Optima™ L-100 XP Ultracentrifuge) at 124436 x g (32000rpm) for 2 h at 4°C using a Beckman SW 32 Ti rotor. After centrifugation, the bottoms of the centrifuge tubes were punctured with a syringe needle and 500uL fractions were collected in microcentrifuge tubes. A sample of each fraction was boiled in 1x SAB for

10 min at 95°C before analysis of protein expression by Western blots and Coomassie-blue stained SDS polyacrylamide gels (section 2.2.5).

The ST-AP205 protein was detected on a Western blot with polyclonal rabbit anti-ST-AP205 primary antibody (1:20 000) and 1:10 000 dilution of goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugated antibody (Sigma-Aldrich).

3.2.5 *In vitro* coupling of purified BTV1-SC and ST-AP205

The purified BTV1-SC and ST-AP205 proteins were quantified by gel densitometry of a Coomassie-stained gel using GeneTools software (Synoptics Inc.). Concentrations of the proteins were estimated from a standard curve constructed from known concentrations of bovine serum albumin (BSA, Separations) ranging from 0.25mg/mL to 0.01mg/mL.

Using the estimated concentrations of the proteins determined from the standard curve, molar calculations were carried out to determine the volume needed for a 1:3, 1:5 and 1:7 (ST-AP205:BTV1-SC) molar ratio. The coupling reactions were aliquoted and stored at room temperature (approx. 22°C) overnight. The coupled reactions were analysed for complex formation by Western blot and Coomassie-stained gels as described in 2.2.5. Controls included each protein (BTV1Tip-SC and ST-AP205) electrophoresed separately. The proteins were detected on a Western blot with polyclonal rabbit anti-ST-AP205 primary antibody (1:20 000) and 1:10 000 dilution of goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugated antibody (Sigma Aldrich).

The coupling efficiency was calculated using the densitometric methods applied by a similar study in our research lab (Stander et al., 2021). Coupling efficiency describes the percentage of binding sites that are occupied on the VLP, therefore the higher the coupling efficiency, the more antigens that are bound to particles. The absorbance readings of the anti-ST-AP205 antibody-detected bands representing the ST-AP205 monomer (16.5kDa) and the coupled complex (60kDa) were measured using GeneTools software (Synoptics Inc.). The coupling efficiency was calculated by dividing the intensity value of the coupled complex band by that of the ST-AP205 monomer and multiplying it by 100.

## 3.3 Results

3.3.1 Preparation of BTV1Tip and SC fragments for In-Fusion® cloning

Before In-Fusion® cloning, the individual BTV1Tip and SC fragments were amplified from individual templates using the primers listed in Table 3.1 and fused to each other before cloning into the pEAQ-*HT* vector. The SC fragment containing a flexible GGS glycine linker at the 5' end was successfully amplified to produce a fragment of 399bp (Appendix: Figure S2 A – red arrow). The BTV1Tip fragment was amplified to include a rigid EAAAK linker at the 5' end and a complementary region to the GGS linker at the 3' end was successfully amplified to produce a fragment of 786bp (Appendix: Figure S2 B – red arrow). These products were extracted from the gel and purified.

3.3.2 Assembly of BTV1Tip-SC fragment for insertion into pEAQ-*HT* by In-Fusion® cloning

The purified BTV1Tip and SC fragments were successfully fused by assembly PCR, yielding a single 1154bp fragment, BTV1Tip-SC, using FP1 and RP5 primers (Table 3.1) (Appendix: Figure S3). The amplified product was extracted from the agarose gel and purified for its use in In-Fusion® cloning into the pEAQ-*HT* vector.

3.3.3 In-Fusion® cloning of BTV1Tip-SC fragment into pEAQ-*HT*

The amplified BTV1Tip-SC was successfully cloned into pEAQ-*HT* by In-Fusion® cloning and transformed into *E. coli*. Colony PCR screening of the recombinant *E. coli* resulted in the expected band sizes of 1401bp (Figure 3.4 red arrow).

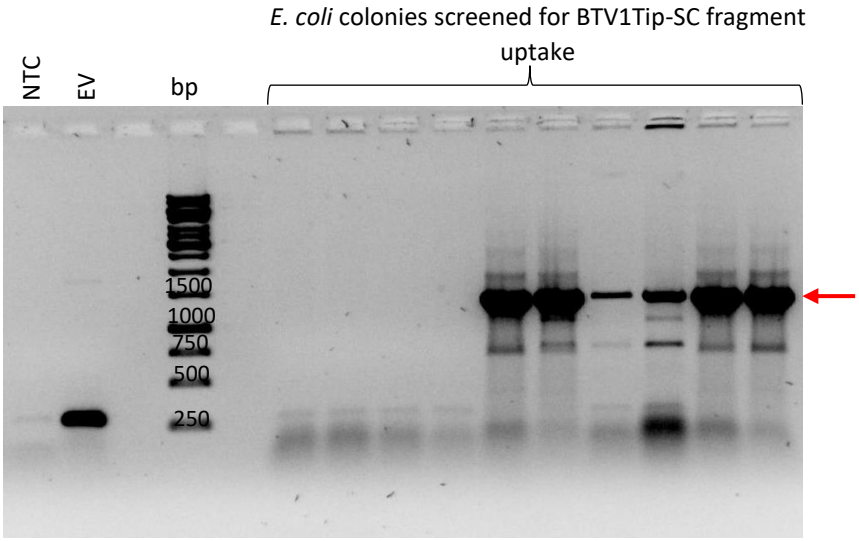



**Figure 3.4: Colony PCR to screen *E. coli* colonies for successful transformation with pEAQ-*HT* BTV1Tip-SC construct.** Amplified products visualised on 0.8% agarose gel stained with ethidium bromide. Of the transformed colonies, ten were selected for colony PCR. Red arrow indicates the BTV1Tip-SC product amplified by pEAQ-*HT* specific primers: 1401bp. EV: empty vector control (275bp). NTC: no template control. Bp: molecular weight marker (in base pairs).

3.3.4 Transformation of BTV1Tip-SC into *A. tumefaciens* for use in plant-expression

Following the screening of *E. coli* colonies, pEAQ-*HT* BTV1Tip-SC, from a selected colony, was purified and transformed into electrocompetent *A. tumefaciens* AGL-1. The transformed AGL-1 cells were screened for successful uptake of pEAQ-*HT* BTV1Tip-SC by colony PCR using vector-specific primers (Table 3.1). All the colonies screened contained the plasmid encoding the BTV1Tip-SC fragment as indicated by the bands corresponding to 1401bp (Figure 3.5 indicated by red arrow).

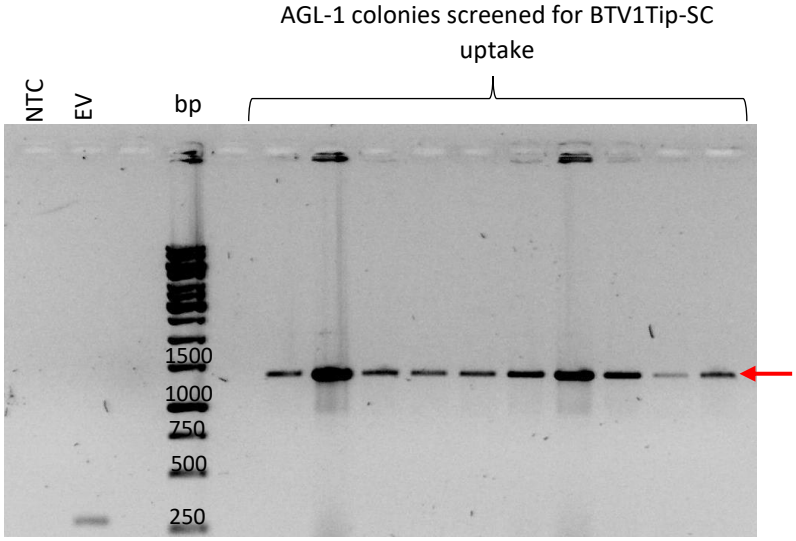


**Figure 3.5: Colony PCR to screen AGL-1 colonies for successful transformation with pEAQ-*HT* BTV1Tip-SC construct.** Of the transformed colonies, ten were selected for colony PCR. Red arrow indicates the BTV1Tip-SC product amplified by pEAQ-*HT* specific primers: 1401bp. EV: empty vector control (275bp). NTC: no template control. Bp: molecular weight marker (in base pairs).

### 3.3.5 Small-scale optimisation of BTV1Tip-SC extraction from *N. benthamiana*

To determine the optimal harvest day and extraction buffer for maximum BTV1Tip-SC protein yield, a small-scale infiltration was conducted. Plants were infiltrated with *Agrobacterium* AGL-1 transformed with pEAQ-*HT* BTV1Tip-SC at an $OD_{600}$ of 0.5 and harvested on 3 and 5 dpi. Three different Tris-HCl based buffers were used to extract the protein: Tris-HCl or Tris-HCl augmented with addition of urea or Tween™ 20. A construct containing a His-tagged *NS1* gene from West Nile virus (48kDa) was used as a positive control for detection by anti-His antibodies.

The BTV1Tip-SC protein was detected on the Western blot as a band of 43.5kDa (Figure 3.6 red arrow). At 3 dpi, the protein was detected when all three buffers were used but band intensity of BTV1Tip-SC extracted with Tris-HCl buffer alone and with 0.5% Tween® 20 was much lower than that of BTV1Tip-SC extracted in Tris-HCl with 8M urea. At 5 dpi, there was a slightly greater density for sample extracted with Tris-HCl buffer than at 3 dpi, but almost no protein was detected in the sample extracted with 0.5% Tween® 20. Based on this qualitative assessment of band intensity, it was concluded that harvesting leaves at 5 dpi and extraction of BTV1Tip-SC with Tris-HCl buffer containing 8M urea resulted in the highest BTV1Tip-SC protein yields. These parameters were used for further experimentation.

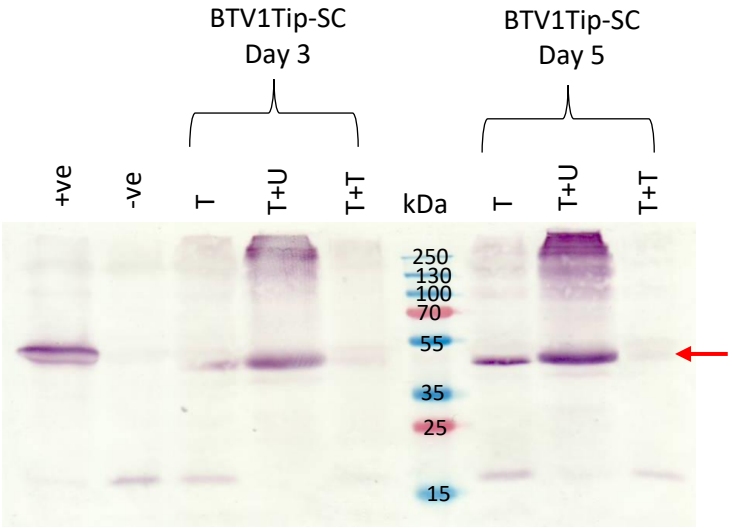



**Figure 3.6: Small-scale expression of BTV1Tip-SC protein in plants.** Leaves harvested on 3 and 5 dpi and protein extracted with three Tris-HCl buffers (pH8.4): Tris-HCl (T), Tris-HCl with 8M Urea (T+U) or Tris-HCl with 0.5% Tween 20 (T+T). Equal volumes of crude extract loaded in each lane. Protein detected by anti-Histidine antibodies (1:2000). Red arrow indicates BTV1Tip-SC protein at 43.5kDa. Positive control for anti-His antibody (+ve): pTRAkc-NS1-His-SEKDEL construct (48kDa). Negative control (-ve): crude leaf extract from plants infiltrated with pEAQ-*HT* vector without an insert. kDa: Molecular weight marker in kilodaltons.

### 3.3.6 Purification of BTV1Tip-SC protein by nickel-affinity chromatography

Once harvest and extraction parameters had been optimised based on the qualitative analysis of crude plant extracts, purification by nickel-affinity chromatography was investigated. Approximately 20g of leaf material was harvested at 5 dpi and homogenised in 0.1M sodium phosphate buffer (suggested

by affinity gel manufacturer, Sigma-Aldrich) containing 8M urea. The BTV1Tip-SC protein was eluted in the same buffer containing 250mM imidazole. The elution fractions were analysed by Western blot and Coomassie-staining of gels (Figure 3.7).

The BTV1Tip-SC protein (43.5kDa) was detected by Western blot by anti-His antibodies in elution fractions 2-8 (Figure 3.7A red arrow). The darkest bands were observed in fractions 2 - 4 with a smaller non-specific band of 40kDa observed in fractions 2 to 5. On the Coomassie-stained gel, BTV1Tip-SC was observed in fractions 2-5 with the greatest band intensity in fractions 3 and 4 (Figure 3.7B red arrow). Based on these observations, fractions 3 and 4 were deemed as having the greatest yield of protein and were selected for dialysis to remove urea and imidazole. The concentration of BTV1Tip-SC was then quantified by gel densitometry and found to be 52.65ug/mL which translates to 18mg/kg fresh leaf weight (FLW).

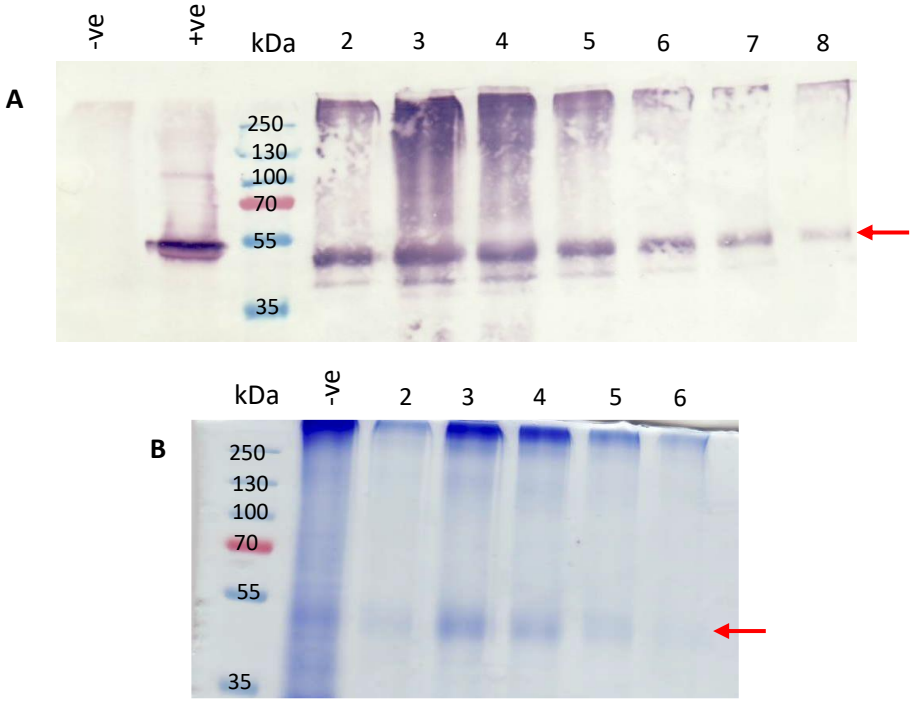


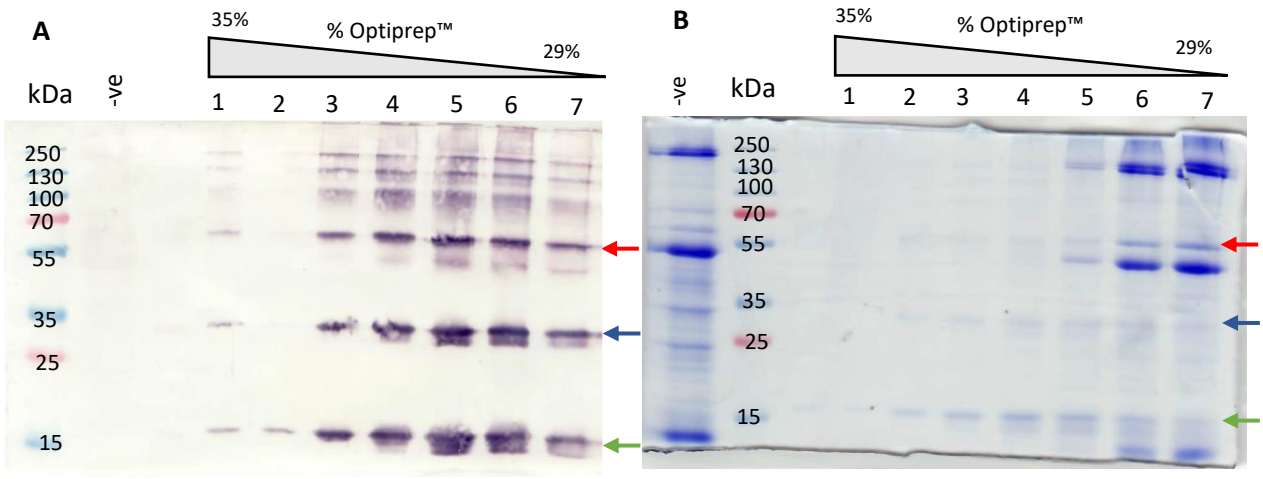

**Figure 3.7: Purification of BTV1Tip-SC protein by nickel-affinity chromatography.** Leaves harvested on 5 dpi and extracted, equilibrated and washed in 0.1M sodium phosphate buffer containing 8M urea. Protein eluted in 0.1M sodium phosphate buffer containing 8M urea and 250mM Imidazole. Elution fractions of 1mL each were collected and equal volumes of eluted samples were loaded in each lane and visualised on **(A)** a Western blot (elution fractions 2 to 8) and **(B)** Coomassie-stained SDS-PAGE gel (elution fractions 2-6). Protein detected by anti-Histidine antibodies (1:2000). Red arrows indicate BTV1Tip-SC protein at 43.5kDa. Positive control for anti-His antibody (+ve): pTRAkc-NS1-His-SEKDEL construct (48kDa). Negative control (-ve) crude leaf extract from plants infiltrated with pEAQ-*HT* vector without an insert. kDa: Molecular weight marker in kilodaltons.

### 3.3.7 Extraction and purification of ST-AP205 VLPs from *N. benthamiana*

The AP205 SpyVLP (Thrane et al., 2016) was selected for display of the BTV1Tip protein as it has previously been successfully assembled in and purified from *N. benthamiana* in our laboratory (Dennis, 2019). No optimisation was required as the established methods were followed in their entirety.

ST-AP205 CP was detected on the Western blot in all fractions (Figure 3.8A). The monomer (16.5kDa indicated by green arrows) was detected in all fractions while the dimer (33kDa indicated with blue arrows) and tetramer (66kDa indicated with red arrows) were detected in all fractions except fraction 2. Band intensity increased with the decreasing Optiprep™ concentration. The monomer and dimer were visualised on the Coomassie-stained gel in fractions 2-7 while the tetramer was visualised in fractions 4-7 (Figure 3.8B). A band corresponding to approximately 55kDa was also visualised on the Coomassie gel in fractions 5-7 and in the negative control which is likely the RuBisCO protein (Figure 3.8B). Fractions 4 and 5 were quantified by gel densitometry and the concentration of ST-AP205 monomers was found to be 26.37ug/mL which translates to 2mg/kg FLW.

A sample from fraction 6 was visualised by TEM. Electron micrographs showed that ST-AP205 VLPs assembled from expressed CP in plants after agroinfiltration (Figure 3.8 C and D). The particles were approximately 28nm in diameter and appeared to be expressed at yields similar to those previously purified in the laboratory (Dennis, 2019). These results confirmed the successful expression, assembly and purification of ST-AP205 VLPs in plants.



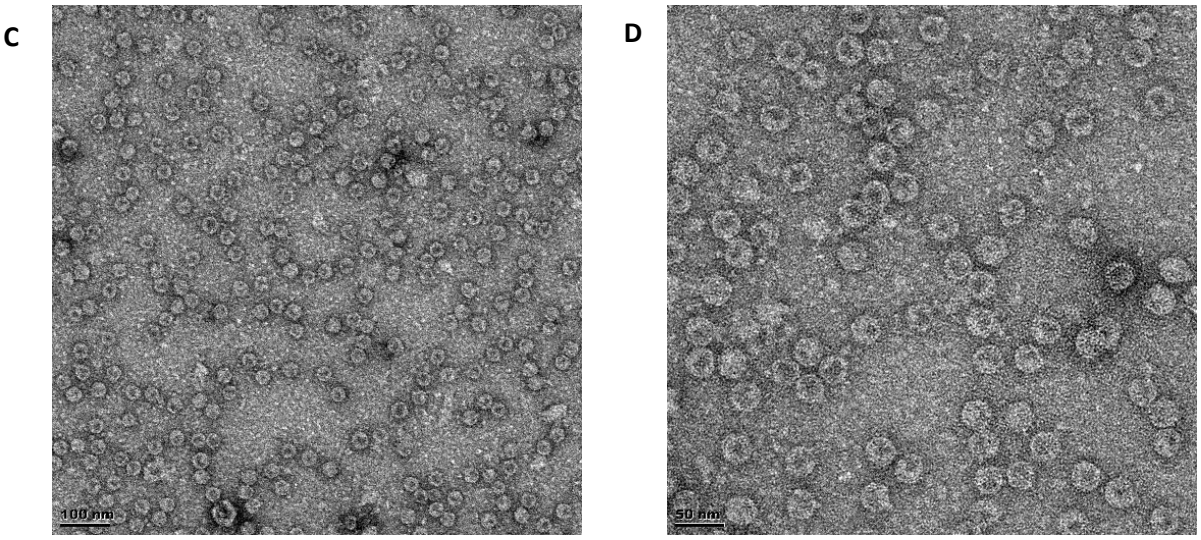



**Figure 3.8: Purification of ST-AP205 VLPs from *N. benthamiana***. Leaves were harvested on 4 dpi and extracted in 1x PBS (pH7.4) buffer. Proteins were purified by ultracentrifugation through a discontinuous Optiprep™ density gradient and 500uL fractions were collected from the bottom of the tube. Protein in purified fractions was detected on **(A)** a Western blot and **(B)** a Coomassie-stained gel. Protein detected by anti-ST-AP205 antiserum (1:20000). Green arrows indicate the ST-AP205 monomer (16.5kDa), blue arrows the dimer (33kDa) and red arrows the trimer (66kDa). 1-7: 500uL fractions collected from bottom of tube where 1=bottom of 35% gradient and 7=top of 29% gradient. Negative control (-ve) crude leaf extract from plants infiltrated with pEAQ-*HT* vector without an insert. kDa: Molecular weight marker in kilodaltons. **C and D**: Electron micrographs of purified ST-AP205 VLPs (approximately 30nm) from fraction 6. Scale bars represent (C) 100nm and (D) 50nm.

### 3.3.8 *In vitro* coupling of purified BTV1Tip-SC and ST-AP205 VLPs

In addition to the plant-produced ST-AP205 particles, a purified *E. coli*-produced ST-AP205 VLP sample provided by Phindile Ximba (BRU, UCT) and purified in 2020 was also used for coupling to BTV1Tip-SC. The purified BTV1Tip-SC protein and ST-AP205 VLPs were quantified by gel densitometry using a BSA standard curve. The BTV1Tip-SC protein, plant-produced ST-AP205 protein and *E. coli*-produced ST-AP205 protein were estimated as having concentrations of 52.65ug/mL, 26.37ug/mL and 348.73ug/mL, respectively. For the plant-produced proteins this translated to 2mg ST-AP205 per kg FLW and 18mg BTV1Tip-SC per kg FLW. The proteins were then coupled *in vitro* in a 1:3 ST-AP205:BTV1Tip-SC molar ratio. Other molar ratios, 1:5 and 1:7, were tested to ensure that there was an excess of BTV1Tip-SC for optimal coupling efficiency. These ratios were tested because a study in our laboratory had used similar ratios for coupling of the HIV envelope glycoprotein gp140 to SC-AP205 (Ximba, 2021). It was determined, however, that a greater molar ratio does not increase the intensity of the coupled protein band (results not shown) and thus a 1:3 molar ratio was used from hereon.

Protein was detected on a Western blot using the ST-AP205 antiserum. It was found that when the same samples were probed with the anti-His antibodies, no bands were detected, neither the BTV1Tip-SC protein nor the coupled product (data not shown). Complex formation was confirmed by a molecular weight shift on a Western blot or Coomassie-stained gel (Figure 3.9). When coupled to the monomeric form of ST-AP205, there was a molecular weight shift to approximately 60kDa (16.5kDa+43.5kDa).

The Western blot (Figure 3.9A), showed detection of the monomers, dimers and tetramers of the plant (plant ST) and *E. coli* (*E. coli* ST)-produced ST-AP205 CP alone (black arrows). In samples from the coupled reactions with plant-made ST-AP205 VLPs, these forms were no longer detected but the coupled complex of approximately 60kDa was detected (Figure 3.9A red arrow). In the coupled fraction with *E. coli*-made ST-AP205, the monomer and multimers were still detected but at a lower signal intensity (black asterisks) and the coupled protein was also observed at a greater signal intensity than in the coupling reaction with plant-made ST-AP205. The coupled proteins were also visualised on the Coomassie-stained gel (Figure 3.9B red arrow). However, the coupled complex comprising the plant produced ST-AP205 was not visualised. It is likely that the bands corresponding to approximately 55kDa are the RuBisCO protein (black arrow heads). The dialysed sample of BTV1Tip-SC was visualised on the Coomassie gel and seen at 43.5kDa (red asterisk).

Using the densitometric methods, the coupling efficiency for the plant-produced ST-AP205 particles was calculated to be approximately 44%.

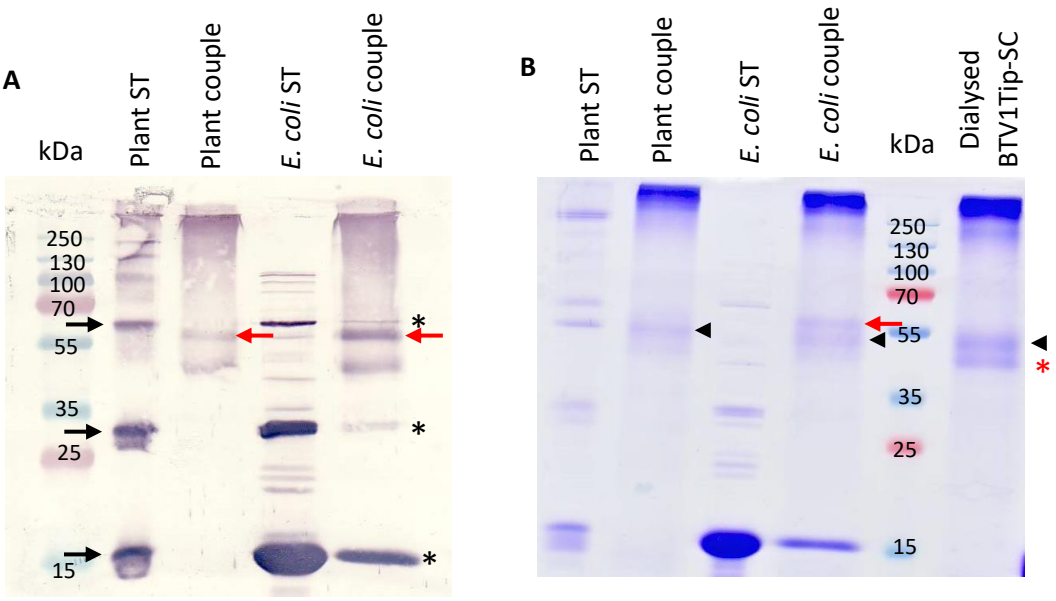



**Figure 3.9: Confirmation of *in vitro* coupling of purified ST-AP205 and BTV1Tip-SC proteins** by **(A)** Western blot and **(B)** Coomasssie-stained SDS-PAGE gel. Protein was detected by anti-AP205 antiserum (1:20000). The coupling reactions were carried out at a molar ratio of 1:3 (ST-AP205:BTV1Tip-SC) using either plant-made (plant couple) or *E. coli*-made (*E. coli* couple) ST-AP205 VLPs. Black arrows and black asterisks indicate the ST-AP205 monomer (16.5kDa), dimer (33kDa) and tetramer (66kDa) which were detected in the fractions containing either the plant-made ST-AP205 VLPs (Plant ST) or *E. coli*-made ST-AP205 VLPs (*E. coli* ST), respectively. Red arrows indicate the coupled complex at approx. 60kDa (43.5kDa+16.5kDa). Red asterisk indicates the BTV1Tip-SC protein at 43.5kDa. Black arrow heads indicate RuBisCO at approx. 55kDa. kDa: Molecular weight marker in kilodaltons.

## 3.4 Discussion

Antigen display on the surface of a particulate structure is a promising approach for the development of vaccines and therapeutics. Antigens presented in a repeating pattern on the surface of a particle can induce a strong immune response which can include the induction of the B-cell response (Bachmann et al., 2010, Frietze et al., 2016, Hill et al., 2018). When an antigen is expressed alone, often a much greater concentration is required, with the addition of an adjuvant, to induce a protective immune response (Feenstra et al., 2017). Although we showed the successful production of BTV8 and BTV1/8 VLPs in plants in chapter 2, we also investigated a different approach to producing particulate BTV vaccines in plants to determine whether there may be a superior method one could develop which would compete with chimeric VLP production in terms of particle yield, immune response and DIVA compliancy.

Antigen display technologies have become more advanced over recent years. Antigen display is no longer limited to genetic fusion or chemical conjugation. The ST/SC technology makes use of bioconjugation of two proteins which are engineered to form an irreversible covalent bond either *in vivo* or *in vitro* once translated into separate proteins (Zakeri et al., 2012). This technology has a number of advantages over other methods of bioconjugation including the versatility of the antigens and particulate structures which can be applied as well as the stability of the reaction under a wide range of buffer, pH and temperature conditions (Zakeri et al., 2012).

In this study we applied the ST/SC technology to display part of the BTV1 VP2 protein on the bacteriophage AP205 VLP. In chapter 2 we were able to produce a chimeric BTV1/8 VLP where the VP2 protein consisted of the BTV8 VP2 sequence with the 221aa immunogenic tip domain substituted with the corresponding region of BTV1 VP2. In this study, we took the same region corresponding to the immunogenic BTV1 VP2 tip domain (BTV1Tip) and designed a construct encoding the tip domain fused to SC (BTV1Tip-SC). Due to time constraints, we were not able to test fusion of SC to both the N and C BTV1Tip termini and thus SC was fused only to the C terminus as it has been found in our research group that greater yields of SC-linked proteins are produced in plants when it is fused to this terminus.

The ST-AP205 VLPs were produced in plants using previously optimised methods (BRU, UCT). Although both the N and C termini of the AP205 capsid protein are exposed on the surface of the AP205 particle, it was found that the fusion of ST on the N terminus resulted in greater yields of protein and a greater density of VLPs when expressed in plants (Dennis, 2019). This was also the case when SC was fused to either of the bacteriophage AP205 N or C termini. The approx. 55bp RuBisCo protein is still detected after purification. Possible methods to combat this include precipitating it out of the fraction by using a combination of calcium ions and phytate with an appropriate pH (Krishnan et al., 2009), which could negatively affect VLP assembly, or using size exclusion chromatography.

BTV1Tip-SC was successfully amplified by PCR and cloned into the pEAQ-*HT* vector by In-Fusion® cloning. This construct was designed so that it could be easily purified and detected on a Western blot by fusion of a His-tag to the N terminus. Primers were designed to amplify this sequence such that it would have a rigid EAAAK linker on the 5' end. This was done to ensure that the His-tag was sufficiently separated from the antigen to prevent steric hindrance which may have prevented binding of the His-tag to the nickel ions during purification and to the anti-His antibodies during detection on Western blots as has been reported by others (Chen et al., 2013). The SC fragment was amplified from a pre-existing construct to facilitate fusion to a flexible GGS glycine linker. The glycine linker was used for separation of the antigen from the SC peptide so that once fused to ST on the AP205 particle, the antigen could extend away from the VLP and have the space to fold correctly into its native conformation without the interference of the SC-ST conjugation. The flexible linker allows slight mobility which can accommodate proper folding so that the fusion protein can be displayed in its biological conformation to maintain functionality (Chen et al., 2013). This was appropriate for the separation of SC from the BTV1 tip domain. The rigid linker, however, has an α-helical conformation which gives it a stiff structure which is more effective at ensuring separation between the two domains (Chen et al., 2013). This is more important for the His-tag as it is a small protein which does not require elaborate folding but simply needs to be exposed for purification and detection purposes.

The final pEAQ-*HT* BTV1Tip-SC recombinant plasmid was constructed through In-Fusion® cloning. This cloning method is able to join any two pieces of DNA which have at least a 15bp overlap at their ends in a single reaction which takes 15 minutes (Zhu et al., 2007). The pEAQ-*HT* BTV1Tip-SC constructs were successfully electroporated into *A. tumefaciens* AGL-1 and cultured, ready for infiltration into plants.

A small-scale infiltration experiment was conducted to determine optimal day of harvest and optimal extraction buffer for the BTV1Tip-SC protein. Leaves were harvested on day 3 and 5 post-infiltration. The plants began to show signs of necrosis on day 5 which is an indication that the plant's defence response had begun to induce the expression of pathogenesis-related genes. It has been previously shown that an accumulation of reactive oxygen species (ROS), plant proteases and other defence responses can lead to necrosis and cell death in the region of infiltrated areas on the leaves

(Wroblewski et al., 2005, Norkunas et al., 2018). In our case, samples were not harvested later than 5 dpi as necrosis on the leaves was beginning to affect the fresh leaf mass available for processing and thus harvesting on a later day would mean more necrosis and less biomass for extraction of proteins.

Tris-HCl is a standard extraction buffer often used to extract SC-fused proteins (He et al., 2014, Stander et al., 2021). Extraction with this buffer including either 8M urea, a strong chaotropic denaturant, or 0.5% Tween® 20, a moderate strength non-ionic denaturant, was also investigated. Sometimes His-tagged proteins can form aggregates and subsequently insoluble inclusion bodies (Spriestersbach et al., 2015). Extracting and purifying under denaturing conditions can improve the detection of protein and efficiency of purification as it ensures the His-tag is fully exposed. It must also be noted that when urea is heated above 37°C, carbamylation of the protein can occur which may result in the protein having a greater molecular weight than expected (Rabilloud et al., 2007). This may explain why the bands corresponding to the BTV1Tip-SC protein on Western blots do appear to be slightly larger than 43.5kDa as these samples were denatured at 95°C before electrophoresis. Although the BTV1Tip-SC protein was detected in samples on all days post-infiltration and was not dependent on extraction buffer, the signal intensity of the band representing the protein expressed in plants harvested on day 5 and extracted in buffer containing 8M urea, was the greatest and thus these conditions were applied for further experimentation.

The large-scale expression and purification of the BTV1Tip-SC protein was achieved through nickel affinity chromatography. Here we harvested plants on 5 dpi but changed the extraction buffer to a 0.1M sodium phosphate buffer as suggested by the manufacturer of the HIS-Select® Nickel Affinity Gel (Sigma-Aldrich). Clarification of the plant extract was optimised to ensure that there was no 'slime' layer forming on top of the column during purification as was found by the first attempts at this purification. This was to ensure the column was not disturbed during the wash or flow-through steps so that the protein is only eluted in the final elution fractions. It is also suggested by the manufacturer that all buffers as well as clarified plant extract should be at a pH of between 7 and 8. We therefore extracted, equilibrated and washed samples in 0.1M sodium phosphate buffer with 8M urea (pH7.1) and eluted protein in the same buffer but with added 250mM imidazole.

The ST-AP205 CP was successfully expressed and particles assembled *in planta* were purified using the previously optimised methods (Dennis, 2019). The monomeric (16.5kDa), dimeric (33kDa) and tetrameric (66kDa) forms of the CP were clearly detected on the Western blot using the AP205 antiserum as was observed by Stander et al. (Stander et al., 2021). A high density of particles was also observed under the TEM (Figure 3.8).

The purified BTV1Tip-SC and ST-AP205 proteins were quantified by gel densitometry using a BSA serial dilution for construction of a standard curve. The quantities were then used to calculate the necessary volumes to make up *in vitro* coupling reactions with different molar ratios. These included 1:3, 1:5 and 1:7 ST-AP205:BTV1Tip-SC. These ratios were chosen to ensure that as much of the BTV1-SC protein would be available for binding to the ST-presenting AP205 particles as possible. Similar studies have also investigated different molar ratios for coupling SpyVLPs starting at a 1:1 ratio and then increasing the concentration of the antigen (Brune et al., 2016, Yenkoidiok-Douti et al., 2019, Tan et al., 2021b). The coupling ratios chosen for this study all resulted in the same band intensity of the coupled protein on a Western blot and thus a 1:3 ratio was used in further experiments.

The BTV1Tip-SC protein was either coupled to plant-produced ST-AP205 or *E. coli*-produced ST-AP205. The *E. coli* produced ST-AP205 was included in the coupling reactions as it has been found in our case that when detecting *E. coli*-produced AP205 on a Western blot, the multimers of the protein (dimer and tetramer) are not usually observed (Thrane et al., 2016). This is unlike the case of plant-produced ST-AP205 where bands representing the dimers and tetramers are usually observed (Stander et al., 2021). This would mean there would be no confusion between the size of coupled protein complex (approx. 61kDa) and the size of the tetramer (66kDa). It has been found that when proteins are expressed in plants, during extraction and purification, there is an increased chance of cross-linking occurring (Castells-Graells et al., 2021). However, it was found that the multimers were detected on the Western blot (Figure 3.9). This may be due to the long storage of the sample resulting in the aggregation and multimerization of the ST-AP205 proteins. We were able to detect the coupled BTV1Tip-SC-ST-AP205 complex on the Western blot at the expected size (approx. 60kDa), running slightly below the tetramer (66kDa). The same pattern was observed for both plant and *E. coli*-produced ST-AP205 (Figure 3.9).

The coupling efficiency was calculated as being approximately 44%. Although the BTV1Tip-SC antigen was present in molar excess in the coupling reaction, it is likely that due to steric hindrance, not all of the binding motifs on the AP205 capsid could be occupied by the antigen. The AP205 VLP is made up of 180 subunits and is between 27-30nm in diameter (270-300 angstrom) (Klovins et al., 2002, Tissot et al., 2010). This translates to 180 potential antigen binding sites. Although it is outside of the scope of this research, one could calculate the number of BTV1Tip antigen proteins that could bind to the AP205 VLP by determining the size of the tip domain in angstroms and determining how many could fit on the total surface area of the AP205 VLP. This would also be dependent on how far the STs are extended from the particle surface. It is likely that even if there was 100% coupling efficiency, each of the 180 potential binding sites would not be able to bind one whole BTV1Tip antigen molecule.

It has been found that larger antigens result in lower coupling efficiencies. Thrane et. al found that a 15kDa antigen coupled to ST-AP205 with 88% coupling efficiency while a 118kDa antigen coupled with 34% efficiency (Thrane et al., 2016). Additionally, Stander et al. found that the 25kDa WNV-EDIII antigen coupled to ST-AP205 with a 51% efficiency (Stander et al., 2021). They point out that this is likely an overestimation of coupling efficiency as the calculation does not take the uncoupled dimer and tetramer forms of AP205 into account. Despite this, however, Stander et al. found that the AP205 particle displaying the antigen still resulted in a greater immune response than the soluble antigen alone, further supporting the fact that display of at least some antigen on a particulate structure increases immunogenicity of the antigen.

Although the *in vitro* coupling shown in this work was promising in its ability to form the desired particulate complexes, there is still optimisation to be done. When coupled protein was probed with the anti-His antibodies, no bands were detected; neither the BTV1Tip-SC protein nor the coupled product. This may be due to the His-tag on the BTV1Tip-SC protein being hidden within the protein complex. Although the rigid linker that separated the His-tag from the BTV1Tip antigenic domain did successfully keep the His-tag exposed for purification and detection of the BTV1Tip-SC protein, it seems that when BTV1Tip-SC was coupled to ST-AP205, the His-tag was not exposed for detection by anti-His antibodies. It has been found that, in rare instances, the His-tag can be hidden by the tertiary structure of a protein (Crowe et al., 1996, Block et al., 2009). Making use of an antibody other than

anti-ST-AP205, such as anti-BTV1 VP2, would help to confirm that the band observed is in fact the coupled protein and not a multimer of the ST-AP205 VLP.

In addition to *in vitro* coupling, there are two other strategies that could be used to couple the ST-AP205 VLPs to the BTV1Tip-SC proteins. One method of coupling is known as co-expression, or co-infiltration, which involves the SpyVLP components being co-infiltrated into the same leaves for coupling to occur *in vivo.* This has been found to be successful in plants expressing the Hepatitis B core antigen (HBcAg) and GFP (Peyret et al., 2020). The second method - co-extraction, or co-purification, - involves the leaf material of individually infiltrated constructs being harvested together in the same extraction buffer, and subsequently purified together. This method has also been successfully conducted in plants (Roder et al., 2017). *In vitro* coupling involves each construct being individually infiltrated into leaves, extracted and purified separately before being mixed together *in vitro*. This is a lengthy process requiring complex purification methods which take time and money to carry out. The co-extraction and co-expression methods are more feasible as they do not require each component to be extracted and purified separately. A previous study conducted in our laboratory found that all three methods were successful in coupling the West Nile virus EDIII protein to the ST-AP205 capsid protein however the co-extraction method resulted in the greatest yield of coupled complexes (Stander et al., 2021). Due to time constraints, this study could not explore these methods to the same extent as was done for *in vitro* coupling, however, future work should investigate whether better coupling of BTV1Tip-SC to ST-AP205 could be achieved using these alternative methods. At present, the *in vitro* method tested here does not lend itself to commercialisation as large-scale expression and purification would be too time-consuming and expensive.

Having investigated the production of chimeric BTV1/8 VLP and the SC/ST-based BTV1 vaccine candidates in plants, we concluded that both approaches have the potential to be used in future vaccine regimes. However, the ST/SC-based approach requires further optimisation to improve the coupling efficiency and yield of the coupled complexes. We obtained yields of 2mg/kg FLW and 18mg/kg FLW of ST-AP205 and BTV1Tip-SC proteins, respectively, which are both much lower than the 34mg/kg FLW yield of chimeric VLPs measured in chapter 2. These yields would likely not be sufficient for dosing due to their low concentration and the fact that there is a maximum dosing volume of 2mL for subcutaneous injection of guinea pigs (Wolfensohn et al., 2008). It was thus decided that the chimeric VLP was the more promising candidate vaccine between the two and was selected for use in animal immunogenicity trials (chapter 4).

# Chapter 4: Evaluating the immunogenicity of plant-produced BTV8 and BTV1/8 VLPs in guinea pigs

## 4.1 Introduction

BTV vaccines are able to mount an immune response which induces the production of neutralising antibodies which protect the animal from disease if they become infected with the virus against which they were immunised. Because there is low cross-protection between BTV serotypes, the ideal vaccine strategy should protect against more than one serotype. The commercially available vaccines for BTV include the modified live virus (MLV) vaccine and the inactivated vaccine. Although the MLV consists of three doses containing five serotypes each to protect against the prevalent serotypes, and the inactivated vaccine is a safer alternative, there are a number of associated risks which are undesirable in terms of an ideal BTV vaccine (Batten et al., 2008, Nomikou et al., 2015, Feenstra et al., 2017). The limitations presented by risks of reassortment and reversion to virulence by the MLV and short-lived immunogenicity of the inactivated vaccines has encouraged the development of alternative methods which are safer, more cost-effective, efficacious, protective against multiple serotypes and DIVA compliant. Although it is difficult to develop a vaccine which addresses every one of these concerns, there have been a number of promising vaccine candidates which have the potential to be developed into more desirable BTV vaccines than those currently in use.

Vaccines can be considered to be 'multivalent' when they protect against challenge by more than one serotype of a given virus, where there are low levels of cross-protection (Lauer et al., 2017, He et al., 2021). For example, multivalency can be achieved through the expression of multiple neutralising epitopes on a single VLP where some are specific to one serotype and the others specific to another (Nunes et al., 2014, Feenstra et al., 2015, Mokoena et al., 2019). Alternatively, individual VLP preparations could be designed to elicit a single serotype-specific immune response with the vaccine comprising a cocktail of the VLPs representing different serotypes. For example, Rojas et al. investigated the use of recombinant adenoviruses in producing a BTV vaccine that would provide protection against challenge by multiple BTV serotypes (Rojas et al., 2021). Their candidate vaccine consisted of the replication-defective human adenovirus serotype 5 (Ad5) expressing VP7 of BTV8 (Ad5VP7-8) in combination with Ad5 expressing VP2 of BTV1 (Ad5VP2-1). This vaccine was able to protect vaccinated mice against BTV1 and BTV8 challenge. However, the authors attributed this to the cross reactive anti-BTV IgG antibody response induced by the T-cell epitopes on the more conserved VP7 protein as there were no significant neutralising antibody titres detected. This study suggests that the inclusion of more conserved BTV proteins, such as VP7, in a vaccine strategy is important as protection is dependent on both the humoral and cellular immune response and T-cell immunity may be a key component of creating multivalent vaccines. A similar strategy investigated the use of a pentavalent DISA vaccine as a potential BTV vaccine that could provide protection against multiple serotypes. This strategy made use of the live-attenuated viruses from five different serotypes where there is a deletion of 72 amino acid codons in NS3/NS3a (van Rijn et al., 2021). There was only full protection provided against BTV8 challenge and partial protection against BTV2 challenge.

Although the above vaccines have had success in providing protection against BTV, there is still a need for multivalent BTV vaccines which provide a long-lasting protective neutralising antibody response. VLPs are ideal vaccine candidates as they present the VP2 epitopes to the immune system in the same

way that the native virus would during natural infection (Crisci et al., 2012). They are also completely safe as they do not include any genetic material.

A number of BTV VLPs have been developed using the baculovirus insect cell expression system, some of which address the need for multivalent vaccines. Stewart et al. used a standard VLP core (BTV17 VP3 and BTV10 VP7) and exchanged the outer capsid proteins (VP2 and VP5) to change the serotype it was targeting (BTV2, BTV4 or BTV9). They found that the BTV2 VLPs (where BTV2 VP2 and VP5 were used) induced neutralising antibodies in sheep which protected them from BTV2 challenge. Additionally, there were no clinical signs of viremia (Stewart et al., 2010, Stewart et al., 2012). Perez de Diego et al. took a different approach and included VLPs for BTV1 and BTV4 in a single vaccine. Although there were neutralising antibodies produced against both VLPs, the BTV4 response was not strong enough to provide complete protection to sheep challenged with both virus serotypes (Perez de Diego et al., 2011). Feenstra et al. investigated chimeric DISA vaccines where either entire VP2 proteins were exchanged or just part of the VP2 protein was exchanged with the corresponding region of another. One of the vaccines where there was a chimeric VP2 protein including parts from BTV1 and BTV16 resulted in serotype-specific antibody production in sheep and the ability to neutralise both serotypes (Feenstra et al., 2014).

In addition to the vaccine candidates produced in the traditional cell expression systems, there have also been great strides made in the production of similar vaccines in plant expression systems. Some promising plant-produced BTV VLPs include BTV8 VLPs which were able to produce serotype-specific antibodies in sheep and provide protection against BTV8 challenge (Thuenemann et al., 2013) and chimeric VLPs targeting serotype 3 and 4 which induced seroconversion in sheep (Mokoena et al., 2019).

Although many of these studies provide immunogenicity data from sheep immunised with the candidate vaccines, many of the preliminary animal work for these and similar studies make use of small animal models to determine preliminary immunogenicity data which will determine whether trials will continue in the target animal. The most common animal models include mice and guinea pigs. Using small animal lab models saves time and money and is ethically beneficial as it decreases the need for the larger animal models at the early stages of vaccine development (Denayer et al., 2014, Perrin, 2014).

Because there is a plethora of research which demonstrates the successful induction of an immune response in lab animal models when immunised with VLP candidate vaccines, this study investigated the ability of the chimeric BTV1/8 VLP to induce an immune response in guinea pigs. There are numerous studies which have shown that guinea pigs have high levels of neutralising antibodies when immunised with BTV candidate vaccines (French et al., 1990, Loudon et al., 1991, Legisa et al., 2015). Guinea pigs are used as they are easy to work with and are susceptible to infection while having an immune system similar to that of humans (Vicari, 2003). They are also preferred over mice as their larger size means that more serum can be collected and used in analyses.

In this chapter we evaluated the ability of the plant produced BTV8 and BTV1/8 VLPs to elicit an immune response in guinea pigs by producing antibodies against the two VLP candidate vaccines. The antisera were analysed by their ability to detect the VLP proteins on a Western blot and bind to the VLP antigens in an indirect enzyme-linked immunosorbent assay (iELISA).

## 4.2 Materials and Methods

### 4.2.1 Immunisation of guinea pigs

Fifteen 10–12-week-old female guinea pigs of the Hartley strain were obtained from the South African Vaccine Producers (SAVP, Johannesburg, South Africa). They were housed in the Research Animal Facility at the University of Cape Town (RAF, UCT) in the Large/Farm animal unit for the duration of the study. This study was approved by the Animal Ethics Committee at UCT (AEC020-023).

The guinea pigs were acclimatised for 10 days prior to vaccination and randomly separated into three groups of five guinea pigs each (n=5). The candidate vaccines included the plant produced BTV8 and BTV1/8 VLPs prepared as described in chapter 2 section 2.2.3.2 and section 2.2.4. The negative control vaccine group was immunised with a vaccine which was prepared in the same way as the VLP vaccines but comprised purified extract from plants infiltrated with *A. tumefaciens* harbouring pEAQ-*HT* vector lacking any insert DNA (essentially all components of the experimental vaccine except for VLP protein). Vaccine dose details are given in Table 4.1.

**Table 4.1: Details of plant produced vaccine candidates used in immunogenicity study**

| Vaccine Name | Group Number | Adjuvant | Total VLP protein dose (ug) |
|---|---|---|---|
| pEAQ-*HT* | 1 | Montanide™ ISA 50 V2 | - |
| BTV8 VLPs | 2 | Montanide™ ISA 50 V2 | 15 |
| BTV1/8 VLPs | 3 | Montanide™ ISA 50 V2 | 15 |

The purified VLP samples were quantified by gel densitometry of Coomassie-stained gels using GeneTools software (Syngene) and a BSA standard curve. Each vaccine was 400uL in volume and prepared to contain 15ug protein and 5% Montanide™ ISA 50 V2 (Seppic) adjuvant. The remaining volume was made up with bicine buffer (50mM bicine, 400mM NaCl, pH8.4).

On day 0, after 10 days of acclimatisation, 100uL of pre-immunisation sera were collected from each guinea pig via the saphenous vein. On the same day, the first vaccine doses were administered by injecting 100uL subcutaneously in 4 places on the back: two to the left and two to the right of the spine (total dose of 400uL). The guinea pigs were boosted using the same method at 13 days post-immunisation. Final bleeds were collected on day 41 post-immunisation by cardiac puncture. Serum was separated from the blood by centrifugation and stored at -20°C. One of the guinea pigs from group 3 was terminated on day 31 due to insufficient weight gain as stipulated by the approved ethics agreement. The animal was autopsied after collection of blood and serum was harvested as described above and stored at -20°C.

### 4.2.2 Detection of VLP antigens with final bleed guinea pig serum on Western blot

To determine whether the final-bleed antisera collected from the guinea pig vaccination groups could detect the VLP antigens on a Western blot, plant purified BTV8 and BTV1/8 VLPs were separated by SDS-PAGE. The methods described in section 2.2.5 were followed for Western blot analysis. The serum collected from each guinea pig in the experimental groups (BTV8 VLP and BTV1/8 VLP immunisations) was diluted 1: 20 000 and used to probe a single lane containing the relevant antigens VP2, VP3, VP5 and VP7. The goat anti-guinea pig IgG (whole molecule) alkaline phosphatase conjugated secondary antibody was used at a dilution of 1:30 000. The negative control lanes were probed with serum from the guinea pigs which were immunised with the negative pEAQ-*HT* control and the positive control

lanes were probed with sheep antiserum raised against BTV8 VLPs and anti-goat/sheep secondary antibody which were used in protein detection as in section 2.2.5.

### 4.2.3 Indirect ELISAs to evaluate antibody response to candidate vaccines

#### 4.2.3.1 Evaluating the post-immunised antibody response compared to pre-immunisation

To evaluate the immune response to each of the candidate vaccines, an iELISA was conducted to compare absorbance of pre-immunised sera (day 0) to post-immunised sera (day 41). The 96-well Maxisorp® microtitre plates (Nunc, ThermoFischer Scientific) were coated with 100uL of 100ng plant-produced BTV8 or BTV1/8 VLPs (purified as described in 2.2.4) diluted in coating buffer (10mM Tris, pH8.5). The plates were incubated at 4°C overnight with gentle agitation. The plates were blocked with 200uL blocking buffer (3% BSA in 1x TBS (50mM Tris, 150mM NaCl, pH7.5)) for 1 h at 37°C with gentle agitation.

The pre-immunisation (day 0) and post-immunisation (day 41) sera collected from each of the five guinea pigs in a group were pooled so that there was a single pre-immunised sample and a single post-immunised sample for each vaccine group. The pooled serum samples were diluted to 1:10 000 in TBS before loading 100uL into the wells containing the respective antigens against which they were raised, and the plates were incubated at 37°C for 1 h with agitation. The pooled serum from the negative control group was added to wells coated with the chimeric BTV1/8 VLPs. Each sample was evaluated in technical triplicates. Blank wells with no antibody were used as a background control.

After incubation with the guinea pig antisera, the wells were washed four times with 200uL 1x TST (0.05% Tween-20 in 1x TBS). The goat anti-guinea pig IgG (whole molecule) alkaline phosphatase conjugated secondary antibody was diluted to 1:30 000 in 1x TBS and 100uL was added to each well and plates were incubated at 37°C for 1 h with agitation. The wells were washed four times with 200uL 1x TBS (pH9.0) and subsequently, 200uL of the SIGMAFAST™ p-Nitrophenyl phosphate substrate (pNPP, Sigma) was added to each well and the plates were developed in the dark for 30 min. The absorbance readings were measured at 405nm using a Multiskan™ GO microplate spectrophotometer (ThermoFisher Scientific).

#### 4.2.3.2 Evaluating the antibody response between vaccine groups

An additional iELISA was conducted to compare the immune response between the two vaccine groups and to conduct an antiserum titration to determine the endpoint titre. Here, the same protocol was followed as in section 4.2.3.1 but using the antiserum of the individual guinea pigs in each vaccination group. Antisera were diluted 4-fold in 1x TBS and used at 1:10000, 1:30000, 1:90000 and 1:270000 dilutions. The absorbance readings were used to plot a titration curve with those of 1:10 000 diluted antiserum used to construct a graph comparing the absorbance between the vaccination groups.

### 4.2.4 Statistical analysis

Statistical analysis was conducted using GrapPad Prism version 9.3.1 (GraphPad, CA, USA). The error bars on the graphs represent the mean ±SEM (standard error of the mean) from three independent experiments with technical triplicates for each group. The comparison of the response between groups was calculated using the Student's two-tailed t-test. The significance threshold (p-value) was set at 5% ($p=0.05$) thus $p < 0.05$ representing a statistically significant result.

## 4.3 Results

4.3.1 Detection of VLP proteins by Western blot using guinea pig post-immunisation antiserum

The BTV8 VLP antisera from all five guinea pigs (1-5) detected all four BTV8 VLP proteins (111kDa VP2, 103kDa VP3, 59kDa VP5 and 38kDa VP7) on a Western blot (Figure 4.1A indicated by red arrows). The proteins were not detected in the negative control lane and all four proteins could be seen in the positive control lane although VP3 was very faint. All four BTV1/8 VLP proteins were detected strongly by the BTV1/8 VLP antisera from guinea pigs 1 and 2 and there was faint detection of the VP5 and VP7 protein in guinea pigs 4 and 5 (Figure 4.1B indicated by red arrows). Guinea pig 3 was put down early in the experiment (31 days post-immunisation) due to liver disease and the antiserum did not detect any of the chimeric VLP proteins. Lanes 2 and 3 of Figure 4.1A and 1 and 2 of Figure 4.1B were re-probed with a 1:10 000 dilution of antiserum to determine if a lower dilution would improve detection of the VLP proteins. These re-probed lanes show a higher signal detection of the proteins than the other lanes. There was some non-specific binding by the antibodies which can be attributed to the detection of host cell contaminants.

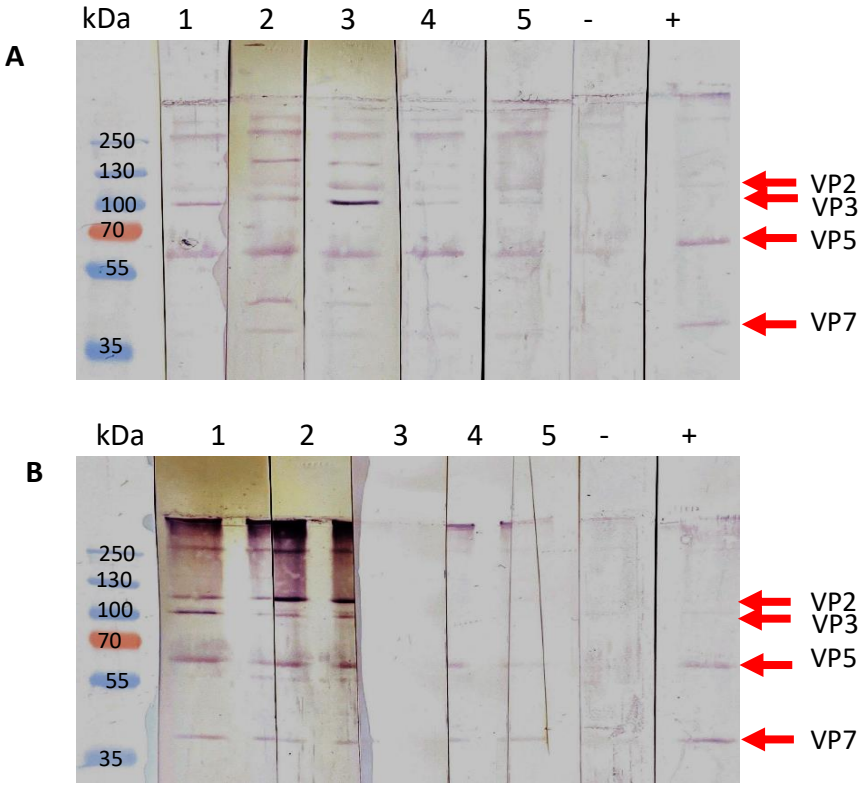



**Figure 4.1: Western blot to determine if guinea pig antiserum can detect plant produced BTV8 and BTV1/8 VLP proteins.** Post-immunisation antiserum (day 41) collected from each guinea pig (1-5) from the groups immunised with BTV8 or BTV1/8 VLPs was diluted to 1:20 000 and used to detect **(A)** BTV8 VLP proteins or **(B)** BTV1/8 VLP proteins on a standard Western blot with goat anti-guinea pig secondary antibody (1:30 000). Lanes 2 and 3 of (A) and 1 and 2 of (B) were re-probed with a 1:10 000 dilution of antiserum. Red arrows indicate the VLP proteins where VP2 is 111kDa, VP3 is 103kDa, VP5 is 59kDa and VP7 is 38kDa. Negative control (-) represents proteins detected with antiserum from guinea pigs immunised without VLP antigen (1:20 000). Positive control (+) represents proteins detected with sheep produced anti-BTV8 VLP anti-serum (1:2000). kDa: molecular weight marker in kilodaltons.

4.3.2 Indirect ELISA analysis of guinea pig antiserum against BTV1 and BTV1/8 VLPs

4.3.2.1 Comparison of pre-immunisation and post-immunisation antibody responses

To determine whether there was a significant difference in the antibody response after vaccination compared to before vaccination, an iELISA was conducted for each vaccine candidate. Wells were coated with 100ng of either plant-produced BTV8 VLP or BTV1/8 VLP antigen and probed with a 1:10 000 dilution of the pooled sera from the corresponding vaccination groups. There was a statistically significant difference ($p<0.01$) in the response between pre- and post-immunised sera collected from guinea pigs immunised with both VLP vaccines (Groups 2 and 3) whereas there was no difference in the response to the negative control (Group 1) before and after vaccination (Figure 4.2).

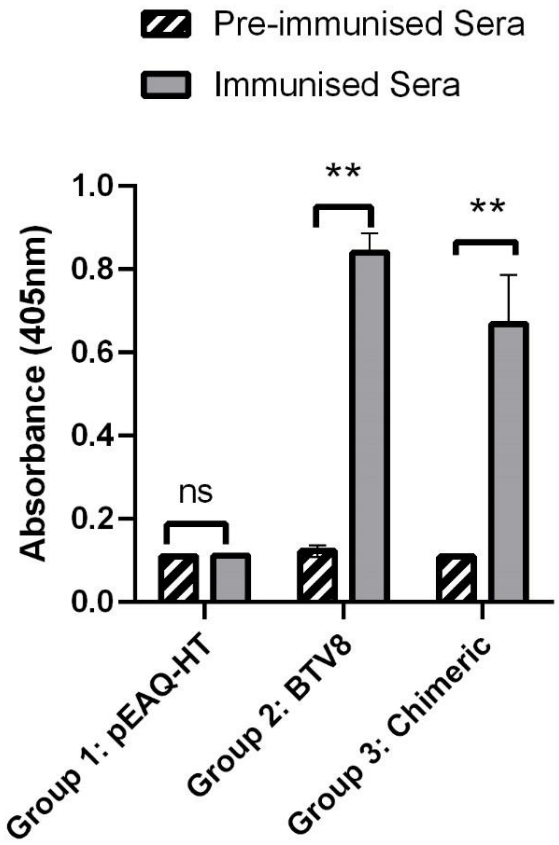



**Figure 4.2: Indirect ELISA of guinea pig serum to determine the difference in antibody response before and after vaccination.** Wells were coated with 100ng plant-produced BTV8 or BTV1/8 VLP antigen and probed with 1:10 000 dilution of pooled antiserum from each group (n=5 per group) before (pre-immunised sera) and after (immunised sera) vaccination to investigate the presence of anti-BTV8 and anti-BTV1/8 VLP antibodies. Error bars represent the mean ±SEM from three independent experiments with technical triplicates for each group. Y-axis represents absorbance at 405nm. X-axis shows each vaccination group where Group 1: negative control group immunised without VLP antigen, Group 2: immunised with BTV8 VLPs, Group 3: immunised with BTV1/8 VLPs. ns: non-significant difference where $p > 0.05$. **: Significant difference where $p < 0.01$.

4.3.2.2 Comparison of immune responses to vaccines

Having determined that there was a significant immune response to each of the VLP vaccines, an iELISA was carried out to compare the antibody responses between the two vaccines (Figure 4.3). Here, the post-immunisation sera from each individual guinea pig in the vaccination groups were diluted to 1:10 000 and evaluated against 100ng of plant-produced BTV8 VLP or BTV1/8 VLP antigen. The VLP vaccination groups, group 2 and 3, both have significantly greater (at least 5-fold) mean $OD_{405}$ absorbance values, 0.997 and 0.633 respectively, than the negative control group ($OD_{405}$ 0.119). Additionally, the BTV8 VLP vaccine induced a significantly greater (1.5-fold) immune response than the chimeric VLP group (see p-values on graph).

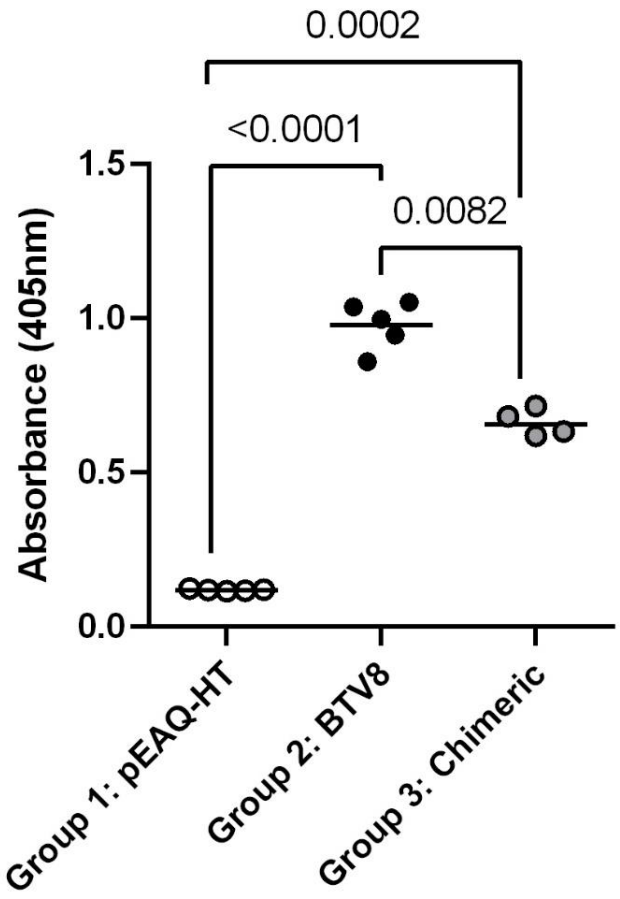



**Figure 4.3: Indirect ELISA of guinea pig serum to compare the post-immunisation antibody response between the three vaccination groups.** Wells were coated with 100ng plant-produced BTV8 or BTV1/8 VLP antigen and probed with 1:10 000 dilution of post-immunisation antiserum (day 41) from each guinea pig (n=5 per group) to investigate the difference in the response of anti-BTV8 and anti-BTV1/8 VLP antibodies. Points represent the individual absorbance readings for each guinea pig averaged from three independent experiments with technical triplicates for each individual. Lines represent the group means. Student's t-test determined significance levels (p-values indicated between groups). Y-axis represents absorbance at 405nm. X-axis shows each vaccination group where Group 1: negative control group immunised without VLP antigen, Group 2: immunised with BTV8 VLPs, Group 3: immunised with BTV1/8 VLPs.

4.3.2.3 Antiserum titration curve to determine endpoint titre for VLP vaccines

A four-fold titration was conducted to determine the antibody endpoint titre for the vaccines tested. The final bleed serum of individual guinea pigs from each vaccination group was diluted 1:10 000, 1:30 000, 1:90 000 and 1:270 000 and used to probe wells containing the corresponding VLP antigen. The $OD_{405}$ absorbance values were averaged and plotted on the curve. Low, close to zero (approx. 0.11), absorbance values were measured for the negative control antisera at all dilutions which was expected as there should be no VLP protein-specific antibodies elicited in this vaccination group. The samples probed with VLP antiserum diluted to 1:270 000 measure an $OD_{405}$ absorbance reading just above that of the negative control (0.190 for group 2 and 0.197 for group 3) (Figure 4.4). This is regarded as the endpoint titre for both VLP vaccination groups as it represents the greatest dilution of antiserum at which the $OD_{405}$ absorbance is greater than the negative control.

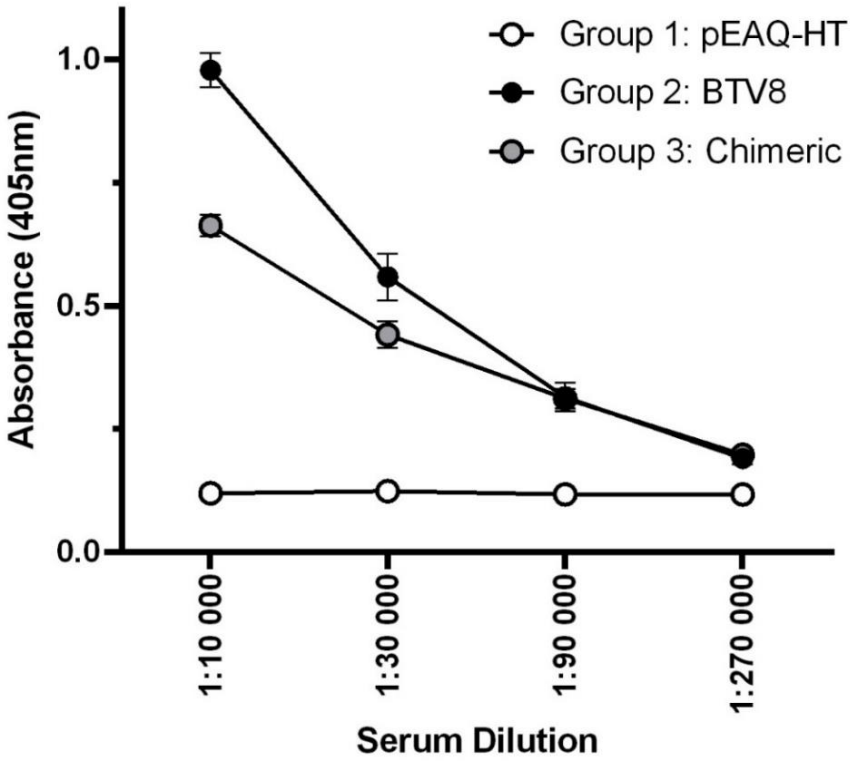



**Figure 4.4: Four-fold titration of antiserum conducted by indirect ELISA to determine titration endpoint.** Wells were coated with 100ng plant-produced BTV8 or BTV1/8 VLP antigen and probed with post-immunisation antiserum with dilutions ranging from 1:10 000 to 1:270 000 from individual guinea pigs (n=5 per group) to determine the endpoint titre. Points represent the mean absorbance readings for each group from three independent experiments with technical triplicates. Error bars represent the mean ±SEM. Y-axis represents absorbance at 405nm. X-axis shows the dilutions of antiserum at which absorbance was measured. Group 3: negative control group immunised without VLP antigen, Group 1 immunised with BTV8 VLPs and Group 2 immunised with BTV1/8 VLPs.

## 4.4 Discussion

Because there is no cure for BTV, vaccination is still the most effective method for controlling spread of the virus and preventing severe symptoms of the disease. There have been numerous studies which have investigated recombinant vaccine strategies which improve on the limitations of the current BTV vaccines.

This study compared the immunogenicity of two plant produced BTV VLP vaccines and their ability to induce a humoral immune response in guinea pigs. This preliminary investigation aimed to determine whether a chimeric BTV VLP could be a potential candidate vaccine for BTV. A chimeric VLP, where a chimeric VP2 protein has been designed, lends itself to multivalency as it is known that there are multiple epitopes present on the VP2 protein, some of which are neutralising (White et al., 1990, DeMaula et al., 2000, Xie et al., 2018). Thus, a chimeric VLP has the potential to provide protection from both the serotypes included in its design (Feenstra et al., 2015, Mokoena et al., 2019). However, in this case, the antibodies would be raised against different domains of the VP2 protein and thus the immune response elicited by each of the two serotypes would not be completely equivalent. Alternatively, in the case of the BTV1/8 chimeric VLP investigated in this study, the chimeric region could be easily manipulated to target any serotype by replacing the chimeric region. We have shown that using a BTV8 backbone results in the spontaneous assembly of stable particles in plants and thus the serotype that the vaccine targets can be easily changed by exchanging the chimeric VP2 tip domain. A vaccine containing a cocktail of chimeric VLPs of this nature could then be administered to protect against multiple circulating serotypes.

Here we have investigated the plant produced BTV8 and BTV1/8 VLPs which were expressed in plants and purified from clarified plant extract as described in chapter 2. BTV8 VLPs were used as a positive control as these particles have been purified from plants previously and shown to induce seroconversion in sheep providing protection against challenge with BTV8 (Thuenemann et al., 2013, van Zyl et al., 2016). These BTV8 VLPs are expressed and purified easily in plants which makes them a stable and reliable backbone for the chimeric BTV1/8 VLPs. This is not the case for all BTV serotypes. Nunes et al. designed inactivated synthetic BT viruses where VP3 and VP5 were based on BTV1, and VP2 and VP5 were based on different serotypes (Nunes et al., 2014). Not all combinations of the exchanged VP2 and VP5 proteins were able to form the synthetic viruses with the BTV1 backbones. Additionally, it has been found that BTV10 VLPs, for example, do not easily form stable VLPs to the same extent that the BTV8 VLPs do (Thuenemann, 2010). Furthermore, the closely related African horse sickness *Orbiviruses* have also been found to form stable VLPs by co-expression with the four corresponding structural proteins in some cases, but not for every serotype. For example, it has been found that AHSV serotype 7 VLPs do not assemble when the four serotype 7 structural proteins are transiently expressed in plants (Rutkowska et al., 2019).

Guinea pigs were immunised subcutaneously with 15ug of candidate vaccine and boosted with the same dose 13 days subsequently. Final bleed antisera collected on day 41 post immunisation were evaluated for the presence of anti-BTV VLP protein antibodies by Western blot and iELISAs using the respective VLPs as antigens. iELISA analysis of the pre-immunisation and final-bleed sera showed no anti-BTV VLP protein response in the negative control serum or the pre-immunisation sera (Figure 4.2). The final-bleed sera from both the BTV8 and BTV1/8 VLP vaccinated guinea pigs had potent antibody responses that were significantly greater than that of the pre-immunised sera ($P<0.01$). This result

confirms the successful induction of the anti-BTV humoral immune response by the plant-produced VLP candidate vaccines.

In comparing the antibody responses between the two vaccines, both induced significant responses in immunised animals compared to those immunised with the negative control. Additionally, the BTV8 VLP vaccine elicited a response significantly greater than the chimeric VLP vaccine. Although this was only a 1.5-fold difference, it does suggest that the chimeric vaccine is not as potent as the BTV8 vaccine. A strong neutralising antibody response requires the VP2 protein and its epitopes to be displayed in the correct conformation. The chimeric nature of the VP2 protein may affect the epitope display or the interaction of VP2 with VP5 making it less effective in inducing an antibody response (Fay et al., 2019). Therefore, the chimeric particles may be less stable than the BTV8 VLPs as the chimeric VP2 protein may not be able to form the native folded VP2 protein with the same level of stability as the monomeric BTV8 VLPs. If there are fewer fully formed chimeric particles, the immune response would not be as high as the BTV8 vaccine. Because gel densitometry was used for the quantification of VLP proteins and subsequently to determine the volume of antigen to include in the vaccine, we do not know the concentration of fully formed particles included in the vaccine doses as this quantification method is based on the protein concentration being measured by protein band density on a Coomassie stained gel. Therefore, although the same concentration of BTV8 and BTV1/8 protein was included in the vaccines, there may have been a difference in the concentration of fully formed VLPs. This may explain the less potent immune response from the guinea pigs immunised with chimeric VLPs. However, although this difference exists when comparing the immune response between vaccination groups, the 4-fold titration showed that both VLP vaccines had the same endpoint titre of 1:270 000.

Additionally, it is possible that the BTV8 region of the chimeric VP2 protein induced a neutralising antibody response via additional neutralising epitopes on the VP2 protein that were external to the BTV1 tip domain. These may be, in part, what was visualised binding to the chimeric VP2 protein on the Western blot. It is also possible that the BTV8 VP5 protein contributed to the humoral response as it has been found to improve neutralising antibody responses when immunised in combination with VP2 (Roy et al., 1990, Lobato et al., 1997). The only way to confirm if the chimeric VLPs can provide protection against BTV1 challenge is to carry out a serum neutralisation assay with BTV1.

The BTV8 VLP antigen was detected clearly by the final-bleed anti-BTV8 VLP antiserum of all five guinea pigs on a Western blot indicating that antibodies were raised against all four VPs of the BTV8 VLP vaccine. The antiserum from two of the guinea pigs in the chimeric vaccine group detected all four VLP proteins on the Western blot while serum from two of the others only detected VP5 and VP7. The detection of the chimeric VLP proteins is not as clear as in the BTV8 group which supports what was seen in the iELISA experiments where the absorbance values for the chimeric group were slightly lower than those for the BTV8 group. One guinea pig in the chimeric VLP group was terminated early due to liver disease and this antiserum could not detect any of the VLP proteins. This may be because the immune system was weakened due to infection and could thus not mount a response to the vaccines. It has been found that cirrhosis causes abnormalities in B-cell function which can lead to a low vaccine response and susceptibility to bacterial infection (Doi et al., 2012). Alternatively, it is possible that specific antibody titres had not yet reached detectable levels at day 31 and thus could not be detected on the Western blot.

The results from this experiment do not prove definitively that the chimeric VLPs provided protection against both BTV1 and BTV8 viruses. Due to time constraints, there were a number of experiments that could not be carried out for this study. Future research should investigate whether the antibodies raised against the chimeric VLPs could bind to non-chimeric BTV8 and BTV1 VP2 proteins or alternatively, anti-BTV1 antibodies could be used to probe the chimeric VLP proteins to determine if there is detection of chimeric BTV1/8 VP2. This research group (BRU, UCT) has attempted to make BTV1 VLPs previously, however there were no appropriate anti-BTV1 antibodies available which could detect such VLPs. Because this was a preliminary study only 3 groups are guinea pigs were used (n=5). Using a greater number of guinea pigs per group could increase reliability of the results and adding another group immunized with BTV1 VLPs would also increase the number of analyses that could be carried out. Ultimately, however, serum neutralisation assays (SNA) would be the only way to confirm whether the chimeric VLP vaccines can induce an immune response that protects animals against both BTV8 and BTV1. A number of studies have found, through SNA, that chimeric BTV vaccines, where the VP2 protein is chimeric, are neutralised by antibodies from both serotypes included in the chimeric construct (Nunes et al., 2014, Feenstra et al., 2015).

In conclusion, the plant produced VLP vaccines were able to induce a humoral immune response in guinea pigs. Both the Western blots and iELISAs were able to illustrate that the antibodies raised against the BTV8 and BTV1/8 VLP vaccines were able to bind the relevant VLP antigens. This result suggests that these antibodies would also be able to bind to the native virus and thus have the potential to protect animals against infection. Overall, this vaccine strategy shows great potential for further development of a BTV vaccine candidate that is safe, scalable and has potential for multivalency.

# Chapter 5: General discussion and concluding remarks

BTV has become a globally prevalent virus which is a challenge to mitigate due to the extensive number of serotypes which have limited cross-reactivity and most of which cause disease. To curb the spread of the virus and minimise fatalities, vaccine regimes have been implemented in a number of countries which frequently experience BTV outbreaks. The currently available commercial vaccines, the MLV and inactivated vaccine, have had success in providing protection against a number of BTV serotypes (Dungu et al., 2004, Patta et al., 2004, Zientara et al., 2013, McVey et al., 2015). However, the limitations of these vaccine strategies have led to the development of a number of recombinant BTV vaccines which are safer, scalable, efficacious and potentially more economically viable, multivalent and DIVA compliant.

BTV VLPs have become particularly promising recombinant vaccine alternatives as they are able to mimic the conformation of the native virus. VLPs have the potential to induce a potent immune response as they are recognised by the immune system as native viruses and have self-adjuvanting properties (Roy et al., 2008, Crisci et al., 2012). Additionally, they have been found to not only induce a B cell mediated immune response but also a cytotoxic T lymphocyte response (Roy et al., 2008). Many BTV subunit and VLP vaccines are expressed in baculovirus insect cell or *E. coli* expression systems where genes are often pre-integrated into the baculovirus genome (Stewart et al., 2010, Perez de Diego et al., 2011). Although these systems have been useful for BTV VLP assembly, they are still too costly to compete with the traditional methods of passage in embryonated chicken eggs and BHK-21 cell culture or inactivation by heat, UV radiation or chemicals used by the current vaccine strategies (Coetzee et al., 2012, Feenstra et al., 2017, Mayo et al., 2017).

Plants have also been used as BTV VLP vaccine expression systems. They are more favourably regarded than conventional fermentation type expression systems as they are more cost effective, easy to work with, and do not require sterile environments and expensive infrastructure to maintain (Merlin et al., 2014, Marsian et al., 2016, Nandi et al., 2016, Moon et al., 2019, Fischer et al., 2020, Rybicki, 2020). A number of BTV VLP vaccine candidates have successfully been produced in *N. benthamiana* and shown to be immunogenic (Thuenemann et al., 2013, van Zyl et al., 2016, Thuenemann et al., 2018). Plant made BTV VLPs comprising the four major structural proteins VP2, VP3, VP5 and VP7 specific for serotype 8 were shown to be protective against BTV8 challenge in sheep. More recently there has been investigation into the production of chimeric BTV VLPs. These VLPs are often designed so that VP2 and VP5, or VP2 only, differ in serotype specificity to the inner capsid proteins (Stewart et al., 2010, Nunes et al., 2014, Mokoena et al., 2019). Chimeric VLPs have the potential to provide protection against multiple serotypes either through cross protection by a single chimeric VLP or through a cocktail of chimeric VLPs targeting different serotypes. This is important for geographical areas which experience multi-serotype outbreaks.

Previous investigations into chimeric BTV VLPs and the need for BTV vaccines protecting against multiple serotypes led to the inspiration for this study. The aim was to develop and compare two different particulate BTV candidate vaccines made in plants and determine their ability to elicit specific immunity in guinea pigs. The first vaccine approach was to develop a chimeric BTV VLP vaccine. Unlike many of the previously developed chimeric VLPs, where the entire VP2 protein is exchanged, here we substituted only the immunogenic tip domain of the *VP2* gene of BTV serotype 8 (BTV8) with that of the corresponding *VP2* domain of BTV serotype 1 generating a chimeric VP2 which, when co-expressed

in plants with the remaining BTV8 VP3, VP5 and VP7, resulted in chimeric BTV1/8 VLPs. The second approach involved the display of the same immunogenic BTV1 VP2 tip domain on the surface of a bacteriophage AP205 particle through the application of the SpyTag (ST)/ SpyCatcher (SC) bioconjugation method. It was anticipated that both these particulate vaccine candidates would be safe to use and allow for rapid production and scalability. Moreover, only a small fragment of the BTV8 *VP2* gene encoding the highly immunogenic epitopes would need to be modified to allow for a VLP to be made against a new serotype.

The first part of this study addressed the optimisation of the expression and purification of chimeric BTV1/8 VLPs. The BTV8 backbone was specifically selected as there is a well-established method for producing BTV8 VLPs (Thuenemann et al., 2013, van Zyl et al., 2016) which are readily assembled in plants. As a proof of concept, the VP2 tip domain of BTV1 was selected for substitution into BTV8 VP2. Serotype 1 was selected for several reasons. Annual vaccination with the commercially available MLV vaccine includes 3 doses, each containing five different serotypes with BTV8 and BTV1 in separate bottles as they have low serological cross-reactivity (Feenstra et al., 2017) and different rates of replication (Dungu et al., 2004). Additionally, BTV1 has been present in a number of the major outbreaks of BTV in recent years (de Diego et al., 2014, Conte et al., 2016). Several studies have investigated chimeric BTV vaccines where either the entire VP2 protein is of a different serotype or only a portion is different. It has been found that when the entire VP2 protein is different, stable particles cannot always be assembled (Feenstra et al., 2015). This is thought to be due to the interaction of the VP2 and VP5 proteins and the importance of the correct display and conformation of VP2. Here, we have made a chimeric particle where only the tip domain, which extends outwards from the native virion, is different from the BTV8 backbone. We were able to improve the protein concentration and yield of particles by increasing the salt concentration in the extraction and purification buffers and harvesting at five days post-infiltration. The optimised methods resulted in BTV8 and BTV1/8 VLP protein yields of, 35mg/kg fresh leaf weight (FLW) and 34mg/kg FLW, respectively. This was sufficient for use in immunisation of guinea pigs at doses of 15ug each. This yield was approximately half the 70mg/kg FLW yields of BTV8 VLPs reported by Thuenemann et al. (Thuenemann et al., 2013) but slightly more than the 27mg/kg FLW reported by van Zyl (van Zyl, 2014) for BTV8 VLPs. These differences in yield may be due to varied methodologies, for example there were differences in the design of constructs and infiltration and purification techniques used by these groups.

With further investigation into improving yields, these chimeric VLPs could become contenders for future BTV vaccine regimes. Some of the methods used by Thuenemann et al. could be applied to improve VLP yields. To increase the yield of VLPs over CLPs and other assembly intermediates, this group expressed the core proteins (VP3 and VP7) from one vector and the outer capsid proteins (VP2 and VP5) from another. Additionally, they expressed VP3 from the wild-type 5' UTR instead of the *HyperTrans* mutation. There was also a two-step gradient centrifugation method to ensure the greatest purity and quality of VLPs (Thuenemann et al., 2013). Additionally, van Zyl et al. found that changing the infiltration ratios of the recombinant *Agrobacterium* harbouring the VLP constructs could improve VLP assembly (van Zyl et al., 2016). Some further investigation is also required into the diagnostic kits for BTV.

One of the main limitations of the above VLP approach is that it would not be able to distinguish between infected and vaccinated animals (DIVA compliant) as the commercial diagnostic kit relies on

detection of the VP7 protein as an indication of infection and this protein is included in the plant-made VLPs (Hamblin, 2004, Rojas et al., 2019). An alternative test kit targeting a conserved and non-structural BTV protein, such as NS3 for example, would need to be developed and commercialised for the plant-produced VLPs to be considered DIVA capable (Barros et al., 2009b). However, our alternative particulate BTV display vaccine could potentially allow for DIVA compliancy as it does not comprise any VP7.

We used the ST/SC antigen display technology to display the BTV1 VP2 tip domain on the surface of bacteriophage AP205 particles. Although, the use of this bioconjugation method for antigen-display is relatively new, there are a number of studies which show the successful application of this system (Brune et al., 2016, Thrane et al., 2016, Roder et al., 2017, Peyret et al., 2020, Stander et al., 2021). Additionally, it was recently used in the development of particulate COVID-19 vaccines where the spike trimer or spike receptor binding domain of the virus was displayed (Zhang et al., 2020, Tan et al., 2021a). Similar to the chimeric VLPs, this antigen display method could provide multi-serotype protection through the administration of a vaccine cocktail where particles displaying antigens from different serotypes are included in a single vaccine.

There are several factors which influence yields of fully coupled vaccine products including the type of coupling method utilised as well as the actual efficiency of coupling. Due to time constraints, we were only able to investigate coupling by the *in vitro* method where the separate components were expressed, harvested and purified separately before coupling *in vitro*. This is a lengthy and costly process which does not lend itself to commercialisation. However, alternative coupling methods such as co-expression or co-extraction of the SpyVLP components should be investigated as these methods require fewer steps and are thus potentially more cost effective than *in vitro* coupling. Stander et al. found that co-extraction resulted in the greatest yield of coupled WNV EDIII protein when compared to the other coupling methods (Stander et al., 2021). To improve yield of the ST/SC-based BTV1 vaccine, one would need to increase the concentrations of each component through optimization of infiltration and purification methods, in addition to investigating the other coupling methods mentioned above. We only investigated coupling of SC to the C-terminus of BTV1Tip. Fusion to the N-terminus could potentially improve coupling and thus yield. A greater concentration of the coupled complex is required to carry out immunogenicity trials.

Coupling efficiency describes the percentage of binding sites that are occupied on the VLP, therefore the higher the coupling efficiency, the more antigens that are bound to particles. This may be influenced by the type and size of core particle used for display. There are a variety of core particles which can be selected to suit the antigen that needs to be displayed. In addition to AP205 particles, other studies have used the mi3 synthetic nanoparticles (Bruun et al., 2018, Tan et al., 2021a), and the surface or core antigens of hepatitis B virus (Marini et al., 2019, Peyret et al., 2020), among others (Roder et al., 2017, Zhang et al., 2020). In our study, coupling efficiency was estimated to be approximately 44% which is fairly low compared to some reports. This could be due to the fact that the AP205 VLP has 180 16.5kDa subunits and the antigen was relatively large at 43.5kDa. A particle with larger spaces between each binding motif may be better suited to the display of the BTV1 tip antigen. This could reduce steric hinderance, thereby increasing coupling efficiency and induce a protective immune response, as found by Bruun et al. (Bruun et al., 2018). One would need to investigate different display particles, such as the 36nm mi3 60-mer, and determine which would be optimal for the size of the antigen that is to be displayed. Cryogenic electron microscopy could also be

used to determine an approximate size of the antigen itself so that the exact antigen binding capacity could be determined and help to inform the choice of display particle. There is a fine balance between keeping the antigen small enough to accommodate maximum binding capacity while including enough of the antigen to ensure correct folding and conformation so that the neutralising epitopes are displayed in their native form.

Finally, the immunogenicity trials showed that the VLPs were able to induce a potent antibody response in guinea pigs that was specific for all four BTV8 or BTV1/8 VLP proteins. Ideally, we would have liked to have purified a high enough yield of the recombinant AP205 particles displaying the BTV1 tip domain so that the two vaccine strategies could be compared, not only in their ability to induce an immune response, but also in their ability to produce antibodies which can neutralise native viruses. The next step in the immunogenicity investigation of the chimeric VLPs, will be to carry out a neutralisation assay to determine whether serum from immunised animals can neutralise BTV1 and/or BTV8 viruses. This would give a good indication of the potential for the chimeric VLPs to be used as a vaccine. If so, these VLPs could potentially be administered as a vaccine cocktail where serotype-specificity could be easily manipulated by the exchange of the tip domain present on the VP2 protein.

Overall, this study was able to achieve its main aim to develop two particulate BTV vaccines in plants by completing the following objectives:

- To optimise expression, extraction, and purification protocols for chimeric BTV1/8 VLPs with BTV8 VLPs used as a control.
- To investigate the potential for the SpyTag/SpyCatcher technology to be applied for the display of the BTV1 antigenic domain on the surface of the AP205 VLP.
- To determine the immunogenicity of the plant produced vaccines in guinea pigs.

Immunogenicity was only investigated for the VLPs due to low yields of display particles being achieved; however the immunogenicity results of the chimeric particles are promising and imminent serum neutralisation assays will enable determination of the extent of serotype-specific immunity elicited and whether these VLPs can be considered as potential vaccine candidates for BTV.

# Appendix

**FP1**

5’-*CATCACCATCATCCC*GGGGAAGCCGCTGCTAAGGAAGCCGCTGCTAAGTGTAGCCAGGAGGCCGCCTAC-3’

**RP1**

5’-TCCAGATCCTCCTCCAGCAGTTCCAGATCCGGAGTCAAACAGATTGATCCTGCTATAGATGTA-3’

**FP5**

5’- GGATCTGGAACTGCTGGAGGAGGATCTGGATCTGGAGCTATGGTTGATACTCT-3’

**RP5**

5’- *GTTAAAGGCCTCGAG*CTAAATGTGAGCATCCCCTTTTGT-3’

**pEAQ-HTf**

5'-TTCTTCTTCTTGCTGATTGG-3'

**pEAQ-HTr**

5'-CACAGAAAACCGCTCACC-3'

**Figure S1:** Primer sequences of primers used for preparation for and completion of InFusion® cloning of the BTV1Tip-SC fragment. Underlined regions indicate the linkers: EAAAK linker (FP1) and GGS linker (RP1 and FP5). Italicised regions indicate where the primer overlaps with the pEAQ-*HT* vector sequence. All other regions represent the overlap with the region being amplified.

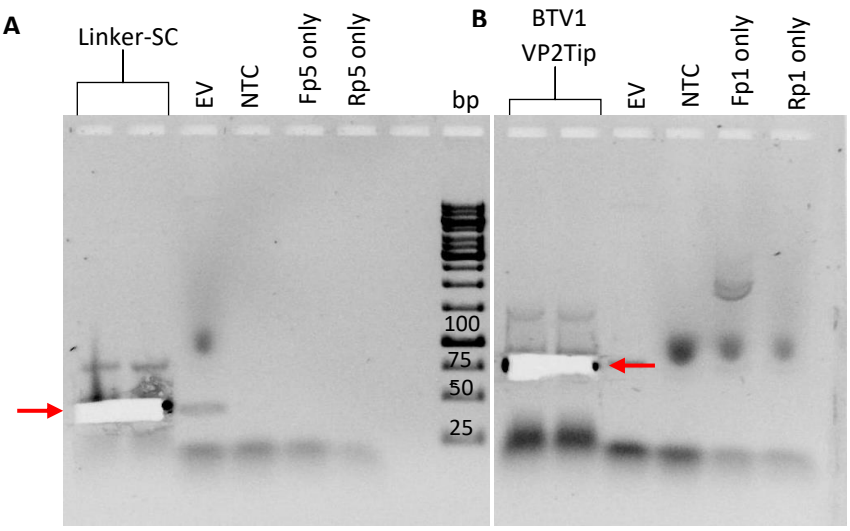



**Figure S2: PCR amplification of SC and BTV1Tip fragments to be used for In-Fusion® cloning**. Amplified products visualised on 0.8% agarose gel stained with ethidium bromide. **(A):** Amplification of SC fragment (Linker-SC) where the reverse primer adds 15bp overlap with pEAQ-*HT* vector at the 3' end. **(B):** Amplification of BTV1Tip fragment (BTV1 VP2Tip) where the forward primer adds a rigid EAAAK linker, SmaI restriction enzyme site and 15bp overlap with pEAQ-*HT* at the 5' end and a 30bp complementary sequence to the SC fragment's flexible GGS linker on the 3' end. Red arrows indicate the amplified products: 399bp Linker-SC fragment and 786bp BTV1 VP2Tip fragment which were extracted from the agarose gel for purification. EV: empty pEAQ-*HT* vector control. NTC: no template control. Fp only: Forward primer only control. Rp only: reverse primer only control. Bp: molecular weight marker (in base pairs).

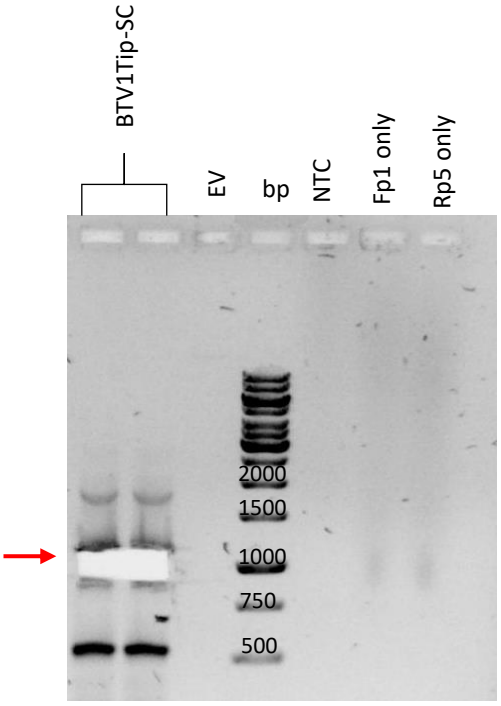



**Figure S3: Assembly PCR for amplification of BTV1Tip-SC construct to be used for In-Fusion® cloning.** Amplified product visualised on 0.8% agarose gel stained with ethidium bromide. PCR reaction including SC and BTV1Tip fragments generates C terminal SC-linked fragment: BTV1Tip-SC. Final product flanked by 15bp overlapping pEAQ-*HT* at 5' and 3' termini to facilitate In-Fusion® cloning. Red arrow indicates the amplified product: 1154bp BTV1Tip-SC. EV: empty vector control. NTC: no template control. Fp1 only: Forward primer only control. Rp5 only: reverse primer only control. Bp: molecular weight marker (in base pairs).

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