

**Development of a potential challenge model and plant-produced vaccine candidate for beak and feather disease virus**

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## Abstract

Psittacine beak and feather disease (PBFD), the most prevalent viral disease affecting psittacines, is caused by beak and feather disease virus (BFDV). An outbreak of the disease has been reported in wild endangered Cape parrots (*Poicephalus robustus*), which is endemic to South Africa. No treatment or vaccine is commercially available. In this study, an investigation into the outbreak was undertaken. BFDV diversity was assessed and viral load and clinical signs correlated. A plant-produced BFDV subunit vaccine was produced in parallel with a corresponding challenge model.

Cape parrots were assessed and 53 blood samples collected. Viral load was determined using quantitative real-time PCR (qPCR), and 22 BFDV full-length genome sequences acquired to infer phylogenetic relatedness. The capsid gene (*cp*) was optimised for transient *Agrobacterium*-mediated expression in whole-plant *Nicotiana benthamiana* (*N. benthamiana*). Virus-like particles (VLPs) were purified and analysed using transmission electron microscopy. Virions from a Palm cockatoo (*Probosciger aterrimus*) were purified and a BFDV dsDNA molecular clone was synthesised and replication assessed in 293TT mammalian cells and *N. benthamiana* using rolling circle replication and qPCR.

Two distinct BFDV phylogenetic clusters were reported for Cape parrots, and a direct correlation was seen between viral load in the blood and clinical signs in PBFD-afflicted birds. The CP was successfully expressed in *N. benthamiana*, and increased through optimisation of *Agrobacterium* infiltration density and the inclusion of the NSs silencing suppressor. The CP formed VLPs, which were shown to be morphologically similar to infectious virions. The dsDNA molecular clone was shown to replicate autonomously in mammalian 293TT cells, and in plants with the assistance of the Bean yellow dwarf virus replication associated protein (Rep).

BFDV genetic diversity in Cape parrots highlights the importance of ensuring new strains are not inadvertently introduced into the wild. This is the first systematic investigation of virus diversity in Cape parrots and assessment of BFDV viral load in a wild psittacine population. The CP was successfully produced *in planta* and presence of VLPs suggests the possibility of developing pseudovirions. This is the first reported replication of BFDV in tissue culture, and will greatly expand the scope of available research.

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'Opportunities multiply as they are seized' - Sun Tzu

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## Abbreviations

BCIP	5-bromo, 4-chloro, 3-indolylphosphate
BFDV	beak and feather disease virus
BeYDV	bean yellow dwarf virus
CP	capsid protein
CaMV	cauliflower mosaic virus
CAV	chicken anaemia virus
DuCV	duck circovirus
ER	endoplasmic reticulum
ELISA	enzyme-linked immunosorbent assay
GoCV	goose circovirus
GuCV	gull circovirus
HA	haemagglutination assay
HI	haemagglutination inhibition
HEK	human embryonic kidney
HPV	human papillomavirus
ICTV	International Committee on Taxonomy of Viruses
LIR	long intergenic region
LB	Luria-Bertani
MIQE	minimum information for publication of quantitative real-time PCR experiments
NBT	nitroblue tetrazolium
NLS	nuclear localisation signal
ORF	open reading frame
ori	origin of replication
PBS	phosphate buffered saline
PiCV	pigeon circovirus
PEG	polyethylene glycol

PCV porcine circovirus  
PK-15 porcine kidney 15  
PBFD psittacine beak and feather disease  
qPCR quantitative real-time PCR  
Rep replication-associated protein  
RCR rolling circle replication  
RCA rolling-circle amplification  
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis  
tco tobacco codon optimised  
TEV tobacco etch virus  
TMV tobacco mosaic virus  
TBSV tomato bushy stunt virus  
TSWV tomato spotted wilt virus  
TTSuV torque teno sus virus  
TTV-1a torque teno virus 1a  
TSP total soluble protein  
TEM transmission electron microscopy  
VLPs virus-like particle  
wt/vol weight per volume

# Chapter 1: Literature Review

## 1.1. Introduction

Psittacines, which are more commonly known as parrots, belong to the family *Psittacidae*, which consists of 85 genera and 356 species. At present almost 30% of psittacine species are threatened with extinction due to the loss of habitat, persecution as crop pests and illegal capture for the wild-caught bird trade; this is further exacerbated by disease (BirdLife International, 2013; Boyes, 2011). Population decline as a result of genetic bottlenecks decreases the immunocompetence of the population, thus increasing the risk of infection (Varsani *et al.*, 2010). A four year study of wild Sulphur-crested cockatoos (*Cacatua galerita*) in Australia showed a decline in population which was in part caused by disease (McOrist *et al.*, 1984). Infectious disease can result in extinction if it is introduced to a naive host species, if the pre-epidemic population is small, or the pathogen has abiotic or biotic reservoirs (Peters *et al.*, 2014).

Beak and feather disease virus (BFDV; family *Circoviridae*, genus *Circovirus*) is one of the most common disease organisms to infect psittacines. The virus infects both wild and captive birds, and has been detected in at least 10% of species (Ortiz-Catedral *et al.*, 2009; Rahaus and Wolff, 2003). The earliest description of the disease was reported in 1907, affecting *Psephotus melanogaster* in Australia (De Kloet and De Kloet, 2004).

Since then, BFDV has been rapidly disseminated around the world through the international trade in wild-caught birds (Doneley, 2003; Hsu *et al.*, 2006). Of particular concern to breeders is the spread of disease amongst captive birds, as aviaries often experience a high turnover and more than one species can share a small enclosure, which is an ideal environment for viral transmission (Bert *et al.*, 2005; Rahaus and Wolff, 2003; Shearer *et al.*, 2008a). It has also threatened conservation efforts of wild psittacines (Kundu *et al.*, 2012; Peters *et al.*, 2014). It has been postulated that the convergence of multiple species and virus strains in breeding facilities could result in the emergence of high virulence genotypes (Varsani *et al.*, 2010).

In South Africa the virus is a significant cause of mortality and has resulted in an annual loss of between 10-20% of psittacine breeding stocks (Albertyn *et al.*, 2004; Heath *et al.*, 2004). The virus also threatens indigenous African wild populations such as the endangered South African Cape parrot and the vulnerable Zambian Black-cheeked lovebirds (*Agapornis nigrigenis*) (Heath *et al.*, 2004).

## 1.2. Beak and feather disease virus

The prevalence of BFDV is widespread and can be detected anywhere psittacines are traded (Albertyn *et al.*, 2004; Kiatipattanasakul-Banlunara *et al.*, 2002; Varsani *et al.*, 2010) (Table 1.1).

Table 1.1: Reported prevalence of BFDV.

Africa	Cameroon	Bert <i>et al.</i> (2005)
	Ivory Coast	Bert <i>et al.</i> (2005)
	Mauritius	Kundu <i>et al.</i> (2012)
	South Africa	Heath <i>et al.</i> (2004)
	Zambia	Warburton and Perrin (2002)
	Zimbabwe	Kock (1990)
	Americas	Costa Rica
United States of America		Bert <i>et al.</i> (2005)
Asia-Pacific	Australia	Shearer <i>et al.</i> (2008a)
	Indonesia	Stewart <i>et al.</i> (2007)
	Japan	Sanada <i>et al.</i> (1999)
	New Caledonia	Julian <i>et al.</i> (2012)
	New Zealand	Ha <i>et al.</i> (2007)
	Papua New Guinea	Shearer <i>et al.</i> (2008a)
	Thailand	Katoh <i>et al.</i> (2010a)
	Taiwan	Hsu <i>et al.</i> (2006)
	Europe and the Middle East	Germany
Israel		Bendheim <i>et al.</i> (2006)
Italy		Bert <i>et al.</i> (2005)
Netherlands		Tomasek and Tukac (2007)
Poland		Julian <i>et al.</i> (2013)
Portugal		Henriques <i>et al.</i> (2010)
Qatar		Raue <i>et al.</i> (2004)
Spain		Raue <i>et al.</i> (2004)
	United Kingdom	Sa <i>et al.</i> (2014)

### 1.2.1. Circoviridae

The species *Beak and feather disease virus* belongs to the family *Circoviridae*, which consists of three genera: these are *Gyrovirus*, *Circovirus* and the proposed *Cyclovirus* (Li *et al.*, 2011; Rahaus *et al.*, 2008; Todd *et al.*, 2001). A novel circo-like genus tentatively called *Krikovirus* has recently been described (Garigliany *et al.*, 2014). Viruses within the family are the smallest known animal viruses and are characterised by having small isometric virions, consisting of a single capsid protein, containing circular ssDNA genomes which replicate via intermediate dsDNA forms (Cheung, 2006; Studdert, 1993). Genera are differentiated according to their genome organisation (Crowther *et al.*, 2003). Viruses in the genus *Gyrovirus* are represented by the type species *Chicken anaemia virus*.

They differ from generic circoviruses in having a negative sense circular ssDNA genome which produces a single polycistronic unspliced transcript and a virion that is different in size and morphology (Mahé *et al.*, 2000; Todd *et al.*, 2007). Viruses in genus *Circovirus* have non-enveloped icosahedral virions which protect an ambisense circular ssDNA genome (Scott *et al.*, 2006). Viral replication is initiated within a conserved nonanucleotide motif (5'-TAGTATTAC-3') situated on a stem-loop within the origin of replication (*ori*)(Mankertz *et al.*, 1998). Adjacent and within this stem-loop structure are direct repeat sequences which are potential binding sites for the replication-associated protein (Rep)(Todd *et al.*, 2001). The numbering of the genome is based on that agreed for geminiviruses, in which the first nucleotide position of the genome is the adenine residue at the eighth position of the nonanucleotide motif (5'-TAGTATT/AC-3')(Meehan *et al.*, 1997; Todd *et al.*, 1991).

Circoviruses have restricted host ranges and it has been suggested that this may be due to the lack of transmission vectors (Niagro *et al.*, 1998; Todd, 2004). The viruses are associated with major diseases and often target the lymphoreticular tissue resulting in immunosuppression (Hattermann *et al.*, 2002). It is within this genus that the smallest known autonomously replicating virus resides (Duchatel *et al.*, 2006). The type species is *Porcine circovirus* and was first identified as a contaminant in a porcine kidney 15 (PK-15) cell line (Karuppannan and Kwang, 2011; Mahé *et al.*, 2000). Two types have been identified: these are porcine circovirus 1 (PCV-1), which is non-pathogenic, and PCV-2, which is associated with postweaning multisystemic wasting syndrome in piglets (Karuppannan and Kwang, 2011; Studdert, 1993). A third type, PCV-1/2a, which is a recombinant between PCV-1 and PCV-2 has also been described (Gagnon *et al.*, 2010). The disease is of significant economic importance and can be linked to a greater than 50% mortality in piglets (Gillespie *et al.*, 2008).

The PCV virion is approximately 17 nm in diameter and contains a 1.76-kb genome (Gillespie *et al.*, 2008; Mankertz *et al.*, 2000a). In addition to Rep, replication is furthermore dependent on splicing of the *rep* to produce Rep' which is essential in mammalian cells; replication in bacteria however does not require Rep' and suggests that the virus possibly evolved from bacterial episomal replicons (Cheung, 2006). New sequencing technologies and enrichment techniques have increased the rate of discovery of these viruses, with Li *et al.* (2013) for example having recently described a dog circovirus. Numerous circoviruses have been discovered affecting non-psittacine avian species; these cluster into a monophyletic group (Hughes and Piontkivska, 2008)(Table 1.2). Recently symptoms similar to those described for BFDV have been reported in Antarctic penguins; however, the causative agent has yet to be identified (Barbosa *et al.*, 2015).

A number of circoviruses and circo-like viruses have been identified in humans, though there is as yet very little known about them as they were largely identified by metagenomic screening methods (Li *et al.*, 2010). A better characterised group are the anelloviruses (family *Anelloviridae*, 11 genera) such as the human torque teno virus 1a (TTV-1a) and swine torque teno sus virus (TTSuV) (Jiménez-Melsió *et al.*, 2013).

Table 1.2 **Circoviruses infecting non-psittacine avian species and reported potential hosts.**

<i>Budgerigar circovirus</i>	Varsani <i>et al.</i> (2011)
<i>Canary circovirus</i>	Phenix <i>et al.</i> (2001)
<i>Duck circovirus</i>	Hattermann <i>et al.</i> (2003)
<i>Finch circovirus</i>	Todd <i>et al.</i> (2007)
<i>Goose circovirus</i>	Soike <i>et al.</i> (1999)
<i>Gull circovirus</i>	Todd <i>et al.</i> (2007)
<i>Pigeon circovirus</i>	Mankertz <i>et al.</i> (2000b), Todd <i>et al.</i> (2001)
<i>Raven circovirus</i>	Todd <i>et al.</i> (2007)
<i>Starling circovirus</i>	Johne <i>et al.</i> (2006)
<i>Swan circovirus</i>	Studdert (1993)
Ostrich	Eisenberg <i>et al.</i> (2003)
Penguin	Barbosa <i>et al.</i> (2015)

Three plant virus species were originally included in genus *Circovirus*: these were *Banana bunchy top virus*, *Coconut foliar decay virus* and *Subterranean clover stunt virus* (Fenaux *et al.*, 2003). However, these viruses have previously been described as having multipartite genomes and were reclassified as members of the family *Nanoviridae* on the master species list of 2012 by the International Committee on Taxonomy of Viruses (ICTV). The geographic distribution of these plant viruses compared to that of circoviruses suggest a possible common ancestor (Niagro *et al.*, 1998). The origin of circoviruses has been speculated to be through recombination between a ssRNA calicivirus and a ssDNA nanovirus, as circoviruses have distant sequence similarities to nanoviruses and geminiviruses (Gibbs and Weiller, 1999).

### 1.2.2. Virus structure

BFDV was the first avian *Circovirus* to be characterised (Ritchie *et al.*, 1989b). The BFDV virion is a non-enveloped icosahedral particle of T = 1 symmetry that consists of 60 subunits arranged into 12 pentameric units (Crowther *et al.*, 2003; Ritchie *et al.*, 1990)(Figure 1.1). When viewed using an electron microscope the virion lacks any surface structure, and its appearance is smooth and spherical (Crowther *et al.*, 2003; Todd *et al.*, 1991). The virus particles can vary in size depending on the staining technique, and Todd *et al.* (1991) reported that virions stained with uranyl acetate were

20% larger than those stained with phosphotungstic acid. The literature reports the size of the virion to be on average between 14-20 nm in diameter, with the smallest reported size being 10 nm and the largest being 22 nm (Figure 1.2). The range of virion sizes appeared to be independent of whether uranyl acetate or phosphotungstic acid was used during the staining procedure. The size range of 14-20 nm is similar to the 20.5 nm determined using the three dimensional structure determined by cryoelectron microscopy (Crowther *et al.*, 2003). The BFDV virion is small in comparison to other psittacine viruses such as avian polyomavirus (40 nm), papovavirus (40-50 nm), avian reovirus (75-80 nm), adenovirus (70-90 nm) and herpesvirus (120-200 nm) which are all considerably larger (Davis *et al.*, 1981; Gaskin, 1989; Johne and Müller, 1998; Pass, 1987; van den Brand *et al.*, 2007). As with other circoviruses, the virion is extremely stable and is capable of withstanding heating for 30 minutes at 80 °C, which has important consequences for disinfection control (Davidson *et al.*, 2008; Raidal and Cross, 1994b).

Enclosed within the virion is a circular ssDNA genome of approximately 2 000 nucleotides (Bassami *et al.*, 1998)(Figure 1.3A). The genome is ambisense and consists of as many as seven putative open reading frames (ORFs); however, only three ORFs have been consistently identified among isolates (Bassami *et al.*, 2001; Hsu *et al.*, 2006; Niagro *et al.*, 1998).

The first ORF found on the virion strand encodes the Rep; however, unlike PCV the alternatively spliced Rep' has not been identified (Bassami *et al.*, 2001; Heath *et al.*, 2006). The Rep is involved in the initiation of rolling circle replication (RCR) of the genome, and the gene contains motifs I, II and III involved in RCR and common to all Rep (Bassami *et al.*, 1998; Bassami *et al.*, 2001; Todd *et al.*, 2007). The protein has a specific nucleotide binding motif, which is believed to have helicase and topoisomerase activity, that recognises and binds genomic DNA (Niagro *et al.*, 1998).

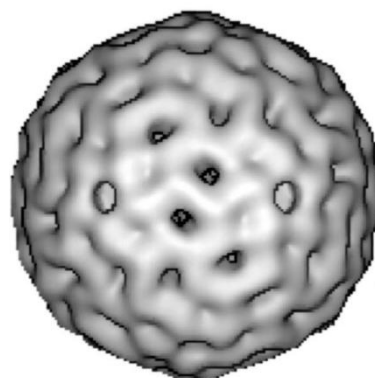


Figure 1.1: **Three dimensional structure for BFDV virions, determined by cryoelectron microscopy from Crowther *et al.* (2003).** Reprinted with permission from Dr. RA Crowther.

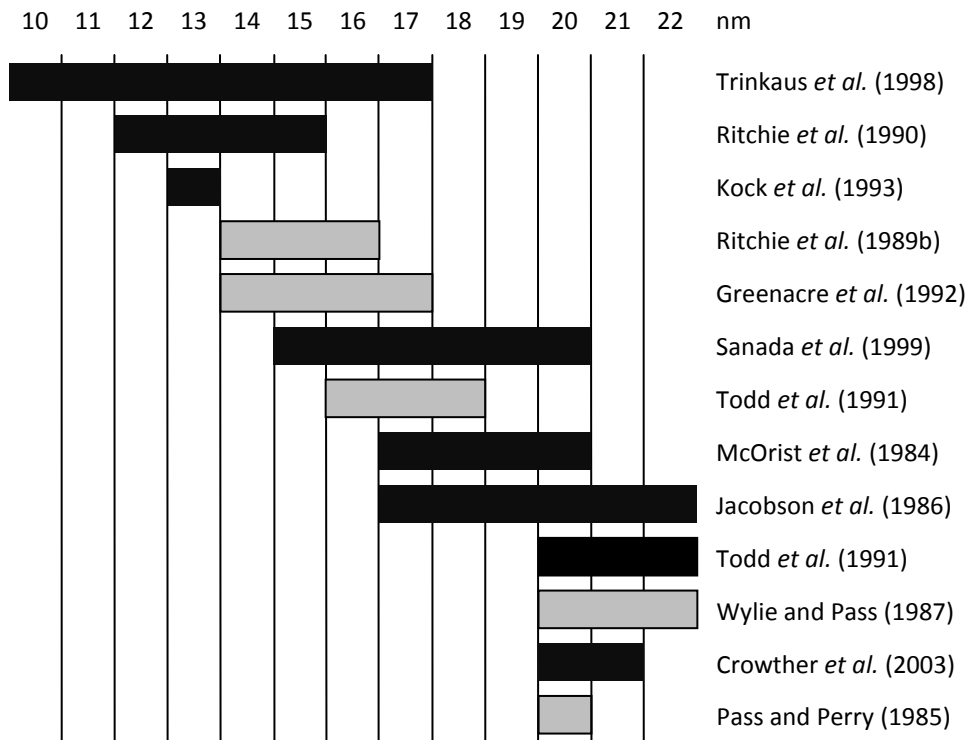


Figure 1.2: **Summary of the BFDV virion size as reported by various authors.** Sizes were determined from electron micrographs of particles stained with either uranyl acetate (black) or phosphotungstic acid (grey).

The second ORF is found on the complementary strand and encodes the capsid protein (CP) (Bassami *et al.*, 2001; Heath *et al.*, 2006). This ORF is unusual in that it has an alternative start codon of either TCT or CTG (Bassami *et al.*, 2001; Niagro *et al.*, 1998). Similar alternative start codons have been detected in the genome sequences of gull circovirus (GuCV), pigeon circovirus (PiCV) and canary circovirus (Phenix *et al.*, 2001; Todd *et al.*, 2008; Todd *et al.*, 2007). The N-terminal region of the CP contains nuclear localisation signals (NLSs) together with an overlapping DNA binding region which is enriched for basic amino acid residues (Heath *et al.*, 2006). The CP is the sole protein constituent of the virion and is also responsible for shuttling the genome and Rep into the nucleus of the host cell for RCR (Heath *et al.*, 2006; Nawagitgul *et al.*, 2000; Stewart *et al.*, 2007; Tribble *et al.*, 2011).

The function of the third ORF has yet to be determined for BFDV; however, in both PCV and chicken anaemia virus (CAV) it is associated with apoptosis (Bassami *et al.*, 2001; Heath *et al.*, 2004; Karuppanan and Kwang, 2011). Between the *rep* and *cp* is the intergenic region which contains a stem-loop structure important for the initiation of RCR (Bassami *et al.*, 2001). Downstream of the stem-loop is a repeated octanucleotide motif (5'-GGGCACCG-3') which has been identified as a putative Rep binding site (Johne *et al.*, 2006; Todd *et al.*, 2007). The stem-loop itself contains the conserved nonanucleotide motif (5'-TAGTATTAC-3') which is identical to that of PCV-1. The motif

which differs slightly in PCV-2 (5'-TAAGTATTAC-3') has been demonstrated to be critical for replication (Cheung, 2004) (Figure 1.3B). The Rep has been shown in the species *Banana bunchy top virus* to bind to the nonanucleotide motif situated on the stem-loop and facilitate DNA nicking and ligation (Mankertz *et al.*, 1998).

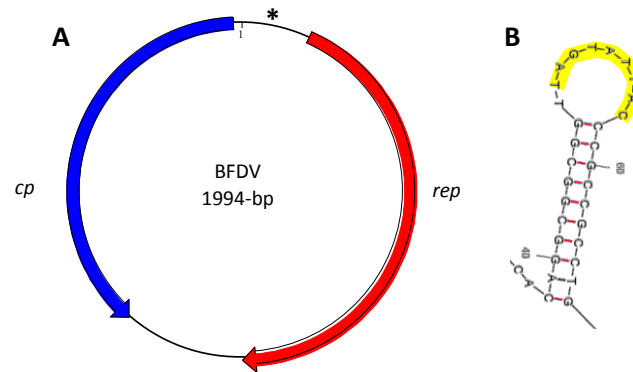


Figure 1.3: **Genome organisation of BFDV.** (A) The ambisense circular ssDNA genome consists of the *rep* (red), *cp* (blue), and a stem-loop structure (\*) located in the intergenic region. (B) The stem-loop structure contains the nonanucleotide motif (yellow; 5'-TAGTATTAC-3') from which RCR is initiated by Rep (yellow).

### 1.2.3. Viral replication

Upon entry into the host cell the CP shuttles both the viral genome and the Rep across the nuclear membrane; once in the nucleus the virus replicates through RCR (Bassami *et al.*, 1998). Replication is dependent on the Rep for initiation and cellular enzymes for DNA polymerisation since the genome does not encode for a DNA polymerase (Heath *et al.*, 2006). The current model for RCR in PCV-2 begins with the conversion of the circular ssDNA genome to a superhelical double stranded DNA replicative intermediate. Binding of the Rep to the direct repeat sequences causes a destabilisation and unwinding of the DNA at the ori. The Rep then nicks the DNA within the nonanucleotide motif as shown (5'-TAAGTATT/AC-3'). The 3'-OH is the site of plus strand DNA replication by cellular enzymes, while the Rep remains covalently attached to the 5' end via a tyrosine residue situated in motif III. Once the first round of DNA synthesis is completed a second cleavage at the nonanucleotide motif between the nascent and original strand occurs. This is followed by ligation at the nonanucleotide motif of the displaced genome by Rep to reconstitute the circular ssDNA genome, thereby releasing Rep (Cheung, 2006).

Virus assembly in circoviruses has been well studied for PCV. In PCV-2, transcripts and antigen can be detected 18 hours post infection, and their presence is correlated with DNA replication (Cheung and Bolin, 2002; Steiner *et al.*, 2008). The CP can be detected 24 hours post infection while cell-free

progeny can be detected as early as 30 hours post infection (Cheung and Bolin, 2002; Liu *et al.*, 2001). Detection of recombinant BFDV CP expressed in *Sf9* insect cells using a polyhedrin promoter has also been reported to be at 24 hours with expression peaking at 72 hours. *In situ* this early expression is thought to be necessary during infection to facilitate the transport of Rep into the nucleus (Stewart *et al.*, 2007). Individual virions are thought to assemble within the nucleus, with the formation of paracrystalline arrays, as inclusion bodies, confined to the cytoplasm (Sanada *et al.*, 1999; Stewart *et al.*, 2007). This confinement to the cytoplasm is inconclusive as intranuclear inclusion bodies in epithelial cells have also been widely reported in the literature; however, psittacines presenting this histopathology often made a spontaneous recovery, and it has been reported that these inclusions are not of viral origin (Gerlach, 1994; Pass and Perry, 1985; Ritchie *et al.*, 1990). These nuclear and cytoplasmic paracrystalline arrays can often persist within the target cell population (Latimer *et al.*, 1990; Todd *et al.*, 2002).

Similarly to virus assembly, virus uptake has also been well documented for PCV-2. Binding to the epithelial cell surface proceeds rapidly in a time dependent manner. The binding occurs in a random distribution indicating that all cells have receptors for virus uptake. The actual process of internalisation is slow, however, and infectious entry into the cell is through an actin polymerisation mediated cholesterol and dynamin independent small Gase regulated caveolin and clathrin independent pathway (Misinzo *et al.*, 2009).

#### **1.2.4. Cell tropism**

The primary sites for BFDV infection are rapidly dividing tissues (Todd, 2000). Apoptosis is seen to facilitate cell to cell spread within these tissues, and the dissemination of the virus leads to the development of viraemia (Latimer *et al.*, 1991; Raidal *et al.*, 1993c; Trinkaus *et al.*, 1998). The presence of virus in the blood and feather has been shown, in unvaccinated-control birds challenged with virus, to increase in a sigmoidal fashion (Bonne *et al.*, 2009). In PCV the threshold for viraemia has been broadly set at  $10^7$  genome copies/mL (Beach *et al.*, 2010). Three main systems are affected by BFDV: these are the epidermis, immune system and the alimentary system (Latimer *et al.*, 1993).

The virus targets replicating cells of the feather follicle and basal epithelium layers, with the resulting cell death being the cause of the characteristic feather abnormalities (Latimer *et al.*, 1991; Latimer *et al.*, 1993; Sanada *et al.*, 1999; Trinkaus *et al.*, 1998). The virus can persist in the epithelial cells and feathers after clinical symptoms have been resolved (Hess *et al.*, 2004).

Within the alimentary system the virus is predominantly found in the intestine and liver, and it has been found to be most concentrated in the basal epithelium layer (Rahaus *et al.*, 2008; Ramis *et al.*,

1994). Lesions found to contain inclusions have been identified in the palate, oesophagus, crop, intestine and liver (Latimer *et al.*, 1990).

In PCV-2 the immune system is seen as the primary target, with the virus infecting dividing macrophages and replicating in the lymph nodes (Fenaux *et al.*, 2002; Tribble *et al.*, 2011). Evidence for the presence of BFDV has been found in both the bursa of Fabricius and the thymus. Both organs have been seen to be depleted, and studies on the bursa of Fabricius have found tissue destruction and lesions containing inclusion bodies (Kock *et al.*, 1993; Latimer *et al.*, 1990; Todd, 2004). Furthermore, inclusion bodies have also been detected in macrophages infiltrating endothelial and epithelial tissue (Kiatipattanasakul-Banlunara *et al.*, 2002; Latimer *et al.*, 1993; Sanada *et al.*, 1999). Macrophages are thought to be the primary target of infection, and phagocytosis of viral particles is suggested to contribute to infection (Latimer *et al.*, 1991). The virus has also been reported to infect embryonated eggs, an observation which has also been identified with PiCV (Duchatel *et al.*, 2006; Rahaus *et al.*, 2008).

#### **1.2.5. Psittacine beak and feather disease**

The disease caused by infection with BFDV is psittacine beak and feather disease (PBFD), previously called French Molt (Pass and Perry, 1985). Wylie and Pass (1987) identified BFDV as the disease agent through experimental transmission studies. Both male and female psittacines are vulnerable to the disease, with there being no difference between the rate of infections, and symptoms are often more pronounced in younger birds which are, also, at a higher risk of infection (Hess *et al.*, 2004; Jergens *et al.*, 1988; Pass and Perry, 1985; Rahaus and Wolff, 2003; Raue *et al.*, 2004). There is no defined time period between infection and onset of disease; however, once symptoms emerge the disease progression is unabating, and the prognosis of symptomatic birds is poor (Doneley, 2003; Greenacre *et al.*, 1992).

Transmission of BFDV between birds can occur either through horizontal or vertical transmission. Horizontal transmission can occur via ingestion or inhalation of the virus, and ingestion is seen as the primary route of transmission (Todd, 2004). The virus can be ingested either directly through preening or feeding of hatchlings, or indirectly through environmental contamination with virus-laden faecal and feather material (Hsu *et al.*, 2006; Latimer *et al.*, 1991; Rahaus *et al.*, 2008). Horizontal transmission is exacerbated by psittacines congregating into flocks and through contamination of nesting sites, which enables transmission between ecologically disconnected species (Peters *et al.*, 2014; Raidal *et al.*, 1993a; Sa *et al.*, 2014).

Overall, transmission is dependent on the locus of persistence and the viral load during acute viraemia (Rahaus *et al.*, 2008). It could be postulated that the high viral loads seen during viraemia may potentially facilitate horizontal spread through blood-feeding transmission vectors, adding an additional route whereby the virus can spread. However, no transmission vectors thus far have been identified for circoviruses, and environmental contamination is still regarded as the main route of transmission. Within breeding facilities, transmission is intensified by close quarters, the concentration of different psittacine species and the movement of breeding stock between facilities (Dolz *et al.*, 2013; Ritchie *et al.*, 2003).

The disease can present in three forms: these are acute, chronic and subclinical. The acute form of the disease is mainly seen in young and neonatal birds; however, it has also been reported in adult African grey parrots (*Psittacus erithacus*) (Schoemaker *et al.*, 2000; Shearer *et al.*, 2009b). The progression of this form is rapid, and an antibody response is detectable within 3-4 weeks (Shearer *et al.*, 2009b). Symptoms of the disease include rapid weight loss, diarrhoea, haemorrhaging in the shaft of developed tail and primary feathers and depression. Beak and feather abnormalities characteristic of the chronic form are absent (Pass and Perry, 1985). The acute form has a high mortality rate (Ha *et al.*, 2007).

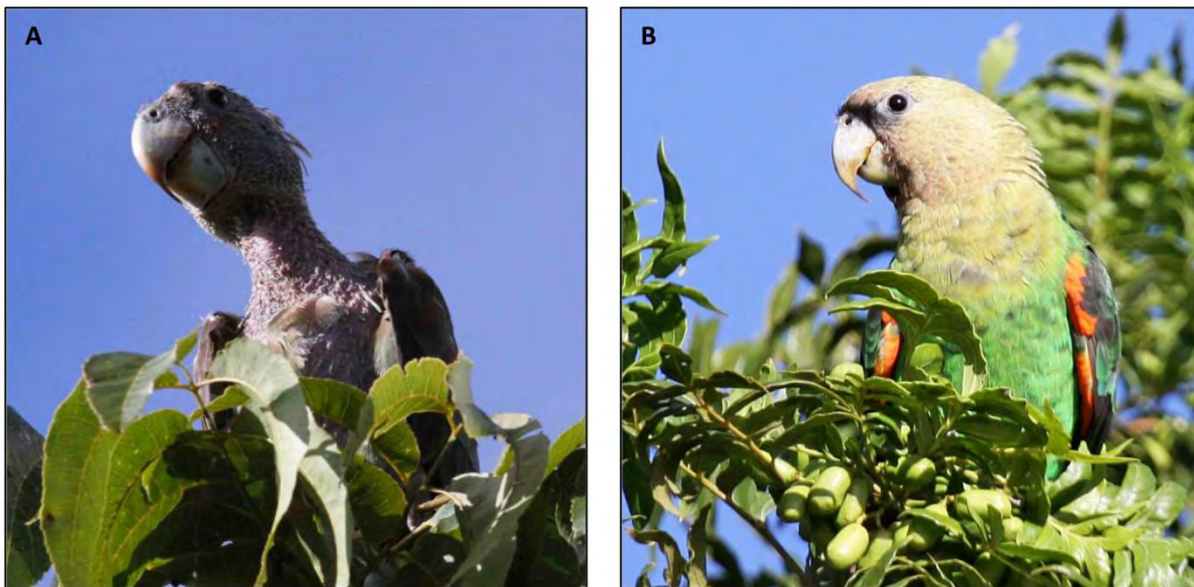


Figure 1.4: **Clinical signs of PBFD.** (A) A Cape parrot with abnormal feathering characteristic of chronic BFDV. (B) A healthy Cape parrot for comparison purposes. Reprinted with permission from Dr. RS Boyes.

By far the most reported form of the disease is the chronic form, which primarily affects adult birds (Schoemaker *et al.*, 2000). Symptoms of chronic disease are the characteristic beak and feather abnormalities associated with PBFD (Ritchie *et al.*, 1990) (Figure 1.4). Generally infection is followed

by a gradual replacement of feathers with abnormal ones after each successive moult (Gerlach, 1994; Latimer *et al.*, 1993; Pass and Perry, 1985). Feather abnormalities are accompanied by symmetric feather loss, this in turn causes the skin to discolour if exposed to sunlight (McOrist *et al.*, 1984; Ritchie *et al.*, 1990). Beak abnormalities include fractures and progressive elongation (Ha *et al.*, 2007). Larger psittacines may also experience claw abnormalities, though beak and claw abnormalities may be associated with specific species (Harkins *et al.*, 2014; Riddoch *et al.*, 1996). The progression to severe disease has been reported to be 6 months in Sulphur-crested cockatoos (Jacobson *et al.*, 1986). Psittacines showing symptoms generally survive for a period of 6-12 months; however, there have been cases of psittacines surviving for 10 years in a featherless state (Gerlach, 1994). There is a correlation between clinical signs of disease and viral load, with the detection of virus in the blood indicative of a recent infection or immunosuppression (Hess *et al.*, 2004; Khalesi *et al.*, 2005; Raidal *et al.*, 1993c). Birds that are infected with BFDV become immunocompromised, which is the primary cause of chronic infection and persistent excretion of the virus in diseased birds (Bonne *et al.*, 2009; Raidal *et al.*, 1993b).

Immunosuppression is an important aspect of BFDV infection, as death of psittacines with PBFD is largely due to secondary infections (Ritchie *et al.*, 1990). Tissue necrosis seen in psittacines infected with BFDV is as a result of secondary bacterial infections (Trinkaus *et al.*, 1998). The development of immunosuppression can be attributed to a depletion of lymphocytes and damage caused by lesions to the thymus and bursa of Fabricius (Latimer *et al.*, 1990; Stanford, 2004). A decreased lymphocyte count would compromise the humoral part of the immune system, and this has been observed in infected psittacines that show an overall decrease in the concentration of serum protein (Jergens *et al.*, 1988). Antibody titres have also been shown to be low in psittacines with active infections, while psittacines with detectable antibody titres test negative for BFDV during PCR and excrete less virus (Khalesi *et al.*, 2005).

The third form of the disease is subclinical infection: this presents the greatest risk for disease spread, as infected psittacines continue to shed virus at low concentrations (Bert *et al.*, 2005; Raidal and Cross, 1994a). Reports vary on the prevalence of subclinical infections in psittacines from approximately 15% to as high as 95% (Bendheim *et al.*, 2006; Ha *et al.*, 2007). Depending on the detection method and whether or not a longitudinal study was undertaken one could argue that in fact these infections are possibly chronic infections still in the asymptomatic stage of development.

## **1.3. BFDV diagnosis**

### **1.3.1. Diagnostic assays**

Previously, diagnoses of PBFV were made using clinical symptoms and histology (Latimer *et al.*, 1992). Clinical symptoms were based on feather loss and abnormalities; however, these could also be the result of infections with other viruses or physiological conditions (Ortiz-Catedral *et al.*, 2009). Histology focused on the identification of inclusion bodies which were either present in the cytoplasm or the nucleus of the feather follicle epithelium (Ritchie *et al.*, 1990). This technique, however, has a low sensitivity and specificity, and in the case of PiCV has been found to result in a significant under-diagnosis of infection (Riddoch *et al.*, 1996; Todd *et al.*, 2002).

### **1.3.2. Serological assays**

The haemagglutination assay (HA) was routinely used to detect BFDV, as BFDV virions agglutinate erythrocytes. This was considered the gold standard assay for feather eluates (Shearer *et al.*, 2009b). The haemagglutination of red blood cells is dependent on the intact virion and hence the assembled CP (Stewart *et al.*, 2007). This assay together with haemagglutination inhibition (HI) test can be used to quantify viral load and detect virus excretion, and the HI test can also to detect capsid-specific antibodies (Riddoch *et al.*, 1996; Shearer *et al.*, 2009a; Shearer *et al.*, 2009b). A disadvantage of these assays is their inherent variability, which is a result of differences in erythrocytes, viral preparations and serum, and that they often fail to detect subclinical and incubating infections (Raidal and Cross, 1994b; Shearer *et al.*, 2009a).

Enzyme-linked immunosorbent assays (ELISA) have recently been developed for the detection of BFDV. This assay has a greater sensitivity when compared to the HI test, and variation can be reduced if monoclonal antibodies are used. Another advantage of ELISA over the HI test is that it can provide a continuous reading of percentage inhibition found between HI test endpoints (Shearer *et al.*, 2009a).

### **1.3.3. PCR-based assays**

The first DNA-based technology to be used in the detection of circoviruses was *in situ* hybridisation, which was regarded as having a greater sensitivity than histology (Todd *et al.*, 2002). This was followed by PCR amplification of viral DNA, which is now preferred over DNA hybridisation and is used in most studies relating to BFDV (Bert *et al.*, 2005; Latimer *et al.*, 1992). One of the first PCR assays used for circoviruses was in fact a nested primer PCR capable of detecting single genome copies of CAV (Soine *et al.*, 1992).

Standard PCR is considered to be the gold standard when testing psittacine blood samples, and most assays use the conserved *rep* as a template for amplification (Khalesi *et al.*, 2005; Shearer *et al.*, 2009b). PCR is favoured when detecting virus because it has a high sensitivity and specificity. These criteria should be determined for each PCR assay to ensure valid data at low DNA concentrations and to reduce the number of false positives (Bert *et al.*, 2005; Rahaus and Wolff, 2003). The assay sensitivity can be increased for samples with low DNA concentrations by using nested-primer PCR (Kiatipattanasakul-Banlunara *et al.*, 2002). The detection limit for standard PCR used to detect PiCV has been shown to be as little 31 fg in one study and  $10^3$  genome equivalents in another (Freick *et al.*, 2008; Todd *et al.*, 2002). The sensitivity of the assay affects its value as a diagnostic in terms of differentiating between disease or infection (Todd *et al.*, 2002). As with any PCR, it is important to include controls to eliminate false negatives: for this purpose a PCR with host genomic DNA as template is used for the detection of total DNA (Bert *et al.*, 2005). Standard PCR has a higher sensitivity than HA; however, a drawback is that non-specific amplification of PCR products of sizes similar to the expected product cannot be differentiated by agarose gel electrophoresis (Katoh *et al.*, 2008; Khalesi *et al.*, 2005). Virus diversity may also affect the reliability of PCR-based assays, as a recent report described the failure of universal primers to detect virus isolated from Budgerigars (Ogawa *et al.*, 2010).

Quantitative real-time PCR (qPCR) is currently being investigated as a suitable assay for BFDV detection. It allows for the measurement of viral load as a copy number ratio of virus and host genes (Lee *et al.*, 2006). The problem of non-specific amplification experienced using standard PCR can be overcome through melting point determination using SYBR Green I, which forms part of the qPCR analysis (Katoh *et al.*, 2008). The advantage of using qPCR is a high sensitivity, which is better than or equal to that of standard PCR for clinical samples, with the limit of detection being between 50-100 copies (Katoh *et al.*, 2008; Ramis *et al.*, 1994; Shearer *et al.*, 2009b).

Another important aspect of PCR-based assays is the sample. Tissue samples can contain PCR inhibitors that can be present after DNA extraction and can affect the reaction resulting in false negatives (Freick *et al.*, 2008; Khalesi *et al.*, 2005; Tomasek *et al.*, 2008). The three routine samples taken from psittacines suspected of being infected with BFDV are blood, feather and faecal samples. Both blood and feather samples can lead to false positives with the detection of non-replicating DNA (Ha *et al.*, 2007; Shearer *et al.*, 2009b). There isn't a consensus as to the use of which sample - blood or feathers - is the more sensitive; however, no significant difference in detection has been found when using either feather or faecal samples (Bert *et al.*, 2005; Hsu *et al.*, 2006; Khalesi *et al.*, 2005). The determination of viral load in the blood using qPCR could be seen as a more reliable diagnostic

for detecting infection and delimiting disease, and further research is needed to create a robust diagnostic assay.

#### **1.4. Phylogenetic analysis of virus relatedness**

Single-stranded DNA viruses have a recombination and nucleotide substitution rate similar to that of RNA viruses (Ortiz-Catedral *et al.*, 2010). In BFDV, recombination is seen as an important driver of diversity (Massaro *et al.*, 2012). The rate of nucleotide substitution in ssDNA viruses is in part due to the high rate of spontaneous deamination that occurs in the absence of the protective double helix (Ritchie *et al.*, 2003). The subsequent rate of evolution, however, differs between the *cp*, *rep* and intergenic regions. The *cp* has been thought to possibly evolve faster than the *rep* and has been described to be subject to neutral or episodic positive selection (Kundu *et al.*, 2012; Varsani *et al.*, 2011). The *rep*, however, is thought to be under purifying selection and no evidence of adaptive selection has been seen (Ritchie *et al.*, 2003; Varsani *et al.*, 2011). Purifying selection is in part due to functional constraints on Rep (Hughes and Piontkivska, 2008). Differences in the rate of evolution on different regions of the genome and the presence of recombination make it essential to analyse the full-length genome when comparing different isolates (Heath *et al.*, 2004; Sarker *et al.*, 2014b; Varsani *et al.*, 2011).

Breeding facilities bring together multiple strains of virus, which increases the risk of recombination which probably leads to the development of new strains (Julian *et al.*, 2013). This is supported by evidence in wild psittacine populations, where isolates are usually genetically uniform (Harkins *et al.*, 2014). Recombination together with point mutations represents a major source of genetic diversity in BFDV, and individual psittacines have been shown to be infected with multiple genetic variants (Heath *et al.*, 2004; Rosario *et al.*, 2009; Sarker *et al.*, 2014b). Recombination hot spots in the genome include the intergenic region and the periphery of the *cp* and *rep* (Varsani *et al.*, 2011). It has been suggested that DNA secondary structure is an important mechanism of site-specific genetic recombination (Sarker *et al.*, 2014b). Therefore, potential similarities in DNA secondary structure could be used to explain the recombination hot spots previously identified by Varsani *et al.* (2011). Recombination complicates the inference of phylogenetic relatedness, especially when break points create sequences of equal lengths (Heath *et al.*, 2004).

The demarcation criteria for BFDV strains as set by the ICTV are isolates sharing greater than 75% genome identity and greater than 70% *cp* identity (Todd *et al.*, 2008). A more stringent demarcation threshold has been suggested by Varsani *et al.* (2011), which is set at 94% genome wide sequence identity. This was later revised to 95% by Julian *et al.* (2013). Isolates are then grouped into subtypes

if they share greater than 98% genome wide sequence identity. Isolates that share less than 89% whole-genome identity fall out of the species BFDV (Varsani *et al.*, 2011).

Based on these demarcations, Varsani *et al.* (2011) proposed 14 distinct strains of BFDV. A previous study identified 8 groups based on a divergence criteria of greater 8% (Hsu *et al.*, 2006). Differences in the number of strains identified may be as a result of the increased stringency and larger dataset used by Varsani *et al.* (2011). The basis of how these strains come to exist is poorly understood. Hughes and Piontkivska (2008) found strong support for separating *Circovirus* species; however, when analysis was performed on clustering patterns within species the support was found to be low. It has been postulated that genetic drift and past selection pressure from the host immune system are reasons for strain evolution (Ritchie *et al.*, 2003). Further reasons are discussed below.

#### **1.4.1. Geographic distribution**

Recent work on the origin of the virus has placed the most recent common ancestor in Australia, an observation which is consistent with historical records (Harkins *et al.*, 2014). A few reports have speculated that strains arose from distinct geographical locations. Heath *et al.* (2004) identified 8 lineages, with Southern African isolates representing 3 unique genotypes, and BFDV in Australia being the most genetically diverse with 4 clusters. They suggested that the introduction from Australia to Southern Africa was ancient since there was already significant genetic divergence between isolates from Southern Africa. In the context of South Africa there is evidence to indicate more than one introduction of BFDV (Kondiah *et al.*, 2006). It has been thought that the disease is absent in Central and South American psittacines since birds imported to Europe all have tested negative; however, they are still susceptible to infection (Bert *et al.*, 2005). The majority of publications, however, suggest that there is very little evidence to support strains arising from distinct geographical locations (Bassami *et al.*, 2001; Heath *et al.*, 2004; Hsu *et al.*, 2006; Ogawa *et al.*, 2005).

Varsani *et al.* (2011) instead propose that the international trade in psittacines has resulted in haphazard strain dissemination, and Heath *et al.* (2004) has suggested that host specificity may predate this dissemination. The importation of psittacines from Australia into New Zealand as pets and for breeding has been blamed for the spread of the disease (Ritchie *et al.*, 2003). The illegal trade in psittacines also exacerbates the spread of BFDV (Bendheim *et al.*, 2006). Phylogenetic analyses of the virus isolates can be used as a tool to counter the growing illegal trade in psittacines, by tracing the source of BFDV infections (Massaro *et al.*, 2012).

### 1.4.2. Species specificity

There is less consensus in the literature on whether strains coevolved alongside host species than has been shown for psittacid herpesvirus (Styles *et al.*, 2005). A high rate of evolution in ssDNA viruses may, however, possibly favour host specific evolution (Johne *et al.*, 2006). This is evident in the high variation seen in the *cp* gene, which may be as a result of virus host interactions or selective pressure from the immune system (Raue *et al.*, 2004). Differences in host factors may also play a role, with Khalesi *et al.* (2005) reporting a higher prevalence of BFDV in lovebirds than Budgerigars.

Some analyses of isolates based on the *cp* sequence have suggested that clades of virus are associated with psittacine subfamilies (Ogawa *et al.*, 2005). This is supported by a study that suggested that there were 3 clades which corresponded with the major psittacine families (Bert *et al.*, 2005). Various studies have potentially identified strains that only affect specific hosts. A strain adapted to lorikeets has been reported in New Zealand; however, this was later shown to also infect other psittacine species (Heath *et al.*, 2004; Khalesi *et al.*, 2005). Viruses isolated from Budgerigars comprised two genotypes which were distinct from other BFDV sequences, and later described as strains of *Budgerigar circovirus*, a novel *Circovirus* species (Ogawa *et al.*, 2010; Varsani *et al.*, 2011). A distinct serotype affecting Cockatiels (*Nymphicus hollandicus*) has been reported (Shearer *et al.*, 2008a; Shearer *et al.*, 2008b). These apparent species specificity could, however, be an artefact of breeding facilities (Varsani *et al.*, 2011). This has been reported in psittacine species housed in separate enclosures, where the virus is then transmitted along pedigree lines or a single breeding stock species (Ritchie *et al.*, 2003).

In contrast to there being evidence for strain specificity, literature exists to suggest there is no host species specificity. Raue *et al.* (2004) was the first to suggest based on *cp* sequence data that isolates do not cluster according to species. Lineages described by Heath *et al.* (2004) were also unrelated to host species. In support of these findings, isolates in Mauritius have been shown to be transmitted between two distinct psittacine host populations (Kundu *et al.*, 2012). Similarly, no species specificity could be detected between Rosella species (Massaro *et al.*, 2012). Cross-reactivity of blood and feather samples from different species when assayed using HI tests has suggested that no distinct serotypes exist amongst different isolates (Khalesi *et al.*, 2005). Virus purification and challenge experiments have shown that viruses share antigenic homology and that virus isolated from one species can be used to infect other species (Ritchie *et al.*, 1990; Ritchie *et al.*, 1992b; Wylie and Pass, 1987). The link between virus isolate and host species appears, therefore, to be largely an artefact of genetic drift in geographically separated psittacines - and whether this would result in the emergence of a species-specific virus remains to be seen.

### **1.4.3. Strain pathogenicity**

It has been suggested that BFDV strains could cluster on the basis of disease severity. Isolates from lorikeets diagnosed with acute PBF, for example, have been shown to cluster separately based on *cp* sequence data. Similar findings have been reported for isolates in African grey parrots, which cluster as separate groups birds affected by immunosuppression, and those with feather disorders (Raue *et al.*, 2004). Gene mutations have been thought to influence strain pathogenicity, although there have been few studies on strain severity to create a consensus (Shearer *et al.*, 2008a). Recent outbreaks of disease in wild birds have been attributed to functional mutations within the BFDV genes. Changes to the *rep* were thought to have been responsible for an outbreak of BFDV in endangered Mauritian Parakeets, while an outbreak of disease in Orange-bellied parrots (*Neophema chrysogaster*) in Australia were thought to be attributed to functional mutations in both the *rep* and *cp* (Kundu *et al.*, 2012; Sarker *et al.*, 2014b).

Other studies suggest that pathogenicity is related to differences in the host immune system and host factors, and are not attributable to the genotype of the isolate (Bassami *et al.*, 2001; Katoh *et al.*, 2010b; Rahaus and Wolff, 2003). Cockatiels have been observed to have an innate resistance to infection, while a study of Budgerigars found no correlation between clinical forms and genotypes (Ogawa *et al.*, 2010; Shearer *et al.*, 2008a). Similarly, younger psittacines are more likely to be infected and to have a higher severity of disease than older birds (Bert *et al.*, 2005; Wylie and Pass, 1987). Further work is obviously required to differentiate between strain pathogenicity and species-specific host factors.

## **1.5. BFDV isolation and purification**

In order to determine the efficacy of a potential vaccine, its ability to protect must ordinarily be tested in a challenge trial. Following vaccination, birds are exposed to virus, either in the form of virus-containing serum or as purified virus. A potential drawback with using serum is that there may be unknown disease agent(s) present which can affect the outcome; therefore, isolation and purification of live virus is preferable as a source of inoculum (Fenaux *et al.*, 2003).

### **1.5.1. Virus purification from infected tissue**

BFDV purified from tissues of infected birds has been used to infect healthy birds and to characterise the virus (Ritchie *et al.*, 1989b). CsCl density gradient centrifugation is useful as a purifying step, as the virus particles have a reported density of 1.35-1.378 g/cm<sup>3</sup> (Raidal and Cross, 1994b; Todd *et al.*, 1991). Final yields from tissue are between 10<sup>9</sup>-10<sup>10</sup> virions/mL in the fraction containing peak HA

activity (Raidal and Cross, 1994b). Accurate quantitation can also be achieved using qPCR (Shearer *et al.*, 2008a). Yields from diseased tissue are low and preparations are often contaminated with host proteins: this could potentially be addressed through the use of affinity purification using monoclonal antibodies specific for BFDV (Latimer *et al.*, 1993; Ritchie *et al.*, 1992a; Shearer *et al.*, 2009a). That said, virus particles purified from diseased tissue as well as crude homogenates have been used successfully to cause Pbfd in neonates (Raidal *et al.*, 1993a; Ritchie *et al.*, 1990). Routes of inoculation have included nasal, oral, intracloacal and intramuscular, all of which have resulted in Pbfd. The severity of disease caused by intramuscular inoculation was found to be dose dependent (Raidal *et al.*, 1993a). There are, however, quite serious ethical considerations around sourcing infected birds in order to purify live virus from diseased tissue; thus, a suitable alternative is required (Shearer *et al.*, 2009a).

### **1.5.2. Growth in tissue culture**

Purification of virus from infected bird tissue could be circumvented by growing the virus *in vitro*; however, attempts to propagate BFDV in tissue culture have been unsuccessful thus far (Shearer *et al.*, 2008a). Attempts to propagate other non-psittacine circoviruses (eg PiCV, goose circovirus [GoCV] and duck circovirus [DuCV]) have also been unsuccessful (Duchatel *et al.*, 2006; Hattermann *et al.*, 2003; Scott *et al.*, 2006). This is in contrast to the genus *Circovirus* type species, PCV, which was first discovered as a PK-15 cell line contaminant (Fenaux *et al.*, 2002). An obstacle to propagation has been the generation of suitable psittacine cell lines, as possibly the virus requires highly specific tissue with stringent growth requirements (Ritchie *et al.*, 1989a). Another consideration has been the use of embryonated chicken eggs, as this has been successfully applied to avian herpesvirus. However, psittacine eggs would almost certainly be required, as previous attempts to infect embryonated chicken eggs with a circovirus isolated from canaries have failed (Panigrahy and Grumbles, 1984; Woods and Latimer, 2000).

### **1.5.3. Infectious DNA clones**

Alternatives to purifying infectious virus have recently been investigated for circoviruses. One approach has been to develop infectious molecular DNA clones, by techniques similar to what has been successfully applied for native geminivirus genomes as well as for constructs used for gene amplification and recombinant protein expression in plants (Regnard *et al.*, 2010). Using full-length genomic DNA clones to elicit an infection overcomes the difficulties and lack of reproducibility associated with virus purification, and was first demonstrated with Hepatitis B virus in chimpanzees (Will *et al.*, 1982). The method also circumvents the need for infectious virus titration assays in cell culture, as DNA can be quantified using a spectrophotometer (Fenaux *et al.*, 2002). For an infectious

molecular DNA clone carried on a theta-replicating vector plasmid to be infectious, two origins of replication (ori) are required, as the circular genome is linearised in the vector backbone. For this purpose, a partial genome repeat is used that incorporates the full genome between two oris.

Infectious molecular DNA clones have been successfully developed for PCV, and have been shown to be infectious in tissue cultures, from which pure virus stocks have been produced (Fenaux *et al.*, 2002; Liu *et al.*, 2001). Both DNA clones and pure virus stocks derived from them were infectious in pigs, and the DNA clone has been suggested as a suitable replacement for infectious virus (Fenaux *et al.*, 2002). Uptake of plasmid DNA into muscle cells after direct intramuscular injection is reportedly between 1-2% (Fan *et al.*, 2008). Chimaeras between PCV-1 and PCV-2 genomes have been created and tested, producing immunity against PCV-2 which is of economic importance while being attenuated in terms of symptoms produced in pigs (Fenaux *et al.*, 2004; Gillespie *et al.*, 2008). The chimaera remained stable in *in vitro* serial passage in PK-15 cells; however, it was found to be unstable after one passage in pigs, with the reversion of an inserted marker mutation (Gillespie *et al.*, 2008). Infectious molecular DNA clones can similarly be developed for BFDV; however, testing would have to be performed on psittacines as no appropriate cell line or embryonated egg system has yet been found.

## **1.6. BFDV vaccine development**

A commercial vaccine for BFDV is currently unavailable, and treatment is principally palliative and supportive (Jergens *et al.*, 1988; Patterson *et al.*, 2013b; Rahaus *et al.*, 2008). Preventing disease spread has focused on identifying and removing infected psittacines for euthanasia (Bendheim *et al.*, 2006; Massaro *et al.*, 2012; Stanford, 2004). Environmental disinfection and improved hygiene when handling psittacines has been suggested; however, Raidal and Cross (1994a) have shown there is an infection incidence of between 2-5% post biocontrol (Duchatel *et al.*, 2006; Stanford, 2004).

Treatments against circoviruses are in development, and antiviral compounds against PCV-2 have been tested. An antiviral trial involving a pan-caspase inhibitor zVAD, known to block the p53 mediated apoptosis, was shown to reduce cell apoptosis during infection in mice, without negatively affecting the immune system and overall health of the mice (Karuppanan and Kwang, 2011). Research into therapeutic treatments against BFDV has focused on cytokines. Gamma interferon isolated from chicken cell culture was shown to be successful in clearing the virus and increasing the total lymphocyte count (Stanford, 2004). Similarly, treatment with  $\beta$ -(1,3/1,6)-D-glucan has been shown to augment interferon production and to have an immunomodulatory effect in Horned

parakeets (*Eunymphicus cornutus*), resulting in the virus being undetectable 7-9 months after commencement of treatment (Tomasek and Tukac, 2007).

These efforts notwithstanding, it has been suggested that eradication of the disease is unlikely due to the prevalence of infection and the stability of the virus (Todd, 2004). A vaccine against BFDV would therefore be desirable, especially due to the presence of subclinical infections which may complicate biocontrol efforts and since the disease progression varies. The likelihood that a single-strain vaccine would be successful is high, due to the absence of obvious serotypes which indicates conservation of major epitopes between isolates: there is therefore a high probability of cross-isolate protection (Ritchie *et al.*, 1992b; Shearer *et al.*, 2009a).

Preliminary reports of the development of a BFDV vaccine have shown that the host immune system does respond to BFDV vaccination, and chickens that have been inoculated with pure virus have been shown to develop high titres without infection (Raidal *et al.*, 1993c; Ritchie *et al.*, 1992b). Vaccination of clinically healthy psittacines with either inactivated virus or recombinant CP has been shown to confer immunity (Bonne *et al.*, 2009; Raidal and Cross, 1994a). This has been used successfully together with other measures to control the prevalence of the BFDV in the USA (Bert *et al.*, 2005). A number of commercial vaccines have been developed for PCV from inactivated and attenuated virus, to subunit, vector and DNA vaccines and development of these for BFDV is described below (Fachinger *et al.*, 2008).

### **1.6.1. Inactivated virus**

The first vaccines tested against BFDV were inactivated whole-virus vaccines. Inactivation was achieved using  $\beta$ -propiolactone which has a minimal effect on the structure of the CP. Paraformaldehyde has also been used in conjunction with  $\beta$ -propiolactone to inactivate virus (Raidal *et al.*, 1993a; Wylie and Pass, 1987). Two adjuvants have been tested: these are a primary oil emulsion using Freund's adjuvant, and a double oil emulsion. Both were effective; however, the Freund's adjuvant was shown to cause undesirable tissue reactions (Raidal *et al.*, 1993a).

Vaccination with inactivated virus was shown to result in seroconversion and a low antibody titre in chicks, which conveyed protection from disease (Raidal *et al.*, 1993a). Maternal immunity from vaccinated hens was passively transferred to neonates that were then challenged and were afforded temporary protection. The vaccine was shown not to prevent virus shedding, however, and is therefore unable to prevent viral replication and new infections (Raidal *et al.*, 1993a).

There are risks associated with inactivated vaccines. Vaccination with inactivated virus may aggravate subclinical infections, resulting in PBFD; partial inactivation may also cause inadvertent

infection (Raidal *et al.*, 1993a). As BFDV cannot be cultured *in vitro* there is no means of assessing complete inactivation (Bonne *et al.*, 2009). A recent study on an emergence of a chimaeric PCV1/2a strain speculated that the source of the outbreak could be traced back to the inclusion of full-length genomic DNA during preparation of a killed chimaeric vaccine. The contaminating genomic DNA, similar to the infectious DNA clones, could produce a viable infection after the vaccine has been delivered (Gagnon *et al.*, 2010). Together with the risks, there are also ethical considerations that must be taken into account when sourcing diseased tissue for virus isolation, as mentioned previously (Bonne *et al.*, 2009).

### **1.6.2. Subunit vaccine**

Recombinant protein expression technologies can potentially be used to circumvent the need for sourcing diseased tissue in order to isolate live virus. Virus-like particles (VLPs) often form spontaneously from capsid proteins of simple viruses expressed in heterologous systems: these lack viral nucleic acid, making them non-infectious (Santi *et al.*, 2006). A number of circovirus CPs, including BFDV CP, have been successfully produced in recombinant expression systems (Heath *et al.*, 2006; Lai *et al.*, 2014; Scott *et al.*, 2006). Vaccination primarily results in a humoral immune response, as has been shown in pigs vaccinated with baculovirus-expressed PCV-2 CP (Fachinger *et al.*, 2008). Furthermore, the expression the PCV-2 and BFDV CPs has been shown to result in spontaneous formation of VLPs (Nawagitgul *et al.*, 2000; Stewart *et al.*, 2007).

Vaccination against BFDV has been shown to reduce the shedding of virus during challenge, and result in only transient viraemia after infection. The subunit vaccine was unable, however, to prevent vertical transmission and replication in chicks born from vaccinated psittacines. This indicated that neonates are still susceptible to infection in the presence of maternal antibodies, as has also been reported for the inactivated vaccine (Bonne *et al.*, 2009; Shearer *et al.*, 2009b).

The use of the insect cell / baculovirus expression system to express the BFDV CP has been successful (Bonne *et al.*, 2009; Heath *et al.*, 2006). Vaccination with full-length CP produced in insect cells has been shown to result in seroconversion in chickens (Stewart *et al.*, 2007). Similarly chickens immunised with insect cell derived truncated CP seroconverted and this was purported to be dose dependent (Heath, 2006). The advantages of using insect cell culture for recombinant virus subunit protein production are a generally high level of recombinant protein expression, the expressed proteins usually maintain immunogenicity and antigenicity, they may assemble into VLPs, and there is no need to handle infectious pathogen during vaccine production (Bucarey *et al.*, 2009; Pérez-Martín *et al.*, 2010).

Fermentation technologies have also been used in the expression of circovirus capsid proteins. Bucarey *et al.* (2009) successfully expressed a codon-optimised PCV-2 CP in yeast, which was administered as an oral vaccine without purification. Bacterial fermentation has been used to express a truncated version of the BFDV CP, which was later purified using affinity and size exclusion chromatography with a reasonably high yield (Heath, 2006; Patterson *et al.*, 2013b). Disadvantages of bacterial expression systems have been low protein solubility, and problems with protein folding and post-translational modifications. Interestingly, protein aggregations were shown to be as a direct result of amino acid sequence and could thus be at least partially avoided during expression by making alterations to the amino acid sequence (Patterson *et al.*, 2013b). The low solubility of the CP could be attributed to the N-terminal region of the CP which is arginine rich and thought to be involved in binding BFDV genome DNA. The absence of viral DNA during recombinant expression may affect the overall stability of the protein resulting in the formation of insoluble aggregates. Extraction buffers designed to negate the charge of the arginine residues may make the proteins more soluble and would be convenient for ion-exchange chromatography.

Plant-based production has gained traction as a complementary system to traditional cell culture and microbial fermentation technologies in the production of biopharmaceuticals. Plants are favourable for the production of region-specific and niche market products (Stoger *et al.*, 2014). This system therefore aligns well with the requirements of a BFDV vaccine and may overcome difficulties associated with CP insolubility in microbial fermentation systems (Patterson *et al.*, 2013b). Initial plant-based production of recombinant proteins focused on the generation of stable transgenic lines; however, transient expression using bacterial, viral or hybrid vectors are now favoured for the rapid optimization of recombinant protein expression (Sainsbury and Lomonosoff, 2014; Stoger *et al.*, 2014).

The use of *Agrobacterium tumefaciens* (*A. tumefaciens*) for transient expression of heterologous proteins in plants is an established technology and involves the transfer of DNA from the *Agrobacterium* into the plant cell nucleus (Sainsbury and Lomonosoff, 2014). Transient expression has been used successfully to produce a number of vaccine antigens against avian viruses such as: infectious bursal disease virus (Chen *et al.*, 2012), avian influenza virus (Kanagarajan *et al.*, 2012; Mortimer *et al.*, 2013), and CAV which is very similar to BFDV in terms of genome and virus particle structure (Lacorte *et al.*, 2007). The BFDV CP fused to an elastin-like polypeptide has been successfully expressed in *Nicotiana benthamiana* (*N. benthamiana*) under transient conditions. Although the authors were able to increase yield of the BFDV CP by fusing it with an elastin-like polypeptide, overall, the yield was low (Duvenage *et al.*, 2013).

A major obstacle for the use of plant-based production has been the relatively low yields when compared with traditional cell culture and microbial fermentation technologies (Stoger *et al.*, 2014). This has led to the development of a number of strategies to improve the overall expression and stability of recombinant proteins (Thomas and Walmsley, 2014). One of these approaches is to optimise codon usage to that of the plant expression system in order to remove rare plant codons present in the gene sequence. Codon optimisation has been successfully applied to PiCV to increase expression in *Escherichia coli* (*E. coli*) (Lai *et al.*, 2014). Codon usage profiles exist for *both N. benthamiana* and *Nicotiana tabacum* (*N. tabacum*) and changing to these profiles has been associated with a significant increase in yield. The expression of human epidermal growth factor and *Plasmodium* antigen have both benefited from plant codon optimisation (Ma *et al.*, 2012; Thomas and Walmsley, 2014). It has further been postulated that changes in the G+C content as a result of codon optimisation are responsible for increasing gene expression, rather than removing rare codons from the sequence (Hitzeroth *et al.*, 2015).

Recombinant proteins expression can be targeted with the addition of signal peptides to different organelles within the plant cell. This localisation can also contribute to an increase in protein stability and yield. Organelles targeted include: the chloroplast, endoplasmic reticulum (ER), vacuole and secretion into apoplastic spaces between cells. Localisation has been used successfully to increase plant expression of human papillomavirus (HPV) L1 capsid protein when targeted to the chloroplast and human epidermal growth factor when targeted to the vacuole (Maclean *et al.*, 2007; Thomas and Walmsley, 2014).

Yield can also be increased with the addition of enhancer elements up- and down-stream of the recombinant gene. This has been successfully applied with the inclusion of *Cowpea mosaic virus* (CPMV) untranslated regions (Sainsbury *et al.*, 2009). However, transcription of recombinant genes with the use of strong promoters can trigger post-transcriptional gene silencing by the host which decreases the overall accumulation of recombinant protein. The inclusion and co-infiltration plant viral silence suppressor genes such as NSs from the tomato spotted wilt virus (TSWV) genome and p19 from tomato bushy stunt virus (TBSV) genome can effectively prevent gene silencing (Takeda *et al.*, 2002; Voinnet *et al.*, 2003). The above optimisations impact the accumulation of recombinant protein at the level of transcription and translation and can be combined to produce higher yields; however, the exact combination is dependent on the protein being expressed.

An additional strategy for increasing protein yield has been to amplify the transient gene copy number. Gene amplification has been demonstrated using viral replication initiated from plasmid DNA after transfer from *Agrobacterium* (Regnard *et al.*, 2010). Both RNA and DNA plant viruses have

been co-opted for this purpose (Gleba *et al.*, 2005). The efficiency of gene amplification using viral replication has been demonstrated using a vector incorporating sequences derived from the bean yellow dwarf virus (BeYDV; family *Geminiviridae*, genus *Mastrevirus*) genome which increased gene DNA copy number by three orders of magnitude. However, this level of amplification does not necessarily translate into a three orders of magnitude increase in protein yield (Regnard *et al.*, 2010). Reasons for lower than expected protein yield may be the result of redirection of cell resources from mRNA transcription and protein translation to DNA replication. The inclusion of gene amplification together with other optimisations that increase protein accumulation could, however, further still improve yield.

### **1.6.3. Live attenuated virus**

The development of infectious molecular DNA and cDNA clones has allowed attenuated viruses, including circoviruses, to be reasonably readily created and tested. With circoviruses there has been a particular focus on PCV, and the development of PCV1-2 chimaeras. These have been successfully tested, with a PCV1-2b live attenuated chimaeric vaccine against PCV2a and 2b which effectively lowered viral load, prevented viraemia, and decreased overall lymphoid lesions in pigs (Beach *et al.*, 2010). This holds significant promise for the development of a similar BFDV infectious molecular clone.

## **1.7. Project aims**

This project aims at understanding the current status of BFDV diversity in South Africa with particular focus on the endangered wild Cape parrot population. The diversity of the virus will be investigated, and a diagnostic assay will be assessed. In terms of vaccine production, an experimental subunit vaccine produced in plants will be explored and a suitable infectious molecular DNA clone challenge model developed.

## Chapter 2: Psittacine beak and feather disease outbreak in Cape parrots (*Poicephalus robustus*) in South Africa

### 2.1. Introduction

Research into PBF in Africa remains limited and is focused on captive psittacines in South Africa. The first significant study of PBF investigated the genetic diversity of BFDV in captive birds from southern Africa (Heath *et al.*, 2004). This coincided with a report in South Africa on PBF affecting Budgerigars and Ring-necked parakeets (*Psittacula krameri*), and was followed later by a study into the genetic diversity of *rep* sequences isolated in South Africa (Albertyn *et al.*, 2004; Kondiah *et al.*, 2006). During this time HA routinely used in research in Australia was introduced and the reagents adapted for the psittacines species in South Africa (Kondiah *et al.*, 2005). Additional BFDV isolates continued to be described, with Varsani *et al.* (2010) reporting unique genomes of BFDV isolates from Budgerigars sampled at a breeding farm in South Africa. The complete set of full-length BFDV genomes isolated from psittacines in South Africa was later included in analysis of all available full-length genomes on databases, and used to characterise the global distribution of BFDV (Varsani *et al.*, 2011).

Apart from South Africa, there have been two reported cases of PBF affecting psittacines in neighbouring countries. These were reported for captive flocks of Black-cheeked and Lillian's lovebirds (*Agapornis lilianae*) in Zimbabwe, and for a single wild Black-cheeked lovebird in Zambia (Kock, 1990; Warburton and Perrin, 2002). In addition, reports of captive psittacines imported from Africa and infected with BFDV have been reported in Italy (Bert *et al.*, 2005). The only study on wild psittacines has been an extensive longitudinal study of BFDV introduced into Echo parakeets (*Psittacula eques*) indigenous to Mauritius (Kundu *et al.*, 2012). However, no studies on wild psittacine populations on the African continent have been reported.

Cape parrots (*Poicephalus robustus*) are endemic to South Africa and are endangered by the loss of forest habitat, persecution as a crop pest, and illegal capture for the wild-caught bird trade (Boyes, 2011; Downs, 2005; Martin *et al.*, 2014). Heath *et al.* (2004) suggested that Cape parrots could be threatened by PBF after a unique viral strain was found in southern Africa. In 2008, a number of birds showing physical signs consistent with BFDV infection, including feather loss and discolouration, were observed in a wild Cape parrot population in the Amathole Region of the Eastern Cape Province. In 2010, similar signs of PBF were also noted in the Transkei regions of the Eastern Cape Province, southern KwaZulu-Natal Province, and the Magoebaskloof area of Limpopo Province (Boyes, 2011). Understanding the BFDV outbreak in the endangered wild Cape parrot

population is important as it can be directly applied to the development of an effective conservation strategy. Furthermore, analysis of BFDV genetic diversity in wild species of African psittacines can contribute towards the development of a region-specific vaccine.

Various techniques have been used to detect BFDV, and antibodies to it, in infected birds. Antibodies against the virus have been detected using HI assays and more recently by ELISA (Riddoch *et al.*, 1996; Shearer *et al.*, 2009a). Detection of the virus has been routinely performed using both HA and viral DNA amplification by PCR; however, these methods can fail to detect latent or incubating infections (Raidal and Cross, 1994b; Ypelaar *et al.*, 1999). The use of qPCR assays can be seen as a reliable and more sensitive alternative to HA and PCR, and to date there have been two qPCR assays developed for the quantification of BFDV (Katoh *et al.*, 2008; Shearer *et al.*, 2009b). The first was developed to detect multiple DNA viruses in psittacines, and was successfully applied to determine viral load in clinical samples (Katoh *et al.*, 2008). The technique was also applied in a BFDV vaccination and challenge trial to track viraemia as reflected by viral copy number over the course of the experiment (Shearer *et al.*, 2009b). Thus far, however, there have been no reports looking at the relationship between clinical signs and viral load in wild birds.

In this study, 26 BFDV genomes were isolated and sequenced from blood samples of 22 wild and four captive Cape parrots caught between 2010 and 2011 in order to characterise the genomes and determine the possible origins of the virus through phylogenetic analysis. In addition, the viral load in 53 BFDV-infected Cape parrots was determined using qPCR DNA amplification from blood samples to test whether there was a useful correlation between viral load and clinical signs. This study represents the first detailed report of BFDV infecting wild psittacine populations in South Africa and the first to report on BFDV viral load in a wild psittacine population.

## **2.2. Materials and methods**

### **2.2.1. General molecular techniques**

Agarose gel electrophoresis was performed in a 1x working solution of Tris-borate-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA at pH 8). DNA was resolved on TBE agarose gel containing 2.5 mg/mL ethidium bromide. Agarose gels were visualised under either short-wavelength UV light (254 nm) or long-wavelength UV light (360 nm) illumination depending on requirements.

Restriction enzyme digestions were performed according to manufacturer's instructions, and all restriction enzymes were purchased from Roche (Switzerland), unless otherwise stated.

Gel fragments were excised under long-wavelength UV light and gel purified using the QIAquick® Gel Extraction kit (Qiagen, Netherlands) as per manufacturer's instructions.

Chemically competent *E. coli* DH5- $\alpha$  cells (*E. cloni*™, Lucigen, WI, United States of America) were transformed as described by Sambrook *et al.* (1989) and plated on Luria-Bertani (LB) agar supplemented with ampicillin (100  $\mu$ g/mL), and incubated for 16 hours at 37 °C. *E. coli* DH5- $\alpha$  capable of growth on ampicillin were then inoculated into 10 mL LB broth supplemented with 100  $\mu$ g/mL ampicillin and incubated with agitation for 16 hours at 37 °C.

Plasmid isolations were performed on overnight cultures using the QIAprep® Spin Miniprep kit (Qiagen) as per manufacturer's instructions.

### **2.2.2. Sample collection<sup>1</sup>**

A total of 49 wild Cape parrots were caught using mist nets during the early morning. The captured parrots were immediately removed from the net, and each bird was handled by teams of two to minimise stress on the bird. Strict precautions were taken to minimise the risk of transmitting diseases between birds and the risk of cross-contamination of samples: each holding bag (cotton pillow cases) was only used once; new latex gloves were used for each parrot; samples were placed immediately into airtight bags and delivered as soon as possible to the lab for analysis. Sex and biometric measurements were recorded, and an overall physical condition score based on the rounded average of six scores (1-5) for clinical signs attributable to PBFD was assessed (Table 2.1). Blood samples were taken from the brachial vein and stored on FTA™ Classic Card (Whatman, United Kingdom) at 4 °C. Parrots were released at the location of capture within 10 minutes. This research was conducted under full approval from the University of Cape Town's Animal Ethics Committee (Ethics clearance number: 2010/V12/RB) and under permits issued by the Department of Economic Development & Environmental Affairs (Eastern Cape) (Permit number: O8071A).

Blood samples, from which BFDV full-length genomes were isolated, were collected from 26 Cape parrots from locations within South Africa. Three of these locations were in the Amathole region in the Eastern Cape Province and represented wild populations: 14 samples were collected from Alice, one from Stutterheim and seven from King William's Town. Due to the close proximity of these

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<sup>1</sup> This work was performed by Dr. RS Boyes, Dr. RO Martin and Mr. D Nkosi

populations to each other (150 km between the furthest two) and the regular nomadic movements of feeding parrots in the area, these samples can be considered to come from a single meta-population of a minimum of 287 parrots in 2011. Blood samples were collected from four captive Cape parrots situated in East London in 2011. Blood was also taken from four captive Cape parrots in Cape Town of unknown origin with no history of BFDV infection and used as a PCR control group. No assessment of physical condition was performed on the captive birds.

Table 2.1: **Summary of the condition scores.** The scores were used to assess overall physical condition of Cape parrots based on possible symptoms of BFDV infection

<b>Clinical symptom</b>	<b>Overall physical condition scores: 1 (Very poor) to 5 (Very good)</b>
Degraded beak	Lesions, cracks and black blotches are: (5) Absent; (4) >15 mm apart or <10 % of beak area; (3) 10-15 mm apart or 10-25 %; (2) 5-10 mm apart or 25-50%; (1) <5 mm apart or >50 %
Darkened cere	Colour of the cere is: (5) White; (4) Light grey to white; (3) Light grey and dry; (2) Dark grey; (1) Dark grey to black with lesions
Degraded feathers	Area degraded/dead feathers estimated on R wing: (5) Absent; (4) < 25 %; (3) 25-50 %; (2) 50-75 %; (1) 100 %
Black blotches	Area black blotches estimated on breast: (5) Absent; (4) Single blotch; (3) 25-50 %; (2) 50-75 %; (1) >75 %
Degraded down feathers	Area degraded or missing down feather estimated on breast: (5) Absent; (4) <25 %; (3) 25-50 %; (2) 50-75 %; (1) 100 %
Excessive feather dust	Feather dust on breast is (5) Absent; (4) Visible on a few feathers; (3) Accumulates on latex glove during handling; (2) Dispersed into air when parrot moves; (1) Visible on all feathers

### **DNA extraction and rolling circle amplification**

A circle (4 mm diameter) of FTA™ Classic Card (Whatman) containing dried blood was detached and resuspended in a final volume of 220 µL phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM KCl, 2 mM KH<sub>2</sub>PO<sub>4</sub> at pH 7.4). DNA was extracted from blood samples using a DNeasy® Blood & Tissue Kit (Qiagen) and prepared as per manufacturer's instructions for the purification of total DNA from nucleated animal blood cells (spin-column protocol). The DNA sample was eluted in a final volume of 200 µL elution buffer. To isolate the BFDV circular ssDNA genome, rolling circle amplification (RCA) was performed on the DNA samples using an Illustra™ TempliPhi™ Amplification Kit (GE Healthcare, United Kingdom) as described by Shepherd *et al.* (2008).

### 2.2.3. DNA sequencing

To clone the full-length BFDV genome, the DNA synthesised during the RCA step was cleaved using *Bam*HI (Fermentas, MA, United States of America) and resolved on a 0.8 % (wt/vol) TBE agarose gel and gel purified. The 2-kb BFDV genome was then cloned into the pGEM<sup>®</sup>-3Zf(+)vector (Promega, WI, United States of America) as per manufacturer's instructions. pGEM<sup>®</sup>-3Zf(+) constructs were transformed into competent *E. coli* DH5- $\alpha$ , and plasmid DNA was isolated from overnight cultures. The DNA sequence was determined using primer walking (Macrogen Inc., South Korea). The sequences were assembled using DNAMAN (version 5.2.9; Lynnon BioSoft) and Mega6 MEGA (Build#: 6140122) (Tamura *et al.*, 2011). The GenBank accession numbers for the sequences are KM188440–KM188465.

### 2.2.4. DNA sequence and phylogenetic analysis

*In silico* analysis of the *rep* was performed using the Pfam database classification to identify motifs (Finn *et al.*, 2014), and *in silico* amino acid sequence analysis of the CP was performed using prosite.expasy.org and the SeqNLS algorithm (Lin and Hu, 2013; Sigrist *et al.*, 2002).

The twenty-six BFDV genome sequences were aligned to 208 full-length genomes available in the GenBank database as at February 2014 using the instance of ClustalW available on Mega6, keeping to the default parameters (Varsani *et al.*, 2011). Alignments were also performed separately for the *rep* and *cp* using the above sequences. Maximum likelihood phylogenies were inferred using Mega6 with 1 000 nonparametric bootstrap replicates for the full-length genome (general time-reversible model using a discrete gamma distribution with five rate categories and by assuming that a certain fraction of sites are evolutionarily invariable), the *rep* (Tamura-Nei model using a discrete gamma distribution with five rate categories and by assuming that a certain fraction of sites are evolutionarily invariable) and the *cp* (Tamura-Nei model using a discrete gamma distribution with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable). Nodes with a bootstrap value of less than 0.60 were collapsed using Mesquite (version 2.75 (build 564)).

### 2.2.5. Quantitative real-time PCR

qPCR analysis was performed following the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines as described by Bustin *et al.* (2009). For further information regarding MIQE, details can found in Minimum information for publication of quantitative real-time PCR experiments.

Each qPCR reaction was performed in triplicate using a LuminoCT® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich, MO, United States of America) as per manufacturer's instructions and primers (5'-CAGTTAAGGGCGCTTTTGTGGAG-3' and 5'-TTCGGGTACAGTCCTCCTTG-3') specific to a 97-bp region of the BFDV *rep* (GenBank accession number GQ165756) together with primers specific to a 97-bp region of the *P. robustus* reference gene, TGF beta 2 (GenBank accession number EU660286) (5'-TCCCATCTGGCACTGTCTCTG-3' and 5'-ACAGAGCTTTCACCCTCATTTATGG-3'). The Cape parrot TGF beta 2 has been used previously to describe phylogeny in psittacines (Wright *et al.*, 2008). This gene is present in both humans and mice as a single-copy gene, and it is therefore our assumption that this is also true for psittacines (Barton *et al.*, 1987). A BLAT search of the recently sequenced Budgerigar genome using Cape parrot TGF beta 2 produced only one alignment, which supports our assumption (Ganapathy *et al.*, 2014). Cloned plasmid DNA containing the amplicons in serial dilution served as the standard curve for the determination of gene copy number for both *rep* and *TGF beta 2*. Thermocycling was performed using a Rotor-Gene RG-6000 (Qiagen), and the parameters consisted of a 10 minute hold at 95 °C followed by cycling (40 repeats) between a 15 s hold for denaturation at 95 °C, a 15 s hold at 55 °C for annealing, and a 15 s hold 60 °C for elongation. Template specificity was confirmed from the reaction melt curve analysis. The  $C_q$  values from the no template controls fell below the lower limit. The qPCR efficiencies and  $r^2$  values for each reaction were 0.990 (SD, 0.0418) and 0.997 (SD, 0.00250) for the BFDV *rep* and 1.01 (SD, 0.0577) and 0.996 (SD, 0.00307) for the TGF beta 2 reference gene, respectively.

## 2.3. Results

### 2.3.1. Genome isolation

Full-length BFDV genomes were isolated from 22 wild-caught Cape parrots sampled from three sites in the Amathole region of the Eastern Cape Province (South Africa) in 2010 ( $n = 6$ ) and 2011 ( $n = 16$ ) (Table 2.2). Blood samples were collected from wild Cape parrots at sites in King William's Town ( $n = 7$ ), Stutterheim ( $n = 1$ ) and Alice ( $n = 14$ ) (Figure 2.1). Parrots captured at each site are thought to belong to a single meta-population of a minimum of 287 parrots in 2011. Many of the parrots captured for sampling appeared to be malnourished and displayed clinical signs of PBF.

BFDV genomes were isolated from an additional four captive Cape parrots in East London with a known history of PBF (Table 2.2). These Cape parrots had been removed from the Amathole population in 1995 and subsequently kept together with two breeding pairs added from a captive source in KwaZulu-Natal.

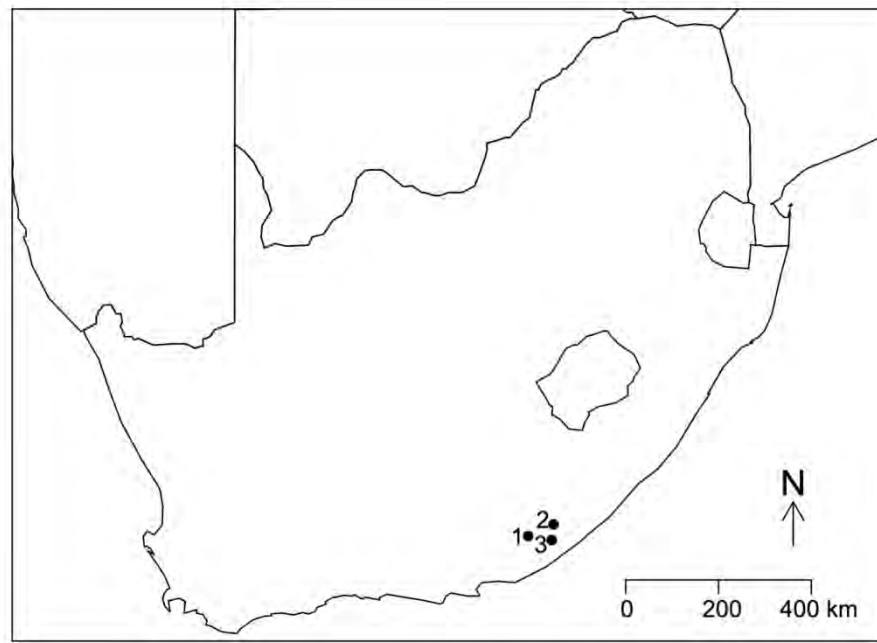


Figure 2.1: **The sampling sites of wild Cape parrots in the Eastern Cape, South Africa.** 1, Alice; 2, Stutterheim; 3, King William's Town

Table 2.2: **Data relating to each sequenced BFDV isolate.** Listed are the GenBank accession number, isolate name and genome length; together with when the host Cape parrot was sampled, and whether the parrot was wild or captive.

Accession #	Isolate name	Genome length (bp)	Sampling year	Wild/Captive
KM188440	BFDV_ZA_FH01_2010	1990	2010	Wild
KM188441	BFDV_ZA_FH05_2010	1993	2010	Wild
KM188442	BFDV_ZA_FH07_2010	1992	2010	Wild
KM188443	BFDV_ZA_FH14_2010	1992	2010	Wild
KM188444	BFDV_ZA_FH15_2010	1992	2010	Wild
KM188445	BFDV_ZA_FH16_2010	1991	2010	Wild
KM188446	BFDV_ZA_FH21_2011	1992	2011	Wild
KM188447	BFDV_ZA_FH22_2011	1992	2011	Wild
KM188448	BFDV_ZA_FH23_2011	1992	2011	Wild
KM188449	BFDV_ZA_FH24_2011	1989	2011	Wild
KM188450	BFDV_ZA_FH27_2011	1992	2011	Wild
KM188451	BFDV_ZA_FH37_2011	1991	2011	Wild
KM188452	BFDV_ZA_FH38_2011	1989	2011	Wild
KM188453	BFDV_ZA_FH42_2011	1991	2011	Wild
KM188458	BFDV_ZA_KWT01_2011	1990	2011	Wild
KM188459	BFDV_ZA_KWT02_2011	1991	2011	Wild
KM188460	BFDV_ZA_KWT03_2011	1990	2011	Wild
KM188461	BFDV_ZA_KWT04_2011	1991	2011	Wild
KM188462	BFDV_ZA_KWT06_2011	1991	2011	Wild
KM188463	BFDV_ZA_KWT07_2011	1988	2011	Wild
KM188464	BFDV_ZA_KWT09_2011	1991	2011	Wild
KM188465	BFDV_ZA_STUT01_2011	1992	2011	Wild
KM188454	BFDV_ZA_GT02_2011	1995	2011	Captive
KM188455	BFDV_ZA_GT07_2011	1994	2011	Captive
KM188456	BFDV_ZA_GT10_2011	1994	2011	Captive
KM188457	BFDV_ZA_GT11_2011	1995	2011	Captive

### 2.3.2. Genome analysis

All of the sequences derived from the 26 genomes successfully isolated and sequenced from Cape parrot blood samples were confirmed to be BFDV, with genome lengths varying between 1988-bp and 1995-bp (Table 2.2). All sequences were identified as having a characteristic circovirus nonanucleotide sequence (5'-TAGTATT/AC-3') situated within a stem-loop structure followed, by a direct octanucleotide repeat as described by Bassami *et al.* (1998).

A putative start codon (ATG) was identified for ORF1, encoding *rep*, together with an upstream TATA box and downstream AATAAA polyadenylation signal. A putative start codon (TCT) was identified, as described by Niagro *et al.* (1998), for ORF2 encoding *cp*, together with a downstream AATAAA polyadenylation signal, while no TATA box could be identified.

Within the amino acid sequence of the Rep, three motifs involved in RCR were identified in the N-terminus: motif I (FTLNN, residues 14-18), motif II (GxxHLQGY, residues 48-55) which matches the motif found in the geminivirus, banana bunchy top virus, and motif III (YxxK, residues 89-92). A putative DNA-binding motif (GKS, residues 170-172) was also identified (Bassami *et al.*, 1998). These motifs were also confirmed through analysis of the protein sequence for matches in the Pfam database, which identified a region belonging to the viral *rep* family and a DNA helicase domain (Finn *et al.*, 2014).

Within the amino acid sequence of the CP, a putative bipartite NLS (RRYRRRRRYFxxRRRRF, residues 29-43) was identified in the N-terminal region (Figure 2.2). This region was also identified as having an arginine-rich profile and was found to lack acidic groups. An additional NLS (residues 16-56) was identified using the SeqNLS algorithm. Between amino acid residues 16 and 43, the following putative NLSs as described by Liu *et al.* (2001) for PCV were identified in BFDV: one pat7 (PxxRRR), four pat4 (RRRR/H) and three bipartite NLSs (Figure 2.2).

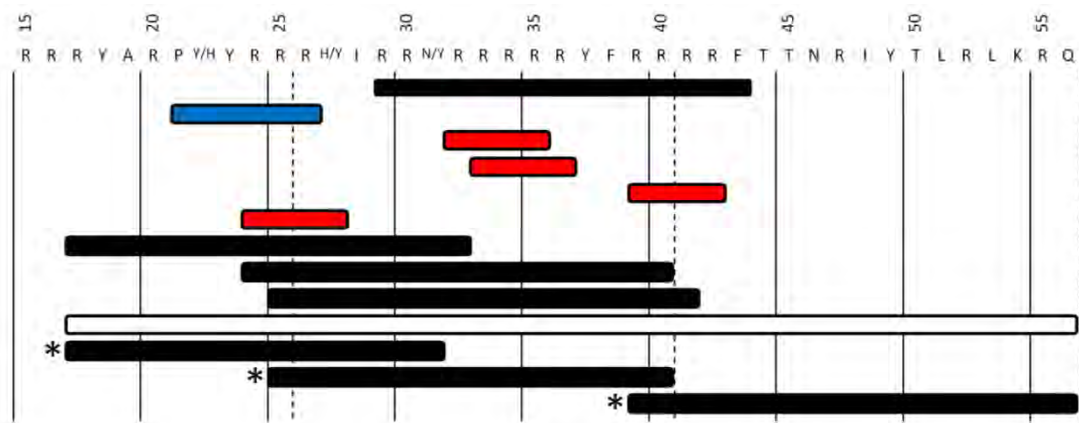


Figure 2.2: **Putative NLS identified within the N-terminal region of BFDV CP through *in silico* amino acid analysis.** Four different NLSs were identified: bipartite NLSs (black), pat7 NLSs (blue), pat4 NLSs (red) and an undefined NLS (white). NLSs previously reported by Heath *et al.* (2006) are indicated by an asterisk (\*). Dashed lines indicate iterative N-terminal gene deletions at residues 25, 40 and 56 previously performed by Heath *et al.* (2006). Residues 41-56 were required for nuclear localisation.

### 2.3.3. Phylogenetic analysis

The 26 BFDV isolates were aligned with and compared to 208 full-length BFDV genomes available in the GenBank database (Figure 2.3). The 22 isolates from wild Cape parrots formed a separate cluster that did not include the four isolates from captive Cape parrots, indicating a different origin (Figure 2.3, circles and branch lines in red). Within each cluster, the nucleotide pair-wise similarity between isolates was greater than 99 %, and for the isolates from wild Cape parrots, this was irrespective of location and year of sampling. The wild Cape parrot isolates were most closely related to sequences from captive psittacines sampled in Poland and Portugal, collected between 2003 and 2009, and in South Africa, collected in 2003 from the KwaZulu-Natal Province, which borders the Eastern Cape Province. Two of these sequences were isolated from captive Cape parrots from South Africa and Poland in 2003 (Figure 2.3, circles in blue). The four BFDV isolates from captive Cape parrots (“GT”) were most closely related to sequences derived from captive parrots sampled in Japan and Poland between 2006 and 2008, and South Africa from KwaZulu-Natal Province in 2003, and all except one were isolated from Budgerigars (*Melopsittacus undulatus*)(Figure 2.3, circles and branch lines in blue). Additional BFDV GenBank sequences of viruses isolated from Cape parrots not from this study clustered separately in the maximum likelihood phylogeny and were all sampled in the KwaZulu-Natal Province, South Africa, in 2003 and 2008 (Figure 2.3, blue circles).

Inference of phylogenetic relatedness based on the *rep* separated the 22 wild Cape parrot isolates from the four captive Cape parrot isolates. The isolates from wild Cape parrots formed a distinct cluster from the rest of the sequences and shared a greater than 99 % nucleotide pair-wise similarity, irrespective of location and year of sampling (Figure 2.4, circles and branch lines in red). The *rep* from isolates from captive Cape parrots shared a greater than 99 % nucleotide pair-wise similarity and were most closely related to sequences from captive psittacines from Poland sampled in 2007 (Figure 2.4, circles and branch lines in blue). A similar pattern emerged when phylogenetic relatedness was inferred based on the *cp* (Figure 2.5).

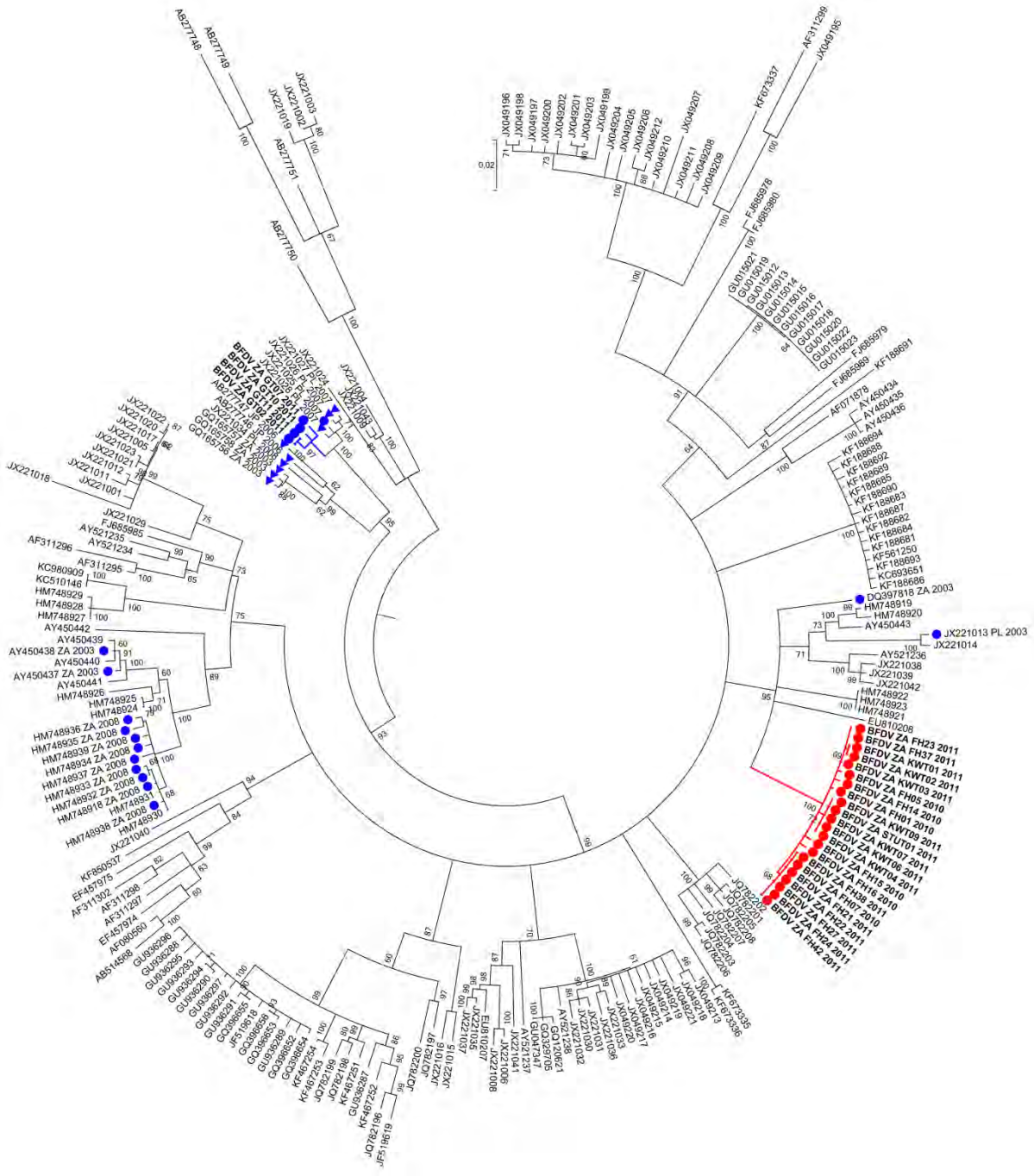


Figure 2.3: Maximum likelihood phylogenetic relationships of the full-length genomes of Cape parrot BFDV isolates with all publicly available BFDV genomes. Wild-caught Cape parrot (●), captive Cape parrot (●) and Budgerigars (▲). For emphasis, sequences from this study are highlighted in bold, and the branch lines are in colour. Sequences isolated from Cape parrot and Budgerigars include the two letter country code and year of sampling. The tree was constructed in Mega6 MEGA (Build#: 6140122), and the numbers associated with the tree branches are indicative of the percentage of 1 000 full maximum likelihood bootstrap replicates that support the existence of the branches.

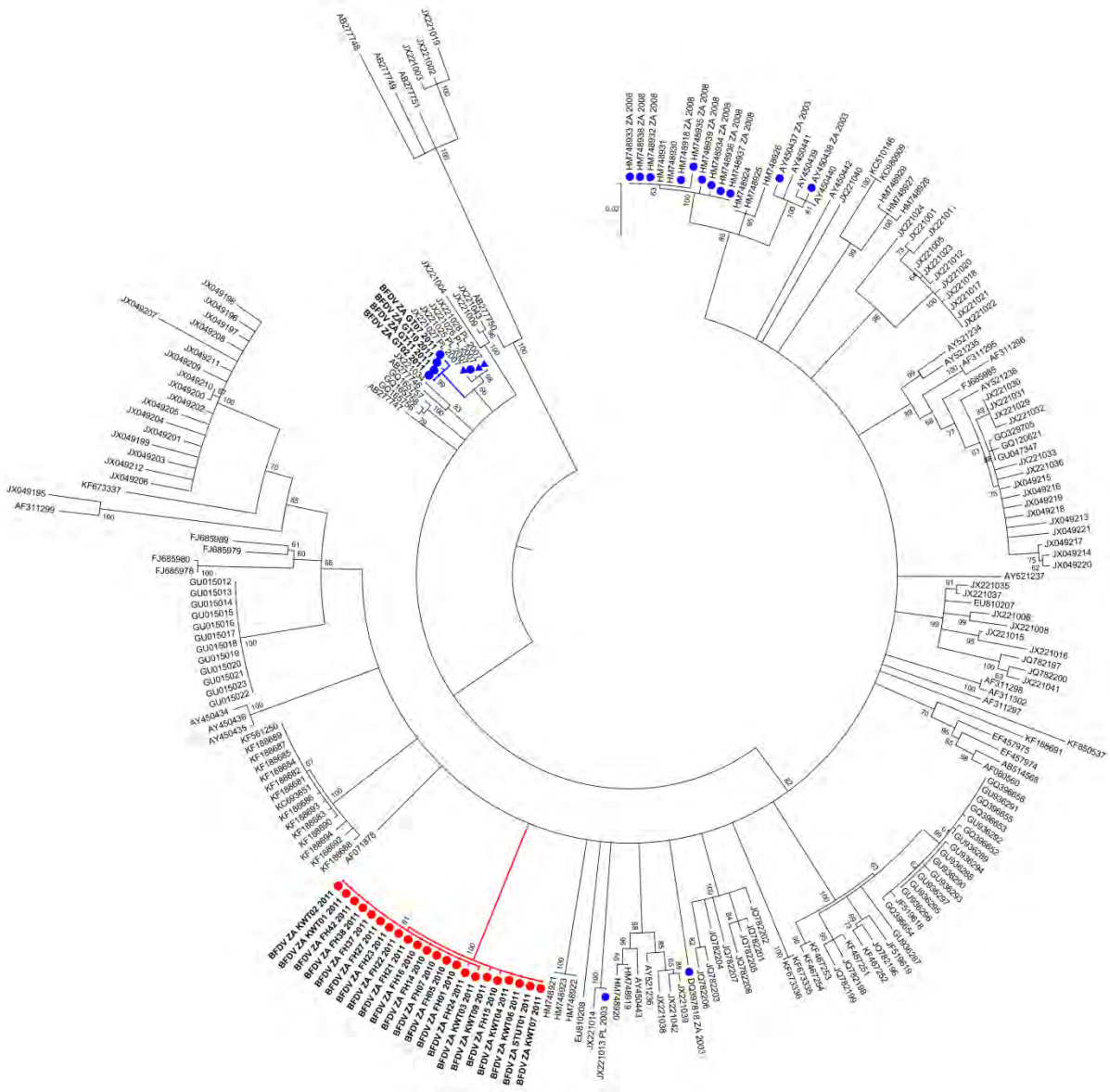


Figure 2.4: Maximum likelihood phylogenetic relationships of the *rep* of Cape parrot BFDV isolates with all publicly available BFDV *rep* sequences. Wild-caught Cape parrot (●), captive Cape parrot (●) and Budgerigars (▲). For emphasis sequences from this study are highlighted in bold and the branch lines are in colour. Sequences isolated from Cape parrot and Budgerigars include the two-letter country code and year of sampling. The tree was constructed in Mega6 MEGA (Build#: 6140122), and the numbers associated with the tree branches are indicative of the percentage of 1 000 full maximum likelihood bootstrap replicates that support the existence of the branches.

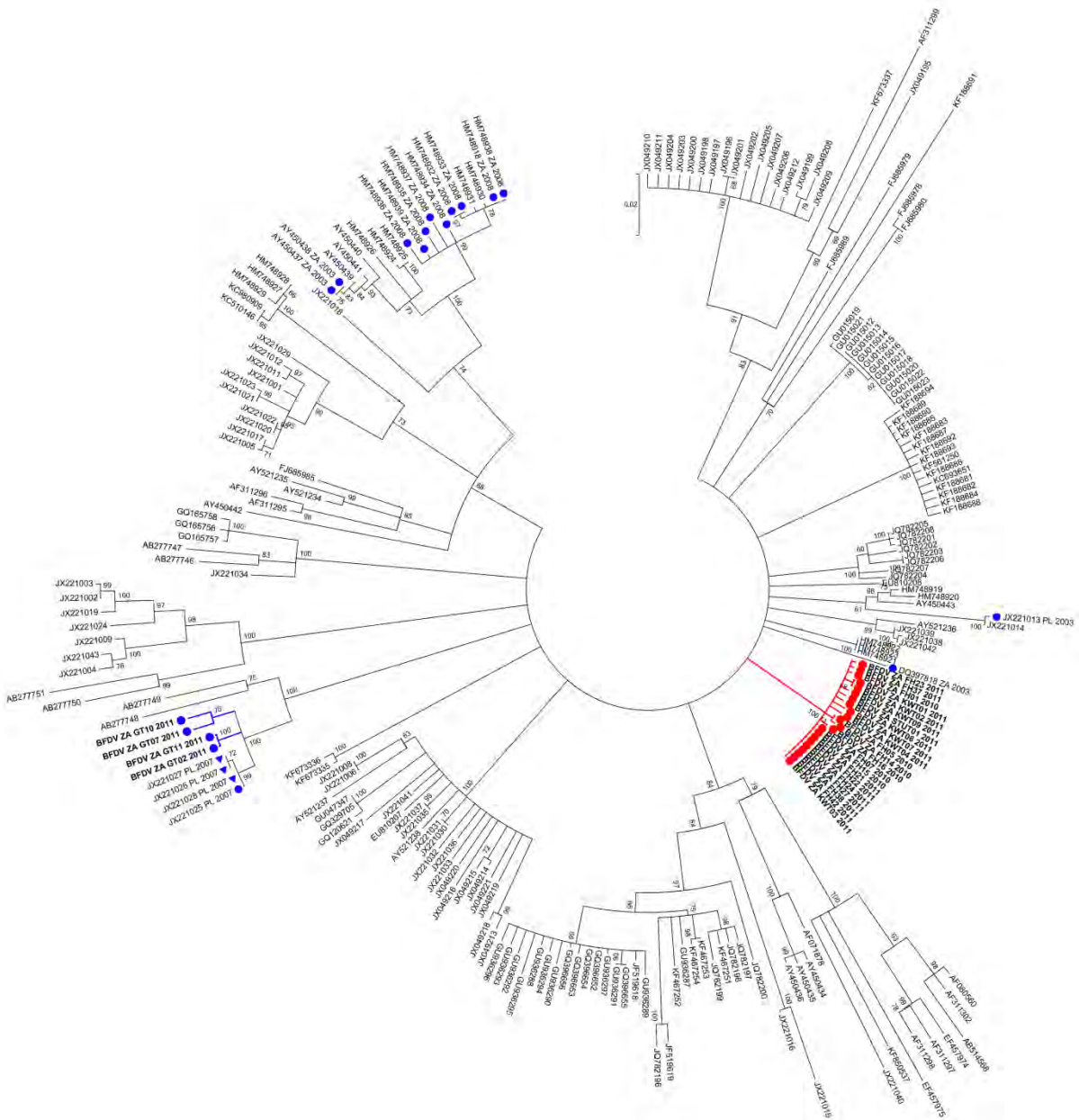


Figure 2.5: **Maximum likelihood phylogenetic relationships of the *cp* of Cape parrot BFDV isolates with all publicly available BFDV *cp* sequences.** Wild-caught Cape parrot (●), captive Cape parrot (●) and Budgerigars (▲). For emphasis sequences from this study are highlighted in bold and the branch lines are in colour. Sequences isolated from Cape parrot and Budgerigars include the two-letter country code and year of sampling. The tree was constructed in Mega6 MEGA (Build#: 6140122), and the numbers associated with the tree branches are indicative of the percentage of 1 000 full maximum likelihood bootstrap replicates that support the existence of the branches.

#### **2.3.4. Correlation between signs of PBFD and viral load**

Viral load was determined as a ratio of BFDV *rep* copies to Cape parrot TGF beta 2 (Table 2.3). The condition of the Cape parrots sampled varied, and many appeared to be malnourished and to display clinical signs of PBFD (Figure 1.4). There was a significant difference in viral load between the different physical condition groups (Kruskal-Wallis;  $df = 4$  ( $N = 49$ ),  $H = 25.563$ ,  $p < 0.0001$ ) (Figure 2.6A). Post hoc testing using multiple comparisons of mean ranks for all groups indicated that parrots with an overall condition score of 1 and 2 had viral loads that were significantly higher than birds in symptom score groups 4 and 5. Birds with an overall condition score of below three had a mean viral load ratio of greater than  $10^3$ , while those with a condition score of three and greater had a mean viral load ratio of less than one. Males and females showed a similar correlation between viral load and overall condition. Two females with an overall physical condition score of 1 but with a viral load ratio of less than 1 were found to be infected with a *Pseudomonas* sp. (Figure 2.6B).

#### **2.4. Discussion**

To date, there have been nine studies of PBFD in wild parrot populations in New Zealand and Australia and in New Caledonia (Ha *et al.*, 2007; Jackson *et al.*, 2014a; Jackson *et al.*, 2014b; Massaro *et al.*, 2012; McOrist *et al.*, 1984; Ortiz-Catedral *et al.*, 2009; Peters *et al.*, 2014; Raidal *et al.*, 1993b; Sarker *et al.*, 2014a), one in the United Kingdom (Sa *et al.*, 2014), and two in Africa and associated islands in Black-cheeked lovebirds and Echo parakeets (Heath *et al.*, 2004; Kundu *et al.*, 2012). The first evidence of the emergence of PBFD in wild Cape parrot populations in South Africa was reported in 2008, via photographs and field observations. In 2009, 32 Cape parrots were sighted with aberrant yellow feathers and observed colour changes in the plumage have previously been reported in parrots affected with PBFD (Doneley, 2003; Gerlach, 1994). This prompted the initial capture of parrots in 2010 (Boyes, 2011). For the present study, I isolated and sequenced BFDV genomes from blood samples on filter paper from 22 wild and four captive Cape parrots collected in 2010 and 2011 to analyse the genome and determine possible source(s) of the outbreak.

Table 2.3: **Data relating to each sampled Cape parrot.** Listed for each parrot are the sample code, qPCR data (presented in log10 scale), sex and overall physical condition score.

Sample code	Viral load ratio (log10)	rep copy number (log10)	TGFB2 copy number (log10)	Sex	Condition
FH02	-2.8	1.0	3.8	M	5
FH13	-2.6	0.9	3.5	M	4
FH11	-2.4	0.5	2.9	M	4
FH08	-2.3	1.3	3.6	F	4
FH12	-1.8	1.3	3.1	M	3
FH10	-1.7	1.0	2.7	F	5
FH09	-1.6	0.8	2.4	M	5
FH07	-1.2	2.7	3.9	F	3
FH06	-1.2	1.9	3.1	M	3
FH39	-0.8	2.9	3.7	M	4
FH17	-0.6	2.9	3.5	M	4
KWT02	-0.6	2.4	3.0	F	3
FH30	-0.6	2.8	3.3	M	3
KWT03	-0.6	2.6	3.2	M	4
KWT05	-0.5	2.2	2.7	F	3
KWT09	-0.3	2.7	3.0	F	1
FH19	-0.3	2.9	3.2	M	5
FH20	-0.2	2.4	2.6	F	3
FH27	-0.2	2.4	2.7	F	3
FH32	-0.2	2.8	2.9	F	4
FH43	-0.2	2.7	2.8	F	4
FH42	-0.1	3.2	3.3	M	4
FH34	-0.1	2.5	2.6	M	4
KWT10	-0.1	2.7	2.8	F	1
FH41	0.0	3.1	3.1	M	3
FH18	0.0	2.3	2.2	F	4
FH40	0.4	3.6	3.3	M	4
FH26	0.4	2.9	2.5	M	3
KWT04	0.5	3.2	2.7	F	3
FH03	0.9	3.5	2.5	F	2
FH33	1.0	3.0	2.0	M	2
FH29	1.0	3.5	2.5	M	3
FH01	1.3	5.3	4.0	M	2
FH23	1.9	4.7	2.8	F	3
FH37	2.1	4.8	2.7	F	2
FH31	2.5	5.3	2.8	F	2
FH04	2.7	6.2	3.4	F	1
KWT07	3.3	6.0	2.7	M	1
KWT01	3.4	6.3	2.9	M	1
FH28	3.5	6.8	3.3	M	2
FH38	3.6	6.7	3.0	M	1
FH05	3.6	6.9	3.3	F	2
KWT06	3.7	6.8	3.1	M	1
FH35	3.9	6.7	2.8	M	2
STUT01	3.9	6.3	2.4	M	1
FH21	4.1	7.0	2.9	F	3

FH16	4.4	6.7	2.3	M	3
FH14	4.6	7.8	3.2	M	2
FH24	4.7	7.6	2.9	F	2
SE04 (captive)	-3.0	0.2	3.2	-	-
SE03 (captive)	-2.8	0.4	3.2	-	-
SE01 (captive)	-2.7	0.6	3.3	-	-
SE02 (captive)	-2.5	0.5	3.0	-	-

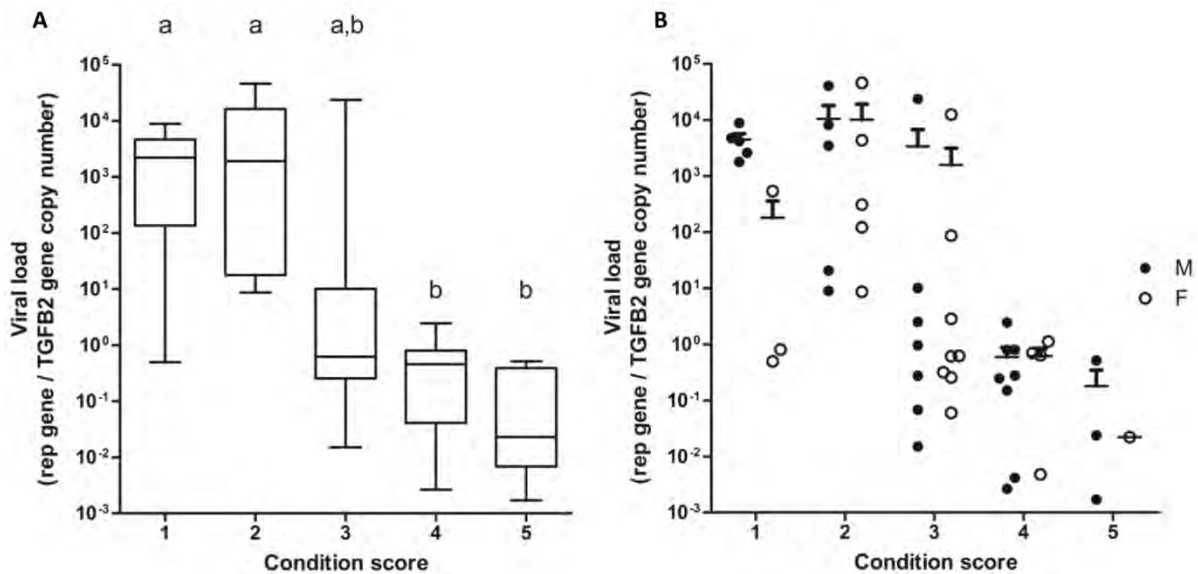


Figure 2.6: **BFDV viral loads in Cape parrot.** (A) A box plot of viral load in the blood as determined by qPCR using the BFDV *rep* and the Cape parrot TGF beta 2 gene in relation to overall physical condition score for Cape parrots. Overall condition was scored as one through five based on observable physical condition, with one being poor condition ( $p < 0.0001$ ). There is no statistical difference within groups 'a' and 'b', while groups 'a' and 'b' are significantly different from each other. (B) A discontinuous plot of viral loads for males (●) and females (○) for each overall physical condition score.

The isolated genomes were analysed to characterise the virus infecting wild Cape parrots. The sequenced BFDV genomes were found to be similar in length to those described in the literature (Bassami *et al.*, 1998), and putative motifs identified in the *rep* sequence resemble those of PCV (Mankertz *et al.*, 1998). The CP has been shown to transport the Rep into the nucleus to initiate RCR (Heath *et al.*, 2006). Three bipartite NLSs have previously been identified in the N-terminal region of the CP; however, only one was found to be required to facilitate localisation. Deletion experiments showed that nuclear localisation was active without N-terminal amino acid residues 1-40 but was lost when amino acid residues 1-56 were removed, resulting in the loss of a bipartite NLS found between residues 39 and 56 (Heath *et al.*, 2006) (Figure 2.2). Our *in silico* analysis of the CP sequence suggests that the NLSs are located between residues 16 and 56. Two bipartite NLSs (RRYRRRRRYFxxRRRRF) between residues 16 and 41 described here match those previously

identified by Heath *et al.* (2006). An additional NLS (residues 16-56), identified using the SeqNLS algorithm, encompasses all three NLSs identified by Heath *et al.* (2006). This NLS, together with one pat4 and two bipartite NLSs overlap the functional region required for nuclear localisation (residues 39-56) described previously by Heath *et al.* (2006). However, none of the motifs that were identified fall exclusively within the functional region (Figure 2.2). This may suggest that an alternative motif may be present within region 39-56, which is required for nuclear localisation. This finding is not surprising, considering that only a limited number of NLSs have been described in the literature (Lin and Hu, 2013).

The BFDV genomes isolated from 26 blood samples from infected birds were successfully cloned and sequenced in order to determine the source of the first reported outbreak of the disease in wild Cape parrots. These sequences fell into two distinct clades, separating viruses of wild parrots from those of captive parrots (Figure 2.3). This suggests that the captive parrots were infected after being removed from the wild population in 1995. The captive parrot sequences cluster with sequences isolated from Budgerigar samples originating from KwaZulu-Natal Province, South Africa, in 2003. This group of isolates was previously described as being unique to Budgerigars (Varsani *et al.*, 2010). The captive Cape parrots were kept together with two Cape parrot breeding pairs added from a captive source in KwaZulu-Natal Province. It could be suggested that the source of this infection originated from these Cape parrot breeding pairs and is related to the isolates previously described in Budgerigars from the KwaZulu-Natal Province. Should these captive Cape parrots be reintroduced into the wild this poses a threat of potentially introducing a new strain of BFDV into the wild population. A similar introduction of BFDV has been suggested to have occurred in Mauritian Echo parakeets, in which aviaries used during the initial recovery of the wild population may have served as the source of the BFDV infection seen in the wild population (Kundu *et al.*, 2012). As was seen in Mauritius, the potential for introducing a new BFDV strain through reintroduction of rehabilitated Cape parrots poses a significant threat to the wild population.

The 22 BFDV isolates sequenced from the wild Cape parrot population shared a high degree of similarity irrespective of year or location, as seen from the maximum likelihood phylogenetic relationships (Figure 2.3). This similarity suggests that the disease outbreak occurred recently from a single introduction, as it has been previously estimated that BFDV has a high evolutionary rate, consistent with similar evidence for other small single-stranded DNA viruses (Kundu *et al.*, 2012; Sarker *et al.*, 2014b). The presence of BFDV in wild New Caledonian rainbow lorikeets from New Caledonia has also been suggested by Jackson *et al.* (2014b) to be the result of a single introduction

with the virus potentially having adapted to the host. A follow-up study of the wild Cape parrot population would be useful to assess the evolution of the virus within this population

A recent outbreak in a wild population of Rose-fronted parakeet (*Pyrrhura roseifrons*) in New Zealand has also been described, stressing that wild endangered populations are susceptible to infection from external sources, especially from introduced species such as Eastern rosellas (*Platycercus eximius*) (Jackson *et al.*, 2014a; Massaro *et al.*, 2012). Considering the Cape parrot is South Africa's only endemic psittacine, there is the potential for the emergence feral populations of imported parrots to pose a threat to the endemic populations and contribute to the viral diversity. The BFDV strain in the wild Cape parrot population probably has a single common ancestor throughout their distributional range, while BFDV in the captive population has a different common ancestor from a separate disease introduction event. The captive Cape parrots were, however, not the source of the outbreak seen in the wild Cape parrots. The origin of the BFDV in the wild Cape parrot population is unclear, but due to the lack of divergence among sequences across the distributional range, it is assumed to be recent. The clustering of the two clades of BFDVs within other, distinct clusters of viruses with diverse origins could indicate that both the captive and wild Cape parrots were infected from contacts with other captive parrots. The source of the outbreak may have originated from KwaZulu-Natal. To date, this is the most extensive study of Pbfd in a wild parrot population on the African continent, and the first systematic investigation of virus diversity in Cape parrots. The only previous reports of Pbfd in wild parrots on the African continent are from a single sample from a single Black-cheeked lovebird chick in Zambia and samples taken from Cape parrots that had been caught but housed in an aviary prior to being tested, and therefore, a wild origin of the virus could not be confirmed.

Studying BFDV in wild Cape parrots provided a unique opportunity to investigate the relationship between viral load and overall physical condition, as wild populations are homogenous and larger than those normally found in captivity, and birds in the flock are at varying stages of disease progression. Most reports of viruses in captive birds have included small sample sizes and multiple species, which complicates assessing overall physical condition (Bert *et al.*, 2005; Katoh *et al.*, 2008). In this study, overall physical condition of wild Cape parrots was compared to BFDV viral load in the blood, measured by qPCR; previous studies of wild psittacine populations have relied on standard and non-quantitative PCR assays to detect the presence of BFDV (Ha *et al.*, 2007; Kundu *et al.*, 2012; Ortiz-Catedral *et al.*, 2009).

The results presented in the current study resemble that reported in a controlled challenge experiment in long-billed corellas, in which a peak viraemia of around  $10^6$  copies/ $\mu$ L was detected

from week two after challenge and continued past week six (Bonne *et al.*, 2009; Shearer *et al.*, 2009b). This is similar to the maximum *rep* copy number found in this study (Table 2.3). This high viral load suggests that a proportion of Cape parrots in the present study had an active viral infection, corresponding to a greater severity of clinical signs. A similar finding for a similar virus has been reported in pigs infected with PCV-2 (Brunborg *et al.*, 2004). Cape parrots with a viral load of less than one *rep* copy per reference gene, corresponding to  $10^2$  *rep* copies/ $\mu$ L, had a high overall condition score: this result was similar to the vaccinated long-billed corellas study, where a value of around  $10^2$  copies/ $\mu$ L was seen. This could indicate that these Cape parrots are mounting a successful immune response against the virus. This is supported by a previous study, where birds with a high HI score had a negative blood sample PCR result (Khalesi *et al.*, 2005). Katoh *et al.* (2008) showed an overall viral load range of between  $10^{-2}$  and  $10^5$  copies per cell in clinical samples obtained from a range of captive psittacine species; however, no comparison with overall physical condition was made. The viral loads determined in the present Cape parrot study also closely mirror these findings. It is worth mentioning that in this study, the isolation of full-length BFDV genomes amplified using RCA was only successful in samples with a *rep* copy number of greater than  $10^2$  *rep* copies/ $\mu$ L, and suggests a possible cut-off value for the current extraction protocol. Interestingly, there have been no literature reports of a viral load ratio less than  $10^{-3}$ . In the present study, the four captive birds with no history of Pbfd had *rep* copy numbers in this range. Similarly, healthy wild Cape parrots also had low *rep* copy numbers, and this indicates that  $10^{-3}$  is possibly a useful cut-off value for assessing birds as being disease-free. Two female Cape parrots with a low overall physical condition score were found to be co-infected with a *Pseudomonas sp.* This secondary infection may have contributed to the low overall physical condition score considering that the viral load in these birds was low. This is the first reported assessment of BFDV viral load in a wild psittacine population.

In summary *in silico* analysis of BFDV CP sequences from this study could not confirm a previously identified bipartite NLS situated within residues 39-56, which is required for nuclear localisation. This may suggest that an alternate NLS is involved in shuttling the BFDV CP into the nucleus. Phylogenetic analysis of the BFDV isolates from wild and captive Cape parrots indicated that there were two separate infection events in different populations, and this highlights the potential risk of introducing new strains of the virus back into the wild population. Knowledge of isolates infecting both captive and wild psittacines in South Africa will assist in the development of a locally produced vaccine. It was demonstrated that the presence and degree of observed clinical signs of Pbfd in Cape parrots correlate strongly with BFDV infection, and more specifically, with viral load. Therefore, clinical signs assessed as described may confidently be used to diagnose the presence and relative severity of BFDV infections in wild Cape parrots in the field. This will be very useful in ongoing Cape

parrot population surveys, and will make it only periodically necessary to capture birds to track the incidence of BFDV in the wild population – which will significantly decrease their stress and capture- and handling related injuries.

## Chapter 3: Expression and optimisation of BFDV CP in *N. benthamiana*

### 3.1. Introduction

At present there are no commercially available treatments for or vaccines against BFDV-related disease (Patterson *et al.*, 2013b). However, there is a commercial inactivated chimaeric vaccine currently available for the distantly-related PCV which infects pigs and is a major threat to the global pork industry (Pérez-Martín *et al.*, 2010; Urniza *et al.*, 2008). Research is ongoing into finding a treatment against circoviruses in general. A pan-caspase inhibitor, zVAD, has recently been tested against PCV in mice and has demonstrated a reduction in cell apoptosis without impacting on the functioning of the immune system (Karuppanan and Kwang, 2011). Considering apoptosis is seen as a possible mode of dissemination of BFDV in the gastrointestinal tract and epidermis, this antiviral compound may have potential as a treatment against PBFV (Latimer *et al.*, 1991; Trinkaus *et al.*, 1998).

Stanford (2004) described the use of interferon produced in chicken cell culture in the treatment of PBFV in African grey parrots. Results suggested that total lymphocyte count was increased and the virus was successfully cleared from the system. However, no further work has been published on the use of interferon, possibly suggesting that the cost of producing a commercial product for the treatment of PBFV is prohibitively expensive. Another antiviral that has been tested against BFDV is  $\beta$ -(1,3/1,6)-D-glucan. Results showed that viral DNA was absent in the blood after 7-9 months of treatment with  $\beta$ -(1,3/1,6)-D-glucan (Tomasek and Tukac, 2007). A treatment regime of this duration would not be a viable solution, as it would not prevent the disease from spreading and may not prevent repeat infection once treatment has been concluded. Thus, it is likely that the best treatment for PBFV is the development of a prophylactic vaccine to prevent the infection of parrots with BFDV.

The virion of BFDV is extremely stable in the environment, therefore treatment of existing infections may not prevent re-exposure and infection once the treatment has ended (Latimer *et al.*, 1991). A vaccine, however, would hopefully prevent infection from occurring, and would also contain the spread of the disease through undiagnosed subclinical infections (Ritchie *et al.*, 1992b). A number of vaccine candidates have been developed against BFDV. The first were inactivated virus particles purified from whole animal tissue and feathers. The purified virions were inactivated using  $\beta$ -propiolactone and paraformaldehyde and administered together with a double oil emulsion adjuvant (Raidal *et al.*, 1993a). Vaccination resulted in seroconversion; however, the risk of incomplete inactivation and ethical considerations associated with isolating virus have made the use

of inactivated vaccines undesirable (Bonne *et al.*, 2009; Woods and Latimer, 2000). An alternative to inactivated vaccines has been the use of recombinant expression systems for the production of subunit vaccines.

Vaccination of parrots with an insect cell derived BFDV subunit vaccine has been shown to elicit an immune response, and reduce to viraemia and shedding after challenge (Bonne *et al.*, 2009). This confirms that subunit vaccines are as effective as inactivated vaccines against BFDV. The vaccine was, however, unable to prevent viral replication or the vertical transmission of the virus to chicks, and would make unvaccinated birds susceptible to disease from vaccinated birds which continue to shed virus into the environment (Bonne *et al.*, 2009; Shearer *et al.*, 2009b). Furthermore, these recombinant expression systems are costly, and with bacterial fermentations, solubility and post-translational modifications can adversely affect both protein yield and structural epitopes (Bucarey *et al.*, 2009; Patterson *et al.*, 2013b). An additional hindrance in the production of CP in recombinant expression systems has been the low yields, especially for the full-length CP.

Yields of the BFDV CP can be increase if the 5'-end of the gene sequence is removed. This domain of the CP has been shown to be arginine rich and contains a number of NLSs, and has been demonstrated to be involved in interacting with the viral genome (Heath *et al.*, 2006). Deletion of this region could potentially increase expression through relieving the translation bottleneck created by repeating arginine residues or by preventing the accumulation of the BFDV CP in the nucleus where it could potentially interfere with the cell cycle. The deletion of the N-terminal region of the CP may, however, affect the ability of the protein to elicit virus neutralising antibodies. This could be through the loss of epitopes specific to the N-terminal region; however, considering the N-terminal region is situated within the virion and interacts with the genomic DNA, this would appear unlikely (Heath *et al.*, 2006; Rosenberg *et al.*, 1993). An alternative could be that the deletion results in a configurational change in the tertiary structure of the BFDV CP, thereby destroying epitopes present on the virion surface. A previous investigation by Heath (2006) into the expression of a truncated CP using insect cell culture and bacterial fermentation, demonstrated that the recombinant antigen reacted poorly to serum derived from infected psittacines. This despite antibodies produced against the insect cell and bacterial-produced truncated CP reacting positively when tested against antigen derived from the other recombinant expression system. Recent use of plant expression systems for the production of full-length BFDV CP show considerable promise for the production of low-cost BFDV vaccines (Duvenage *et al.*, 2013).

In addition to improving BFDV protein expression and accumulation through regulating gene copy number, transcription and translation in plants, extracting the protein in a soluble form is important

as this increases the final yield after purification. Before extraction, the day on which CP accumulation peaks in leaf tissue must be determined. This can be determined empirically through evaluating protein accumulation at different time points after initial *Agrobacterium* infiltration (Regnard *et al.*, 2010). Increasing protein solubility during extraction is dependent on the components present in the extraction buffer. A number of buffers have been tested for the extraction of BFDV CP from insect cell culture and bacterial fermentations. Solubility was found to increase when the buffer pH was higher than 7 and a high salt concentration (greater than 100 mM) was used (Heath *et al.*, 2006; Patterson *et al.*, 2013b). The inclusion of a detergent has also been seen to increase solubility of the protein (Johne *et al.*, 2004; Patterson *et al.*, 2013b). An optimisation of extraction buffer would be required for plant-expressed BFDV CP to increase the final yield of soluble protein.

In conclusion, the expression of the BFDV CP as a potential subunit vaccine has been investigated using a number of expression platforms. The BFDV CP has been successfully expressed in *N. benthamiana* by Duvenage *et al.* (2013); however, the yield was considered too low. Increasing recombinant protein accumulation in plants remains a challenge and must be determined empirically. A number of optimisations have been devised to increase accumulation of recombinant proteins and include: protein truncation, codon optimisation, directed protein localisation, transcription enhancer elements, suppressing silencing and gene amplification. Optimisation of the extraction time and buffer components can also be used to increase the final soluble protein yield.

The aim of this study was to improve the expression and accumulation of the BFDV CP in *N. benthamiana* and to improve the yield after extraction. For the purposes of the study it was decided to design a vaccine candidate based genome sequence isolated from Budgerigars (*M. undulatus*). Cape parrots are considered endangered and are only found in South Africa, and as such Budgerigars are preferable for vaccine trials. Expression and accumulation of the BFDV CP in *N. benthamiana* was investigated through comparing and combining different expression optimisations, and through optimisation of the extraction buffer.

## **3.2. Materials and methods**

### **3.2.1. General molecular techniques**

High-fidelity amplification of PCR fragments was performed using *Pfu* DNA Polymerase (Promega) or ACCUZYME™ DNA Polymerase (Bioline, United Kingdom) where stated as per manufacturer's instruction. *Pfu* DNA Polymerase-generated PCR products are blunt-ended; therefore in order to prepare PCR products for TA cloning A-tailing was performed. Amplified PCR products were first

resolved on a 0.8 % (wt/vol) TBE agarose gel and gel purified. The A-tailing reaction comprised of 10 ng of the PCR product, 2.5 mM MgCl<sub>2</sub>, 1x Taq DNA polymerase reaction buffer, 0.2 mM dATP (Bioline), 5 units of GoTaq® DNA polymerase (Promega) and deionised water to a final reaction volume of 10 µL. The reaction was incubated at 72 °C for 15 minutes; thereafter the PCR product was ready for TA cloning.

Vector DNA was dephosphorylated using 1 U rAPid Alkaline Phosphatase (Roche) as per the manufacturer's instructions.

Ligation of DNA fragments was performed using T4 DNA ligase (Roche) according to the manufacturer's instructions.

### **3.2.2. Molecular cloning**

#### **3.2.2.1. BFDV isolates**

Capsid genes from two BFDV isolates were used for transient protein expression in plants. The first isolate was AFG3-ZA (GenBank accession number AY450443) that had previously been isolated from an African grey parrot (*P. erithacus*) by Heath *et al.* (2004). To determine whether codon usage affected expression, the *cp* from isolate AFG3-ZA was codon optimised for *N. tabacum* (*tco*) and synthesised *in silico* by Geneart (Germany). Protein expression based on this isolate was used to compare the levels of expression between full-length and truncated capsid genes, and codon-optimised and wild-type genes.

A second *cp* used for transient protein expression in plants was from isolate BKS1ZA\_84 (GenBank accession number GQ165756) previously described by Varsani *et al.* (2010) from a Budgerigar. The full-length BKS1ZA\_84 genome was synthesised *in silico* by Geneart. Protein expression based on this isolate was used to compare the levels of expression based on different localisation signals, and between replicating and non-replicating plant expression vectors.

#### **3.2.2.2. Agrobacterium plant expression vectors**

The plant expression vectors used in this study were based on the pTRA binary *Agrobacterium* plant expression vector suite which was provided by Prof. Rainer Fischer of the Fraunhofer Institute for Molecular Biology and Applied Ecology in Germany (Maclean *et al.*, 2007). The vector suite consists of three plant expression vectors: pTRAc, pTRAc-ERH and pTRAc-rbcs1-CTP (Figure 3.1A-C). All vectors contain an expression cassette consisting of a cauliflower mosaic virus (CaMV) 35S promoter with a duplicated transcriptional enhancer, chalcone synthase 5'-untranslated region and a CaMV 35S polyadenylation signal. The expression cassette is flanked by two scaffold attachment region of

the tobacco Rb7 gene which is in turn flanked by the left and right borders required for T-DNA integration. Each vector contains origins of replication for *E. coli* and *A. tumefaciens*, and a *bla* gene conferring ampicillin/carbenicillin resistance. In addition the pTRAc-ERH and pTRAc-rbcs1-CTP vectors also contain an *nptII* gene that confers kanamycin resistance required during production of transgenic plants. Expression of the *nptII* gene is controlled by the promoter and polyadenylation signal of the nopaline synthase gene, and the entire sequence is situated between the left border and scaffold attachment region.

Each vector is responsible for targeting protein expression to different locations within the cell. Protein expression from pTRAc, in the absence of any intrinsic signal sequences encoded by the recombinant gene, accumulates in the cytoplasm. The pTRAc-rbcs1-CTP vector contains an N-terminal *Solanum tuberosum* chloroplast-transit peptide sequence of the rubisco small-subunit gene *rbcs1* that is responsible for accumulating recombinant protein within the stromal compartment of the chloroplast. The pTRAc-ERH vector contains an N-terminal plant codon-optimised signal peptide sequence from the murine mAB24 heavy chain gene and a C-terminal SEKDEL signal peptide sequence that is responsible for accumulating recombinant protein within the ER. Removal of the SEKDEL-encoding sequence for pTRAc-ERH produces pTRAc-AH, and allows for the secretion of the recombinant protein to the apoplast.

A fourth plant expression vector that was used for the expression of the BFDV CP is pRIC 3.0 (Figure 3.1D). This vector incorporates sequences of the BeYDV genome that allow for vector replication *in planta* (Regnard *et al.*, 2010). The BeYDV short intergenic region with promoter activity and gene encoding the Rep are included adjacent to the expression cassette. This sequence is flanked by two BeYDV long intergenic regions (LIRs). The Rep and the two LIRs are responsible for initiating recircularisation and amplification of a replicon containing the expression cassette.

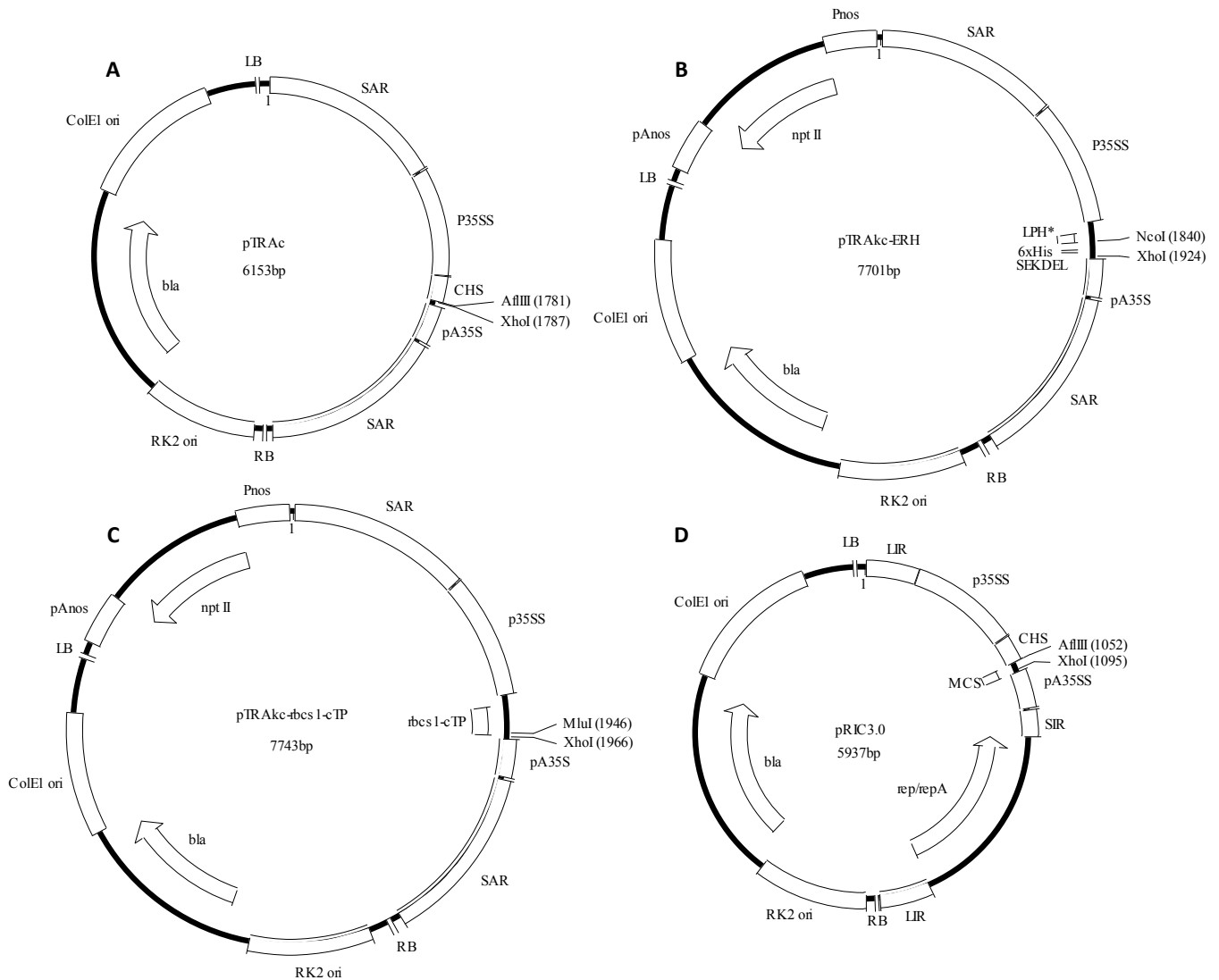


Figure 3.1: *Agrobacterium* pTRA plant expression vectors: pTRAc (A), pTRAc-ERH (B), pTRAc-rbcs1-cTP (C) and pRIC 3.0 (D). LB and RB, left and right borders for T-DNA integration; SAR, scaffold attachment region of the tobacco Rb7 gene; P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; CHS, chalcone synthase 5' untranslated region; pA35S, CaMV 35S polyadenylation signal; RK2 ori, origin of replication for *A. tumefaciens*; bla, ampicillin/carbenicillin-resistance bla gene; ColEI ori, origin of replication for *E. coli*; LPH, signal peptide sequence from the murine mAb24 heavy chain; 6xHis, 6 His tag sequence; SEKDEL, ER-retention signal sequence; rbcs1-cTP, chloroplast-transit peptide sequence of a Rubisco small-subunit gene (*rbcs1*) from *S. tuberosum*; npt II, kanamycin-resistance npt II gene; Pnos and pAnos, promoter and polyadenylation signal of the nopaline synthase gene; LIR, BeYDV long intergenic region; rep/repA, BeYDV rep/repA gene; SIR, BeYDV short intergenic region.

### **3.2.2.3. pTRAc 6xHis TEV vector**

Initial expression of the BFDV CP was based on previous work performed by Heath (2006) in insect cell culture. The Bac-to-Bac® Baculovirus Expression System (Life Technologies, CA, United States of America), which included an N-terminal 6xHis tag together with a tobacco etch virus (TEV) protease cleavage site, was used to express the CP. It was initially decided to incorporate this tag and cleavage site into the design of recombinant plant expression vectors containing the BFDV CP.

The 6xHis tag together with a TEV protease cleavage site and in-frame *NcoI* restriction enzyme site was amplified by PCR from pFastBac-HTc and modified to include a 5' *AflIII* site and a 3' *XhoI* site for cloning. The PCR, as described Appendix B.1, was performed using *Pfu* DNA polymerase (Promega) and primers that amplified a 97-bp 6xHis TEV PCR product (Table 3.1).

The 6xHis TEV PCR product was ligated into pGEM®-T-Easy Vector (Promega) as per manufacturer's instructions. The ligated plasmid constructs were transformed into competent *E. coli* DH5α. Plasmid DNA was isolated from overnight cultures, and the integrity of the clones was confirmed through sequencing (Macrogen Inc.) using M13 forward and reverse primers.

The 6xHis TEV sequence was excised from the pGEM®-T-Easy backbone by digesting the plasmid DNA with restriction enzymes *AflIII* and *XhoI*, after which the DNA was gel purified. The digestion from pGEM®-T-Easy yielded an 82-bp 6xHis TEV fragment with a 5' *AflIII* and 3' *XhoI* cleavage sites. The pTRAc vector was linearised by restriction digestion with *AflIII* and *XhoI* (Figure 3.1A) and dephosphorylated. The 6xHis TEV fragment was directionally sub-cloned into pTRAc to produce pTRAc 6xHis TEV, and the ligated plasmid constructs were transformed into competent *E. coli* DH5α. Plasmid DNA was isolated from overnight cultures, and the integrity of the clones was confirmed through sequencing (Macrogen Inc.) using primers specific to the CHS (5'-CATTTCATTTGGAGAGGACACG-3') and pA35SS (5'-GAACTACTCACACATTATTCTGG-3') regions of the expression cassette.

Table 3.1 Primers for PCR amplification used during molecular cloning. Underlined sequence indicates the position of the restriction enzyme sites used during cloning.

Product Description	Size (bp)	Sense primer Sequence (5' - 3')	Cloning site	Antisense primer Sequence (5' - 3')	Cloning site
6xHis TEV	97	GATGATC <u>CATGTC</u> GTACTACCATCACCAT	<i>AflIII</i>	CTC <u>TCGAG</u> ACCATGGCGCCCTGAAAATAC	<i>XhoI</i>
BFDV AFG3-ZA cp	781	GGAACGCGTTAGGT <u>ACATGT</u> TGGGGCACCTCTAACTGC	<i>AflIII</i>	CCTAGATCTCGAGTTAAGTACTTGGATTGTTGGGGCAAAC	<i>BglII, XhoI</i>
BFDV AFG3-ZA ΔN40 cp	658	GGAACGCGTTAGGT <u>ACATGT</u> TCTCAACCAATAGAATTTACAC	<i>AflIII</i>	CCTAGATCTCGAGTTAAGTACTTGGATTGTTGGGGCAAAC	<i>BglII, XhoI</i>
BFDV tco AFG3-ZA ΔN40 cp	673	GGAACGCGTTAGGT <u>ACATGT</u> TCTCTACTAACAGGATCTAC	<i>AflIII</i>	CATGAGCTCGAATTCTCTAGAAG	
		<u>GTAACGCGTTAGGTACATGT</u> TGGGGCACCTCTAAC	<i>AflIII/MluI</i>	GTGATGGTGATGCCCTTCAGTTCTGGGATTATTGG	
BFDV BKS1ZA_84 cp 6xHis	796	<u>GTAACGCGTTAGGTACATGT</u> TGGGGCACCTCTAAC	<i>AflIII/MluI</i>	CATCTCGAGCTAGTGATGGTGATGGTGATGCCCTTC	<i>XhoI</i>
rbcS1-cTP BFDV BKS1ZA_84 cp 6xHis	959	<u>GGACCATGGCTTCCTCTGTTATTTCTC</u> T	<i>NcoI</i>	CATCTCGAGCTAGTGATGGTGATGGTGATGCCCTTC	<i>XhoI</i>
		<u>GTAACGCGTTAGGTACATGT</u> TGGGGCACCTCTAAC	<i>AflIII/MluI</i>	CTCATCTTTCTCAGAGTGATGGTGATGGTGATGCC	
BFDV BKS1ZA_84 cp 6xHis SEKDEL	814	<u>GTAACGCGTTAGGTACATGT</u> TGGGGCACCTCTAAC	<i>AflIII/MluI</i>	CCTCTCGAGCTAGAGCTCATCTTTCTCAGAGTGAT	<i>XhoI</i>
LPH BFDV BKS1ZA_84 cp 6xHis SEKDEL	860	<u>GGACCATGGAGTGGAGCTGGATCTTC</u>	<i>NcoI</i>	CCTCTCGAGCTAGAGCTCATCTTTCTCAGAGTGAT	<i>XhoI</i>
LPH BFDV BKS1ZA_84 cp 6xHis	840	<u>GGACCATGGAGTGGAGCTGGATCTTC</u>	<i>NcoI</i>	CATCTCGAGCTAGTGATGGTGATGGTGATGCCCTTC	<i>XhoI</i>

#### **3.2.2.4. Subcloning**

The *cp* for isolate AFG3-ZA had previously been cloned into the pGEM<sup>®</sup>-T Easy Vector (Promega) by Heath *et al.* (2004) and served as template DNA for PCR amplification of a full-length and truncated *cp* with modifications for cloning into the pTRAc 6xHis TEV vector. The tobacco-codon-optimised AFG3-ZA *cp* synthesised by Geneart included restriction enzyme sites for direct cloning of the full-length gene into the pTRAc 6xHis TEV vector and served as template for PCR amplification of a truncated version with modifications for cloning into the pTRAc 6xHis TEV vector.

The AFG3-ZA *cp* was amplified by PCR and modified to include a 5' *Afl*III site and a 3' *Xho*I and *Bgl*II site after the stop codon. The truncated *cp* that would express a 40 residue N-terminal deletion product was produced by PCR amplification and modified to include a 5' *Afl*III site and a 3' *Xho*I and *Bgl*II site after the stop codon. The tobacco-codon-optimised AFG3-ZA truncated *cp* was produced by PCR amplification and modified to include a 5' *Afl*III site.

PCR reactions, as described Appendix B.2, were performed using *Pfu* DNA polymerase (Promega) and primers that amplified PCR products: 781-bp BFDV AFG3-ZA *cp*, a 658-bp BFDV AFG3-ZA  $\Delta$ N40 *cp* and a 673-bp BFDV *tco* AFG3-ZA  $\Delta$ N40 *cp* (Table 3.1).

The PCR products were individually ligated into the pGEM<sup>®</sup>-T-Easy Vector (Promega) as per manufacturer's instructions. The ligated plasmid constructs were transformed into competent *E. coli* DH5 $\alpha$ , and the plasmid DNA was isolated from overnight cultures. The integrity of the clones was confirmed through sequencing (Macrogen Inc.) using M13 forward and reverse primers.

The modified BFDV AFG3-ZA *cp* variants were excised from the pGEM<sup>®</sup>-T-Easy backbone and Geneart carrier plasmid by digesting the plasmid DNA with *Afl*III and *Xho*I, after which the DNA was gel purified. The digestions yielded fragments: 746-bp BFDV AFG3-ZA *cp*, 623-bp BFDV AFG3-ZA  $\Delta$ N40 *cp*, 749-bp BFDV *tco* AFG3-ZA *cp* and 626-bp BFDV *tco* AFG3-ZA  $\Delta$ N40 *cp* all with 5' *Afl*III and 3' *Xho*I cleavage sites. The pTRAc 6xHis TEV vector was linearised by restriction digestion with *Nco*I and *Xho*I, and dephosphorylated.

The modified BFDV AFG3-ZA *cp* variants were directionally sub-cloned into pTRAc 6xHis TEV (Figure 3.2). The ligated plasmid constructs were transformed into competent *E. coli* DH5 $\alpha$ . Plasmid DNA was isolated from overnight cultures, and the integrity of the clones was confirmed through sequencing (Macrogen Inc.) using primers specific to the CHS and pA35SS regions of the expression cassette.

The full-length genome for isolate BKS1ZA\_84 synthesised by Geneart served as the template for PCR amplification of *cp* with modifications for cloning into the pTRAc, pTRAc-*rbcs1*-cTP and pRIC 3.0. The BKS1ZA\_84 *cp* was amplified by PCR reaction and modified by a second PCR reaction to include a 5' *Mlu*I site, chloroplast targeting linker sequence and *Afl*III site and a 3' 6xHis tag, stop codon and *Xho*I site. The PCR reaction, as described in Appendix B.3, was performed using ACCUZYME™ DNA polymerase (Bioline) and primers that amplified a final 796-bp BFDV BKS1ZA\_84 *cp* 6xHis PCR product (Table 3.1). The amplified PCR product was resolved on 1 % (wt/vol) TBE agarose gel and gel purified. This served as template for the second primer pair under identical thermocycling parameters (Table 3.1).

The amplified 796-bp BFDV BKS1ZA\_84 *cp* 6xHis PCR product was purified and then digested with either *Afl*III and *Xho*I or *Mlu*I and *Xho*I, after which the DNA was gel purified. The digestions yielded BFDV BKS1ZA\_84 *cp* 6xHis fragments of 773-bp (5' *Afl*III and 3' *Xho*I) and 784-bp (5' *Mlu*I and 3' *Xho*I).

The pTRAc and pRIC 3.0 vectors were linearised by restriction digestion with *Afl*III and *Xho*I, while pTRAc-*rbcs1*-cTP was linearised by restriction digestion with *Mlu*I and *Xho*I (Figure 3.1). The BFDV BKS1ZA\_84 *cp* 6xHis fragments were directionally sub-cloned into the dephosphorylated vectors (Figure 3.2). The ligated plasmid constructs were transformed into competent *E. coli* DH5 $\alpha$ . Plasmid DNA was isolated from overnight cultures, and sequence integrity was performed as described above.

The pTRAc-*rbcs1*-cTP BFDV BKS1ZA\_84 *cp* 6xHis construct served as the template for PCR amplification of *cp* with modifications for cloning into the pRIC 3.0. The BFDV BKS1ZA\_84 *cp* 6xHis gene including the 5' chloroplast-transit peptide sequence was amplified by PCR reaction and modified to include a 5' *Nco*I site. The PCR reaction, as described in Appendix B.5 was performed using ACCUZYME™ DNA polymerase (Bioline) and primers that amplified a 959-bp *rbcs1*-cTP BFDV BKS1ZA\_84 *cp* 6xHis PCR product (Table 3.1). The amplified PCR product was resolved on a 1 % (wt/vol) TBE agarose gel and gel purified.

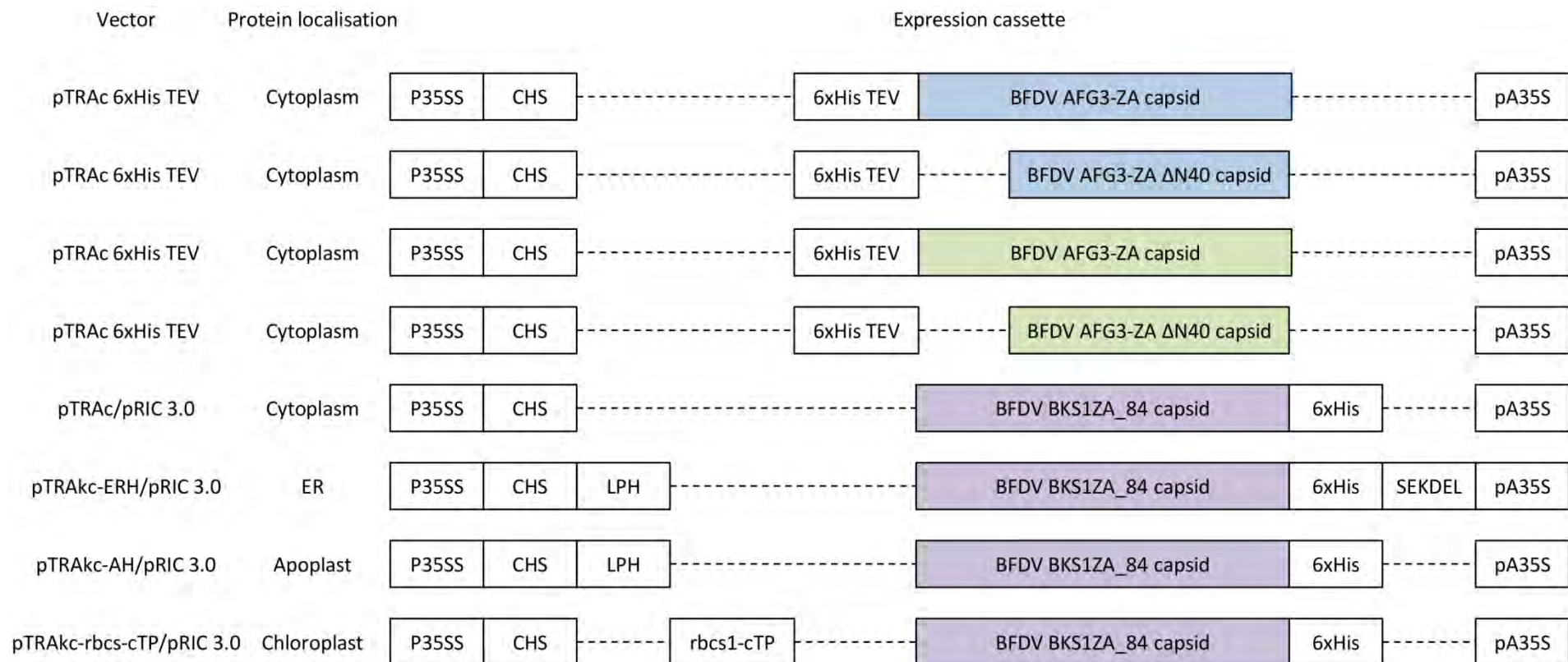


Figure 3.2: **Plant expression cassettes used in this study together with the *Agrobacterium* plant expression vectors they were cloned into and the BFDV CP localisation.** BFDV isolate AFG3-ZA *cp* (blue);  $\Delta$ N40, gene truncated to express a 40 residue N-terminal deletion product; tobacco-codon-optimised BFDV isolate AFG3-ZA *cp* (green); BFDV isolate BKS1ZA\_84 *cp* (purple); P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; CHS, chalcone synthase 5' untranslated region; 6xHis TEV, 6 His tag sequence and TEV protease recognition site; pA35S, CaMV 35S polyadenylation signal; 6xHis, 6 His tag sequence; LPH, signal peptide sequence from the murine mAb24 heavy chain; SEKDEL, ER-retention signal sequence; rbcs1-CTP, chloroplast-transit peptide sequence of a Rubisco small-subunit gene (*rbcs1*) from *S. tuberosum*.

The purified PCR product was then digested with *NcoI* and *XhoI*, after which the DNA was gel purified. The digestion yielded a 951-bp rcb1-cTP BFDV BKS1ZA\_84 cp 6xHis fragment with 5' *NcoI* and 3' *XhoI* cleavage sites. The fragment was then directionally sub-cloned into 5' *AflIII* and 3' *XhoI* linear and dephosphorylated pRIC 3.0 (Figure 3.2). The ligated plasmid construct was transformed into competent *E. coli* DH5 $\alpha$ . Plasmid DNA was isolated from overnight cultures, and sequence integrity were performed as described above.

The pTRAc BFDV BKS1ZA\_84 cp 6xHis construct served as the template for PCR amplification of *cp* with modifications for cloning into the pTRAc-ERH vector. The BFDV BKS1ZA\_84 cp 6xHis gene was amplified by PCR reaction and modified by a second PCR reaction to include a 3' SEKDEL, stop codon and *XhoI* site. The PCR reaction, as described in Appendix B.7 was performed using ACCUZYME™ DNA polymerase (Bioline) and primers that amplified a final 814-bp BFDV BKS1ZA\_84 cp 6xHis SEKDEL PCR product (Table 3.1). The amplified PCR product was gel purified, and served as template for the second primer pair under identical thermocycling parameters (Table 3.1).

The PCR product was purified digested, along with pTRAc BFDV BKS1ZA\_84 cp 6xHis, with *AflIII* and *XhoI*, after which the DNA was gel purified. The digestion yielded a 791-bp BFDV BKS1ZA\_84 cp 6xHis SEKDEL fragment and 773-bp BFDV BKS1ZA\_84 cp 6xHis fragment both with 5' *AflIII* and 3' *XhoI* cleavage sites. The pTRAc-ERH vector was linearised by restriction digestion with *NcoI* and *XhoI*, and dephosphorylated (Figure 3.1B). The BFDV BKS1ZA\_84 cp 6xHis SEKDEL and BFDV BKS1ZA\_84 cp 6xHis fragments were directionally sub-cloned into the pTRAc-ERH vector (Figure 3.2). The ligated plasmid constructs were transformed into competent *E. coli* DH5 $\alpha$ . Plasmid DNA was isolated from overnight cultures, and sequence integrity was performed as described above.

The pTRAc-ERH BFDV BKS1ZA\_84 cp 6xHis SEKDEL construct served as the template for PCR amplification of *cp* with modifications for cloning into the pRIC 3.0. The BFDV BKS1ZA\_84 cp 6xHis SEKDEL gene including the 5' signal peptide sequence from the murine mAB24 heavy chain gene was amplified by two PCR reactions and modified to include a 5' *NcoI* site and with the second PCR reaction to replace the 3' SEKDEL sequence with an *XhoI* site. The PCR reactions, as described in Appendix B.7, were performed using ACCUZYME™ DNA polymerase (Bioline) and primers that amplified an 860-bp LPH BFDV BKS1ZA\_84 cp 6xHis SEKDEL PCR product and an 840-bp LPH BFDV BKS1ZA\_84 cp 6xHis PCR product (Table 3.1). The PCR products were purified using a QIAquick® PCR Purification kit (Qiagen).

The purified PCR products were then digested with *NcoI* and *XhoI*, after which the DNA was gel purified. The digestion yielded an 848-bp LPH BFDV BKS1ZA\_84 cp 6xHis SEKDEL and 830-bp LPH

BFDV BKS1ZA\_84 cp 6xHis fragment both with 5' *NcoI* and 3' *XhoI* cleavage sites. The fragments were then directionally sub-cloned into 5' *AflIII* and 3' *XhoI*, linear and dephosphorylated pRIC 3.0 (Figure 3.2). The ligated plasmid constructs were transformed into competent *E. coli* DH5 $\alpha$ . Plasmid DNA was isolated from overnight cultures, and sequence integrity was performed as described above.

### 3.2.3. Transformation of *A. tumefaciens*

The *A. tumefaciens* GV3101::pMP90RK cells were made electrocompetent using the method described by Shen and Forde (1989). Cells were electroporated using 40–400 ng of recombinant plasmid as described by Maclean *et al.* (2007). Recombinant clones were selected using agar plates containing kanamycin (30  $\mu\text{g}/\text{mL}$ ), rifampicin (50  $\mu\text{g}/\text{mL}$ ) and carbenicillin (50  $\mu\text{g}/\text{mL}$ ) and incubated at 27 °C.

Recombinant *Agrobacterium* clones were confirmed by preparing plasmid isolations from overnight cultures and back-transformation of the DNA into competent *E. coli* DH5- $\alpha$ . Plasmid isolations and sequence integrity were performed as described above.

### 3.2.4. *A. tumefaciens*-mediated transient expression

For infiltration, recombinant *A. tumefaciens* GV3101::pMP90RK were grown up overnight at 27 °C with agitation in induction medium (LB medium, 20 mM acetosyringone and 10 mM 2-morpholin-4-ylethanesulfonic acid [MES] at pH 5.6) supplemented with kanamycin (30  $\mu\text{g}/\text{mL}$ ), rifampicin (50  $\mu\text{g}/\text{mL}$ ) and carbenicillin (50  $\mu\text{g}/\text{mL}$ ). The strain LBA4404 containing pBIN-NSs, provided by Marcel Prins from the Laboratory of Virology, Wageningen in the Netherlands, was supplemented with kanamycin (30  $\mu\text{g}/\text{mL}$ ), rifampicin (50  $\mu\text{g}/\text{mL}$ ) and 2 mM  $\text{MgSO}_4$ . The addition of  $\text{MgSO}_4$  was to prevent cell clumping during incubation (Maclean *et al.*, 2007). The NSs protein has been shown to suppress post-transcriptional gene silencing in plants, leading to an increase in transient protein expression (Takeda *et al.*, 2002). Cells were harvested by centrifugation at 4 000 g for 10 minutes, and resuspended in infiltration medium (3% sucrose, 10 mM  $\text{MgCl}_2$ , 200 mM acetosyringone and 10 mM MES at pH 5.6). The suspensions were diluted to the required absorbance ( $\text{OD}_{600}$ ) using an Ultrospec™ 10 Cell density meter (Amersham Biosciences, United Kingdom) and incubated at 22 °C for 2 hours. The incubation step was required for the expression of the *Agrobacterium vir* genes for T-DNA transfer into the plant cell. The  $\text{OD}_{600}$  at infiltration was optimised in order to determine the best density for the expression of the BFDV CP. The *A. tumefaciens* GV3101::pMP90RK suspensions of each expression construct were infiltrated with and without strain LBA4404 containing pBIN-NSs into 6-week-old *N. benthamiana* plants by injecting the suspension into the abaxial spaces using a needleless 1 mL syringe. The plants were maintained in a greenhouse under a 16 hour light and 8

hour dark photoperiod at light intensity of 60–80  $\mu\text{E}/\text{m}^2/\text{s}$  and 22 °C. Negative controls included plant leaf tissue that was non-infiltrated, infiltrated with infiltration medium only and infiltrated with a suspension containing only strain LBA4404 with pBIN-NSs. Time trials were conducted to evaluate and compare expression of the BFDV CP with the different vectors.

### 3.2.5. Protein extraction and western blot analysis

Leaf discs were harvested using the cap of a microcentrifuge tube at 1, 3, 5 and 7 days post infiltration (dpi). The plant material was prepared into a fine powder using a micro-pestle and liquid nitrogen. A 100  $\mu\text{L}$  of extraction buffer per leaf disc was then added and the leaf material was vortexed. The extraction buffer used to prepare crude leaf extract included PBS and general lysis buffer (50 mM Tris, 100 mM NaCl, 10% glycerol and 1 mM dithiothreitol at pH 7.5). The general lysis buffer has been described as an initial starting buffer from which to further optimise (European Molecular Biology Laboratory, 2015). Individual components of the general lysis buffer were optimised: these included pH (buffer solutions included citric acid – sodium citrate; and glycine – NaOH), salt concentration, reducing agent and the addition of detergent (Triton X-100; BDH Laboratory Supplies, United Kingdom). The suspension of leaf tissue was clarified by centrifugation at 1500 g for 3 minutes and the supernatant representing the crude leaf extract was collected for further analysis. The crude leaf extracts were incubated at 90 °C for 10 minutes in sample application buffer prepared for analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook *et al.*, 1989).

The proteins were resolved on 10% SDS polyacrylamide gels and an equal volume of crude plant extract or equal amount of total soluble protein (TSP) were loaded into each lane. Gels were stained with Coomassie Brilliant Blue stain for 2 hours at 37 °C and destained overnight. To determine the sizes of the resolved proteins in Coomassie stained gels and on nitrocellulose membranes a PageRuler™ Prestained Protein Ladder (Thermo Scientific, MA, United States of America) was used as a molecular weight marker. After gel electrophoresis the proteins were transferred onto nitrocellulose membranes at 15 V for 1 hour using a Trans-blot®SD semi-dry transfer cell (Bio-Rad, CA, United States of America). After electrophoretic transfer the membranes were blocked in blocking buffer (5% non-fat dairy milk and 1x PBST [137 mM NaCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 2.7 mM KCl, 2 mM  $\text{KH}_2\text{PO}_4$  at pH 7.4 and 0.1% Tween-20]). The membranes were probed overnight at 4 °C with 1:2000 anti-His tag mouse IgG antibody (AbD Serotec, NC, United States of America) or 1:2000 anti-BFDV  $\Delta\text{N}40$  CP polyclonal IgG antibody produced in rabbit (Duvenage *et al.*, 2013) diluted in blocking buffer. The membranes were washed four times with blocking buffer for 15 minutes each and afterward incubated in a 1:10 000 dilution of anti-mouse IgG (whole molecule) alkaline phosphatase

antibody produced in goat affinity isolated antibody (Sigma-Aldrich) or anti-rabbit IgG (whole molecule) alkaline phosphatase antibody produced in goat affinity isolated antibody (Sigma-Aldrich) in blocking buffer for 1 hour at 37 °C. Membranes incubated in secondary antibody were washed four times with 1x PBST, with 15 minutes for each wash. Detection was performed with 5-bromo, 4-chloro, 3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) phosphatase substrate (KPL, MD, United States of America). Protein expression, extraction and western blot analysis was repeated at least three times to confirm the expression of the BFDV CP.

### 3.3. Results

#### 3.3.1. Expression of the BFDV CP in *N. benthamiana*

Recombinant *Agrobacterium* containing the BFDV *cp* fused to a histidine tag sequence was infiltrated into *N. benthamiana* to determine whether the gene was expressed. A silencing suppressor, NSs, was co-expressed to improve expression of the *cp*. Leaf tissue was harvested and analysed by western blot using an antibody specific for the histidine tag. Results show that the 30.1 kDa CP was expressed in *N. benthamiana* (Figure 3.3). This was not present in the control lanes and was not as a result of the expression of the silencing suppressor; however, a band of approximately 17 kDa was present in all samples. The antibody also detected a protein of approximately 60 kDa and a fainter band at approximately 120 kDa. The co-infiltration with the silencing suppressor improved expression of the CP as an increase in protein band intensity is apparent for the CP co-expressed with the silencing suppressor.

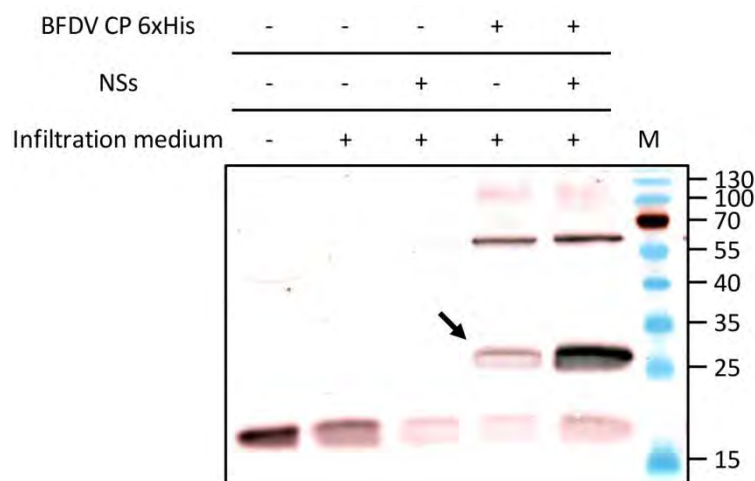


Figure 3.3: **Western blot analysis of BFDV CP expression in *N. benthamiana*.** BFDV CP (GenBank accession number GQ165756) fused with a C-terminal histidine tag (30.1 kDa) was expressed in the leaves of *N. benthamiana* after infiltration with *Agrobacterium* containing the BFDV *cp* in the presence (+) and absence (-) of the silencing suppressor NSs. Negative controls were non-infiltrated plant leaves, leaves infiltrated with medium only, and medium containing NSs only. Arrow indicates BFDV CP with C-terminal histidine tag. TSP was extracted from leaf tissue harvested on day 6 post-infiltration. An equal volume of each sample was loaded into the lanes, the CP was detected using anti-histidine antibody and the BCIP/NBT substrate reaction was performed for 1 hour. M – prestained protein marker.

To confirm that the protein that was expressed was indeed the BFDV CP, antiserum raised in rabbits against BFDV CP produced in bacteria was used as a primary antibody to detect expression of the CP in the leaves of *N. benthamiana* (Figure 3.4). The CP fused to a histidine tag both in the presence and absence of the silencing suppressor was unable to be detected using this antibody. However, the native protein (30 kDa) was readily detected and the intensity of the band was increased in the presence of the silencing suppressor.

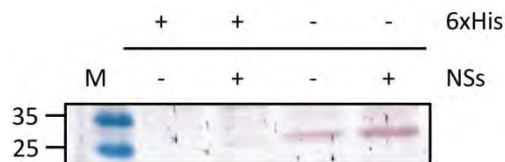


Figure 3.4: **Western blot analysis of BFDV CP using anti-BFDV CP serum raised in rabbits.** The BFDV CP fused to (+) and without (-) a C-terminal histidine tag was expressed in the leaves of *N. benthamiana* infiltrated with *Agrobacterium* containing the BFDV *cp* in the presence (+) and absence (-) of the silencing suppressor NSs. TSP was extracted from leaf tissue harvested on day 3 post-infiltration. An equal volume of each sample was loaded into the lanes, the CP was detected using anti-BFDV CP and the BCIP/NBT substrate reaction was performed for 1 hour. M – prestained protein marker.

### 3.3.2. Optimisation of BFDV CP expression in *N. benthamiana*

To determine whether the expression of BFDV CP in *N. benthamiana* is affected by deletion of the N-terminal region the first 120 nucleotides of the *cp* encoding the first 40 amino acid residues was removed and expression compared to that of the full-length gene (Figure 3.5). The expression of the N-terminal deletion CP (26.5 kDa) was higher than the full-length CP and peaked on day 5 post-infiltration. The plant-produced N-terminal deletion CP corresponded in molecular weight to the positive control which was N-terminal deletion CP (26.5 kDa) previously produced in insect cell culture (Heath, 2006). The expression of the full-length protein was seen to peak on day 3 post-infiltration. The expression of both proteins decreased sharply after the peak was reached.

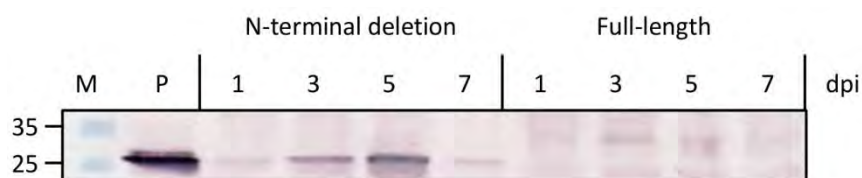


Figure 3.5: **Western blot analysis of plant-produced full-length and truncated BFDV CP.** Transient expression of BFDV CP via *Agrobacterium* infiltration into *N. benthamiana* leaves was assessed over seven days. An equal volume of sample was loaded into the lanes. The full-length (32.1 kDa) and N-terminal deletion (26.5 kDa) BFDV CP, fused to a N-terminal histidine tag and TEV protease cleavage site, was detected using anti-histidine antibody and the BCIP/NBT substrate reaction was performed for 1 hour. M – prestained protein marker; P – positive control (truncated BFDV CP produced in insect cell culture).

The codon usage of the full-length (western blot data not shown) and N-terminal deletion BFDV *cp* were optimised to that of *N. tabacum* to determine whether this would result in an increase in protein expression (Figure 3.6). The expression of the N-terminal deletion of both the codon-optimised and wild-type gene appeared similar and increased over the 5 day period. Further optimisation studies were based on the full-length gene in an attempt to improve expression while retaining the DNA binding domain of the BFDV CP.

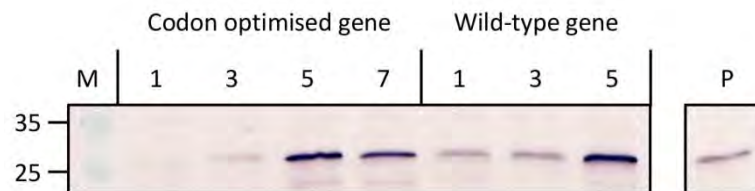


Figure 3.6: **Western blot analysis of BFDV CP encoded by codon-optimised and wild-type *cp*.** Expression over five days of N-terminal deletion BFDV *cp* variants, fused to an N-terminal histidine tag and TEV protease cleavage site. An equal volume of sample was loaded into the lanes. The CP was detected using anti-histidine antibody and the BCIP/NBT substrate reaction was performed for 1 hour. M – prestained protein marker; P – positive control (plant-produced N-terminal deletion BFDV CP).

Another factor to consider when optimising expression is the day on which the leaf tissue is harvested. The consensus for when accumulation of the full-length BFDV CP expressed in leaf tissue reached its maximum was found to be day 3 (Figure 3.7A). The expression peaked on day 3 after which there was a marked decline towards day 7. This expression pattern was mostly observed for the expression of all iterations of full-length CP tested. In comparison to the 30 kDa protein marker, the full-length BFDV CP with the C-terminal His tag was found to be smaller in size and therefore smaller than the size determined through *in silico* analysis (30.1 kDa). To further enhance expression of the full-length BFDV CP, the  $OD_{600}$  at which the *Agrobacterium* was infiltrated into *N. benthamiana* leaves was optimised. Optimisation of  $OD_{600}$  was performed for *Agrobacterium* containing the *cp* and the NSs silencing suppressor (Figure 3.7B-C). Expression of the full-length BFDV CP in the absence of the NSs silencing suppressor was improved when *N. benthamiana* leaves were infiltrated at an  $OD_{600}$  of between 0.50 and 1.00 (Figure 3.7B). When the NSs was co-infiltrated at an  $OD_{600}$  of 0.25 expression was improved when compared to expression without the presence of NSs. Co-infiltration of NSs with an  $OD_{600}$  of 0.50 and 1.00 did not improve expression of the CP. The NSs silencing suppressor infiltrated at an  $OD_{600}$  of 0.25 improved the expression of the CP at all absorbance values (Figure 3.7C). The greatest difference in expression in the presence and absence of NSs was seen when the *cp* was infiltrated at an  $OD_{600}$  of 0.50.

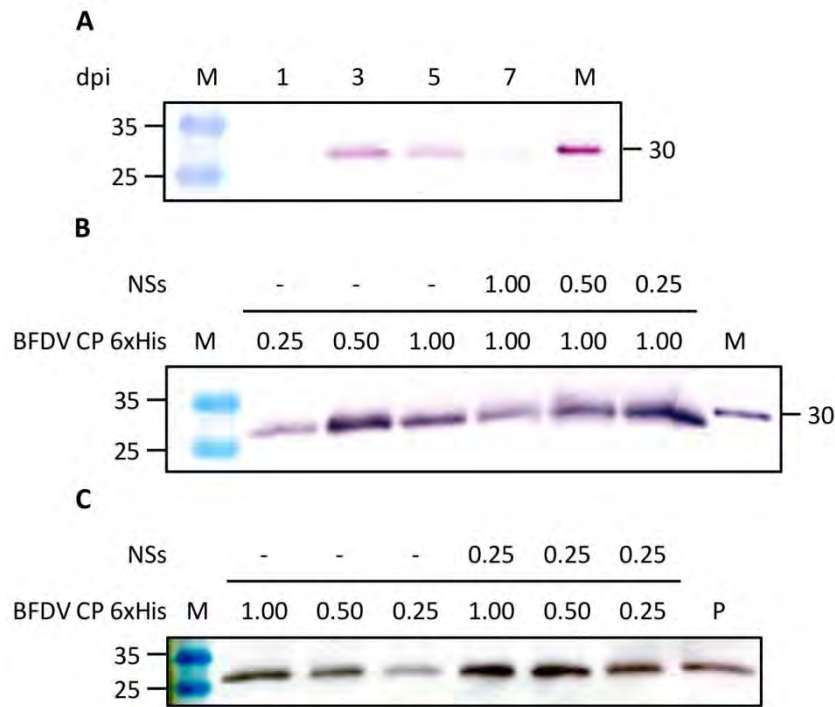


Figure 3.7: **Western blot analysis of BFDV CP expression over a seven day period and on day 3 using different *Agrobacterium* infiltration densities.** (A) A representative western blot of the accumulation over seven days of full-length BFDV CP with C-terminal histidine tag in the presence of the NSs silencing suppressor. (B) Optimisation of absorbance ( $OD_{600}$ ) of *Agrobacterium* suspension infiltrated into *N. benthamiana* leaves and harvested on day 3. Absorbance was assessed for *Agrobacterium* containing the *cp* or NSs silencing suppressor gene. (C) The variation of the infiltration  $OD_{600}$  for *Agrobacterium* containing the *cp* in the presence or absence of NSs silencing suppressor gene infiltrated into *N. benthamiana* leaves. An equal amount of TSP was loaded into each lane, the CP was detected using anti-histidine antibody and the BCIP/NBT substrate reaction was performed for 30 minutes. M – prestained protein marker and histidine tagged protein marker, P – positive control (plant-produced full-length BFDV CP).

The effect of targeting the full-length BFDV CP to different organelles was assessed together with gene amplification. The organelles targeted were the chloroplast and the ER, and secretion to the apoplast was also investigated. The CP was successfully expressed for each plant expression vector (Figure 3.8). Additional proteins with higher molecular weights than the CP were detected in the SDS-PAGE electropherograms, and are similar to bands detected in Figure 3.3. Protein expression in the cytoplasm resulted in histidine-tagged proteins of approximately 55 kDa, 70-100 kDa and 100-130 kDa. Additional histidine-tagged proteins, with approximate molecular weights of 35 kDa, 55-70 kDa and 100 kDa, were also detected when the CP was targeted to the chloroplast. To a lesser extent, the CP targeted to the ER and apoplast resulted in additional histidine-tagged proteins of approximately 55 kDa and 100-130 kDa which were similar to those found in the cytoplasm. The cytoplasm remained the most favourable location for expression, followed by targeting to the chloroplast and the ER. Accumulation of protein when targeted to the apoplast was lower than that in the other locations. Gene amplification had a mixed effect on expression. Expression in the

cytoplasm together with targeting of the CP to the chloroplast appeared to be improved when gene amplification occurred. However, gene amplification appeared to decrease expression when the CP was secreted to the apoplast and had no effect on expression when targeted to the ER. Overall, expression in the cytoplasm with gene amplification was most favourable.

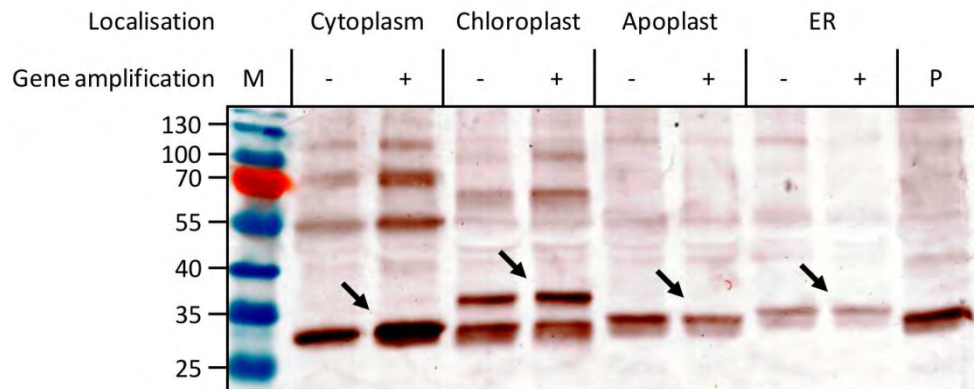


Figure 3.8: **Western blot analysis of the effect of targeted BFDV CP localisation and BFDV *cp* gene amplification on expression.** Protein was extracted on day 3 from *N. benthamiana* leaves. Expression of the CP as indicated by black arrows in the cytoplasm (30.1 kDa), the chloroplast (36.0 kDa), ER (33.0 kDa) and secretion into the apoplast (32.3 kDa). Expression in the presence (+) or absence (-) DNA amplification of the *cp* was assessed in all cases. An equal amount of TSP was loaded into each lane, the CP was detected using anti-histidine antibody and the BCIP/NBT substrate reaction was performed for 30 minutes. M – prestained protein marker, P – positive control (plant-produced full-length BFDV CP).

### 3.3.3. Optimisation of BFDV CP extraction from *N. benthamiana* leaf tissue

Initially TSP from harvested leaf tissue was extracted using PBS buffer (pH 7.6). To determine whether optimisation of extraction buffer could increase the yield of full-length BFDV CP a general lysis buffer (pH 7.5) was tested. This buffer was further optimised for salt and reducing agent concentration, detergent (% v/v) and pH (Figure 3.9). The use of the general lysis buffer increased the yield of CP when compared to the PBS buffer. No improvement in extraction was observed when the salt concentration in the general lysis buffer was increased or removed all together. Neither the addition of detergent nor the removal of the reducing agent had a positive effect on the extraction (Figure 3.9A). When the pH of the general lysis buffer was lowered to pH 6.0 more CP was extracted. At pH 4.5 less CP was extracted from the leaf tissue while at pH 9.0 and pH 10.5 a similar yield was achieved (Figure 3.9B). Combining both pH 6.0 and a high salt concentration did not improve CP extraction over and above the original general lysis buffer (Figure 3.9C).

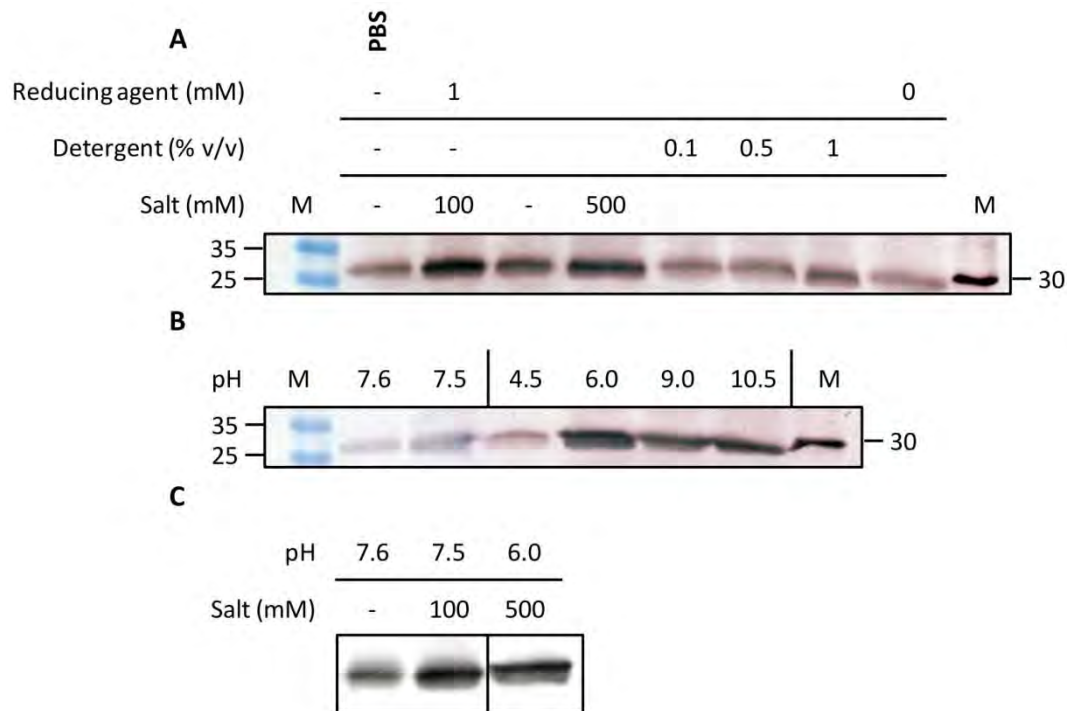


Figure 3.9: **Western blot analysis of buffer optimisation for extracting BFDV CP.** (A) TSP was extracted leaf tissue harvested on day 3 post-infiltration using PBS buffer (pH 7.6) or general lysis buffer (50 mM Tris, 100 mM NaCl, 10% glycerol and 1 mM dithiothreitol at pH 7.5). Optimisation of the general lysis buffer with respect to salt, detergent and reducing agent. (B) Optimisation of the general lysis buffer with respect to pH. (C) Comparison between PBS buffer, general lysis buffer and a buffer (pH 6.0, 500 mM salt, and 1 mM reducing agent) optimised for salt concentration and pH. An equal volume of each sample was loaded into the lanes, the CP was detected using anti-histidine antibody and the BCIP/NBT substrate reaction was performed for between 30 minutes and 1 hour. M – prestained protein marker and histidine tagged protein marker.

### 3.4. Discussion

A number of recombinant expression systems have been successful used to produce the BFDV CP. These include insect cell culture, and bacterial and yeast fermentation expression platforms (Heath *et al.*, 2006; Johne *et al.*, 2004; Patterson *et al.*, 2013b; Sariya and Prompiram, 2014; Stewart *et al.*, 2007). The disadvantage with these recombinant expression systems is that they are generally costly in the case of eukaryotic cells, and with bacterial fermentations solubility and post-translational modifications can affect protein yield and structural epitopes (Bucarey *et al.*, 2009; Patterson *et al.*, 2013b). An increasingly useful alternative technology has been the use of plant expression systems, which can be seen as a complementary system to traditional cell culture and microbial fermentation technologies for the production of region-specific and niche market products (Stoger *et al.*, 2014).

The use of plant expression systems, specifically whole-plant *N. benthamiana*, has already been used successfully in the expression of the BFDV CP fused to a elastin-like polypeptide (Duvenage *et al.*,

2013). In the present study the *cp* from BFDV isolates AFG3-ZA and BKS1ZA\_84 were cloned into plant expression vectors and transiently expressed in plants. Isolate AFG3-ZA was chosen as it had previously been used in the successful expression of BFDV CP through recombinant baculoviruses in insect cell culture (Heath *et al.*, 2006). The choice of isolate BKS1ZA\_84, identified in Budgerigar, follows from the investigation into BFDV affecting endemic Cape parrot. BFDV isolates from captive Cape parrot were found to be closely related to South African Budgerigar isolates, including BKS1ZA\_84, previously identified by Varsani *et al.* (2010). The choice was also based on the parallel development of a suitable Budgerigar challenge model in which a partial repeat of the BKS1ZA\_84 full-length genome was developed.

#### **3.4.1. Expression of the BFDV CP in *N. benthamiana***

In the present study the BFDV CPs for isolates AFG3-ZA and BKS1ZA\_84 were successfully expressed. Analysis *in silico* of the CP predicted a 30.1 kDa protein when fused with a C-terminal 6xHis tag and a 32.1 kDa protein when fused to an N-terminal 6xHis tag together with TEV protease cleavage site. These predicted sizes corresponded with those seen in the western blot analysis (Figure 3.3 and Figure 3.5). However, when the CP fused with a C-terminal 6xHis tag was compared to 30 kDa 6xHis tag protein marker the protein weight was found to be slightly smaller than 30 kDa (Figure 3.7A). A possible explanation for this discrepancy could be due to incomplete denaturation by SDS of the CP (Rath *et al.*, 2009). In comparison, the largest protein associated with purified virus was reportedly 26.3 kDa and alternative recombinant expression systems have produced CP that was approximately 32 kDa when expressed in insect cell culture and 38 kDa when expressed in yeast (Heath *et al.*, 2006; Ritchie *et al.*, 1990; Sariya and Prompiram, 2014). The larger sizes observed for recombinant protein is a result of the inclusion of signal tags which would increase the overall protein size.

In addition to the 30.1 kDa protein, proteins of approximately 60 kDa, 70-100 kDa and 120 kDa were also detected when the CP was expressed without localisation (Figure 3.3 and Figure 3.8). Interestingly, a similar band of 58 kDa considered to be a minor viral protein was reported for purified virus (Ritchie *et al.*, 1990). The molecular weights of the additional proteins detected are approximately multiples of the 30.1 kDa CP. It could be suggested that the protein described in the previous study and the larger proteins detected in this study are potentially CP dimers, trimers and tetramers, and that these could potentially be precursors to the pentameric subunits described by Crowther *et al.* (2003). In addition, this would suggest that the C-terminal 6xHis does not impact protein-protein interactions, and that denaturation by SDS was incomplete; this contributes to previous reports that the virion, and by extension the CP, is extremely stable (Davidson *et al.*, 2008; Raidal and Cross, 1994a).

To counter the potential effect of RNA silencing on BFDV CP yield, the plasmid constructs were co-infiltrated into *N. benthamiana* with the RNA silencing suppressor NSs. The silencing suppressor activity of the NSs protein from TSWV has previously described by Takeda *et al.* (2002). Co-infiltration with the silencing suppressor resulted in an increase in CP yield suggesting that as with most recombinant proteins expressed in plants, the transcription of RNA from the BFDV *cp* is susceptible to RNA silencing (Figure 3.3).

Interestingly, when anti-BFDV  $\Delta$ N40 CP polyclonal IgG antibody was used to detect the BFDV CP fused to a C-terminal histidine tag, the protein was undetectable; however, BFDV CP lacking a C-terminal histidine tag was readily detected by the polyclonal antibody (Figure 3.4). This suggests that the C-terminal histidine tag interferes with the epitope recognition of the antibody. Whether this epitope is located at the C-terminal or disrupted as a result of changes in the protein tertiary structure remains to be seen. It must be noted that the C-terminal histidine tag used in this study was used to detect protein expression and purification while a suitable antibody specific for the BFDV CP was in development.

#### **3.4.2. Optimisation of BFDV CP expression in *N. benthamiana***

To determine the optimum conditions required for BFDV CP expression in *N. benthamiana* a number of parameters were investigated. Expression of circovirus capsid proteins in insect cell culture and *E. coli* have suggested that an N-terminal deletion can greatly improve yield (Daum *et al.*, 2009; Heath, 2006; Johne *et al.*, 2004; Shang *et al.*, 2008). In this study, plant expressed full-length CP was compared to a truncated version in which the first 40 residues were removed (Figure 3.5). The expression of the CP in *N. benthamiana* was greatly improved when the first 40 residues were removed, as has been shown in other recombinant expression systems.

The N-terminal region of the CP has been shown to be responsible for nuclear localisation and contains a DNA binding domain that interacts with the BFDV genome (Heath *et al.*, 2006). It could therefore be suggested that in the absence of genomic DNA the full-length CP may localise to the nucleus where it could interact with the host genome disrupting cellular functions. Removal of the N-terminal region probably consigns the CP to the cytoplasm where it accumulates without interfering with the host genome.

To further investigate improving expression of the BFDV CP the gene was codon optimised for *N. tabacum* (Figure 3.6). Codon optimisation had no effect on protein expression for either the full-length or truncated CP. This result is supported by a previous finding in which plant-codon optimisation of the HPV L1 gene did not result in an increase in expression (Maclean *et al.*, 2007). It

is interesting to note that plant-codon optimisation maintained the overall G+C content of the *cp*. The G+C content of the wild-type gene was 47.63 % whereas the plant-codon-optimised gene was 45.19 %. It has been suggested that increasing the overall G+C content of the sequence can lead to an improve expression alongside codon optimisation, and could possibly explain the absence of any improvement in expression of the BFDV CP (Hitzeroth *et al.*, 2015). A recent result in which the overall G+C content after plant-codon optimisation remained the same has been reported by Thomas and Walmsley (2014); however, expression was improved based on codon optimisation alone, whether the effects are cumulative must still be determined.

Although the N-terminal truncation greatly increased expression further work focused on improving expression of the full-length CP. The full-length was chosen because tentative evidence suggested that plant-produced CP was assembling into pentameric subunits and the DNA binding domain on the N-terminal region would be required in the production of pseudovirions.

The optimisation of protein expression is not only determined by improving the efficiency of protein translation through gene deletions and codon optimisation but can include steps both preceding and following translation. Post-translational accumulation can be affected by post-transcriptional gene silencing and protein degradation pathways. A time trial was performed to determine the day at which BFDV CP accumulation was greatest. After mitigating for post-transcriptional gene silencing by including the NSs silencing suppressor the optimal day post-infiltration for protein accumulation was determined to be day 3 (Figure 3.7A). It could be suggested that after day 3 the CP enters into a protein degradation pathway. Plant leaves expressing the BFDV CP start undergoing leaf tissue damage from day 5 (data not shown). This could also account for the decrease in the total CP present in the leaf tissue as the CP is degraded and plant cell integrity is compromised leading to the release of proteases into the cytoplasm. A similar finding has been reported for the expression of haemagglutinin antigen in *N. benthamiana* in which the highest gene expression was observed on day 6 after which there was a decrease accompanied by plant wilting (Kanagarajan *et al.*, 2012).

The expression of the BFDV CP is also affected by the efficiency at which the T-DNA from *Agrobacterium* is transferred into the plant cells found in the leaf tissue. Increasing the concentration of *Agrobacterium* in the suspension that is infiltrated into air spaces between the leaf tissue may lead to a greater number of T-DNA copies entering plant cells and therefore resulting in an increase in expression. An increase in the absorbance ( $OD_{600}$ ) at which *Agrobacterium* was infiltrated at led to an increase in protein accumulation; however, this increase was reversed with the increase in the  $OD_{600}$  at which co-infiltrated *Agrobacterium* containing the NSs silencing suppressor was infiltrated (Figure 3.8B). This suggests possible competition of transcriptional and

translational resources between the transferred T-DNA containing the BFDV *cp* and the NSs silencing suppressor. Co-infiltration with the NSs silencing suppressor at optimal OD<sub>600</sub> of 0.25; however, resulted in an increase in expression for all absorbance values tested for the *cp* (Figure 3.8C). It is established that silencing suppressors can effectively inhibit post-transcriptional gene silencing of recombinant genes expressed in plants (Voinnet *et al.*, 2003). The optimum infiltration absorbance for *Agrobacterium* containing the BFDV *cp* was 0.50 while for the NSs silencing suppressor it was determined to be 0.25.

Protein localisation can also affect accumulation and various cellular compartments were tested for the expression of the BFDV CP (Figure 3.9). Expression to the cytoplasm led to the greatest accumulation of CP on day 3 when compared to the chloroplast, apoplast and ER. This is to be expected considering the expression of the CP during viral infection in the host occurs in the cytoplasm from where NLSs located on the protein transport the protein into the nucleus (Heath *et al.*, 2006). The nuclear localisation may negatively affect targeting to other cellular compartments. Similarly processing of the chloroplast-targeted signal would appear to be disrupted by the presence of an additional protein band of higher molecular weight. This could be as a result of the CP being transported into the nucleus before the signal tag could be cleaved and CP deposited into the chloroplast. In order to confirm this, fluorescently labelled BFDV CP could be used together with an inverted epifluorescence microscope to trace the localisation of the protein in the plant cell as has been demonstrated by Heath *et al.* (2006). The NLSs are located in the N-terminal region and could compete effectively with the N-terminal signal tags. Interestingly inclusion of the ER retention SEKDEL sequence results in a reduction in CP accumulation. It could be suggested that the retention in the ER resulted in the CP being diverted to proteolytic vesicles. This would reduce the overall accumulation of CP in the plant cell. An additional cellular compartment that could be investigated is the vacuole. Thomas and Walmsley (2014) reported the accumulation of human epidermal growth factor was greatest when targeted to the vacuole when compared to the ER and apoplastic space.

An increase in gene copy number had a variable effect on protein accumulation dependent on the protein localisation. Gene amplification increased the overall protein accumulation in the cytoplasm and chloroplast, while a decrease in accumulation was observed in the apoplastic space and ER. As has been previously reported, an increase in gene copy number by means of gene amplification based on BeYDV RCR resulted in up to three orders of magnitude increase in gene copy number that translated into a marginal increase in protein accumulation (Regnard *et al.*, 2010). This could be as a result of diversion of cell resources away from transcription and protein translation to DNA replication. Analysis of mRNA transcription from a non-replicating plant expression vector has shown

transcript levels to peak at three orders of magnitude (Kanagarajan *et al.*, 2012). Gene amplification would therefore likely compete with transcription for cell resources. A solution to this would be to control the level of gene amplification.

Although optimisations increased the expression of BFDV CP, the protein was undetectable in SDS-PAGE when stained with Coomassie. Coomassie dye reagents can detect as few 25 ng per band for most proteins. This would indicate that the concentration of BFDV CP was below 625 ng/mL. In comparison to TSP the BFDV CP would represent less than 0.00025 % of TSP, and in terms of overall yield of BFDV CP per gram of fresh weight this would be less than 5 mg/kg. In comparison this is similar to recombinant expression levels seen for plant-produced HIV p17 / p24 of 5 mg/kg (Meyers *et al.*, 2008; Regnard *et al.*, 2010). However, in order for plant-produced BFDV to be viable, yields approaching 50 mg/kg are required to commercial production, as has been reported for Influenza VLPs (D'Aoust *et al.*, 2008).

### **3.4.3. Optimisation of BFDV CP extraction from *N. benthamiana* leaf tissue**

The BFDV CP was extracted using PBS. To further increase the yield for small-scale protein extraction a general lysis buffer was tested with individual components optimised including pH, salt concentration, reducing agent and the addition of detergent (Figure 3.9A). An increase in salt concentration had no effect on CP yield, while addition of a detergent or removal of reducing agent resulted in a decreased yield. A pH of 6.0 resulted in the greatest yield of CP (Figure 3.9B). The pI for the BFDV CP based on *in silico* analysis (isoelectric.ovh.org) was determined to range between 10.8-11.3. Decreasing the extraction buffer pH below the pI would result in a positively charged CP. Removing salt from the extraction buffer would decrease the ionic strength of the buffer and would reduce the solubility of the positively charged CP. Combining a pH of 6.0 and salt concentration of 500 mM did, however, not have a cumulative effect on protein yield (Figure 3.9C).

In summary, the BFDV CP can be expressed in whole-plant *N. benthamiana*, and the full-length CP would appear to have the potential to assemble into VLPs. However, the CP was poorly expressed in plants, and various optimisations were undertaken to increase yield. Recombinant expression was greater for the truncated CP than the full-length CP, and these findings are similar to reports for other circoviruses. Optimisation of the codon usage appeared to have little effect; however, this may be more related to the G+C content than removal of rare codons. Localisation of proteins after expression did not improve the expression of the CP; possibly due to NLSs in the CP interfering with the signal tags. Gene amplification was seen to slightly improve expression, and could be diverting cell resources away from protein translation. Changes to the composition of the extraction buffer increased extraction; however, it is unknown how these changes would affect CP stability. The main

differences in expression were seen during co-infiltration of the silencing suppressor NSs and optimisation of the *Agrobacterium* cell density in the infiltration media. Both increased expression, and expression and accumulation of CP was highest on day 3, when *Agrobacterium* containing *cp* was co-infiltrated at an absorbance of 0.5 together with NSs at an absorbance of 0.25.

Further work on plant-produced BFDV CP should continue to optimise expression. Disabling the NLSs within the CP may improve expression, though at what cost to protein stability. Targeting the CP to additional organelles, such as the vacuole, may also counter the potential effect of the NLSs. The *cp* should be codon-optimised such that the G+C content is raised to see whether this would affect expression. Insect cell produced BFDV CP has been shown to readily form VLPs, investigation of VLP formation in plants should be investigated.

## Chapter 4: Plant-produced BFDV CP and virus-like particles

### 4.1. Introduction

The virions of BFDV consist of icosahedral capsids of approximately 20.5 nm in diameter, containing a 2-kb circular ssDNA genome that apparently encodes two genes: these are the *cp*, which produces the CP, and the *rep*, which produces Rep (Bassami *et al.*, 1998; Bassami *et al.*, 2001; Crowther *et al.*, 2003). The virion is extremely stable in the environment and horizontal transmission through ingestion of contaminated material is seen as the primary route of infection (Davidson *et al.*, 2008; Todd, 2004). The persistence of virus outside of the host and a dearth of available treatments have resulted in considerable research towards developing a vaccine against BFDV.

Initial vaccine research centred on inactivated virus vaccines derived from whole tissue of infected birds, purified from feathers or internal organs (Raidal and Cross, 1994a). This was problematic, however, as production of purified virus was reliant on sourcing infected birds, which has ethical implications and can be limiting in terms of availability of harvestable tissue (Bonne *et al.*, 2009). Furthermore, problems arising from incomplete inactivation of the purified virus have made an inactivated BFDV vaccine an unattractive option (Raidal *et al.*, 1993a). The virus has not as yet been propagated in tissue culture; therefore, the application of recombinant DNA technologies has been viewed as an alternative to infected whole tissue as a source of virus (Shearer *et al.*, 2008a). Recent work towards expression of recombinant BFDV CP shows great promise.

Recombinant BFDV CP has been successfully expressed using a number of expression platforms, including bacterial and yeast fermentation, insect cell culture and plants (Duvenage *et al.*, 2013; Heath *et al.*, 2006; Patterson *et al.*, 2013b; Sariya and Prompiram, 2014). Tissue culture technology has also been applied in the expression of PCV and GoCV CPs (Fachinger *et al.*, 2008; Scott *et al.*, 2006). Use of recombinant PCV CP as a vaccine has been shown to result in a humoral response that can produce sterile immunity (Pérez-Martín *et al.*, 2010; Tribble *et al.*, 2011). Limited studies have been conducted on expressed recombinant BFDV CP, however. Bonne *et al.* (2009) reported that vaccination produced a humoral response that failed to produce sterile immunity and instead resulted in a decrease in viraemia, replication and virus shedding. Despite the inability of the vaccine to produce sterile immunity, this research indicates that recombinant BFDV CP could be used to affect a response in the immune system. Further optimisation would be required to improve the strength of the immune response.

One area of focus has been in the production of VLPs. These lack viral nucleic acids and are therefore non-infectious, while retaining the same structural characteristics of the infectious virion (Santi *et*

*al.*, 2006). VLPs are highly immunogenic and stimulate both the humoral and cellular response pathways, and have been successfully applied in poultry against influenza (Nerome *et al.*, 2015; Scotti and Rybicki, 2013). The BFDV virion has a T = 1 symmetry and is assembled from 60 CP subunits that are arranged into 12 pentamer units (Crowther *et al.*, 2003). The virion of circoviruses is comprised of a single protein, making it an attractive target for investigation of the production of VLPs – and these have in fact been demonstrated for PCV and insect cell produced BFDV CPs (Nawagitgul *et al.*, 2000; Stewart *et al.*, 2007; Wu *et al.*, 2012).

Recombinant BFDV CP expressed in insect cell culture has been found to spontaneously assemble into VLPs. These particles ranged in size from 16-22 nm in diameter, which falls within most reported sizes for infectious virions (10-22 nm in diameter; Figure 1.2)(Stewart *et al.*, 2007). Attempts to produced BFDV VLPs using other recombinant systems have been limited. The expression of the full-length BFDV CP using bacterial fermentation resulted in insoluble protein (Patterson *et al.*, 2013b). Although the expression of a truncated version was successful, this may hinder the assembly of intact VLPs. Plant-based expression in *N. benthamiana* of BFDV CP by Duvenage *et al.* (2013) included a C-terminal-fused 140 or 255 residue elastin-like polypeptide to improve protein purification. The fusion of the BFDV CP to such purification tag would potentially affect the ability of the CP to assemble into VLPs. The assembly of plant-expressed BFDV CP into VLPs has not been assessed to date.

*Agrobacterium*-mediated expression of BFDV CP in *N. benthamiana* through the use of replicating plant expression vectors may have the unintended consequence of packaging replicon DNA into the VLPs. Plant expression vectors such as those derived from geminiviruses employ gene amplification as a method for increasing protein yield (Regnard *et al.*, 2010). Replication of the derived vector via RCR produces circular ssDNA replicons. These replicons and the BFDV genome are alike in that both are circular ssDNA, therefore the expressed recombinant BFDV CP may recognise the replicon. Depending on the size of the replicon the DNA could potentially be packaged by recombinant BFDV CPs to form pseudovirions.

In summary, the development of a vaccine against BFDV has moved away from inactivated virus towards the recombinant expression and purification of BFDV CP. This has been shown to successfully produce a humoral response in vaccinated birds. Insect cell derived BFDV CP has been reported to assemble into VLPs, but has otherwise not been shown for bacterial fermentations or plant systems.

The aim of this study was to investigate the formation and purification of BFDV VLPs in *N. benthamiana* through the expression of the BFDV CP using a BeYDV-based replicating vector. In addition the incorporation and packaging of replicon DNA by the BFDV CP was explored. The replicating plant expression vector was chosen over the non-replicating vector based on the results seen in Chapter 3 and the possibility of VLP assembly including vector replicons produced *in planta*.

## **4.2. Materials and methods**

### **4.2.1. *A. tumefaciens* growth kinetics**

The expression of BFDV CP for purification was performed using the replicating BeYDV mastrevirus-derived pRIC 3.0 vector without protein localisation signals. The cloning of the BFDV *cp* into pRIC 3.0 was performed as described in Section 3.2.2.4. Infiltration using this construct would result in amplification of the BFDV gene followed by expression and accumulation of the CP in the cytoplasm.

An initial starter culture of the recombinant *A. tumefaciens* GV3101::pMP90RK containing the plasmid pRIC 3.0 BFDV BKS1ZA\_84 cp 6xHis and strain LBA4404 containing pBIN-NSs were grown up overnight at 27 °C with agitation in LB medium and supplemented as described in Section 3.2.4. Gram stains of the cultures were performed, as described by Smith and Hussey (2005) to visualise the *Agrobacterium* strains using an inverted microscope (DIAPHOT-TMD, Nikon, Japan).

#### **4.2.1.1. Growth curve analysis**

Starter cultures were used to inoculate 500 mL induction medium for final OD<sub>600</sub> of less than 0.01 and grown for 48 hours at 27 °C with agitation. Cultures of strain GV3101::pMP90RK strain LBA4404 were supplemented as described in Section 3.2.4. The absorbance for both strains was assessed every 2 hours from 8 hours after inoculation. *Agrobacterium* culture was sampled and diluted in triplicate using sterile induction medium for a 1:10 dilution and the absorbance (OD<sub>600</sub>) was determined using an Ultrospec™ 10 Cell density meter (Amersham Biosciences). From the curve the *Agrobacterium* doubling time based on absorbance could be determined.

#### **4.2.2. *A. tumefaciens*-mediated transient expression**

The *Agrobacterium* cultures were grown in induction medium as described in Section 3.2.4 after which cells were harvested by centrifugation at 4 000 g for 10 minutes, and resuspended in infiltration medium. The absorbance (OD<sub>600</sub>) was adjusted using infiltration medium such that strain GV3101::pMP90RK containing the plasmid pRIC 3.0 BFDV BKS1ZA\_84 cp 6xHis was diluted to an absorbance of 1.00 and strain LBA4404 containing pBIN-NSs was diluted to an absorbance of 0.25

using an Ultrospec™ 10 Cell density meter (Amersham Biosciences). The diluted *Agrobacterium* cultures were then incubated at 22 °C for 2 hours as described in Section 3.2.4 and used for both syringe and vacuum infiltration.

A set of infiltration and recombinant expression controls were established and the relevant *A. tumefaciens* suspensions were infiltrated into 6-week-old *N. benthamiana* using a needleless 1 mL syringe as describe in Section 3.2.4. The following controls were tested: plant leaf tissue only, plant leaf tissue infiltrated with infiltration medium only, plant leaf tissue infiltrated with strain LBA4404 only, plant leaf tissue infiltrated with strain GV3101::pMP90RK only and plant leaf tissue co-infiltrated with both strains. On day 3 protein extraction with PBS and western blot analysis was performed as describe in Section 3.2.5.

#### **4.2.2.1. Vacuum infiltration of *A. tumefaciens* into whole plants**

Vacuum infiltration of *A. tumefaciens* into *N. benthamiana* was performed as described by Maclean *et al.* (2007) with the following modifications. The *Agrobacterium* strains were combined in infiltration medium for a final absorbance (OD<sub>600</sub>) of 1.00 for strain GV3101::pMP90RK and 0.25 for strain LBA4404 making a total OD<sub>600</sub> of 1.25. The 6-week-old *N. benthamiana* plants were prepared for inversion into infiltration medium by sealing the base of the plant through the use of a 130 x 130 mm Perspex sheet that contained a 10 mm channel to the centre to allow for the plant stem to be inserted (Figure 4.1). This prevented soil from falling into the infiltration medium while the plant was inverted and leaves and stem submerged.

Plants were submerged into the bacterial suspension and subjected to a vacuum of -90 kPa for 5–10 minutes, with occasional agitation to release trapped air bubbles. The vacuum was released rapidly (approximately 10 kPa/s). In addition, a recombinant expression control, using strain LBA4044 only, was vacuum infiltrated into plants. The plants were grown as described in Section 3.2.4.



Figure 4.1: **Perspex seal used to enclose soil during vacuum infiltration of *N. benthamiana*.** A Perspex sheet (130 x 130 mm) prevented soil from upturned plants from falling into the infiltration medium.

#### **4.2.3. Extraction and purification of BFDV CP from *N. benthamiana***

On day 3 post-infiltration plant leaves were harvested and approximately 25 g of leaf tissue was used for the extraction of expressed recombinant BFDV CP. Leaves were rinsed in water to remove soil particulates and dried with paper towel to remove excess water, after which the plant material was ground into a fine powder in liquid nitrogen using a mortar and pestle. The plant material was then combined with extraction buffer using ratio of one part plant material (eg 1 g) to two parts extraction buffer (eg 2 mL). The extraction buffer used in the extraction of BFDV CP was PBS containing cComplete™ Mini, EDTA-free protease inhibitor (Roche). PBS has previously been used as a buffer for the extraction of BFDV virions from whole tissue of diseased psittacines (Raidal and Cross, 1994b). The mixture was then homogenised for 5 minutes at 10 000 rpm and 4 °C using a T 25 digital ULTRA-TURRAX® (IKA® Works Inc., NC, United States of America).

##### **4.2.3.1. Slow speed centrifugation**

The homogenate was centrifuged at either 4 000 g for 10 minutes or 15 000 g for 15 minutes at 4 °C using a JA-14 rotor (Beckman Coulter, CA, United States of America) to determine optimum relative centrifugal force required to separate particulate plant material from TSP. The supernatant was filtered through two layers of Miracloth (Merck, Germany) in preparation for sucrose cushion centrifugation. The pellet was resuspended in an equal volume of buffer for analysis. The resuspended pellet was analysed alongside the supernatant to determine the extent of protein solubility.

#### **4.2.3.2. Sucrose cushion centrifugation**

Sucrose cushion centrifugation was based on purification PCV-2 capsid particles by Wu *et al.* (2012). A 40 % by weight sucrose solution (470.6 mg/mL of solution) was prepared and confirmed using a R5000 hand refractometer (Atago, Japan). A 2 mL 40% sucrose cushion was prepared in 5 mL Ultra-Clear™ centrifugation tubes (Beckman Coulter) and layered with supernatant derived from the slow speed centrifugation step. The tubes were centrifuged in a SW 55 Ti rotor (Beckman Coulter) at 40 500 rpm ( $RCF_{max} \approx 200\ 000\ g$ ) for 6 hours at 4 °C. The sucrose cushion was fractionated using a Foxy® Jr. Fraction Collector (Teledyne Isco, NE, United States of America) and the pellet was resuspended in a volume equivalent to one fraction. The fractions were analysed using western blot as described in Section 3.2.5. The pellet was further analysed by transmission electron microscopy (TEM).

#### **4.2.3.3. Transmission electron microscopy**

Analysis of BFDV CP particle assembly was determined using TEM. Copper grids (mesh size 200) were made hydrophilic by glow discharging at 25 mA for 30 s using a Model 900 SmartSet Cold Stage Controller (Electron Microscopy Sciences, PA, United States of America). The grids were then placed on a 1:100 dilution of the samples for 5 minutes and then washed three times in sterile water. The grids were negatively stained on uranyl acetate for 10 s and again for a further 20 s and viewed using a Tecnai™ F20 Scanning Transmission Electron Microscope (FEI, OR, United States of America).

#### **4.2.4. CsCl density gradient centrifugation**

CsCl density gradient centrifugation was performed based on a protocol modified from Ritchie *et al.* (1989b). Leaves containing expressed recombinant BFDV CP were extracted as described above using a 50 mM Tris at pH 7.6 buffer at a ratio of 3:1 buffer to plant material. The sucrose cushion centrifugation was modified such that a 45 % by weight sucrose solution (541.1 mg/mL of solution) was prepared. The sample together with a 5 mL sucrose cushion was centrifuged in a SW 32 Ti rotor (Beckman Coulter) at 32 000 rpm ( $RCF_{max} \approx 175\ 000\ g$ ) for 2 hours at 4 °C. The resulting pellet was used during CsCl density gradient centrifugation.

The pellets were resuspended in Tris buffer containing CsCl at a density of 1.406 g/cm<sup>3</sup>. CsCl density was confirmed using a R5000 hand refractometer (Atago). The resuspended pellets were centrifuged using a SW 55 Ti rotor (Beckman Coulter) at 48 000 rpm ( $RCF_{max} \approx 280\ 000\ g$ ) for 20 hours at 20 °C. The CsCl gradients were performed in duplicate or triplicate and were fractionated using a Foxy® Jr. Fraction Collector (Teledyne Isco). The refractive index for each fraction was determined using a R5000 hand refractometer (Atago) and 10 µL of each fraction was spotted onto nitrocellulose

membrane and western blot analysis was performed as described in Section 3.2.5. The BFDV CP present on the nitrocellulose membrane was then quantified using a Syngene Gene Genius imaging system and GeneTools software (Synoptics Inc., United Kingdom). In addition to quantifying the BFDV CP in each fraction, TSP and total DNA was determined using a ND-1000 Spectrophotometer (NanoDrop®, DE, United States of America).

Fractions containing the greatest concentration of BFDV CP were pooled and dialysed using dialysis tubing cellulose membrane (Sigma-Aldrich) against 50 mM Tris at pH 7.6. Dialysed pooled fractions were analysed using western blotting and standard PCR. No TEM was performed on the dialysed pooled fractions.

The PCR reaction, as described in Appendix B.7, was performed using GoTaq® DNA Polymerase (Promega) according to manufacturer's instructions and primers (5'-AGTGAAATCAGACTGGCAACC-3' and 5'-GTAAATTCCACGTCATTTTCC-3') that amplified a 1-kb BeYDV gene fragment. The amplified PCR products were resolved on 1 % (wt/vol) TBE agarose gels.

### 4.3. Results

#### 4.3.1. Growth optimisation of *Agrobacterium* strains for vacuum infiltration

To culture sufficient *Agrobacterium* for the volumes required for vacuum infiltration a starter culture is required and followed by inoculation into a larger volume. Two strains of *Agrobacterium* were used, the first being GV3101::pMP90RK that contains the plant expression plasmid with the BFDV *cp*. The second strain, LBA4404, contains the NSs silencing suppressor. The growth rates of these strains differ considerably requiring each strain to be cultured separately. In order to optimise this process the individual strains were analysed and their doubling times determined. Gram stain analysis of the two strains showed differences in cell morphology (Figure 4.2). Culture of strain LBA4404 consisted of uniform bacilli that were present as singles, pairs or aggregates (Figure 4.2A), whereas culture of strain GV3101::pMP90RK consisted of irregularly sized coccobacillus that didn't appear as aggregated as strain LBA4404 (Figure 4.2B). Budding daughter cells were readily observed for strain GV3101::pMP90RK.

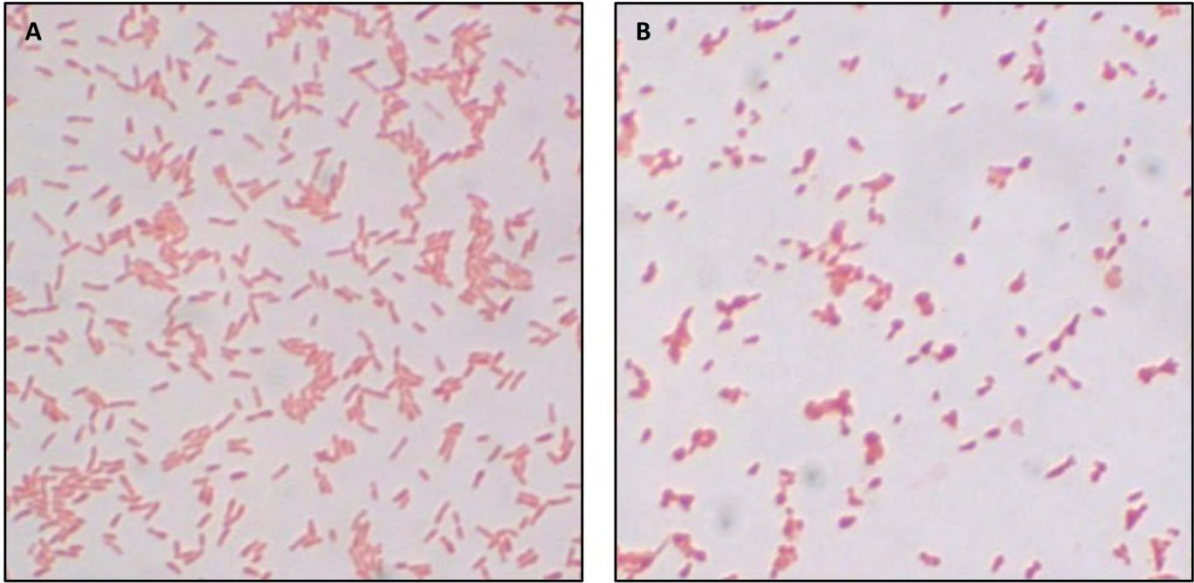


Figure 4.2: **Light microscopy of Gram-negative *A. tumefaciens* strains.** (A) LBA4404 and (B) GV3101::pMP90RK at 100 x magnification.

These strains grow at different rates and scale up of both cultures to the correct absorbance ( $OD_{600}$ ) requires that the cultures be inoculated at separate time intervals or absorbance values. To determine the generation time of both strains growth curve analysis was performed (Figure 4.3). As the spectrophotometer used to measure absorbance at 600 nm had a detection limit of 0.01 units the lag phase for both strains was not recorded, the strains were therefore already in exponential phase when a change in absorbance was first detected. The strain LBA4404 entered stationary phase at approximately 24 hours with an absorbance reading of 1.77 (SD, 0.06), while GV3101::pMP90RK entered stationary phase at approximately 40 hours with an absorbance reading of 1.67 (SD, 0.06). The generation time for strain LBA4404 was calculated to be approximately 115 minutes and was based on the exponential phase taken from 12 hours to 24 hours. The generation time for strain GV3101::pMP90RK was calculated to be approximately 180 minutes and was based on the exponential phase taken from 16 hours to 26 hours. The approximate  $\mu_{max}$  was determined to be  $0.29 \text{ h}^{-1}$  for strain LBA4404 and  $0.27 \text{ h}^{-1}$  for strain GV3101::pMP90RK.

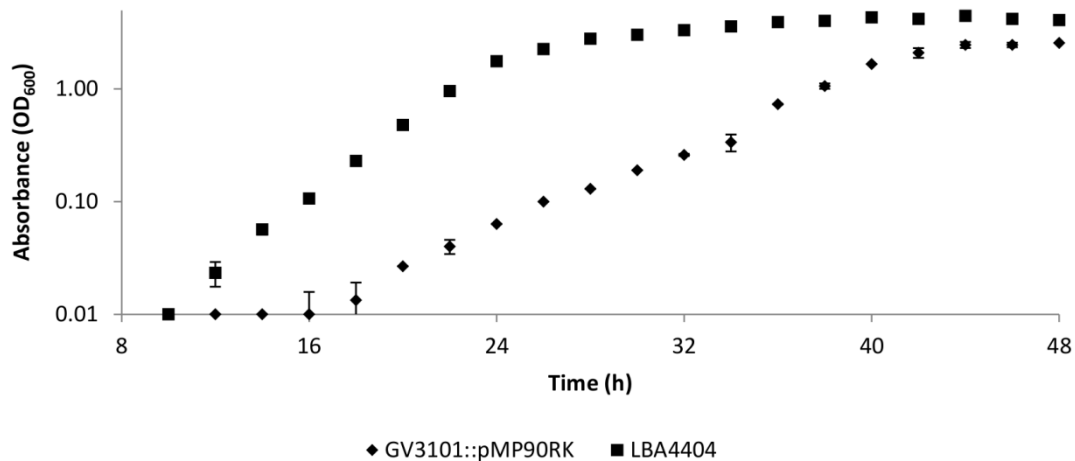


Figure 4.3: ***Agrobacterium* cell density as measured by absorbance at 600 nm over time.** Comparison between strain LBA4404 containing the NSs silencing suppressor gene and GV3101::pMP90RK containing the full-length BFDV *cp*. The detection limit for the spectrophotometer used to measure absorbance was 0.01 units. Error bars represent SD of technical repeats (n = 3).

To produce enough CP for purification requires the infiltration of plants as opposed to individual leaves. *Agrobacterium* infiltration of leaves can be performed using a syringe; however, this method is inefficient should more than one plant be required for expression purposes. Multiple plants can be effectively infiltrated using vacuum infiltration. In this method plants are submerged in infiltration medium containing *Agrobacterium* and a vacuum is created during which time air moves out the intercellular spaces through the stomata. A sudden increase in pressure when the vacuum is released forces infiltration medium containing the *Agrobacterium* through the stomata and into the intercellular spaces. This allows for the entire plant to be infiltrated as opposed to syringe infiltrating individual leaves. Leaves can then be harvested after expression and processed accordingly.

After optimising the *Agrobacterium* strains for vacuum infiltration a comparison was made between the two methods of infiltration (Figure 4.4). Results show that the 30.1 kDa CP is expressed in *N. benthamiana* using both infiltration methods. The CP was not present in the negative control lanes. The antibody also detected protein bands present at approximately 55 kDa, 70 kDa, between 100 kDa and 130 kDa and above the 130 kDa prestained protein marker. The silencing suppressor improved expression of the CP as an increase in band intensity is apparent for the CP co-expressed with the silencing suppressor.

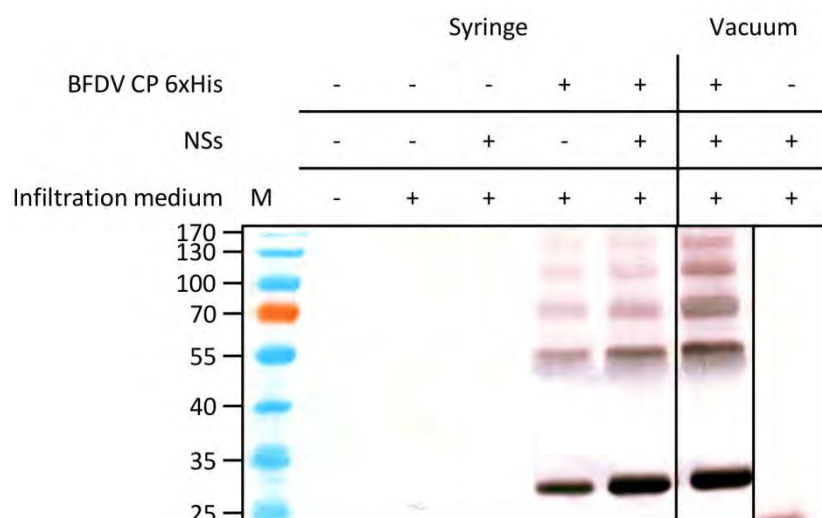


Figure 4.4: **Western blot analysis of BFDV CP expression comparing syringe versus vacuum infiltration of *Agrobacterium*.** Negative controls were non-infiltrated plant leaves, leaves infiltrated with medium and NSs only. The presence (+) or absence (-) of each component is specified. TSP was extracted from leaf tissue harvested on day 4 post-infiltration. An equal volume of each sample was loaded into the lanes, the CP was detected using anti-histidine antibody and the BCIP/NBT substrate reaction was performed for 1 hour. M – prestained protein marker.

#### 4.3.2. Purification of full-length BFDV CP

A larger mass of leaf tissue is required for the purification of VLPs, therefore it was decided to homogenise leaf tissue using a mechanical blender maintaining the preparation at 4 °C. The homogenate was filtered to remove large particulates at the filtrate was centrifuged at either 4 000 g or 15 000 g to determine if plant material can be removed without pelleting the proteins (Figure 4.5). Western blot analysis of TSP extracted from leaf tissue indicated the presence of the BFDV CP and the presence of larger histidine-tagged proteins at regular intervals. The approximate sizes were 60 kDa, 90 kDa, 120 kDa and 150 kDa. The CP was found mainly in the pellet as opposed to the supernatant and the concentration CP in the pellet was increased when the relative centrifugal force was increased.

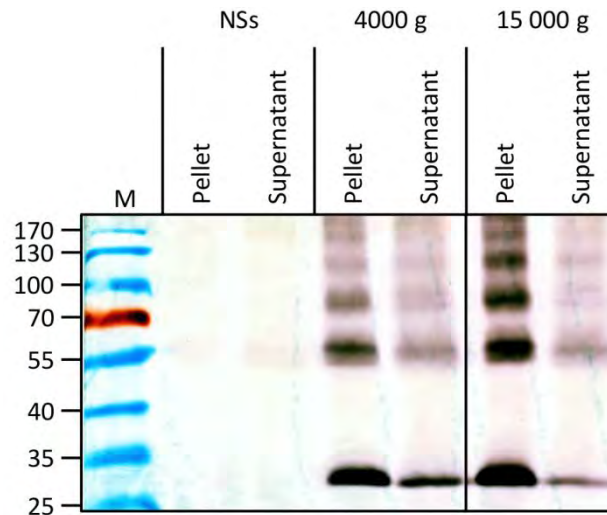


Figure 4.5: **Western blot analysis of the initial centrifugation step in the purification of BFDV CP.** Centrifugation was performed at two different relative centrifugal forces. The *Agrobacterium* was vacuum infiltrated into plants and TSP was extracted from leaf tissue harvested on day 6 post-infiltration. Plants infiltrated with NSs only served as a negative control. An equal volume of each sample was loaded into the lanes, the CP was detected using anti-histidine antibody and the BCIP/NBT substrate reaction was performed for 1 hour. M – prestained protein marker.

After an initial centrifugation the supernatant was layered onto a sucrose cushion and centrifuged. After the centrifugation was complete fractions of the sucrose cushion were collected and the pellet was resuspended for western blot analysis (Figure 4.6). The CP was found mainly in the pellet but was also present at the interface between the sample and sucrose cushion. A green layer was also present at the interface between the sample and sucrose cushion.

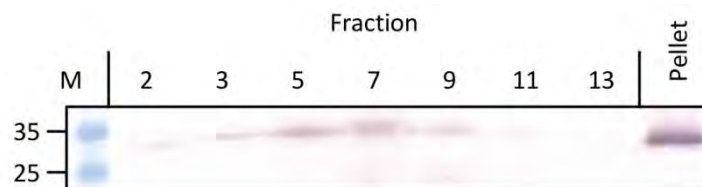


Figure 4.6: **Western blot analysis of fractions collected from sucrose cushion centrifugation during the purification of BFDV CP.** Fractions collected and then analysed are indicated. Fractions were numbered in the order they were collected starting from the bottom of the tube. An equal volume of each sample was loaded into the lanes, the CP was detected using anti-histidine antibody and the BCIP/NBT substrate reaction was performed for 1 hour. M – prestained protein marker.

The pellet from the sucrose cushion was resuspended and analysed using TEM (Figure 4.7). The TEM analysis of the pellet from leaf tissue expressing the BFDV CP together with the silencing suppressor NSs detected regular particles ranging between 13-23 nm in diameter (Figure 4.7A). The predominant size detected was approximately 17 nm in diameter. Analysis of the pellet from leaf

tissue expressing the silencing suppressor NSs only did not find anything consistent with VLPs (Figure 4.7B).

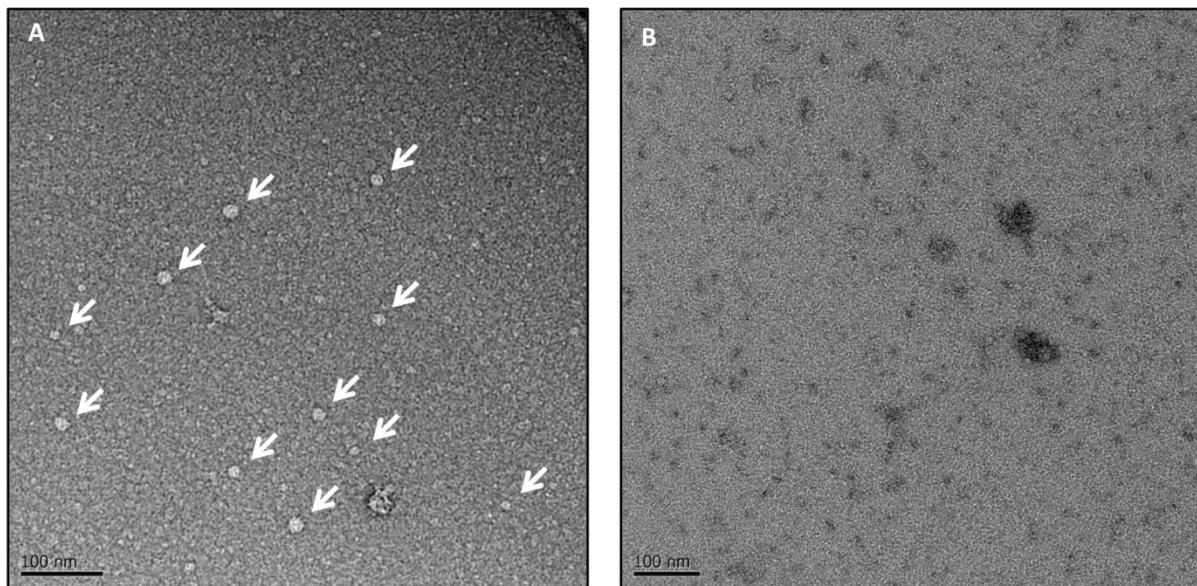


Figure 4.7: **Transmission electron micrographs of the partially purified BFDV VLPs (indicated by white arrows) after sucrose cushion centrifugation.** (A) Resuspended pellet from leaf tissue expressing the BFDV CP with C-terminal histidine tag together with the silencing suppressor NSs. (B) Resuspended pellet from leaf tissue expressing only the silencing suppressor NSs which served as a negative control.

The sucrose cushion pellets for BFDV CP co-expressed with NSs and the NSs control were further analysed using CsCl density gradient centrifugation. The resuspended pellets were combined with CsCl for final CsCl density of  $1.406 \text{ g/cm}^3$  and centrifuged until isopycnic equilibrium was achieved. Fractions of the CsCl density gradients were collected for immunoblotting, and spectrophotometric analysis (Figure 4.8). Dot blot analysis using anti-histidine antibody on CsCl density gradient fractions detected a relative intensity peak at fraction 6 for sample containing the BFDV CP co-expressed with the NSs silencing suppressor and a shoulder peak was detected in fraction 8 (Figure 4.8A). These peaks were absent in the sample in which only the NSs silencing suppressor was expressed. The fractions represent a CsCl density gradient that ranged from  $1.29 \text{ g/cm}^3$  to  $1.58 \text{ g/cm}^3$ . The peak in relative intensity seen in fraction 6 for the sample containing the BFDV CP co-expressed with the NSs silencing suppressor corresponded to a CsCl density of  $1.38 \text{ g/cm}^3$ . The shoulder peak seen in fraction 8 had a CsCl density of  $1.34 \text{ g/cm}^3$ . Fractions 5-7 of for each density gradient were pooled, dialysed and analysed by Coomassie staining and western blot (Figure 4.8B). The BFDV CP was undetectable when stained with Coomassie (data not shown). The 30.1 kDa CP and histidine-tagged protein of approximately 60 kDa were detected in the sample containing the BFDV CP co-expressed

with the NSs silencing suppressor. No histidine-tagged proteins were detected for the pooled fractions of the NSs silencing suppressor expressed on its own.

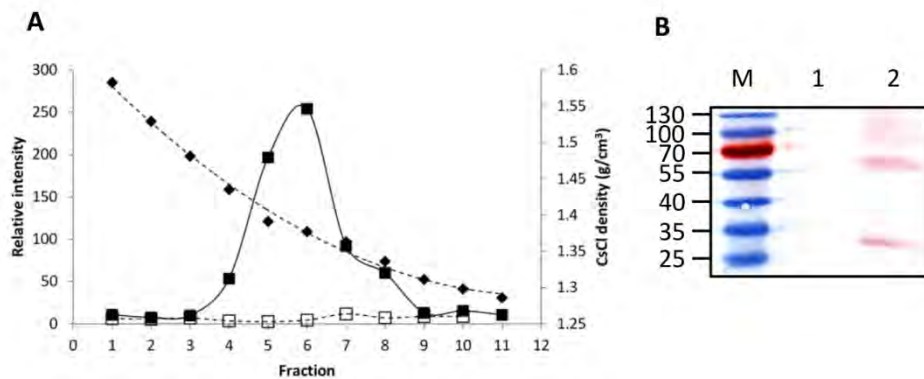


Figure 4.8: **CsCl density gradient centrifugation profile of BFDV CP.** (A) The CsCl density (◆) and relative intensity of histidine tagged protein as determined by dot-blot from leaf tissue co-expressing the BFDV CP and NSs silencing suppressor (■) or the NSs silencing suppressor alone (□). (B) Western blot analysis of pooled fractions 5-7 after dialysis. M – Prestained protein marker, 1 – NSs control, 2 – BFDV CP and NSs. An equal volume of each sample was loaded into the lanes, the CP was detected using anti-histidine antibody and the BCIP/NBT substrate reaction was performed for 1 hour.

PCR analysis on pooled, dialysed fractions was performed to determine whether DNA replicons produced by the expression vector were present in the observed relative intensity peak seen between fractions 5 and 7 (Figure 4.9 lane 1). Amplification of a 1-kb DNA product of the expression vector *BeYDV rep* was successful for the pooled fractions and corresponded to that seen in the positive control.

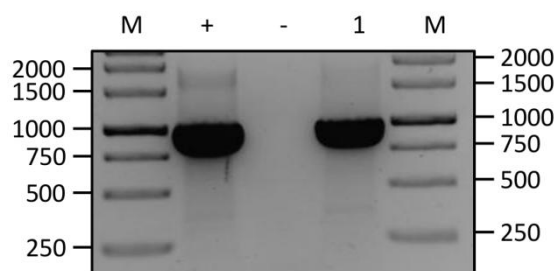
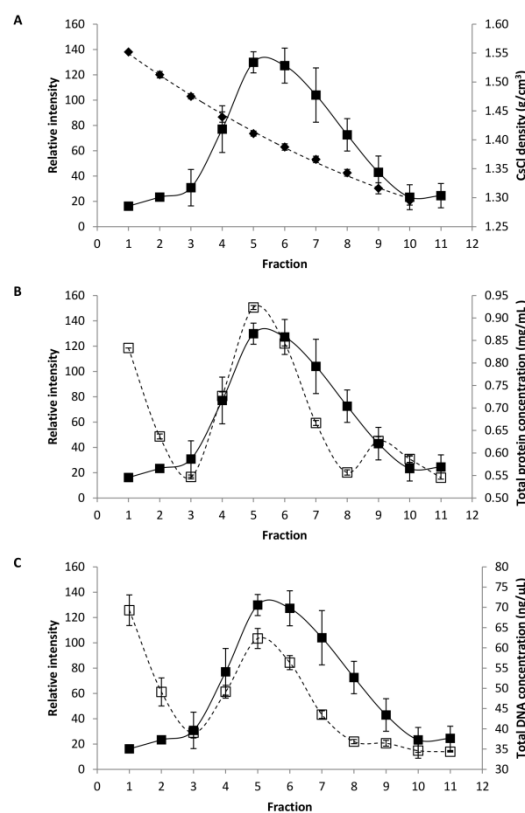


Figure 4.9: **The detection of *BeYDV* replicon DNA in dialysed BFDV CP fractions.** Pooled and dialysed fractions that correspond to relative intensity peak of BFDV CP seen between fraction 5 and 6 were tested. M – DNA marker, (+) – positive control with vector DNA, (-) - negative control with no template DNA, 1 – pooled and dialysed fractions.

To further analyse the CsCl density gradient fractions spectrophotometric readings for protein and DNA concentration were performed. Three densities gradients for the BFDV CP co-expressed with the NSs silencing suppressor were prepared and analysed (Figure 4.10). The relative intensity

determined by dot blot analysis using anti-histidine antibody was compared to CsCl density, TSP concentration, and total DNA concentration. A similar relative intensity peak was observed between fraction and 6 and corresponded to a CsCl density of between 1.41 g/cm<sup>3</sup> and 1.44 g/cm<sup>3</sup> (Figure 4.10A). The shoulder peak seen in the previous results was absent. The CsCl density gradient ranged from 1.29 g/cm<sup>3</sup> to 1.55 g/cm<sup>3</sup>. A peak in TSP of 0.92 mg/mL at fraction 5 was observed that corresponded to the relative intensity peak seen between fraction 5 and 6 (Figure 4.10B). A second smaller peak for TSP was observed at fraction 9 and corresponded to a CsCl density of 1.34 g/cm<sup>3</sup>, while a final peak was present at a density of 1.55 g/cm<sup>3</sup>. Similar peaks were detected for total DNA concentration (Figure 4.10C).



**Figure 4.10: CsCl density gradient centrifugation profiles of BFDV CP, protein and DNA concentration.** Comparison of relative intensity of histidine tagged protein as determined by dot-blot from leaf tissue co-expressing the BFDV CP and NSs silencing suppressor (■) with (A) CsCl density (◆), (B) TSP concentration (□) and (C) total DNA concentration (□).

## 4.4. Discussion

### 4.4.1. Growth optimisation of *Agrobacterium* strains for vacuum infiltration

One method broadly employed for the recombinant expression of proteins in plants is through the use of *Agrobacterium*. The transfer of the recombinant gene from the *Agrobacterium* into the plant cell requires a direct interaction between cells. In order to facilitate this, *Agrobacterium* is infiltrated between the intercellular spaces of *N. benthamiana* leaves through either needleless-syringe or vacuum. In the case of needleless-syringe infiltration a relatively small volume of infiltration medium containing *Agrobacterium* is needed. However, the drawback to this method is that each leaf on the plant has to be infiltrated separately, reducing the throughput time of plants that can be infiltrated. This, however, can be overcome through the use of vacuum infiltration in which plants are directly submerged into infiltration medium. This greatly enhances the throughput of plants that can be processed.

Vacuum infiltration requires considerably larger volumes of infiltration medium owing to the large volume that is required to fully submerge an entire plant. Maclean *et al.* (2007) noted that between 1-7 L of infiltration medium was required for vacuum infiltration of large numbers of plants in a laboratory scale setting, considerably more than if syringe infiltration were used. However, vacuum infiltration would be necessary for scale-up of operations for convenience and has been successfully used to produce Influenza VLPs for clinical trials on a large scale (D'Aoust *et al.*, 2010).

In the expression of BFDV CP in plants two strains of *Agrobacterium* were utilised. The strain GV3101::pMP90RK containing the plasmid pRIC 3.0 BFDV BKS1ZA\_84 cp 6xHis was used together with strain LBA4404 containing pBIN-NSs. These strains grew at different rates requiring separate inoculation schedules. In order to streamline the process, the strains were analysed and the doubling time determined. These two strains are morphologically different (Figure 4.2) and had very different growth rates (Figure 4.3). In order to produce the volumes required for vacuum infiltration, these strains would need to be inoculated at different absorbance values in order for the correct final absorbance for both strains to be reached and to avoid cultures from entering stationary phase. The doubling time of strain GV3101::pMP90RK was approximately 50% longer than that of strain LBA4404, and this could be based on the strains themselves (Figure 4.3). Alternatively, the difference in doubling time may be explained by the selection pressures of the two strains: strain GV3101::pMP90RK was under the selective pressure of three different antibiotics, two antibiotics selected for the *Agrobacterium* and helper plasmid, while the third antibiotic, selected for the plant expression plasmid. In contrast, strain LBA4404 was under the selective pressure of two antibiotics.

The diversion of cellular energy into the production of enzymes that inactivate the antibiotics would affect the overall growth rate of the cell. Similarly it could explain the difference in morphologies considering under no selective pressure *Agrobacterium* are bacillus-like in shape (Schaad *et al.*, 1988). An alternate explanation of the difference in observed morphologies could be the difference in plant expression vectors. It has been demonstrated that a the mammalian PCV can replicate via RCR in *E. coli* (Cheung, 2006). The replicating BeYDV plant expression vector could similarly be replicating via RCR in *Agrobacterium* and this may place additional strain over and above antibiotic selection pressure causing changes in the overall morphologies of the cell. Both strains of *Agrobacterium* enter stationary phase at an absorbance reading of between 1.67 and 1.77 (Figure 4.3). Whether this phase results in the dormancy or cell death would have to be determined through viable cell counts by determining the number of colony forming units. It could also be postulated that these upper absorbency readings are the result of depletion of nutrients. Whether infiltrating *Agrobacterium*, that have entered stationary phase, into plant leaves affects the transfer of T-DNA remains to be determined.

In order to ensure reliable viability of the *Agrobacterium* strains during infiltration induction medium should be inoculated at an absorbance that would result in maximum growth of viable cells before entering stationary phase. To do this strain LBA4404 would require inoculation into induction medium for a final absorbance of 0.04 while strain GV3101::pMP90RK would require inoculation into induction medium for a final absorbance of 0.06. At these absorbance values the exponential phase would approach the stationary phase in 16 hours.

Vacuum infiltration results in the same level of expression as when *Agrobacterium* suspensions are infiltrated using a needleless syringe, and as has previously been demonstrated the co-expression of the silencing suppressor protein NSs results in an increase in BFDV CP accumulation (Figure 4.4).

#### **4.4.2. Purification of full-length BFDV CP**

Initial low speed centrifugation prior to sucrose cushion centrifugation revealed that the majority of expressed BFDV CP was in the pellet (Figure 4.5). This could indicate the presence of particulate plant tissue that bypassed the filtration step, or it could indicate an insoluble fraction. This insoluble fraction has similarly been reported for expression of full-length CP using bacterial fermentation (Patterson *et al.*, 2013b). Increasing the absolute centrifugal force resulted in further accumulation of the BFDV CP in the pellet fraction.

The supernatant containing the soluble BFDV CP was used for sucrose cushion centrifugation (Figure 4.6). The majority of soluble BFDV CP accumulated as part of the pellet; however, the protein was

also seen to concentrate at the interface between the sample and the sucrose cushion. Previous work on purification of PCV particles used sucrose cushion centrifugation to effectively pellet VLPs (Wu *et al.*, 2012).

TEM analysis of the sucrose cushion pellet revealed negatively stained particles were circular and featureless. This description fits that used to describe infectious BFDV particles by Crowther *et al.* (2003). The plant-produced BFDV particles ranged between 13-23 nm in diameter and were similar to BFDV VLPs produced in insect cell culture which range between 16-22 nm diameter (Stewart *et al.*, 2007). The predominant diameter measured was approximately 17 nm which falls within the 10-22 nm range reported for infectious virions (Figure 1.2). These findings are comparable to insect cell-produced PCV VLPs that averaged 20 nm in diameter, closely resembling the infectious PCV virion that consists of a 1.7-kb genome encapsidated within a 20.5 nm non-enveloped capsid (Crowther *et al.*, 2003).

Fractionation of CsCl gradients of the pellet produced during sucrose cushion centrifugation produced two peaks of BFDV CP. The major peak was found to have an approximate density of 1.38 g/cm<sup>3</sup>, while a shoulder peak at 1.34 g/cm<sup>3</sup> was also detected (Figure 4.8). It could be that the major peak represents VLPs containing plant expression vector replicon DNA generated from RCR and the shoulder peak could represent empty VLPs. A similar density of 1.365 g/cm<sup>3</sup> has been reported for PCV VLPs produced in Tn5 insect cells (Liu *et al.*, 2008). Considering the BeYDV replicon is approximately 3.3-kb it is questionable whether it could be successfully packaged by the BFDV CP. A BeYDV replicon that was similar in size to the 2-kb BFDV genome would be more suitable for encapsidation. The higher observed density could also be the result of the presence of the C-terminal 6xHis tag. The absence of viral DNA has been seen to affect the density of the VLPs. Infectious BFDV virions when purified using CsCl have a density that ranges between 1.35-1.378 g/cm<sup>3</sup>, while intact VLPs range between 1.215-1.325 g/cm<sup>3</sup> (Raidal and Cross, 1994b; Stewart *et al.*, 2007; Todd *et al.*, 1991). A similar decrease in density has been reported for PCV VLPs (Nawagitgul *et al.*, 2000).

Although the BeYDV replicon used to express the BFDV CP is larger than the BFDV genome, standard PCR analysis of dialysed sample containing BFDV CP was found to be associated with the plant expression vector BeYDV *rep* (Figure 4.9). In order to confirm the presence of the replicon, RCA could be performed on the dialysed sample to retrieve the circular ssDNA replicon. Spectrophotometric analysis of protein and DNA concentrations in samples from CsCl gradient fractions revealed similar peaks corresponding to the BFDV CP and BeYDV DNA. This suggests that the BFDV CP could indeed be packaging the amplicon DNA. Plant viral CPs have been shown to effectively and spontaneously

package nucleic acids of mammalian viruses (Azizgolshani *et al.*, 2013). These pseudovirions have then been shown to release their nucleic acids into the cytoplasm of mammalian cells. This technology has also been used successfully using a plant-produced bamboo mosaic virus particles containing infectious bursal disease virus antigens for the immunisation of chickens (Chen *et al.*, 2012). Plant-produced CP derived from circoviruses could be similarly be used to package nucleic acids that have been replicated *in planta* using the BeYDV replication system. These pseudovirions could then potentially be used to create a potent vaccine capable of eliciting a strong humoral and cellular response in the target host.

In conclusion, different *Agrobacterium* strains or antibiotic selection pressures can have an effect on the growth rate of cultures required for infiltration into *N. benthamiana*. The *Agrobacterium* strain GV3101::pMP90RK doubling time was approximately 50% longer than that of strain LBA4404 and the morphology appeared different. This could be as a result of differences between the strains, in the level of antibiotic selection pressure or plant expression vectors. The strains enter stationary phase at an absorbance of between 1.67-1.77. A better understanding of *Agrobacterium* growth kinetics is required to determine whether the stationary phase has an impact on T-DNA transfer and optimise culture scale up for vacuum infiltration. The BFDV CP has been shown to be successfully expressed in *N. benthamiana* via vacuum infiltration. It has also been shown to self-assemble into VLPs that can be detected using electron microscopy and purified using CsCl centrifugation; however the yield was low, as the CP was undetectable when stained with Coomassie. The detection of VLPs in plants allows for refinement of the purification method, possibly by incorporating the sedimentation coefficient determined for PCV and CAV to improved isolation by centrifugation. These plant-produced BFDV VLPs resemble those produced in insect cells and infectious virions. It is possible that the VLPs are spontaneously incorporating amplicon DNA produced from the replicating BeYDV plant vector. The resulting pseudovirions could be used to further the efficacy of vaccines against BFDV.

## Chapter 5: The production of infectious BFDV agents

### 5.1. Introduction

In order for the efficacy of a viral vaccine to be established, the vaccine must be tested against an infectious virus during a challenge trial (Fenaux *et al.*, 2003). For BFDV the preparation of infectious virus has until now relied on the extraction of virus from whole tissue of deceased parrots known to be infected with the virus. This technique is still in use as the virus has yet to be grown *in vitro* using tissue culture (Patterson *et al.*, 2013a). The difficulties in culturing the virus have been put down to tissue specificity and *in vivo* growth requirements (Ritchie *et al.*, 1989a). Similar problems have been reported for other avian circoviruses such as GoCV and PiCV (Duchatel *et al.*, 2006; Scott *et al.*, 2006). Recently DuCV has been shown to replicate - albeit with a low efficiency - in continuous cell lines derived from Muscovy ducks (*Cairina moschata*) (Mészáros *et al.*, 2014). Research advances have therefore been focused on the economically important and culturable PCV, while developments with avian circoviruses have lagged behind.

PCV-1 was first described as a tissue culture contaminant in PK-15 cells and has been extensively studied (Karuppanan and Kwang, 2011). Considering the simple genome and replication strategy of circoviruses, an infectious molecular clone of PCV-2 could be readily synthesised and tested in tissue culture. A dsDNA replicative form of the PCV genome was shown to produce virus particles in a foetal porcine retina cell line (VIDO R1) (Liu *et al.*, 2001). Similar results have been reported for PK-15 cells that were transfected with circular dsDNA full-length PCV-2 genomes (Dezen *et al.*, 2010). In addition infectious molecular PCV-2 DNA clones have been used to produce active infections in pigs through direct injection into the liver and lymph nodes (Fenaux *et al.*, 2002). The ability to uncouple viral replication and virion production has enabled researchers to create PCV1-2 chimeras that produce immunity against PCV-2 but are attenuated in pigs (Fenaux *et al.*, 2004; Gillespie *et al.*, 2008). This technology can potentially be directly applied to BFDV and would circumvent the difficulties associated to virus purification from birds (Fenaux *et al.*, 2002).

A BFDV infectious molecular DNA clone could be made as for PCV. RCR of BFDV is dependent on the expression of the Rep and presence of the ori whose sequence contains a nonanucleotide motif (5'-TAGTATTAC-3') displayed on a stem-loop (Cheung, 2006; Heath *et al.*, 2006). A BFDV infectious molecular DNA clone would consist of a partial genome repeat, in which the *ori* sequence is repeated, cloned into a theta-replicating plasmid. The benefits of using an infectious molecular DNA clone for BFDV challenge experiments would be the ease of producing endotoxin-free DNA and spectrophotometric quantification. In comparison the quantification of purified virus requires

titrations of the virus in tissue culture or potentially in animals in order to calculate the viral titre (Fenaux *et al.*, 2002). Pure BFDV virus isolates could also be produced from infectious molecular DNA clones, thus ensuring viral preparations are free of other pathogens. A suitable system for generating virus particles would need to be explored.

Thus far infectious molecular DNA clones of PCV have been successfully used in pigs and porcine cell lines to produce infectious virus particles (Dezen *et al.*, 2010; Fenaux *et al.*, 2002; Liu *et al.*, 2001). As no continuous psittacine cell lines exist an infectious BFDV molecular DNA clone would require testing in psittacine embryonated eggs or an alternate system. Cheung (2006) demonstrated that PCV was capable of replicating in *E. coli*, suggesting that BFDV could potentially replicate outside of avian host cells. The mechanism of RCR has been successfully harnessed from work with geminiviruses in our laboratory to create a replicating plant expression system based on BeYDV capable of amplifying heterologous genes in *N. benthamiana* (Regnard *et al.*, 2010). Considering the replication strategies of both BeYDV and BFDV are similar, BeYDV could be capable of assisting the replication of BFDV *in planta* and the prospect is worth investigating.

The aim of this study was to prepare potentially infectious BFDV agents which can be used as challenge material for vaccine testing. First, virus particles were purified from whole tissue taken from organs and visualised using TEM. Second, a putatively infectious BFDV molecular DNA clone was designed and tested in mammalian cell culture. Replication was investigated using RCA, qPCR and replication-deficient clones. Replication of the infectious BFDV molecular DNA clone was also assessed using qPCR in *N. benthamiana* in the presence of BeYDV replication components.

## **5.2. Materials and methods**

### **5.2.1. Extraction of BFDV virions from whole tissue organs**

A deceased Palm cockatoo (*Probosciger aterrimus*) known to have succumbed from Pbfd was received by the laboratory for necropsy. Blood and feather samples were collected and internal organs weighing approximately 20 g collected. The organs were immediately stored on ice after which they were added to 150 mL extraction buffer (PBS and 0.5 % [wt/vol] SDS). The tissue was homogenised for 10 minutes at 4 °C using a T 25 digital ULTRA-TURRAX® (IKA® Works Inc.), after which the homogenate was filtered through Miracloth (Merck). The filtrate was centrifuged at 5 000 g for 10 minutes at 4 °C. The supernatant was then adjusted to include 1 M NaCl and 10% (wt/vol) polyethylene glycol (PEG) 6000 and incubated for 1 hour at 4 °C. The solution was then centrifuged at 11 000 g for 20 minutes after which the supernatant was discarded and pellet resuspended in PBS containing CsCl at a density of 1.30 g/cm<sup>3</sup>. CsCl density was confirmed using a R5000 hand

refractometer (Atago). The resuspended pellets were centrifuged in 5 mL Ultra-Clear™ centrifugation tubes (Beckman Coulter) using a SW 55 Ti rotor (Beckman Coulter) at ≈ 40 500 rpm (RCF<sub>max</sub> 200 000 g) for 20 hours at 4 °C. After isopycnic equilibrium visible bands present in the CsCl gradient were collected by piercing the side of the tube using a needle and 1 mL syringe. Samples were then dialysed in PBS using a Slide-A-Lyser™ Dialysis Cassette, 10K MWCO (Thermo Scientific). The BFDV genomic DNA concentration of the dialysed sample was determined using *rep* specific qPCR as described in Section 2.2.5.

### 5.2.2. DNA extraction, rolling circle amplification and PCR

Total DNA from feather, blood and organ samples were extracted and analysed as described in Section 0. Total DNA from dialysed samples was extracted as described in Section 0. The DNA from the dialysed samples was analysed using qPCR and PCR. The reaction, as described in Appendix B.7 was performed using GoTaq® DNA Polymerase (Promega) according to manufacturer's instructions and primers (5'-AGATCTAGTCCGTCCTCAAGGAGGGATCTG-3' and 5'-AAGCTTCTAATAATTGATGGGGTGGGCGAG-3') that amplified a 900-bp BFDV *rep* fragment. The amplified PCR products were resolved on 0.8 % (wt/vol) TBE agarose gels.

### 5.2.3. Transmission electron microscopy

Analysis of dialysed BFDV virus particles was performed using TEM. Copper grids (mesh size 200) were made hydrophilic by glow discharging at 25 mA for 30 s using a Model 900 SmartSet Cold Stage Controller (Electron Microscopy Sciences). The grids were then placed on a solution with a 1:1 ratio of sample and 0.5 mg/mL tobacco mosaic virus (TMV) for 30 s and then washed twice in sterile water for 5 s. The grids were negatively stained on uranyl acetate for 30 s and again for a further 30 s and viewed using a Tecnai™ F20 Scanning Transmission Electron Microscope (FEI).

### 5.2.4. Design and synthesis of the BFDV infectious molecular DNA clone

The infectious molecular DNA clone was based on isolate BKS1ZA\_84 (GenBank accession number GQ165756) previously described by Varsani *et al.* (2010) from a Budgerigar (*M. undulatus*), given that the probable challenge model would involve this animal. The partial genome repeat of BKS1ZA\_84 was synthesised *in silico* by Geneart (Figure 5.1). The sequence included a 5' *Pst*I and 3' *Hind*III and *Xba*I restriction enzyme sites for cloning purposes. The stem-loop as described by Bassami *et al.* (1998) was identified and was confirmed through ssDNA secondary structure analysis using the mfold web server (Zuker, 2003)(Figure 5.2).

### 5.2.5. Cloning and mutation strategy

The BFDV infectious molecular DNA clone was modified using PCR to disrupt either the *rep* or the nonanucleotide motif situated in the first stem-loop. To disrupt the nonanucleotide motif the 5'-TAGTATTAC-3' was modified to include a *Bam*HI restriction enzyme site (Figure 5.3). An initial PCR reaction, as described in Appendix B.8, was performed using ACCUZYME™ DNA polymerase (Bioline) and primers that amplified a 401-bp PCR product (Table 5.1). The amplified PCR product was resolved on 1 % (wt/vol) TBE agarose gels and gel purified. A final PCR amplified a 421-bp BFDV BKS1ZA\_84 N9 *Pst*I mutant PCR product under identical thermocycling parameters (Table 5.1). The amplified PCR product was resolved on 1 % (wt/vol). The gel-purified BFDV BKS1ZA\_84 N9 *Pst*I mutant fragment was then used to replace the existing sequence through restriction enzyme digestion with *Pst*I and *Sac*I. The ligated constructs were transformed into competent *E. coli* DH5 $\alpha$ , and the plasmid DNA was isolated from overnight cultures. The integrity of the clones was confirmed through sequencing (Macrogen Inc.).

```

1      CTGCAGAGGT GCCCCACAGG CGGCGGTTAG TATTACCCGC CGCCTGGGGC ACCGGGGCAC
61     CGCAGCCATT GGCTGCCGTG CCGAGGTGCC CCGCCTTAGG GAGGAGTAAA TGGCGCCGTT
121    AAACCGGTGCC GTAATTTCCG GAGGATCACA GTCGCCCGGG AACAATGCCG TCCAAGGAGG
181    GCTCTGGCTG TCGCCGTTGG TGTTTCACCC TTAACAACCC TACAGACGGC GAGATCGAAT
241    TCGTCCGTAC TCTCGGGCCT GACGAATTCT ACTATGCCAT CGTTGGACGG GAAAAGGGCG
301    AGCAAGGTAC CCCCATTG CAAGGCTACT TTCATTTCAA AAATAAGAAG CGACTGAGCG
361    CGCTTAATAA AATGCTGCCG CGAGCTCATT TTGAGCGCGC TAAAGGGAGT GATGCAGATA
421    ATGAGAAGTA TTGCAGTAAA GAGGGGGACG TTATACTTAC CCTGGGCATT GTGGCGAGAG
481    ATGGTCACCG CGCTTTCGAC GGAGCTGTTG CTGCCGTGAT GTCCGGACGC AAAATGAAGG
541    AAGTCGCGCG AGAGTTCCCA GATATCTACG TCAGGCATGG GCGGGGCTTG CATAACCTCT
601    CTCTATTGGT CGGTTCCCGC CCACGTGATT TCAAGACAGA AGTTGACGTC ATCTACGGGC
661    CTCTGGGTG TGGCAAGAGT AAATGGGCCA ATGAGCAGCC TGGGACTAAA TATTATAAAA
721    TGCGCGGTGA ATGGTGGGAT GGATATGATG GGGGAAGATGT TGTCAATTG GACGACTTTT
781    ATGGGTGGCT ACCTTATTGT GAGATGCTCC GCCTTTGCGA CCGTTATCCA CATAAAGTGC
841    CAGTTAAGGG CGCTTTTGTG GAGTTTACCA GCAAGAGGAT CATTATCACG AGCAATAAGT
901    CCCCCGAGAC CTGGTACAAG GAGGACTGTG ACCCGAAGCC ACTGTTCCGG AGATTCACTC
961    GTGTTTGGTG GTACACTGAC AAGTTGGAAC AAGTCCGGCC TGATTTCCTT GCCCACCCCA
1021   TCAATTTTG ATACCCCCCG GAGGTTATTA ATAAAAGCCG GCCGTCGGGC CGAAGGCCCG
1081   ATGGCGCAGG GCGGGACGCC CTGCCGGAGG GCTCGCAGGG CCGTCAGGCC CGAGAGCCCG
1141   ACCAGCCCG AGGGCCTAGT CTGTGTCGGG GGGGGGGGCC CAGGGGGGTC CCCCCGACAC
1201   GACGAGAATG GTAGCGCCGA AGCGCCAAT AAACACTCGA AAAGGTATTT GCCGTTTGAG
1261   TCTTTATTCA GTTCTGGGAT TATTGGGGGC AAACTGACGG AATTGAACAT ACAAGTAAG
1321   TTGACATACA TAAGATATCG TTACGTCTGG CTGAGGAAG CTGAAGGCCA GGCCGTAGTG
1381   CTTGACGTTT GCTCCTGCCG CGTCGGGTCC TCCTTGTAGT GGGATCCATC CGGTTCTGAC
1441   GCTGTTGAGC CACAATGCCG CAGACTGGTT CGTTGTGCTC AGGTCGTTTA TTGTTATTTG
1501   TGGTTTTGGT CTGAGGAGGC GTTTGAATCC TCTGCTAAGA TACCATTCTT TTGCACCGTC
1561   GAAAGGTGCC ACCGGTCTT GTGTTGGTC AATCTTAGTT TTAAATTTAG TTATTCTGGA
1621   GTCTTGGATT ACGGCAGTGT GTCCGAATCC GGCTGAACTA ATGGTGTAGT GGCCCATGT
1681   GGGCCTGAGT TCCATTTTAG CTAACTTGAT CCGGTAGTCT TCAAATTTCA GTGTTTGTGG
1741   GTTTGGTGTG TTTGTAAAGGA AGTCTGACAA TGTAAAGGTT ATGTAGTCAG AGTTCAAAT
1801   TACTTGGCCA GTACTCGTTG TTTGTTTTTG AATATCGAAT TTGAATTGGC GCTTGAGTCT
1861   GAGCGTGTAA ATTCTATTGG TCGAGAAACG GCGTCTGCGG AAGTATCTAC GTCGACGGCG
1921   GTATCGCCGG ATATGACGTC TGCGGTAGTA TGGGCGGGCA TATCTTCGTT TAATCTGAAA
1981   TATAGCGCAT GCGCAGTTAG AGGTGCCCCA CAGGCGGCGG TTAGTATTAC CCGCCGCCTG
2041   GGGCACCGGG GCACCGCAGC CATTGGCTGC CGTGCCGAGG TGCCCCGCTT TAGGGAGGAG
2101   TAAATGGCGC CGTTAAACGG TGCCGTAATT TCCGGAGGAT CACAGTCGCC CGGGAACAAG
2161   CTTCTAGA

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Figure 5.1: Sequence of infectious molecular BFDV DNA clone based on isolate BKS1ZA\_84 (GenBank accession number GQ165756). BFDV *cp* (blue), stem-loop (black box) as described by Bassami *et al.* (1998), nonanucleotide motif (yellow), and *rep* (red).

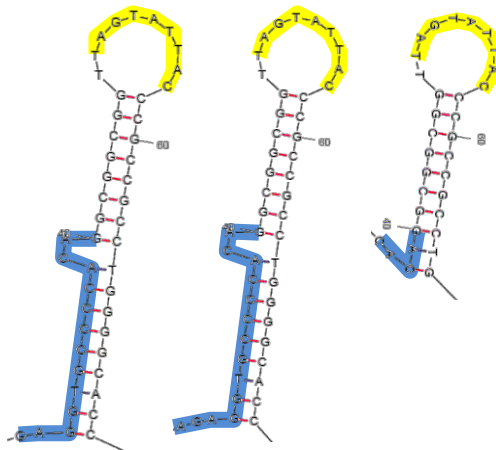


Figure 5.2: **Predicted BFDV stem-loop structures as determined through the use of the mfold web server.** BFDV *cp* (blue), and nonanucleotide motif (yellow).

To disrupt translation of the *rep* the ATG start codon was replaced by a *SpeI* restriction enzyme site (Figure 5.3). Initial PCR reactions, as described in Appendix B.9, introduced the modifications and amplified a 265-bp BFDV BKS1ZA\_84 *rep SacI* mutant and 239-bp BFDV BKS1ZA\_84 *rep PstI* mutant PCR product (Table 5.1). These PCR products were gel purified and amplified, as described in Appendix B.10, together to produce a 504-bp BFDV BKS1ZA\_84 *rep SpeI* mutant PCR product (Table 5.1). The gel purified BFDV BKS1ZA\_84 *rep SpeI* mutant fragment was then used to replace the existing sequence through restriction enzyme digestion with *PstI* and *SacI*. The ligated constructs were transformed into competent *E. coli* DH5 $\alpha$ , and the plasmid DNA was isolated from overnight cultures. The integrity of the clones was confirmed through sequencing (Macrogen Inc.).

The BFDV genome constructs were then inserted into a modified pTRAc plant expression vector. The plant expression cassette of pTRAc was removed through restriction enzyme digestion with *SapI* and *PmeI*. The resulting linearised plasmid, without the plant expression cassette, contained the left and right borders for T-DNA transfer into plant cells. The linear DNA fragment was ligated together with a 30-bp DNA fragment containing a MCS with the restriction enzyme sites: *SapI*, *PstI*, *HindIII*, and *PmeI* (Table 5.1). The BFDV infectious molecular DNA clones were then cloned into the modified pTRAc using *PstI* and *HindIII*. The ligated constructs were transformed into competent *E. coli* DH5 $\alpha$ , and the plasmid DNA was isolated from overnight cultures. The integrity of the clones was confirmed through sequencing (Macrogen Inc.).

Table 5.1: Primers for PCR amplification used during molecular cloning of BKS1ZA\_84 mutants. Underlined sequence indicates the position of the restriction enzyme sites used during cloning.

Product Description	Size (bp)	Sense primer	Antisense primer		Cloning site
		Sequence (5' - 3')	Cloning site	Sequence (5' - 3')	
BFDV BKS1ZA_84 N9 <i>Bam</i> HI mutant	401	AGGCGGCGGTTAGGATCCGCCGCCCTGGGGCAC	<i>Bam</i> HI	TCTGCATCACTCCCTTTAGCGC	-
BFDV BKS1ZA_84 N9 <i>Pst</i> I mutant	421	GCCCTGCAGAGGTGCCCCACAGGCGGCGGTTAGGA	<i>Pst</i> I, <i>Bam</i> HI	TCTGCATCACTCCCTTTAGCGC	-
BFDV BKS1ZA_84 rep <i>Pst</i> I mutant	239	TGAGCGCGACGTAATACGACTC	-	GACGGACTAGTTCCCGGGC	<i>Spe</i> I
BFDV BKS1ZA_84 rep <i>Sac</i> I mutant	265	GCCCGGGA <u>ACTAGT</u> CCGTC	<i>Spe</i> I	TCTGCATCACTCCCTTTAGCGC	-
BFDV BKS1ZA_84 rep <i>Spe</i> I mutant	504	TGAGCGCGACGTAATACGACTC	-	TCTGCATCACTCCCTTTAGCGC	-
pTRAc linker <i>Sap</i> I <i>Pme</i> I	30	<u>CATTGAAGAGCCTGCAGGTGAAGCTTGTTT</u>	<i>Sap</i> I, <i>Pst</i> I, <i>Hind</i> III, <i>Pme</i> I	<u>AAACAAGCTTCACCTGCAGGCTCTTCA</u>	<i>Sap</i> I, <i>Pst</i> I, <i>Hind</i> III, <i>Pme</i> I

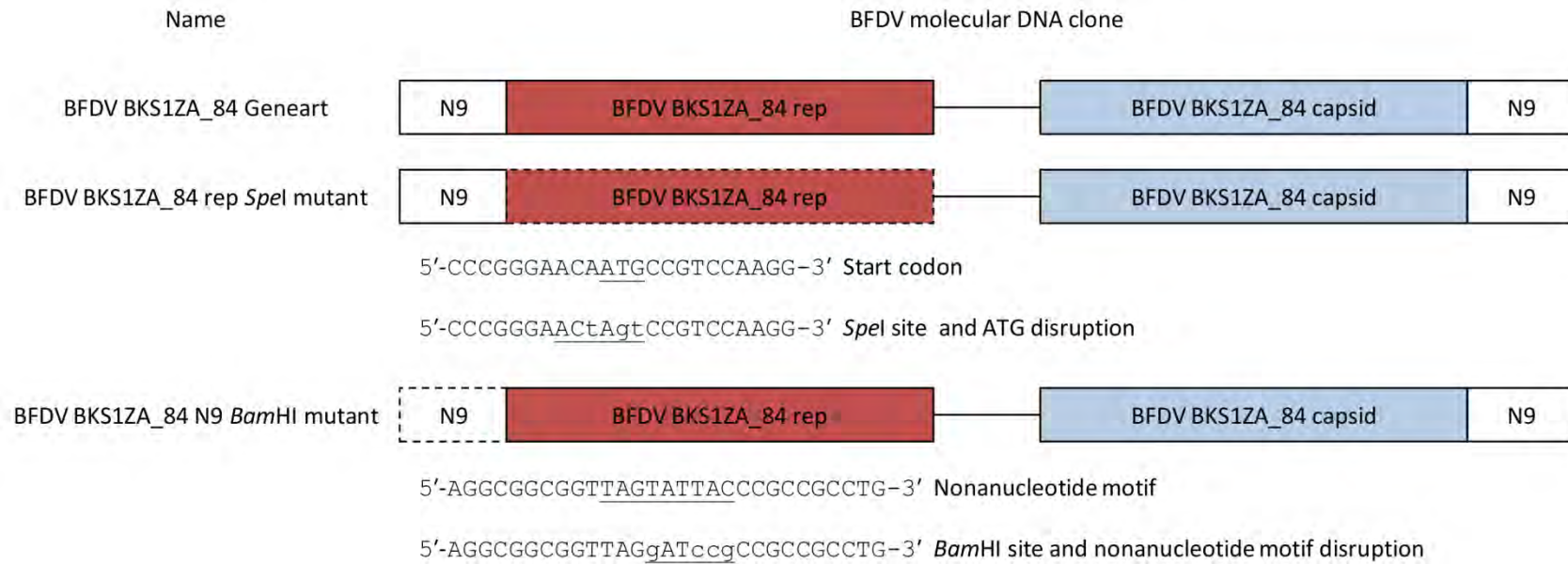


Figure 5.3: **BFDV BKS1ZA\_84 infectious molecular DNA clones used in this study.** N9 – nonanucleotide motif (5'-TAGTATTAC-3'), BFDV *rep* (red), BFDV *cp* (blue). Elements subject to disruption (dashed boxes) with sequence description below. Nucleotides changed in the sequence are indicated by lower-case lettering.

### **5.2.6. Transfection of 293TT cells**

Endotoxin-free plasmid DNA was prepared using a NucleoBond® Xtra Midi EF (Macherey-Nagel, Germany) as per manufacturer's instructions from *E. coli* cultures containing the BFDV BKS1ZA\_84 infectious molecular DNA clones.

The 293TT cell line, kindly provided by Dr. J Schiller, was originally generated from a human embryonic kidney (HEK) cell line, HEK 293T, that was stably transfected with an SV40 Large T Antigen cDNA expression cassette. The 293TT cell line was maintained at 37°C, in a 5 % atmospheric CO<sub>2</sub> and 95 % humidity incubator. The nutrient medium was exchanged every 2-3 days, with DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (Life Technologies) supplemented with 10 % HyClone™ Fetal Bovine Serum (South America), Research Grade (GE Healthcare), 1 % Penicillin/Streptomycin (100x, Sigma-Aldrich), 1 % MEM Non-essential Amino Acid (NAA) Solution (100x, Sigma-Aldrich), 0.5 % 50 mg/mL Hygromycin B (Roche).

A day prior to transfection, cells in 3 mL of complete growth medium (DMEM and 10% Fetal Bovine Serum) were plated in a 6-well plate for a final density of  $5 \times 10^5$  cells per well. A FuGENE®HD:DNA ratio of 3.0:1 was used for all transfections. A 931 µL solution was prepared in sterile deionised water for each BFDV infectious molecular DNA clone at a concentration of 0.020 µg/µL. To this, 59 µL of FuGENE® HD reagent (Promega) was added, briefly vortexed and incubated for between 5-10 minutes at room temperature. A 150 µL of the complex was added per well to the cells, and mixed thoroughly and incubated for 24 hours. After incubation the nutrient medium was exchanged and for day 0, cells were immediately harvested for total DNA extraction using DNeasy® Blood & Tissue Kit (Qiagen), while the remaining cells were incubated for 3 days before being harvested.

### **5.2.7. Transfer and analysis of BFDV infectious molecular DNA in *N. benthamiana***

BFDV infectious molecular DNA clones in the modified pTRAc plant vector were transformed in *A. tumefaciens* as described in Section 3.2.3 and then infiltrated into *N. benthamiana* as described in Section 3.2.4. Total DNA was extracted from plant leaf tissue using an Extract-N-Amp™ Plant PCR Kit (Sigma-Aldrich) and used in RCA, as described in Section 0, and qPCR analysis.

### 5.2.8. Quantitative real-time PCR

Each qPCR reaction was performed in triplicate using a LuminoCT® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich) as per manufacturer's instructions and primers (5'-TATTGGTTGAGAAGCGGCGTCT-3' and 5'-GACGTAGATATGCCCGCCCAT-3') specific to a 96-bp region of the BFDV *cp*. Cloned plasmid DNA containing the amplicons in serial dilution served as the standard curve for the determination of gene copy number for *cp*. Thermocycling was performed using a Rotor-Gene RG-6000 (Qiagen), and the parameters consisted of a 10 minute hold at 95 °C followed by cycling (40 repeats) between a 15 s hold for denaturation at 95 °C, a 15 s hold at 53 °C for annealing, and a 15 s hold 60 °C for elongation. Template specificity was confirmed from the reaction melt curve analysis. In addition BFDV *rep* primers were also used as described in Section 2.2.5. SD and SE were calculated were applicable and probability was calculated using a Student's t-Test with two-tailed distribution and two-sample unequal variance.

## 5.3. Results

### 5.3.1. Extraction and purification of virus particles from diseased whole tissue

To determine the presence of BFDV in each Palm cockatoo sample, DNA was extracted and RCA performed after which the DNA was cleaved using *Bam*HI. The RCA reaction was confirmed by the amplification and cleavage of the pUC19 plasmid DNA control to produce a linear 2.7-kb dsDNA fragment (Figure 5.4A lane 5). An additional control, a 2-kb circular BFDV genomic dsDNA, was also successfully amplified using RCA and cleaved to produce a linear fragment (lane 4). The extracted DNA from all three samples indicated the presence of a linear 2-kb dsDNA fragment that is consistent with the size of the BFDV genome used as a control (lane 1-3). A 2-kb fragment was subsequently cloned, sequenced and confirmed to be BFDV.

Approximately 25.57 g of internal organs were collected and used for extraction of virus particles. The extract was fractionated using CsCl gradient centrifugation. After equilibrium sedimentation two white bands were visible in the CsCl density gradient (Figure 5.4B). These bands were isolated and dialysed to remove CsCl. PCR analysis of the dialysed samples indicated that the white band with a higher CsCl density tested positive for BFDV DNA (Figure 5.4C). The concentration of virus present in dialysed sample was determined using qPCR to be approximately  $10^9$  genomes/ $\mu$ l. TEM of the dialysed sample testing positive for BFDV indicated the presence of virus particles with a diameter of approximately 21 nm (Figure 5.5).

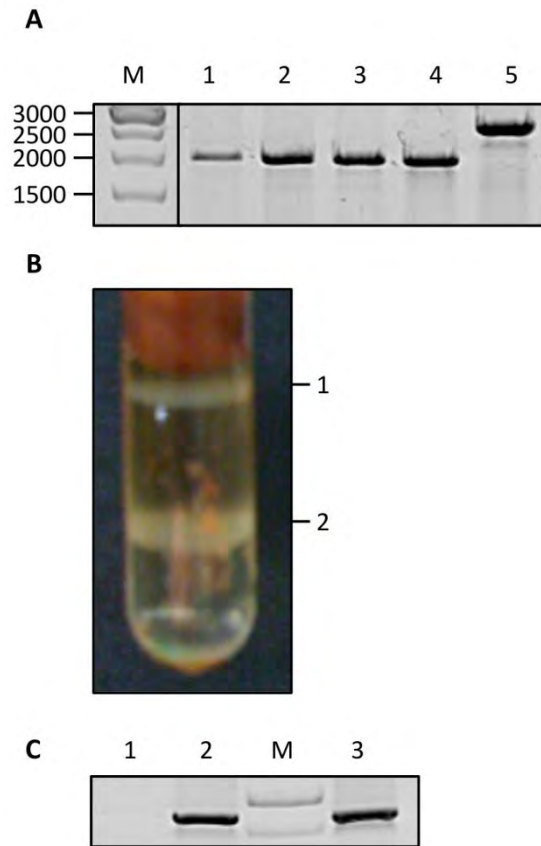


Figure 5.4: **Purification of BFDV virus particles from whole tissue taken from a Palm cockatoo (*P. aterrimus*).** (A) RCA product linearised with *Bam*HI to detect the presence of BFDV genomic DNA in various tissue samples, M – DNA marker, 1 – feather, 2 – blood, 3 – organs, 4 – positive control (circular dsDNA BFDV genome), 5 – RCA control (pUC19 plasmid). (B) CsCl gradient centrifugation to purify BFDV virus particles from internal organs, band 1 and 2 collected for analysis. (C) Standard PCR analysis of dialysed CsCl bands collected 1 – band 1, 2 – band 2, M – DNA marker, 3 – positive PCR control.

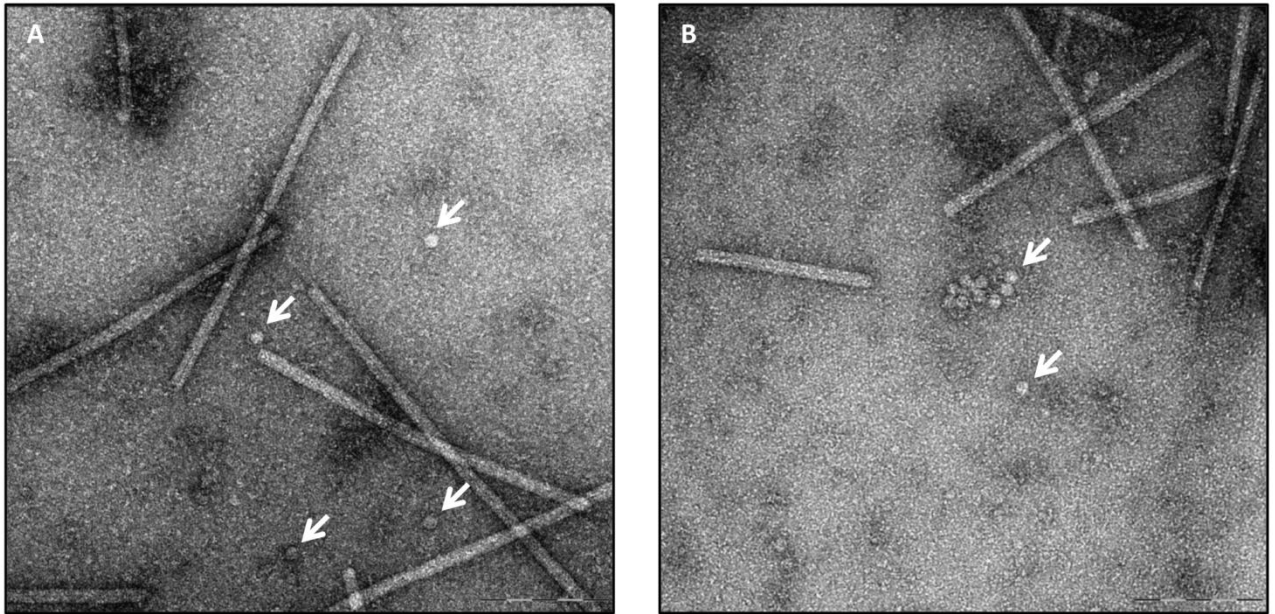


Figure 5.5: **Transmission electron micrographs of purified BFDV virus particles after CsCl gradient centrifugation.** (A and B) BFDV virus particles indicated by white arrows, TMV (18 nm width) included as a size control. Scale bar represent 200 nm.

### 5.3.2. Replication of a BFDV dsDNA molecular clone in 293TT cells

To determine whether a synthesised BFDV dsDNA molecular clone is functional at the level of DNA replication, endotoxin-free DNA was used to transfect 293TT cells. Total DNA from cells extracted on day 0 and day 3 after transfection were analysed using RCA and qPCR. Amplified DNA produced during RCA was linearised with *Bam*HI and resolved using gel electrophoresis (Figure 5.6A lane 1). The RCA reaction was confirmed by the presence of a linear 2.7-kb dsDNA fragment for the reaction containing the pUC19 plasmid control DNA. The presence of a linear 2-kb BFDV full-length genome was absent on day 0 and present on day 3 for the BFDV dsDNA molecular clone (Figure 5.6A lane 4 and 5). This circular genome was cloned, sequenced and confirmed to be identical in sequence to the input DNA. A linear 2-kb BFDV genome was absent on both day 0 and 3 for the negative control that did not contain sequence elements responsible for BFDV replication (Figure 5.6A lane 2 and 3). Analysis of the total DNA samples using qPCR confirmed that there was a significant increase in the copy number of the *cp* from day 0 to day 3 for the BFDV clone (Figure 5.6B). There was a significant decrease in the copy number of the *cp* from day 0 to day 3 for the negative control DNA.

To confirm whether the 2-kb genome detected using RCA and the increase in gene copy number observed using qPCR were as a result of BFDV replication, the synthesised BFDV dsDNA molecular clone was altered at either the *rep*, to prevent translation, or at the nonanucleotide motif found within the stem-loop, to prevent DNA nicking by Rep. Endotoxin-free DNA was prepared for the

mutants and used to transfect 293TT cells. Total DNA from cells extracted on day 0 and day 3 after transfection were analysed using RCA. Amplified DNA was linearised with *Bam*HI and resolved using gel electrophoresis (Figure 5.6C). The presence of a linear 2-kb BFDV full-length genome was absent on day 0 and present on day 3 for the BFDV dsDNA molecular clone (Figure 5.6C lane 3 and 4). A linear 2-kb BFDV genome was absent on both day 0 and 3 for the negative control (lane 1 and 2) together with the mutant containing the *rep* alteration (lane 5 and 6) and the nonanucleotide alteration (lane 7 and 8).

### **5.3.3. BeYDV-assisted replication of a BFDV dsDNA molecular clone in *N. benthamiana***

To determine whether the BFDV dsDNA molecular clone was capable of replicating in *N. benthamiana*, the clone was inserted into a plant expression vector and *Agrobacterium*-infiltrated into plant leaves in the presence or absence of the BeYDV replicating vector. Total DNA from leaf tissue was extracted on day 1-4 after infiltration and analysed using qPCR (Figure 5.7A). In the absence of the BeYDV vector the BFDV *cp* copy number remained constant over the 4 day period. In the presence of the BeYDV vector the BFDV *cp* increased by two orders of magnitude from day 1 to day 3, after which the copy number remained constant.

The BFDV genome mutant containing the altered nonanucleotide sequence was tested to determine whether the observed replication was as a result of the BFDV dsDNA molecular clone (Figure 5.7B). No difference in *cp* copy number in the presence of the BeYDV vector was observed between the mutant and the BFDV dsDNA molecular clone. To confirm this was result was not an artefact of BeYDV replicating, further analysis was performed.

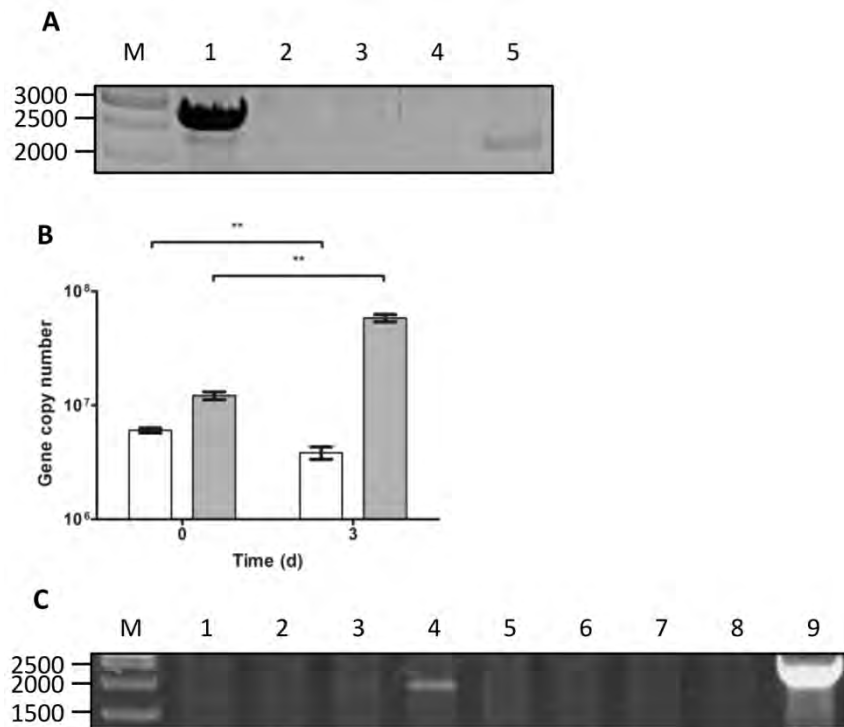


Figure 5.6: **Replication of a BFDV dsDNA molecular clone in 293TT cells.** (A) RCA product linearised with *Bam*HI to detect the presence of BFDV genomic DNA on day 0 and day 3 post transfection, M – DNA marker, 1 – RCA control (pUC19 plasmid), non-replicating control 2 – day 0, 3 – day 3, BFDV dsDNA molecular clone 4 – day 0, 5 – day 3. (B) qPCR analysis of *cp* copy number on day 0 and day 3 of non-replicating control (white) and BFDV dsDNA molecular clone (shaded), \*\* -  $p < 0.01$ . (C) RCA product linearised with *Bam*HI to detect the presence of BFDV genomic DNA on day 0 and day 3 post transfection, M – DNA marker, non-replicating control 1 – day 0, 2 – day 3, BFDV dsDNA molecular clone 3 – day 0, 4 – day 3, BFDV dsDNA molecular clone (*rep* mutant) 5 – day 0, 6 – day 3, BFDV dsDNA molecular clone (nonanucleotide sequence mutant) 7 – day 0, 8 – day 3, 9 – RCA control (pUC19 plasmid).

The BFDV molecular clone and BeYDV vector were infiltrated into plants and total DNA was extracted on day 5 after infiltration and analysed using RCA. Amplified DNA produced during RCA was linearised with *Bam*HI and resolved using gel electrophoresis (Figure 5.8A). No 2-kb linear fragment was detected for the BFDV dsDNA molecular clone only or when co-infiltrated with the BeYDV replicating vector (lane 1 and 2). A 2.5-kb BeYDV replicon was detected in plants infiltrated with the BeYDV vector (lane 2 and 3).

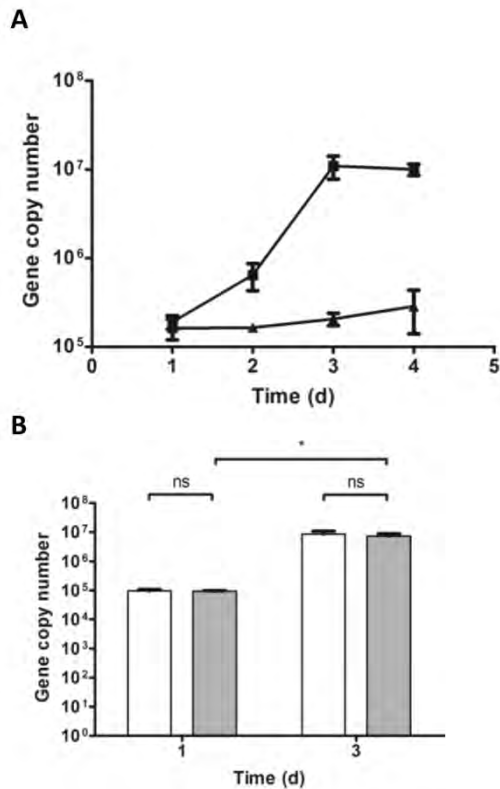


Figure 5.7: **BeYDV-assisted replication of a BFDV dsDNA molecular clone in *N. benthamiana*.** (A) qPCR analysis of *cp* copy number between day 1 and day 4 post infiltration, BFDV dsDNA molecular clone in the presence (■) and absence (▲) of the BeYDV replicating vector. (B) qPCR analysis of *cp* copy number on day 1 and day 3 of BFDV dsDNA molecular clone (white) and BFDV dsDNA molecular clone (nonanucleotide sequence mutant, shaded) in the presence of the BeYDV plant replicating vector, ns – not significant, \* -  $p < 0.05$ .

To distinguish between the replication of the BFDV genome and the BeYDV replicon, the copy number for both was determined using *cp* and *rep* specific qPCR. A BeYDV replicating vector containing *cp* would allow for the copy number of the replicon to be determined. The BFDV dsDNA molecular clone was co-infiltrated into plant leaves in the presence of an empty BeYDV replicating vector or one containing a copy of the BFDV *cp*. Total DNA from leaf tissue was extracted on day 1 and 4 after infiltration. The total DNA was analysed using qPCR to determine gene copy number of *rep* for the BFDV genome and *cp* for both the BFDV genome and the BeYDV replicon containing *cp* (Figure 5.8B and C). Analysis using primers specific for the *cp* indicated an increase in gene copy number from day 1 to day 4 for both infiltrations (Figure 5.8B). The *cp* copy number increased by over an order for magnitude when the BFDV dsDNA molecular clone was infiltrated alongside an empty BeYDV replicating vector. However, when infiltrated with the vector containing a *cp* the BFDV gene copy number increased by three orders of magnitude.

Analysis of the *rep* copy number produced a similar increase in gene copy number for both infiltrations and served as a control (Figure 5.8C). An increase of one order of magnitude was

observed for the BFDV dsDNA molecular clone infiltrated alongside an empty BeYDV replicating vector. A similar increase in copy number of *rep*, by an order of magnitude, was observed when infiltrated alongside the BeYDV replicating vector containing a *cp*. When compared to the empty BeYDV replicating vector, the vector, containing *cp*, had a *rep* copy number on day 4 that was significantly lower.

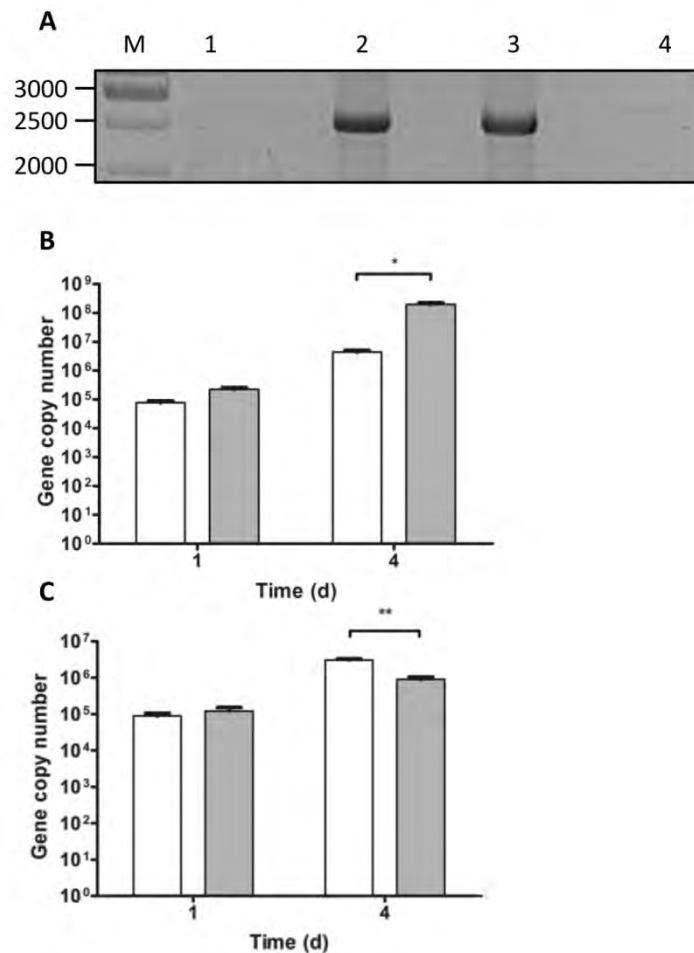


Figure 5.8: **Distinguishing between BFDV dsDNA molecular clone and BeYDV replication in *N. benthamiana*.** (A) RCA product linearised with *Bam*HI to detect the presence of BFDV genomic DNA on day 5 post infiltration, M – DNA marker, 1 – BFDV dsDNA molecular clone only, 2 – BFDV dsDNA molecular clone and BeYDV replicating vector, 3 – BeYDV replicating vector only, 4 – RCA control (pUC19 plasmid). (B) qPCR analysis of *cp* copy number on day 1 and day 4 of BFDV dsDNA molecular clone in the presence of the BeYDV plant replicating vector only (white) or containing the BFDV *cp* (shaded), \* -  $p < 0.05$ . (C) qPCR analysis of *rep* copy number on day 1 and day 4 of BFDV dsDNA molecular clone in the presence of the BeYDV plant replicating vector only (white) or containing the BFDV *cp* (shaded), \*\* -  $p < 0.01$

## 5.4. Discussion

### 5.4.1. Extraction and purification of virus particles from diseased whole tissue

Whole tissue collected from psittacines infected with BFDV can be used to successfully isolate virion particles. In this study whole tissue from internal organs were used for virus purification as has been previously described (Wylie and Pass, 1987). Virus particles have also been successfully extracted from feathers of diseased animals (Raidal and Cross, 1994b; Ritchie *et al.*, 1990; Shearer *et al.*, 2008a). Before the production of recombinant BFDV CP, purified virus was the sole source of BFDV antigen for use as a reagent in HI tests (Raidal and Cross, 1994a; Shearer *et al.*, 2009a). As the virus cannot be cultured, virus preparations from whole tissue samples are still required for material used in challenge experiments. The virus isolated from this study was from a Palm cockatoo that had succumbed to Pbfd. The virus was detected using RCA in the feathers, blood and organs and could be said to be systemic (Figure 5.4). Virus was purified using CsCl density gradient centrifugation from internal organs collected during necropsy.

The yield for purified virus in this study was  $10^9$  genome copies/ $\mu\text{L}$  which is three orders of magnitude higher than previously reported values. BFDV particle yields for feathers after extraction and density gradient centrifugation have been reported to be between  $10^6$  particles/ $\mu\text{L}$  and  $10^7$  particles/ $\mu\text{L}$  (Raidal and Cross, 1994a). These yields are reportedly low making the process unsuitable for the large scale preparation of reagent used in HI testing (Shearer *et al.*, 2009a). Interestingly peak viraemia during BFDV infection has been reported as being in the region  $10^6$  genome copies/ $\mu\text{L}$  (Bonne *et al.*, 2009). Reasons for the difference in virus quantity could be in the choice of tissue used for the extraction or the assay used to quantify the virus preparation. Approximately 220 g of feather material from eight diseased psittacines was used in the extraction of virus as described in Raidal and Cross (1994b). It is unclear whether the author used whole feathers or a specific region of the feather. The BFDV virus is known to affect the replicating cells in the feather follicle and basal epithelial layer resulting in feather dystrophy (Latimer *et al.*, 1993). The use of whole feathers would therefore not result in high yields as only a small portion of the feather contains viral particles and any healthy feathers would suggest that any infection in the follicle is relatively new. Previous studies have relied on HA to quantify virus concentration whereas in this study qPCR was used (Raidal *et al.*, 1993c). The variability in results produced from these assays is high as a result of batch differences in the erythrocytes, viral preparations and polyclonal antibodies used (Raidal and Cross, 1994a). Quantification using qPCR is seen as a more reliable method of determination (Shearer *et al.*, 2009b).

Analysis of virus particles using TEM indicated that that the diameter of particles was approximately 21 nm (Figure 5.5). This diameter is larger than most of the particle sizes described in the literature (Figure 1.2) and falls within the range described in two publications and one chapter (Crowther *et al.*, 2003; Gerlach, 1994; Jacobson *et al.*, 1986). The size of 20.5 nm as put forward by Crowther *et al.* (2003) was calculated based on the three dimensional structure for BFDV virions, as determined by cryoelectron microscopy. These values are higher than expected and may have been as a result of the preparation. For comparison purposes TMV was combined with the sample and the widths of the particles were also found to be approximately 21 nm. This value is larger than the literature values which describe the TMV virion as having a width of 18 nm and length of 300 nm (Tidona and Darai, 2011). This further supports the suggestion that the preparation of the sample on the copper grids may have affected the staining of the particles. The purified virions (21 nm) were also larger in comparison to the plant-produced BFDV VLPs that were on average 17 nm in diameter (Section 4.3.2). The size reported for the BFDV particles purified in this study, however, falls within range of reported values in the literature. To confirm infectivity the purified virus could be tested in embryonated eggs before being used as challenge material for vaccine testing.

There are a number of disadvantages to using virus purified from whole tissue from infected psittacines. Firstly the purification process is expensive and time consuming and more often than not produces low yields (Latimer *et al.*, 1993; Shearer *et al.*, 2009a). The yields are low in part because of the availability of infected whole tissues used for extraction. This also hampers consistency as birds sourced from different locations may have different BFDV isolates. Secondly, the purified virus can be contaminated with host proteins affecting the perceived yield and use in serological assays or with other viruses, considering psittacines infected with BFDV more often than not have accompanying secondary infections (Stewart *et al.*, 2007; van den Brand *et al.*, 2007). Finally there is the ethical dilemma faced with producing purified virus from whole tissue of diseased psittacines. Continual harvesting of feather material from birds with chronic PBFV or the intentional infection of these birds for the purpose of producing infectious virus is ethically questionable, therefore researchers mainly rely on naturally infected birds that succumb to the PBFV as a source of virions (Shearer *et al.*, 2009a). Although there is an absence of any obvious serotypes for BFDV and a high probability of cross-isolate protection, the testing of vaccine efficacy would be better served using virus and vaccine antigen originating from the same isolate (Ritchie *et al.*, 1992b; Shearer *et al.*, 2009a).

#### **5.4.2. Replication of a BFDV dsDNA molecular clone in 293TT cells**

To circumvent the disadvantages faced when using BFDV virus purified from whole tissue, a synthetic BFDV dsDNA molecular clone was synthesised and tested in tissue culture. 293TT cells were transfected and the presence of circular BFDV genomic DNA three days later was identified using RCA (Figure 5.6A). This is the first time BFDV has been demonstrated to replicate in mammalian tissue culture. Using qPCR analysis an approximately ten-fold increase in DNA copy number could be observed (Figure 5.6B). This increase is modest in comparison to active viraemia seen in psittacines infected with purified virus in which a viral load increases by six orders of magnitude; however this is over a two week period. The underperformance in replication efficiency could be attributed to the cell type used, considering that mammalian cells are not the natural host for BFDV. In addition, since the synthetic BFDV dsDNA molecular clone was transfected into the 293TT cells it is also likely that replication is confined to the transfected cells. The absence of cell-to-cell spread would also explain the modest increase in genome copy number.

To confirm that the increase in genome copy number was driven by the synthetic BFDV DNA molecular clone, the clone was altered either to prevent translation of the BFDV *rep* or to prevent the initiation of replication by Rep at the nonanucleotide motif situated on the stem-loop. The modification of the nonanucleotide motif was based on results obtained through single-site mutations along the nonanucleotide motif of PCV-2 (Cheung, 2004). These were then transfected into 293TT cells to determine whether circular BFDV genomic DNA was generated (Figure 5.6C). Both altered clones failed to produce a 2 kb circular BFDV genome as has previously been shown for the synthetic BFDV DNA molecular clone. This is the first confirmation that Rep and the nonanucleotide are required in BFDV for initiation of replication.

#### **5.4.3. BeYDV-assisted replication of a BFDV dsDNA molecular clone in *N. benthamiana***

To explore alternative systems for replicating BFDV and ultimately producing virus particles, the synthetic BFDV dsDNA molecular clone was tested in *N. benthamiana*. It has previously been reported that replication elements of BeYDV have been successfully used for amplification of heterologous genes in plants (Regnard *et al.*, 2010): these authors reported that gene copy number increased by three orders of magnitude over a three day period as the vector replicated. For the present study it was decided to measure gene copy number over a four day period.

The synthetic BFDV dsDNA molecular clone was unable to replicate on its own after *Agrobacterium* T-DNA transfer into the plant cell nucleus (Figure 5.7A). This would be expected, considering the promoters in the BFDV genome have evolved for an avian host and animal promoters generally do

not work well in plants. When the synthetic BFDV dsDNA molecular clone tested in the presence of the replicating BeYDV-derived plant vector; however, a measurable increase in gene copy number over the four-day period was seen. By day 3 the gene copy number had increased approximately two orders of magnitude, indicating that the BeYDV Rep was able to initiate replication of the BFDV genome from the nonanucleotide motif in the construct. The nonanucleotide motif for geminiviruses is 5'-TAATATTAC-3', while for BFDV it is 5'-TAGTATTAC-3'; however, it has been shown for PCV-2 (5'-AAGTATTAC-3') that the nucleotide in position three (underlined) does not influence replication efficiency (Cheung, 2004; Laufs *et al.*, 1995). This could hold true too for recognition of the BFDV nonanucleotide motif by the BeYDV Rep, as the BFDV genome was replicated reasonably efficiently.

To confirm that the BeYDV Rep was initiating the replication of the synthetic BFDV DNA molecular clone, the BFDV clone with a mutated nonanucleotide motif was tested (Figure 5.7B). Unexpectedly, no difference was observed between synthetic BFDV DNA molecular clone and the altered clone (5'-TAGgATcgg-3'): the BeYDV Rep could apparently recognise the altered nonanucleotide motif. A possible explanation for this is that the changes to the BFDV sequence were based on lethal mutations observed in PCV-2, and did not account for allowed variations in the nonanucleotide motif of geminiviruses (Cheung, 2004). For example, Laufs *et al.* (1995) demonstrated that changing the nonanucleotide motif (5'-TAATATTAC-3') for geminiviruses at position 7 or 8 (underlined) resulted replication efficiency that remained above 50 %; however, mutations at position 9 reduced replication efficiency to below 25 %. It could be suggested the changes to the BFDV nonanucleotide motif in this study may allow replication due to the heterologous Rep.

To confirm that the increase in copy number observed was from the BFDV genome and not the BeYDV plant vector, RCA and qPCR amplifications were performed on the plant total DNA extracts. The RCA analysis confirmed that the synthetic BFDV DNA molecular clone did not replicate in the absence of the BeYDV plant vector (Figure 5.8). However, it was apparent that the RCA reaction involving the amplification of the BeYDV replicon out-competed that of the BFDV genome, as only a 2.5-kb linear BeYDV replicon was detected. This could be due to the much greater replication efficiency of the BeYDV replicon versus the BFDV genome before DNA extraction. As is also the case with PCR, the RCA reaction would preferentially amplify the DNA species having the highest concentration.

To determine the copy number of the BeYDV replicon, synthetic BFDV DNA molecular clone was tested in the presence of an empty BeYDV plant vector and a vector containing a cloned *cp*. Analysis of copy number specifically measuring the *cp* was performed using qPCR (Figure 5.8B). In the absence of the cloned BFDV *cp* the copy number of the BFDV genome increased by approximately

two orders of magnitude, as has previously been shown. This reflects the replication of the BFDV genome and does not detect the BeYDV replicon. On day 4 of the experiment with both the BFDV genome, and the BeYDV replicon containing *cp*, the *cp* copy number increased by three orders of magnitude which is similar to that reported for other heterologous genes cloned into the BeYDV plant vector (Regnard *et al.*, 2010). This increase in *cp* copy number is almost certainly due mainly to the BeYDV amplification, as the contribution of the BFDV genome to the increase in *cp* copy number is expected to be marginal considering the BFDV genome alone increases by only two orders of magnitude compared to three for BeYDV. This would support the observations seen for the RCA amplification. Analysis of copy number specifically measuring the *rep* was performed using qPCR and served as a control (Figure 5.8C). The qPCR reaction would only be able to detect replication of BFDV genome irrespective of whether the BeYDV plant vector contained *cp*. The increase in BFDV genome copy number was lower in the presence of the BeYDV replicon containing *cp*. This could be explained by the downstream translation of the *cp* that would compete for cell resources. There is also the possibility that the expressed CP was interfering in genome amplification by encapsidating the ssDNA form of the genome. This possibility was not tested, however. These results could be further supported by determining the copy number of the BeYDV replicon based qPCR specific to the BeYDV replicon and not the BFDV *cp*.

Further analysis of purified BFDV virions and the synthetic BFDV DNA molecular clone would involve testing of infectivity in embryonated eggs. Considering the BFDV isolate used in this study was isolated from Budgerigars, eggs from this host would probably be suitable. However, the unexpected finding of the replication of BFDV in 293TT cells presents an opportunity for the development of a replicating vector for use in gene delivery. Similarly, the replication of the BFDV genome in *N. benthamiana* coupled with the expression of the BFDV CP could provide an alternative gene delivery vector or vaccine that can generate a strong humoral and cellular response for psittacines.

In conclusion, I found that the BFDV virions could be successfully purified from whole tissue of parrot internal organs, and that a synthetic BFDV DNA molecular clone was able to replicate once transfected into HEK 293 TT cells and is moreover capable of the replicating in *N. benthamiana* in the presence of BeYDV replication elements. This is the first reported instance of BFDV replicating in mammalian cells and the first report of a circovirus replicating with the aid of a geminivirus in *N. benthamiana*. Both these developments could significantly open up possibilities for making reagents and vaccines for BFDV, and possibly even sophisticated virion-based vaccines containing artificial genomes.

## Chapter 6: General conclusion

At present, there is no commercial treatment or vaccine available against PBFV (Patterson *et al.*, 2013b). The development of subunit vaccines is seen as being preferable to inactivated vaccines, as the virion is extremely stable, making incomplete inactivation a potential risk (Raidal and Cross, 1994a). The BFDV CP has previously been expressed in various systems, including bacterial fermentations; however expression of the full-length protein was unsuccessful as the protein was insoluble (Heath, 2006; Patterson *et al.*, 2013b). Thus far, only one challenge trial of a BFDV subunit vaccine has been conducted: this tested insect cell-produced CP against purified infectious virions derived from whole tissue (Bonne *et al.*, 2009). The trial was successful in that an immune response was produced in the vaccinated fledglings; however, the vaccine was unable to prevent viral replication and the challenge material was prepared more than a decade ago. Improvements to the current subunit vaccine are therefore needed, together with the development of a reliable alternative to purified virus as challenge material.

To this end, the aim of this study was to understand the current status of BFDV diversity in South Africa, with particular focus on the endangered wild Cape parrot population. Understanding the regional diversity would thereby direct the development of an experimental subunit vaccine to be produced in plants. This would be accompanied by parallel development of a suitable infectious virus-derived molecular DNA clone challenge model.

Investigation of a recent outbreak of BFDV in endangered Cape parrots endemic to South Africa revealed that wild and captive birds were infected independently, which could result in raising the risk of introducing new virus strains upon successful rehabilitation and release of the captive birds. Developing a challenge model based on Cape parrots would be unfeasible, as the birds are endangered due to dwindling populations and therefore protected (Martin *et al.*, 2014). BFDV isolates from captive Cape parrots were seen to cluster phylogenetically with South African isolates from Budgerigars previously described (Varsani *et al.*, 2010). The development of a suitable challenge model based on Budgerigars is therefore a feasible alternative, considering its relative abundance as a companion species (Parr and Juniper, 2010).

As part of the conservation strategy of the Cape parrot, the relationship between clinical signs of the disease to the BFDV viral load in the blood was characterised, with the aim of developing a reliable tool for use in the field for differentiating diseased and healthy birds. A direct correlation between

viral load in the blood and an increase in clinical signs was shown, allowing researchers to monitor disease spread in wild birds indirectly.

The development of a subunit vaccine focused on plant-based production of CP from a Budgerigar isolate described by Varsani *et al.* (2010). The plant expression platform has been shown to be favourable for the production of region-specific and niche market products (Stoger *et al.*, 2014). This made it viable for a vaccine against BFDV, which would be considered a niche market. The CP was expressed in plants and accumulation could be increased with a variety of optimisations; however, the overall yield was still insufficient to make production commercially viable. This could be due to problems associated with solubility, as has previously been reported. Tentative evidence suggested that the CP was able to form assemble into pentameric subunits, even under SDS denaturation, leading the possibility of the CP forming plant-produced VLPs. The assembly of insect cell produced CP into VLPs has been demonstrated, suggesting that the same could be true for plant-produced CP (Stewart *et al.*, 2007).

Plant-produced BFDV VLPs were demonstrated using purification methods based on the extraction of virions. The VLPs were similar in size and morphology to the genuine virions and insect cell produced VLPs, and it was possible that the VLPs were incorporating geminivirus-derived replicons expressed from the BeYDV-based replicating plant expression vector (Crowther *et al.*, 2003; Regnard *et al.*, 2010; Stewart *et al.*, 2007). The production of VLPs and the inclusion of episomal DNA has the potential to create a potent vaccine capable of eliciting a strong humoral and cellular response, should the *cp* be included and expressed in the host cell. This could overcome the problem of continued viral replication and transient viraemia present in vaccinated birds challenged with live virus.

Parallel development of the challenge vector involved testing of a potentially infectious molecular DNA clone in mammalian cell culture and *N. benthamiana*. The clone, based on the described Budgerigar isolate, was seen to replicate in transfected mammalian 293TT cells. This confirmed that the replication elements used to develop the clone were functional and capable of producing intact BFDV genomes. This would be critical to starting an infection in host cells during challenge. The study explored the possibility of the BFDV clone replicating in plants. Replication was seen only in the presence of a BeYDV replicating vector, suggesting the BFDV Rep was not expressed and therefore RCR was initiated *in trans* by the BeYDV Rep. Successful replication on the BFDV genome in plants in combination with plant expression of the CP could potentially lead to the packaging of infectious plant-produced virions. These virions would be free of bird pathogens, and could be readily quantified and used in a challenge trial. These aspects will be addressed in future studies.

This study was established to investigate the prevalence and diversity of BFDV in the endangered Cape parrot, and if possible to develop a robust challenge model and vaccine for BFDV. I can conclude that I have successfully studied the virus in Cape parrots, and I have produced a potential subunit vaccine in plants, and I have tested replication of a 'live challenge' vector in mammalian tissue culture and in plants.

The present study expands significantly on the current state of knowledge of BFDV. The elucidation of the genetic diversity of BFDV in wild Cape parrots in South Africa can assist further in understanding of BFDV global movement (Harkins *et al.*, 2014). In addition, these data can be used by conservation agencies to trace the illegal capture of wild Cape parrots through analysis of virus isolates (Massaro *et al.*, 2012). Knowledge of BFDV isolates infecting Cape parrots could also advise conservation policies, especially with regards to introducing rehabilitated birds back into the wild population. The insights provided through analysis of viral load in the blood provides a better understanding of viraemia, and is a useful addition in defining the upper and lower limits of virus presence in the blood (Katoh *et al.*, 2008; Shearer *et al.*, 2009b). The correlation between increasing viral load and deteriorating clinical signs can be readily applied in the conservation of wild Cape parrots. Here too, knowledge of the lower limits of viral load found in healthy birds could assist in the diagnosis of PBFD.

Plant-produced BFDV CP and VLPs provide an alternative to CP produced in insect cell culture and bacterial fermentations. As has been demonstrated, the CP can be readily expressed in plants, albeit fused to a large purification/aggregation tag such as the elastin-like polypeptide (ELP)(Duvenage *et al.*, 2013). The present study is the first reported production of BFDV VLPs in plants. This could form the basis of a viable vaccine, and if combined with DNA capable of being packaged by CP, could potentially result in pseudovirions. These would probably elicit a potent humoral and cellular immune response. The synthesis and successful preliminary testing of a BFDV dsDNA molecular clone was based on similar successes shown for PCV (Fenaux *et al.*, 2002; Liu *et al.*, 2001). Not only does the study put forward a convenient challenge vector, it expands the tools available with which to study the replication of BFDV and circoviruses at large. There is potential in creating defective virion particles, opening up a BFDV attenuated vaccine alternative to be explored.

Exploring the following as future research may further develop on these reported findings. Continued monitoring and sampling of the Cape parrot population would provide invaluable data regarding virus genetic evolution and bottlenecks, as well as temporal data on genetic diversity and viral load within the wild bird population. Purification of sufficient quantities of plant-produced CP for testing of immunogenicity in Budgerigars should be done as a matter of urgency. The production

of pseudovirions in plants should be further explored. Combinations of BFDV and BeYDV replication elements together with a genome-sized replicon may allow for packaging and assembly. The replicon design could introduce additional disease antigens, thus allowing for the vaccine to protect against more than one disease. Future work with the BFDV dsDNA infectious molecular clone should focus on testing in embryonated eggs together with exploring plant-produced virion particles. The production of virus particles can be used to test knockout mutants and provide empirical evidence for the mechanism of BFDV replication. Due to the simple replication strategy, the BFDV sequence elements used in this study could be deconstructed and optimised for use as a potential replicating component for gene therapy.

A successful vaccine against BFDV could be integrated into the conservation of wild Cape Parrots during routine capture and release of birds for monitoring of BFDV. The vaccine could be administered either as a subcutaneous injection during handling or the possibility exists to introduce the vaccine via feed however dosage would be unregulated.

There were a number of limitations that were encountered during this study. Sampling of Cape parrots favoured the capture of diseased parrots over healthy ones, as it was observed in the field that these struggled during flight to gain enough height, and thus were more often caught. This may have excluded samples that may have tested positive for BFDV while having no clinical signs of PBFD. Moreover, the isolation of BFDV genomic DNA through RCA may have overlooked samples containing multiple infections with different isolates. Identifying samples with multiple infections would have been a time-consuming exercise, but should however be explored in the future, with the possibility of detecting recombination events. The yield of plant-produced CP was determined to be low in comparison to other proteins. Various optimisations were undertaken to increase expression, with mixed results being achieved. Plant codon-optimisation had little effect on expression; however the overall G+C content of the gene remained unchanged. Increasing the G+C content as opposed to optimising codon usage could possibly increase the expression of CP. The low yield prompted us to explore VLP formation, as a possible explanation.

This study contributes considerably to the overall understanding of BFDV. The virus diversity present in a recent outbreak of BFDV was defined for a wild population of Cape parrots. This was accompanied by the first described assessment of BFDV viral load in a wild psittacine population, and the verification of a field guide for estimation of disease status. This study also considerably furthered the development of a plant-produced BFDV CP vaccine. The expression of CP in *N. benthamiana* was optimised, and thereafter purification of CP VLPs was successful. This is the first report of BFDV VLP production in plants. In parallel to vaccine development, a suitable challenge

vector was explored. A BFDV dsDNA molecular clone was synthesised and demonstrated to replicate in mammalian tissue culture and plants. This is the first report of successful BFDV replication in a tissue culture system. It is to be hoped that others can build on this to complete our team's goal of making a viable BFDV vaccine, together with a reliable challenge model.

## Appendix A: Minimum information for publication of quantitative real-time PCR experiments

Table A.1: **MIQE checklist for authors, reviewers and editors.** All essential information (E) must be submitted. Desirable information (D) should be submitted if available. If using primers obtained from RTPriemerDB, information on qPCR target, oligonucleotides, protocols and validation is available from that source.

Item to check	!	Checklist	
<b>Experimental design</b>			
Definition of experimental and control groups	E	See Section 2.2.5	
Number within each group	E	A total of 49 samples	
Assay carried out by core lab or investigator's lab?	D		
Acknowledgement of authors' contributions	D		
<b>Sample</b>			
Description	E	Blood on FTA™ Classic Card (Whatman)	
Volume/mass of sample processed	D		
Microdissection or macrodissection	E	Not applicable	
Processing procedure	E	Not applicable	
If frozen - how and how quickly?	E	Not applicable	
If fixed - with what, how quickly?	E	Not applicable	
Sample storage conditions and duration (especially for FFPE samples)	E	Storage at 4 °C	
<b>Nucleic acid extraction</b>			
Procedure and/or instrumentation	E	DNA extraction from nucleated blood samples	
Name of kit and details of any modifications	E	DNeasy® Blood & Tissue Kit (Qiagen)	
Source of additional reagents used	D		
Details of DNase or RNase treatment	E	Not applicable (Measurement of virus particles in blood)	
Contamination assessment (DNA or RNA)	E	Not applicable	
Nucleic acid quantification	E	Spectrophotometric analysis	
Instrument and method	E	NanoDrop ND-1000 Spectrophotometer V3.1.0	
Purity (A260/A280)	D		
Yield	D		
RNA integrity method/instrument	E	Not applicable	
RIN/RQI or C <sub>q</sub> of 3' and 5' transcripts	E	Not applicable	
Electrophoresis traces	D		
Inhibition testing (C <sub>q</sub> dilutions, spike or other)	E	No inhibition testing was performed	
<b>Reverse transcription</b>			
Complete reaction conditions	E	Not applicable	
Amount of RNA and reaction volume	E	Not applicable	
Priming oligonucleotide (if using GSP) and concentration	E	Not applicable	
Reverse transcriptase and concentration	E	Not applicable	
Temperature and time	E	Not applicable	
Manufacturer of reagents and catalogue numbers	D		
C <sub>q</sub> s with and without RT	D		
Storage conditions of cDNA	D		
<b>qPCR target information</b>			
If multiplex, efficiency and LOD of each assay.	E	Not applicable	
Sequence accession number	E	GQ165756	EU660286
Location of amplicon	D		
Amplicon length	E	97-bp	97-bp
<i>In silico</i> specificity screen (BLAST, etc)	E	BLAST analysis performed	
Pseudogenes, retropseudogenes or other homologs?	D		

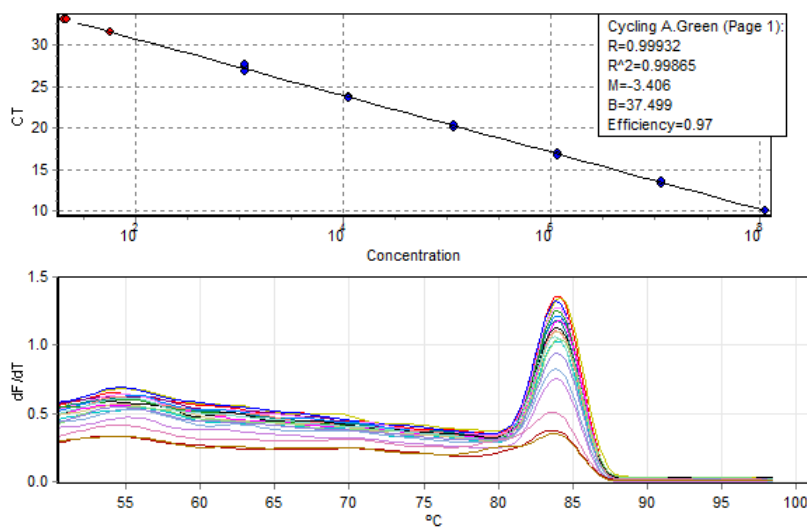
Sequence alignment	D	
Secondary structure analysis of amplicon	D	
Location of each primer by exon or intron (if applicable)	E	Not applicable
What splice variants are targeted?	E	Not applicable
<b>qPCR oligonucleotides</b>		
Primer sequences	E	5'-CAGTTAAGGGCGCTTTTGTGGAG-3'      5'-TCCCATCTGGCACTGTCTCTG-3' 5'-TTCGGGTACAGTCCTCCTTG-3'      5'-ACAGAGCTTTCACCCTCATTTATGG-3'
RTPimerDB Identification Number	D	
Probe sequences	D	
Location and identity of any modifications	E	Not applicable University of Cape Town, Molecular and Cell Biology Department, Synthetic DNA
Manufacturer of oligonucleotides	D	Laboratory
Purification method	D	Post-synthesis desalting
<b>qPCR protocol</b>		
Complete reaction conditions	E	See below
Reaction volume and amount of cDNA/DNA	E	20 µL and 1 µL DNA
Primer, (probe), Mg <sup>++</sup> and dNTP concentrations	E	200 µM each, N/A, manufacturer, manufacturer
Polymerase identity and concentration	E	JumpStart™ Taq DNA Polymerase (Sigma-Aldrich); concentration unknown
Buffer/kit identity and manufacturer	E	LuminoCT SYBR Green qPCR ReadyMix; Sigma-Aldrich Tris-HCl, pH 8.3, KCl, dNTPs (dATP, dCTP, dGTP, TTP), stabilizers, MgCl <sub>2</sub> , SYBR Green I
Exact chemical constitution of the buffer	D	and JumpStart™ Taq DNA Polymerase
Additives (SYBR Green I, DMSO, etc.)	E	None
Manufacturer of plates/tubes and catalogue number	D	Corbet Research 0.1 ml Tubes & Caps 3001-002 Hold @ 95 °C (10 minutes), cycling (40 repeats) step 1 @ 95 °C (hold 15 s), step 2 @
Complete thermocycling parameters	E	55 °C (hold 15 s), step 3 @ 60 °C (hold 15 s)
Reaction setup (manual/robotic)	D	Manual
Manufacturer of qPCR instrument	E	Qiagen
<b>qPCR validation</b>		
Evidence of optimisation (from gradients)	D	
Specificity (gel, sequence, melt, or digest)	E	Melt (See Section A.1)
For SYBR Green I, C <sub>q</sub> of the NTC	E	See Table A.2
Standard curves with slope and y-intercept	E	See Section A.1
PCR efficiency calculated from slope	E	See Table A.2
Confidence interval for PCR efficiency or standard error	D	
r <sup>2</sup> of standard curve	E	See Table A.2
Linear dynamic range	E	See Section A.1
C <sub>q</sub> variation at lower limit	E	See Table A.2
Confidence intervals throughout range	D	
Evidence for limit of detection	E	See Section A.1
If multiplex, efficiency and LOD of each assay.	E	Not applicable
<b>Data analysis</b>		
qPCR analysis program (source, version)	E	Rotor-Gene 6000 real-time rotary analyzer, Software version 1.7 (Build 87)
C <sub>q</sub> method determination	E	Rotor-Gene 6000 real-time rotary analyzer, Software version 1.7 (Build 87)
Outlier identification and disposition	E	Outliers due to pipetting error were removed
Results of NTCs	E	See Section A.1
Justification of number and choice of reference genes	E	See Section 2.2.5
Description of normalisation method	E	See Section 2.2.5
Number and concordance of biological replicates	D	
Number and stage (RT or qPCR) of technical replicates	E	3 qPCR technical repeats
Repeatability (intra-assay variation)	E	See Section A.1
Reproducibility (inter-assay variation, %CV)	D	
Power analysis	D	
Statistical methods for result significance	E	See Section 2.2.5
Software (source, version)	E	StatSoft, Inc. (2011). STATISTICA (data analysis software system), version 10.
C <sub>q</sub> or raw data submission using RDML	D	

Table A.2: **MIQE qPCR validation.** Essential information submitted for each qPCR run:  $C_q$  of the NTC, PCR efficiency,  $r^2$  of standard curve and  $C_q$  variation at lower limit.

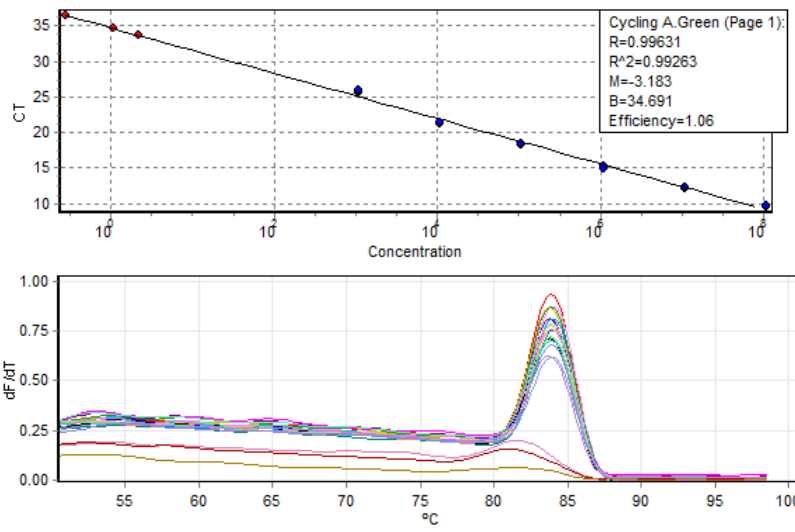
	$C_q$ of the NTC	PCR efficiency	$r^2$ of standard curve	$C_q$ variation at lower limit (SD Orders of Mag.)
<i>rep</i> (mean)	(0.990)	(0.997)		
Run 1	32.49	0.970	0.999	1.34
Run 2	34.88	1.06	0.993	1.08
Run 3	31.9	0.950	0.999	1.21
Run 4	32.78	0.970	0.999	1.23
Run 5	33.8	0.960	0.999	1.18
Run 6	32.05	0.960	0.999	1.19
Run 7	35.69	0.990	0.996	1.20
Run 8	37.33	1.06	0.996	1.33
Run 9	35.67	0.990	0.994	1.21
TGFB2 (mean)	(1.01)	(0.996)		
Run 10	31.67	1.06	0.990	1.31
Run 11	31.97	0.940	0.998	1.28
Run 12	33.39	0.950	0.997	1.28
Run 13	31.52	0.980	0.997	1.16
Run 14	28.24	1.08	0.998	1.45
Run 15	33.68	1.06	0.995	1.41
Run 16	35.67	1.04	0.997	1.22

A.1. qPCR runs. Essential information submitted for each qPCR run: standard curves (blue) with slope and y-intercept, linear dynamic range, evidence for limit of detection, results of NTCs (red), repeatability and specificity.

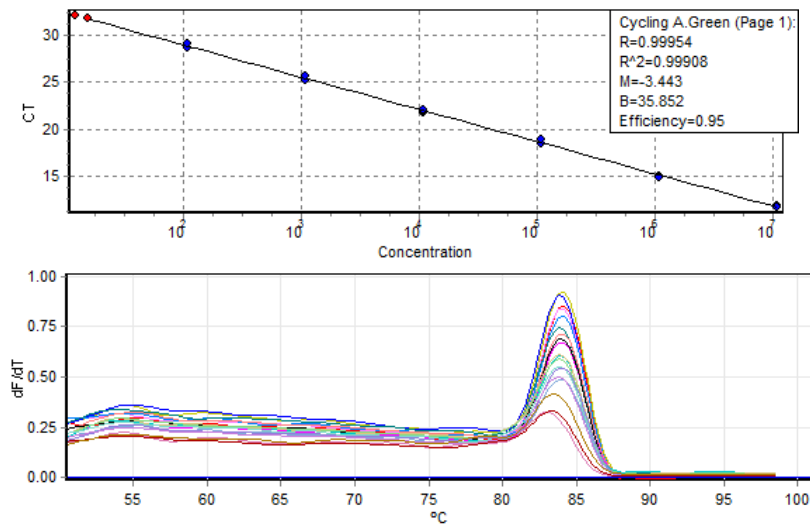
qPCR run 1



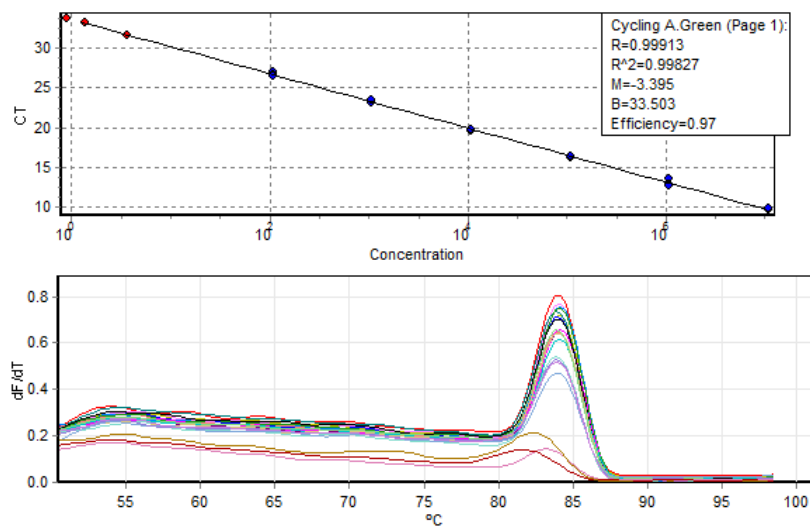
### qPCR run 2



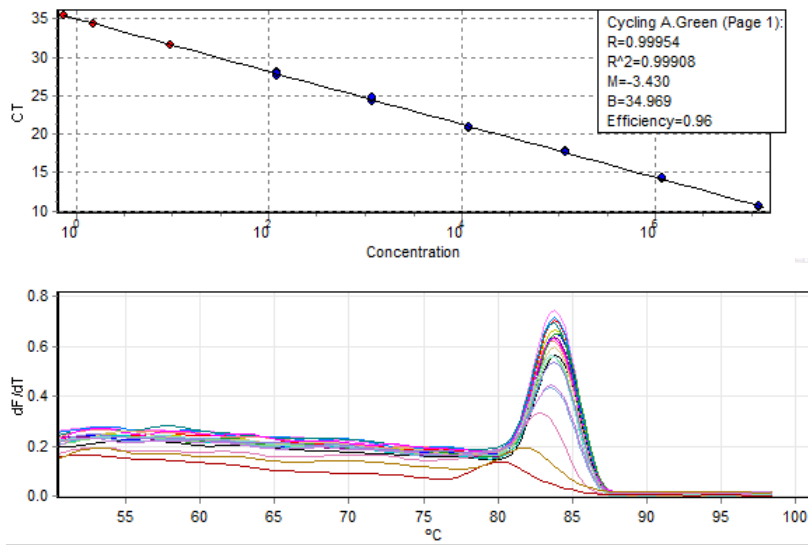
### qPCR run 3



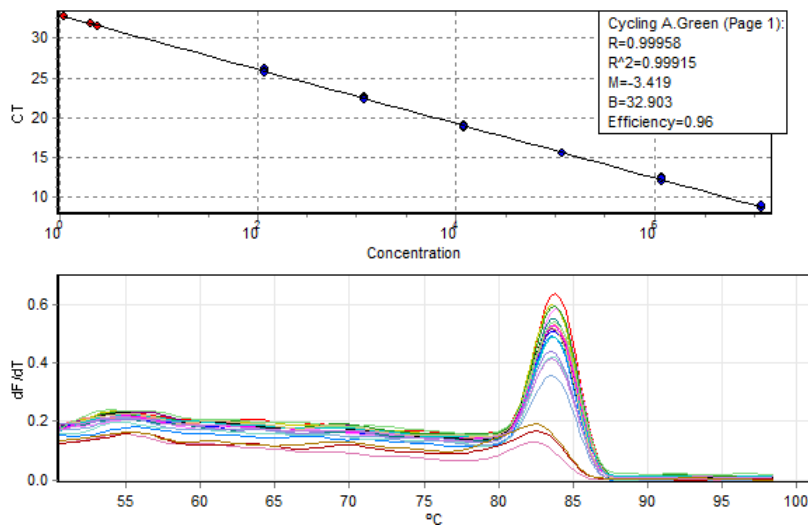
### qPCR run 4



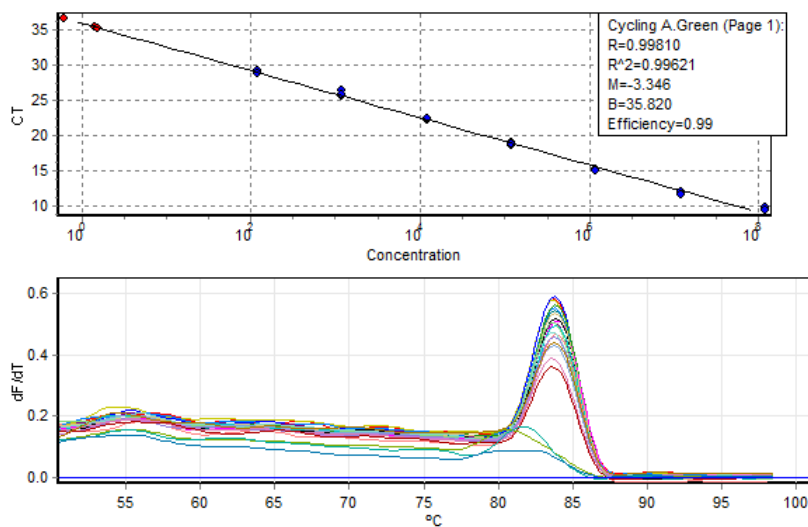
### qPCR run 5



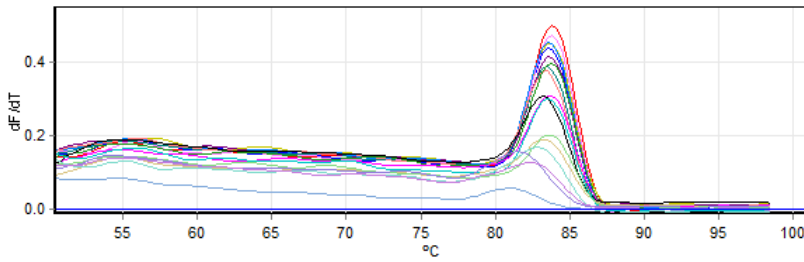
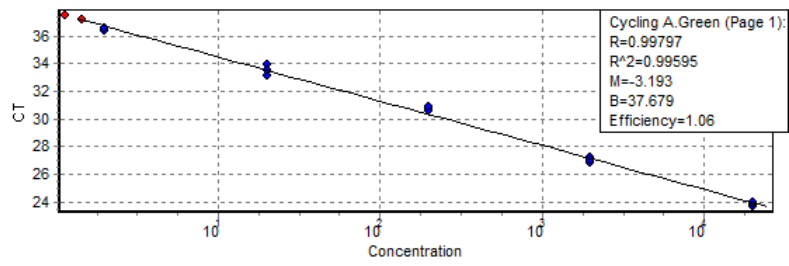
### qPCR run 6



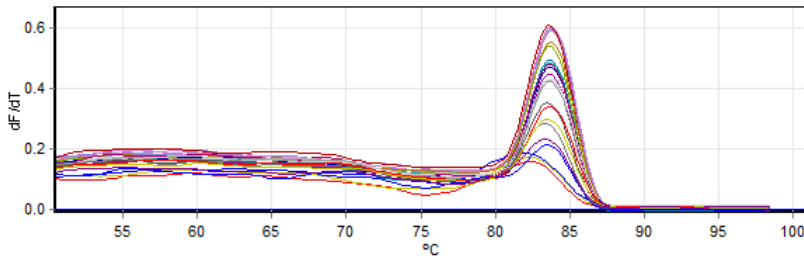
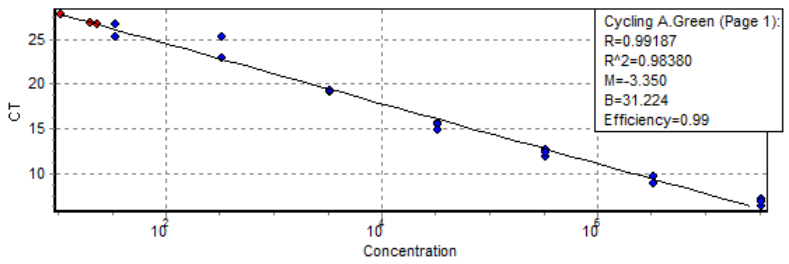
### qPCR run 7



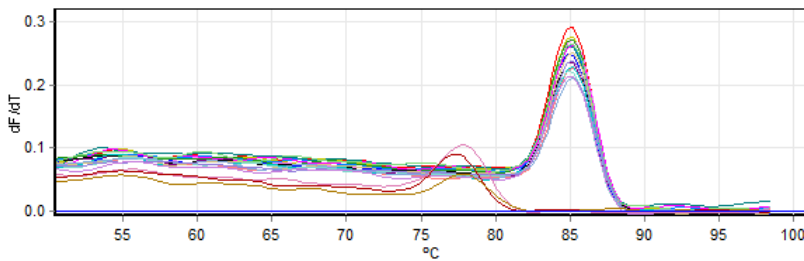
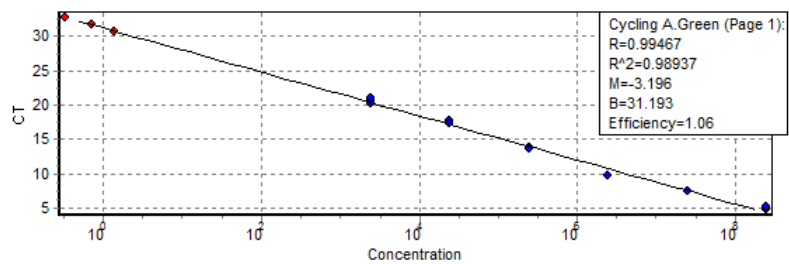
qPCR run 8



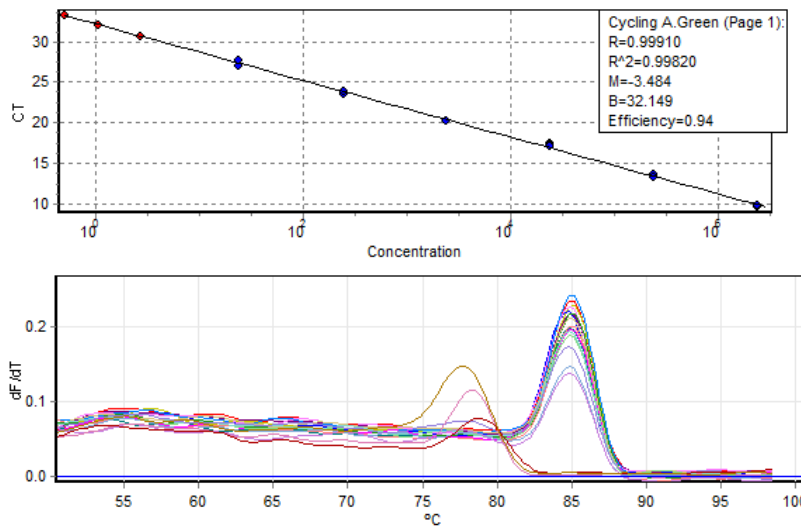
qPCR run 9



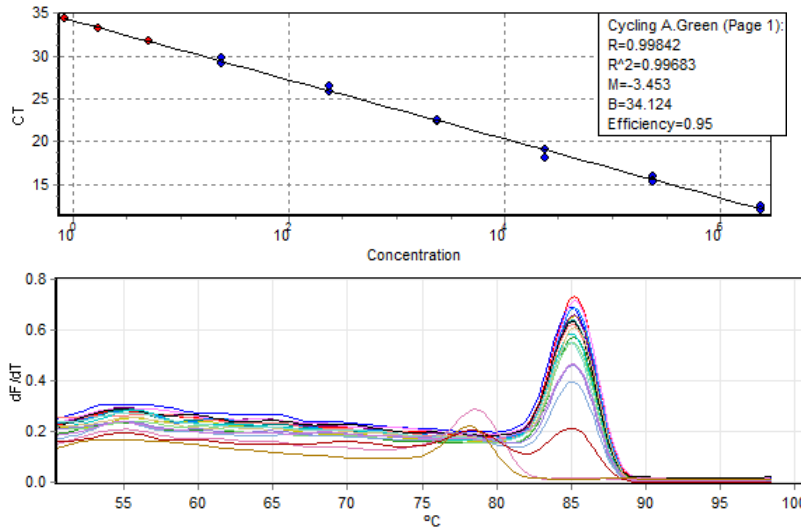
qPCR run 10



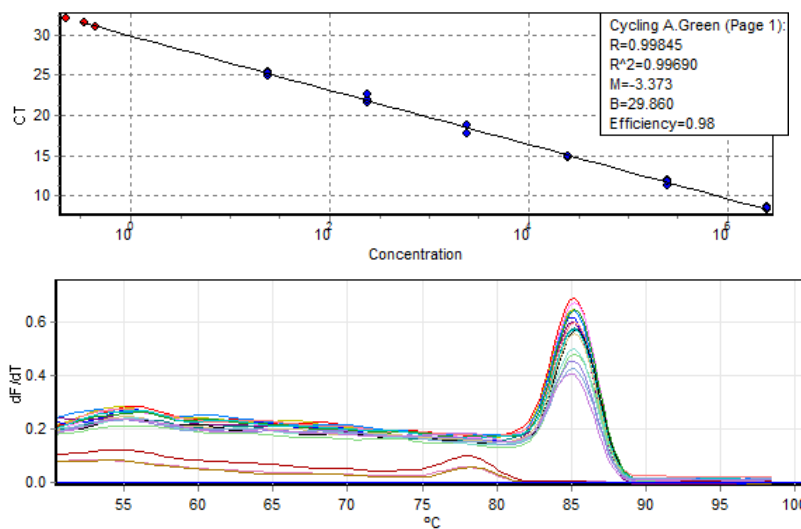
qPCR run 11



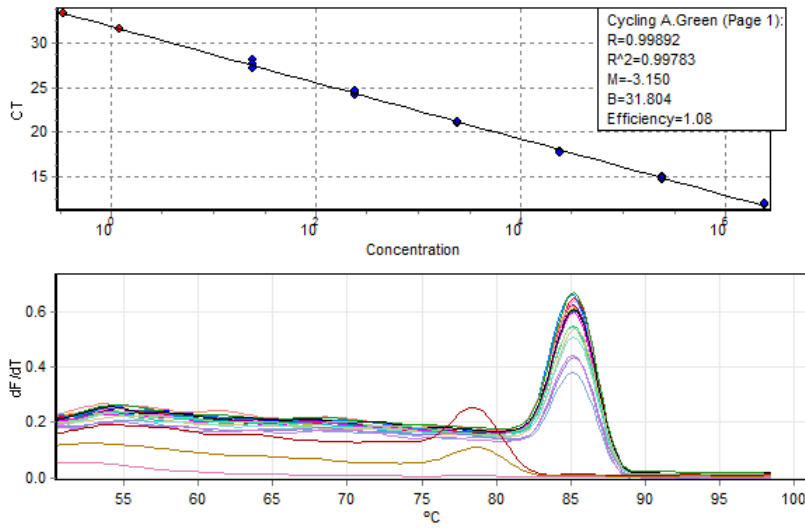
qPCR run 12



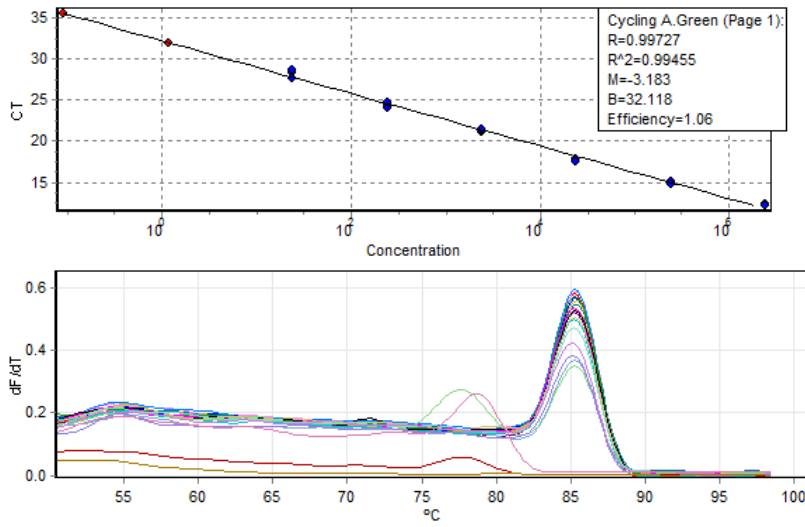
qPCR run 13



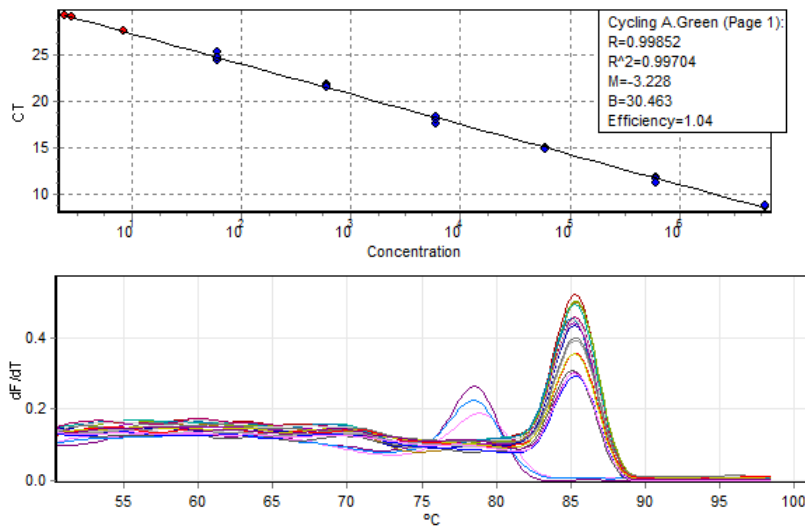
### qPCR run 14



### qPCR run 15



### qPCR run 16



## Appendix B: PCR thermocycling parameters

### B.1. PCR amplification of 97-bp 6xHis TEV

Temperature (°C)	Time (s)	Cycles
95	90	1
95	30	
59	30	5
72	60	
95	30	
67	30	25
72	30	
72	300	1

### B.2. PCR amplification of 781-bp BFDV AFG3-ZA cp, 658-bp BFDV AFG3-ZA ΔN40 cp and 673-bp BFDV tco AFG3-ZA ΔN40 cp

Temperature (°C)	Time (s)	Cycles
95	90	1
95	30	
55	30	5
72	120	
95	30	
61	30	25
72	120	
72	300	1

### B.3. PCR amplification of 796-bp BFDV BKS1ZA\_84 cp

Temperature (°C)	Time (s)	Cycles
95	180	1
95	15	
50	15	5
72	120	
95	15	
56	15	25
72	120	

### B.4. PCR amplification of 959-bp rcbs1-cTP BFDV BKS1ZA\_84 cp 6xHis

Temperature (°C)	Time (s)	Cycles
95	180	1
95	15	
52	15	30
72	120	

### B.5. PCR amplification of 814-bp BFDV BKS1ZA\_84 cp 6xHis SEKDEL, 860-bp LPH BFDV BKS1ZA\_84 cp 6xHis SEKDEL and an 840-bp LPH BFDV BKS1ZA\_84 cp 6xHis

Temperature (°C)	Time (s)	Cycles
95	180	1
95	15	
55	15	30
72	120	

### B.6. PCR amplification of 1-kb BeYDV gene fragment

Temperature (°C)	Time (s)	Cycles
95	90	1
95	30	

54	30	30
72	60	

B.7. PCR amplification of 900-bp BFDV *rep* fragment

Temperature (°C)	Time (s)	Cycles
94	300	1
94	30	
65	30	30
72	60	
72	300	1

B.8. PCR amplification of 421-bp BK1ZA\_84 N9 *Pst*I mutant

Temperature (°C)	Time (s)	Cycles
95	180	1
95	30	
55	30	30
72	120	

B.9. PCR amplification of 265-bp BFDV BKS1ZA\_84 *rep* *Sac*I mutant and 239-bp BFDV BKS1ZA\_84 *rep* *Pst*I mutant

Temperature (°C)	Time (s)	Cycles
95	180	1
95	15	
55	15	30
72	45	

B.10. PCR amplification of 504-bp BFDV BKS1ZA\_84 *rep* *Spe*I mutant

Temperature (°C)	Time (s)	Cycles
95	180	1
95	15	
55	15	30
72	60	

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