

MOLECULAR DETECTION AND GENETIC MANIPULATION OF THE BLACK QUEEN CELL VIRUS

By

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DECLARATION

I declare that "Molecular detection and genetic manipulation of the black queen cell virus" is my own work and that all the resources I have used or quoted have been indicated and acknowledged by means of complete references.



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| <p style="text-align: center;">LIST OF PUBLICATIONS (Emanating from the work reported herein)</p> |
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- **Benjeddou, M., Leat, N., Allsopp, M. & Davison, S. (2001).** Detection of acute bee paralysis virus and black queen cell virus from honeybees by reverse transcriptase PCR. *Applied and Environmental Microbiology* **67**, 2384-2387.
- **Benjeddou, M., N. Leat & S. Davison (2002).** The black queen cell virus genomic RNA is infectious in honeybee pupae. *Journal of Invertebrate Pathology*. In Review.
- **Benjeddou, M., N. Leat, M. Allsopp & S. Davison (2002).** Development of infectious transcripts and genome manipulation of the black queen-cell virus of honeybees. *Journal of General Virology*. In Press.

ABSTRACT

The South African isolate of the Black Queen-Cell Virus (BQCV), a honeybee virus, was previously found to have an 8550 nucleotide genome excluding the poly(A) tail. Its genome contained two ORFs, a 5'-proximal ORF encoding a putative replicase protein and a 3'-proximal ORF encoding a capsid polyprotein.

A reverse transcriptase PCR (RT-PCR) assay was developed for the detection of BQCV and acute bee-paralysis virus (ABPV). Complete genome sequences were used to design unique PCR primers within a 1-kb region from the 3' end of both genomes to amplify a fragment of 700 bp from BQCV and 900 bp from ABPV. The combined guanidinium thiocyanate and silica membrane method was used to extract total RNA from samples of healthy and laboratory-infected bee pupae. In a blind test, RT-PCR successfully identified the samples containing BQCV and ABPV. Sensitivities were of the order of 130 genome equivalents of purified BQCV and 1600 genome equivalents of ABPV.

The ability of the BQCV naked genomic RNA to initiate a complete infectious cycle upon injection into honeybee pupae was tested. Purified BQCV particles, viral RNA, RNase A-treated BQCV particles, RNase A-treated viral RNA and phosphate buffer were injected into bee pupae. The bee pupae were incubated at 30-35°C for 8 days prior to the virus being purified. Virus particles were generated by injection of bee pupae with purified BQCV particles, viral RNA and RNase A-treated BQCV particles. No virus was recovered from bees injected with RNase A-treated viral RNA or with phosphate buffer. An amount of 66 ng of viral RNA per bee pupae was found to be sufficient to cause a regular infection.

Long Reverse Transcription-PCR was used to produce infectious transcripts for BQCV, and to manipulate its genome. Primers were designed for the amplification of the complete genome, the *in vitro* transcription of infectious RNA, and PCR-directed mutagenesis. An 18-mer antisense primer was designed for reverse transcription (RT) to produce full-length single-stranded cDNA (ss cDNA). Unpurified ss cDNA from the RT reaction mixture was used directly as a template to amplify the full genome using long high fidelity PCR. The SP6 promoter sequence was introduced into the sense primer to transcribe RNA directly from the amplicon. RNA was transcribed *in vitro* with and without the presence of a cap analog and injected directly into bee pupae and incubated for 8 days. *In vitro* transcripts were infectious but the presence of a cap analog did not increase the amount of virus recovered. A single base mutation abolishing an *EcoRI* restriction site was introduced by fusion-PCR, to distinguish viral particles recovered from infectious transcripts from the wild type virus (wtBQCV). The mutant virus (mutBQCV) and wtBQCV were indistinguishable using electron microscopy and western blot analysis. The *EcoRI* restriction site was present in wtBQCV and not in mutBQCV.

Genome-length ss cDNA was synthesized by reverse-transcription, and used directly for the amplification of two fragments, 5'-BQCV and 3'-BQCV, extending from the 5' end to base number 7600 and from base number 7526 to the 3' end respectively, using long high fidelity PCR. The sequence encoding for GFP was also amplified using high fidelity PCR. The three fragments were successfully joined together by fusion-PCR following a stepwise strategy and using the overlaps between fragments that were created by relevant primers. The obtained amplicon (gfpBQCV) was used as template in the *in vitro* transcription reactions. The sequence encoding the green fluorescent protein was confirmed by RT-PCR to have been integrated in the *in vitro* transcribed RNA of GFPBQCV. The GFPBQCV transcripts were infectious, but it was not

possible to detect any fluorescence from whole bees or electrophoresed bee extracts. An RT-PCR analysis of the progeny virus recovered showed that no GFP-encoding sequence was present in the viral genome, but the virus was authenticated as being BQCV

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THIS THESIS IS DEDICATED TO MY PARENTS

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CHAPTER 1

GENERAL INTRODUCTION

1. 1. INTRODUCTION

The European honeybee (*Apis mellifera* L.) is found in all parts of the world, except for the extreme Polar Regions (Dietz, 1992). The extreme wide distribution range of this species necessitated that it adapts to a broad range of climates, from cold temperate to tropical conditions and from areas with high rainfall to semi-deserts. Each region has its distinctive floral season, its complement of natural enemies, and its characteristic nesting sites (Eardley *et al.*, 2001). Managed honeybee colonies are the main source of pollinators of cultivated plants in tropical and temperate countries, because of their social structure, their behavior, and their anatomy (Johannesmeier & Mostert, 2001). Bees are effective pollinators due to the combination of quick visits to many flowers of the same species, deliberate gathering of pollen, and hairy bodies (Eardley *et al.*, 2001). A honeybee colony needs from 34-65 kg of honey per year, and from 20-50 kg of pollen. This represents literally millions of visits to flowers by colony foragers (Johannesmeier & Mostert, 2001).

The importance of honeybees to both agriculture and conservation in South Africa exceeds the direct market value of honeybee products derived by beekeepers in terms of honey, bee-collected pollen, royal jelly, wax, venom, and health supplements. Honeybees are considered to be responsible for 60-70 % of all pollination (Johannesmeier & Mostert, 2001). They pollinate garden plants, exotic plants, as well as indigenous plants. As such, honeybees play an important

role in the conservation of floral reserves and maintenance of biodiversity. Commercial honeybees pollinate most deciduous fruits, some sub-tropical fruits, all oilseed crops, most oilseed and vegetable seed production, and lucerne seed. The value of these crops has been estimated to be at least 2.8 Billion Rands per annum (Johannesmeier & Mostert, 2001).

The entry of certain bee pests and diseases into South Africa has led to the honeybee population being under increasing threat. Two species of honeybee parasitic mites, the tracheal mite (*Acarapis woodi*) and the varroa mite (*Varroa destructor*) have recently been detected in South Africa (Buys, 1995; Allsopp *et al.*, 1997). Varroa is of a particular concern as it has decimated honeybee colonies in many parts of the world. In its last continent of conquest, North America, it has been responsible for large-scale colony losses (Kraus & Page Jr., 1995; Finley *et al.*, 1996). Even with extensive use of acaricides, reports from areas of the USA indicated more than 50% commercial colony losses and up to 85% loss of wild honeybee colonies (Finley *et al.*, 1996; Kraus & Page Jr., 1995). However, there are strong suggestions that losses recorded in colonies infested with the mite are a result of an association between varroa and honeybee viruses rather than the mite acting alone (Bailey *et al.*, 1983; Ball & Allen, 1988; Allen & Ball, 1996; Brødsgaard *et al.*, 2000). This has led to the use of the term “bee parasitic mite syndrome” to describe such a disease complex.

1. 2. BEE PARASITIC MITE SYNDROME

The term “bee parasitic mite syndrome” has been used to describe a disease complex in which colonies are simultaneously infested with mites and infected with viruses and accompanied with high mortality (Shimanuki *et al.*, 1994). The relationship between mite

infestation and virus infection is not clearly understood. Although the mite has been demonstrated to act as an activator of inapparent virus infections and as a virus-transmitting vector (Ball & Allen, 1988; Bowen-Walker *et al.*, 1999), no direct link between the actual mite population and colony collapse has been found (Martin, 1998). Furthermore, contradicting results from different studies have caused confusion about the importance and the extent of the damage caused by some honeybee viruses. Bailey & Ball (1991) reported that the acute bee paralysis virus (ABPV) had never been associated with disease mortality in nature, in contrast ABPV was detected in large amounts in dead adult bees and diseased brood from the mite-free countries Belize and Nicaragua (Allen & Ball, 1996). In another study, Hung *et al.* (1996) reported that no virus particles were found in dead adult bees collected from two colonies with bee parasitic mite syndrome. This has led to suggestions that the mite and the virus are part of a complex multiple-factor problem involved in the collapse of mite-infested colonies (Hung *et al.*, 1996; Martin, 2001)

1. 3. VARROA MITE

The varroa mite, *Varroa jacobsoni* (Oudemans), is currently considered the major pest of honeybees in most parts of the world. Only Australia, New Zealand and the state of Hawaii remain free of this pest (Sammataro *et al.*, 2000). It was first described as an ectoparasitic mite of the Asian honeybee, *Apis cerana*, from Indonesia in 1904. This bee has probably coevolved with the parasite, which adapted to keep the mite under control (Sammataro *et al.*, 2000). The mite only infested *Apis mellifera* colonies when these were taken into areas where *A. cerana* were

present. Movement of *Apis mellifera* by humans then ensured that the mite spread throughout the world (Swart *et al.*, 2001).

It has since been shown that *V. jacobsoni* is a complex of at least two different species, with the second species described as *Varroa destructor* (Swart *et al.*, 2001). It is *V. destructor* from Korea that has become the pest of *A. mellifera* worldwide, including South Africa, and not *V. jacobsoni* as previously believed (Swart *et al.*, 2001). *Varroa destructor* is a flat, oval-shaped, 1.1 mm long and 1.5 mm wide, pale brown to reddish-brown ectoparasitic mite. It is often found between the abdominal segments or body regions of the adult bees, particularly adjacent to the wax glands, or in the honeybee brood (Swart *et al.*, 2001).

Varroa infestations have proved difficult to control and impossible to eradicate. This especially the case where there is a massive wild population of bees beyond reach, as in the case of South Africa (Allsopp, 1997; Swart *et al.*, 2001). In Europe and the USA many hundreds of thousands of commercial honeybee colonies have died as a result of varroaosis (pathology caused by varroa), beekeeping is no longer possible without some anti-varroa treatment (Allsopp, 1997). The collapse of colonies however, is not solely attributed to the effect of varroa. Colonies with large numbers of varroa mites are weakened further by other diseases and pests, including viruses (Swart *et al.*, 2001). These secondary infections may have caused the total collapse of the colonies.

In South Africa, the Black Queen Cell Virus, the Acute Bee Paralysis Virus and another two unidentified viruses have been shown to be implicated in increased honeybee mortality in African honeybee colonies infected with varroa mite (Swart *et al.*, 2001). Large numbers of opened cells with dead, pink-eyed pupae were seen in honeybee colonies with high varroa loads.

These pupae had virus levels many times higher than those of healthy pupae removed from the same colonies, suggesting that viruses were the cause of this mortality (Swart *et al.*, 2001).

1. 4. HONEYBEE VIRUSES

1. 4. 1. Overview

A total of eighteen honeybee viruses have been identified and physically characterized (Allen and Ball, 1996). In addition, the complete genome sequences of three honeybee viruses, namely ABPV (Govan *et al.*, 2000), BQCV (Leat *et al.*, 2000) and SBV (sacbrood virus) (Ghosh *et al.* 1999), have been determined. Among these three viruses, only SBV causes symptoms that can confidently be attributed to viral infection (Allen & Ball, 1996).

Most of these viruses are physically similar to picornaviruses, and the term picorna-like is used to describe them. Picorna-like honeybee viruses have small RNA genomes and are isometric in shape, with a diameter of approximately 30 nm. The Filamentous Virus (FV) and the Chronic Paralysis Virus (CPV) however, differ significantly from picornaviruses. FV is ellipsoidal and has a DNA genome, and CPV has an RNA genome but produces an anisometric virus particle (Bailey *et al.*, 1968). Some physical features of honeybee viruses are listed in Table 1. 1.

| Genome | Shape | Size (nm) | Name | Abbreviation |
|--------|-------|-----------|-----------------------------------|--------------|
| DNA | | | Filamentous | FV |
| RNA | | | Chronic Paralysis | CPV |
| RNA | | | Chronic Paralysis Virus Associate | CPVA |
| | | | Cloudy Wing | CWV |
| | | 30 | Acute Bee Paralysis | ABPV |
| | | | Black Queen Cell | BQCV |
| | | | Deformed Wing/Egypt Bee | DWV/EBV |
| | | | Kashmir (various strains) | KBV |
| | | | Sacbrood | SBV |
| | | | Slow Paralysis | SPV |
| | | 35 | Bee Virus X | BVX |
| | | | Bee Virus Y | BVY |

Table 1. 1. Physical features of honeybee viruses isolated from *Apis mellifera*. From Allen & Ball (1996).

1. 4. 2. Taxonomy

Most honeybee viruses resemble picornaviruses, but there is currently not sufficient information for classification. Phylogenetic studies on the ABPV, BQCV and SBV revealed that these viruses are not members of the picornavirus family. SBV appears to be most closely related to the infectious flacherie virus of silkworms. BQCV and ABPV belong to a novel group of insect viruses, the Cricket paralysis-like virus group, which has recently been described.

This novel group of insect infecting RNA viruses includes Cricket paralysis virus (CrPV), *Drosophila* C virus (DCV), *Plautia stali* intestine virus (PSIV), *Rhopalosiphum padi* virus (RhPV), and Himetobi P virus (HiPV) (van Regenmortel *et al.*, 2000). The genomes of CrPV, DCV, PSIV, RhPV and HiPV are monopartite and bicistronic with replicase proteins encoded by a 5'-proximal ORF and capsid proteins by a 3'-proximal ORF. In the case of PSIV, translation

initiation of the 3'-proximal ORF has been demonstrated to be dependent on an internal ribosome entry site (IRES), starting at a CUU codon (Sasaki & Nakashima, 1999). Similarly, it has been suggested that translation initiation of the 3'-proximal ORF of BQCV is facilitated by an IRES at a CCU codon (Leat *et al.*, 2000).

1. 4. 3. Incidence

Most honeybee viruses can remain in a latent state within individuals and may spread within bee populations at this low level of inapparent infection (Allen & Ball, 1996; Ball, 1997). Sacbrood virus (SBV) and chronic paralysis virus (CPV), however, can reliably be diagnosed by symptoms occurring naturally in honeybee colonies (Allen and Ball, 1996). When allowed to undertake their normal activities unhindered, bees may have developed an ability to resist the multiplication and spread of viral infections (Allen & Ball, 1996; Ball, 1997). However, under certain circumstances virus replication is triggered and infection can spread between bees, leading to outbreaks of disease. These outbreaks of overt disease may be spectacular and lead to significant losses (Allen & Ball, 1996; Ball, 1997).

Viruses persistent as inapparent infections in honeybees can be induced to multiply to readily detectable levels by laborious infectivity experiments. In these tests, extracts of apparently healthy live bees are injected into adult bees or pupae from the same colony (Allen & Ball, 1996). Viruses are then detected by immunodiffusion test, any other serological or molecular technique.

1. 4. 4. Detection

Several methods have been used to detect honeybee viruses including immunodiffusion, enzyme-linked immunosorbent assay (ELISA), enhanced chemiluminescent western blotting and RT-PCR (Allen & Ball, 1996; Allen *et al.*, 1986; Stoltz *et al.*, 1995). The most commonly used among these methods is still the immunodiffusion test because it is rapid, inexpensive and specific (Allen & Ball, 1996).

1. 4. 4. 1. Immunodiffusion test

Extracts of dead adult bees or brood are placed in wells made in a thin layer of agar jelly. Different virus antisera can then be placed in adjacent wells. The test extracts and antisera diffuse through the agar and when a virus meets its specific antiserum an insoluble complex is formed which results in the production of a visible line (Ball, 1997). This test is relatively insensitive; a high concentration of virus is required to give a clear positive reaction. Because of its sensitivity however, this test is a useful indicator of the cause of mortality in natural disease outbreaks (Ball, 1997).

1. 4. 4. 2. Enzyme-linked immunosorbent assay (ELISA)

ELISA is a more sensitive serological technique that also enables the quantification of the amount of virus present in test extracts. It relies on the detecting of antibody being conjugated to an enzyme. The amount of enzyme present depends on the amount of virus that will be bound to the antibody. Thus, the intensity of color reaction produced on addition of the enzyme substrate

can give a quantitative estimate of the amount of virus present by reference to standard virus dilutions (Ball, 1997). The ELISA is particularly useful for studying the early events in virus multiplication when the number of particles is small and the technique can also detect and quantify virus in individual mites (Allen *et al.*, 1986; Ball, 1997).

Even with the improved sensitivity of the ELISA technique, the serological methods still have the drawbacks of limited availability of antisera and questions regarding the specificity of some antisera as a result of antiserum production from preparations containing virus mixtures. Raising antisera is also a time consuming process with a large amount of a given virus needed to raise the antiserum. Consequently, the use of the serological method would be limited to laboratories that can produce large amounts of pure virus to raise a library of suitable antisera.

1. 4. 4. 3. Reverse transcriptase PCR

The use of this technique for the detection and identification of honeybee viruses is relatively new because of the lack of availability of sequence data for these viruses. The value of a diagnostic technique using RT-PCR is that it can be rapidly implemented in independent laboratories after the basic protocol and primer sequences are made available. The technique is reliable, specific and sensitive. It is rapidly becoming the method of choice for honeybee viruses' detection, especially to identify viruses present in field samples collected from bees under natural conditions (Stoltz *et al.*, 1995; Hung & Shimanuki, 1999; Benjeddou *et al.*, 2001; Grabensteiner *et al.*, 2001; Bakonyi *et al.*, 2002).

1. 5. BLACK QUEEN CELL VIRUS

Black Queen-Cell Virus (BQCV) was first isolated from queen larvae and pupae found dead in their cells (Bailey & Woods, 1974). The name of the virus was derived from darkened areas on the walls of the cells containing infected pupae. BQCV has been shown to be the most common cause of death of queen larvae in Australia (Anderson, 1993). The virus is also often present in bees infested with the microsporidian parasite *Nosema apis* (Allen & Ball, 1996; Bailey *et al.*, 1983) and may be implicated in the mortality of bees infected with this parasite.

The 30 nm BQCV particles contain a single-stranded RNA genome and four capsid proteins, with molecular masses of 34, 32, 29 and 6 kDa. The South African isolate of BQCV was found to have an 8550 nucleotide genome, excluding the poly(A) tail. The genome contained two ORFs, a 5'-proximal ORF encoding a putative replicase protein and a 3'-proximal ORF encoding a capsid polyprotein (Leat *et al.*, 2000). Three domains characteristic of helicases, 3C-like cysteine proteases and RNA-dependent RNA polymerases (RdRp) have been identified in the 5'-proximal ORF, using multiple sequence alignment analysis (Leat *et al.*, 2000). The translation initiation of the 3'-proximal ORF has been suggested to be facilitated by an IRES at a CCU codon (Leat *et al.*, 2000). Schematic diagrams illustrating the BQCV genome structure, structural polyprotein cleavage pattern, and amino acid cleavage site sequences are presented in Figure

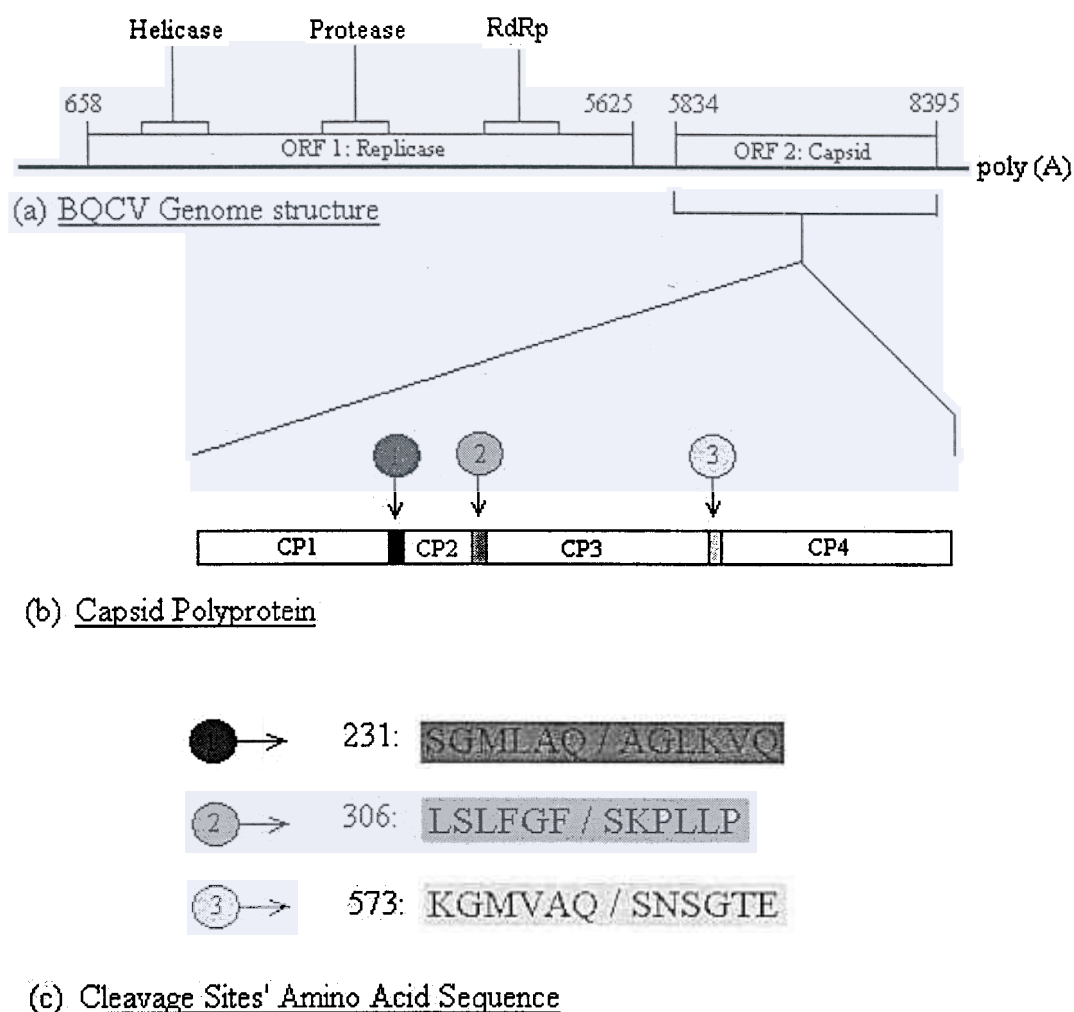


Fig. 1. 1. Schematic diagrams of the BQCV genome structure, structural protein cleavage pattern, and cleavage site sequence. From Leat *et al.* (2000). (a) BQCV (SA) genome structure. Open boxes represent ORF 1 and ORF 2. Numbers at the beginning and end of each ORF indicate the positions of the proposed initiation and termination codons. Regions of the genome proposed to encode a helicase, protease and RdRp are indicated. (b) Capsid polypeptide: schematic representation of the structural protein cleavage pattern. Boxes labels CP1 to CP4 refer to the mature capsid proteins of the BQCV (SA). Numbers 1 to 3 in circles indicate cleavage sites in the BQCV capsid protein. (c) Amino acid sequences of cleavage sites. The position of each cleavage site relative to the corresponding capsid polypeptide sequence is shown at the beginning of each sequence. The scissile bond of the cleavage site is represented by a forward slash.

1. 6. GENETIC ENGINEERING OF POSITIVE-STRAND RNA VIRUSES

1. 6. 1. Reverse genetics

The study of viruses and their interactions with host cells and organisms has benefited greatly from the ability to engineer specific mutations into viral genomes, a technique known as reverse genetics (Pekosz *et al.*, 1999). The ability to genetically engineer animal viruses has dramatically changed the understanding of how these organisms replicate and has allowed the construction of vectors to direct expression of heterologous proteins in different systems (Palese, 1998).

The reverse genetics of RNA viruses originated at about the same time as the dawning of the genomic manipulation of DNA viruses (Lai, 2000). The advent of recombinant DNA technology in the 1970s prompted RNA virologists to convert viral RNA genomes into complementary DNA copies and replicate them as plasmid inserts in bacterial hosts for easier genetic manipulation (Lai, 2000). The first success of this approach was achieved with the 4.5 kb Q β phage RNA (Lai, 2000). A plasmid containing the complete cDNA of this phage RNA was fully infectious when introduced into bacterial hosts, and was capable of completing the full viral replicating cycle. Later this technique was applied to several other viruses, including poliovirus (7.5 kb) and viroids, and for many years was used in poliovirus genetics (Lai, 2000). Subsequently, many more different strategies were employed to obtain infectious clones from RNA viruses.

1. 6. 2. Development of infectious clone systems

The genetic modification of RNA virus genomes depends on the availability of full-length cDNA clones from which functional RNA transcripts can be generated (Van_Dinten *et al.*, 1997). Infectious clone systems have been developed for a number of positive-stranded RNA viruses, including Picornaviruses, Caliciviruses, Alphaviruses, Flaviviruses, and Arteriviruses, whose RNA genomes range in size from ~7 to 15 kb in length (Yount *et al.*, 2000). The development of infectious clone systems has become an essential step in the molecular genetic analysis of these viruses. Infectious clones are useful in studies focused on genetic expression, replication, function of viral proteins, and recombination of viral proteins, as well as the development of new viral vectors and vaccines (Meulenberg *et al.*, 1998). The technology is applicable to positive-strand RNA viruses, whose RNA genomes function as mRNA and initiate a complete infectious cycle upon introduction into appropriate host cells (Meulenberg *et al.*, 1998). Infectious clone systems have been developed as infectious cDNAs (*in vivo*-transcribed RNAs) or as infectious *in vitro*-transcribed RNAs.

1. 6. 2. 1. Infectious cDNAs (*in vivo*-transcribed RNAs)

In this technique, plasmids containing the complete cDNA copy of the viral RNA are introduced into bacterial hosts, and a full viral replicating cycle is achieved. The viral cDNA is expressed by random initiation of transcription, probably from within the plasmid sequences, and the RNA is processed to the correct viral sequence (Lai, 2000). However, the mechanism whereby infectious RNAs are synthesised *in vivo* remains unclear (Boyer & Haenni, 1994). In more recent studies, the viral cDNA was placed under a specific promoter (e.g., cytomegalovirus

immediate-early promoter), and the ends of viral RNA were carefully engineered to match their natural sequences (Lai, 2000).

The expression of infectious viral RNAs through *in vivo* transcription of cDNA-containing vectors has several advantages over the *in vitro* transcription system. Firstly, the replication process can overcome detrimental effects resulting from RNA degradation that presumably occurs only within cells. Secondly, the *in vitro* transcription is not necessary. This is particularly important for RNA viruses for which the production of a good yield of highly infectious full-length transcripts can be problematic. Furthermore, costly reagents such as the cap analogues and RNA polymerases are not required. The third advantage is that the *in vivo*-produced viral transcripts are able to express native or mutant proteins without being replicated. This might be very convenient when studying the role and localisation of proteins expressed by mutant viral RNAs unable to replicate in cells (Boyer & Haenni, 1994).

Despite these advantages and the fact that historically the first infectious clones of RNA viruses were cDNA clones, to date there are only a few examples of animal and plant viruses for which full-length cDNA-containing vectors were successfully used for *in vivo* production of infectious RNA (Boyer & Haenni, 1994). Past examples of the successful use of the cDNA transfection approach for RNA viruses mostly involved viruses that normally replicate in the nucleus, such as hepatitis delta virus, viroids, and more recently, influenza virus (Lai, 2000). Success with some viruses that naturally replicate only in the cytoplasm, such as poliovirus and coronavirus, was intuitively thought to be artificial (Lai, 2000). This approach also involves the cloning and propagation of full-length viral cDNA clones in bacteria. This step has been proven to be very challenging for many viruses, because of high instability of many viral sequences in

bacteria. This instability was possibly due to toxicity of viral sequences in bacteria or to recombination and mutation events during propagation (Boyer & Haenni, 1994).

1. 6. 2. 2. Infectious *in vitro* transcripts

In this technique, viral RNA is made by *in vitro* transcription of viral cDNA templates linked to a promoter recognised by *Escherichia coli* or phage DNA-dependent RNA polymerases. When the *in vitro*-transcribed RNA is transfected into cells, it leads to viral RNA replication (Lai *et al.*, 1991). The fusion of the transcription promoters was made possible either through the use of universal transcription vectors, or by priming the second strand cDNA synthesis with an oligonucleotide containing a promoter directly linked to the 5'end of the viral sequence.

In the latter method, single stranded cDNA is amplified with PCR, and the purified amplicon is used directly for the *in vitro* synthesis of transcripts covering the entire genome. This procedure is now widely used for its simplicity and convenience (Hayes & Buck, 1990; Gritsun & Gould, 1995; Tellier *et al.*, 1996; Gritsun & Gould, 1998; Campbell & Pletnev, 2000), especially with the recent improvement of the RT-PCR procedures. The availability of high fidelity RT and polymerases has significantly decreased the error rate of RT-PCR. This approach overcomes the problem of presence of poison sequences, which make the cDNA sequence-containing plasmids unstable. This instability problem during propagation in *E. coli* is especially manifested in (+) RNA viruses (Yamshchikov *et al.*, 2001). In addition, bacteria also have the capacity to artificially select particular viral sequences; thus, the cloned sequences obtained often are non-randomly biased rather than representative of the majority of the RNA sequences (Lai, 2000). The use of the RT-PCR method helps to minimize the number of non-viral nucleotides at

the extremities of the viral transcripts, which can decrease or even abolish their infectivity. It is noteworthy that among all the commercially available *in vitro* transcription vectors, none leads to the production of transcripts devoid of vector-derived sequences.

1. 6. 2. 3. Parameters affecting infectivity

Although obtaining full-length cDNA and/or the corresponding transcripts is a crucial step, it does not necessarily ensure biological activity, as it depends on many parameters (Boyer & Haenni, 1994). Relative infectivity of the infectious clones is also very difficult to compare since the results obtained are expressed differently by various authors, depending on the system studied and on the method used (Boyer & Haenni, 1994).

The parameters that have influence on the infectivity of viral transcripts are: (1) the heterogeneity of the transcript size, (2) the presence of point mutations, (3) the number and sequence of non-viral nucleotides at the 5' and 3' ends, (4) the presence of a cap structure at the 5' end, and (5) the presence and length of a poly(A) tail at the 3' end (Boyer & Haenni, 1994).

The presence of non-viral nucleotides at the extremities of viral transcripts can reduce their infectivity. As a general rule, 5' extensions substantially decrease or even abolish infectivity, whereas 3' extensions are more easily tolerated (Boyer & Haenni, 1994). In both cases however, it is not clear whether the structure of the additional sequences affects the biological activity of the transcripts. In most cases examined, infectivity is greatly diminished even for short 5' extensions such as 1 or 2 nucleotides (generally G residues). In contrast, the biological activity of viral transcripts appears to be relatively insensitive to short 3' extensions of 1 to 7 nucleotides,

whereas very long extensions abolish infectivity. For many viruses, transcripts bearing more than 30 nucleotide-long additional sequence at the 3' end are infectious (Boyer & Haenni, 1994).

As a general rule, a cap structure (m⁷GpppG) is required at the 5' end of the transcripts for optimum infectivity, possibly because it enhances translation initiation and/or improves their stability by conferring a better resistance to host cell nucleases (Boyer & Haenni, 1994). Previous studies have shown that uncapped transcripts are either not infectious or show a highly reduced level of infectivity. However, in a few cases, both capped and uncapped transcripts proved to be highly infectious (Boyer & Haenni, 1994). In many RNA viruses where a viral-encoded protein (VPg) replaces the cap structure at the 5' extremity of their genome, it has been also suggested that adding a cap structure could compensate for the lack of VPg in the transcripts (Boyer & Haenni, 1994).

For viral genomes presenting a poly(A) tail at their 3' end, the presence of a long homopolymeric adenine sequences at the 3' end of the *in vitro*-produced transcripts increases their infectivity. It seems probable that there is a threshold length of A residues below which the stability of the transcripts would be altered (Boyer & Haenni, 1994).

1. 7. RNA VIRUS-BASED EXPRESSION VECTORS

The development of virus-based vectors for the expression of foreign genes is having a major impact on laboratory research, therapeutic treatments, and commercial biotechnology (Shivprasad *et al.*, 1999). Expression of foreign proteins/antigens has been shown for genetically

engineered influenza viruses, vesicular stomatitis virus, measles, respiratory syncytial, SV5, and Sendai viruses (Palese, 1998). Many of these efforts are aimed at expressing antigens of viruses, bacteria or parasites for which there are no (or no adequate) vaccines available (Palese, 1998). Viral vectors could be constructed to express multiple foreign proteins for use against multiple disease agents. Such single constructs could serve as universal vaccines (Palese, 1998). These novel constructs are being studied and characterised to expand the knowledge of how these chimeric viruses/vectors replicate and how they interact with the host (Palese, 1998).

RNA viruses have evolved to optimal fitness, and as a result most of them have a concise genome and the genetic information is expected to be essential (Bramel-Verheije *et al.*, 2000). Thus, the construction of a viral vector raises basic question as to the regions of the viral genome in which insertions are tolerated and about the maximum uptake capacity for foreign sequences (Bramel-Verheije *et al.*, 2000). Different RNA viruses might be more tolerant of sequence manipulations and may be less prone to insert instability (MacFarlane & Popovich, 2000). New vectors might be developed with increased carrying capacity or with the ability to express more than one non-viral protein (MacFarlane & Popovich, 2000).

Several strategies have been employed in the development of RNA virus-based expression vectors. Foreign antigens/proteins can be expressed as fusions with the viral polyprotein and subsequently cleaved at engineered 3C-protease cleavage sites. The expressed proteins could also be presented on the surface of the virion. Alternatively, the viral genome sequence is used to develop self-replicating RNA systems (or replicons) that express the proteins of interest as replacement of the structural protein genes.

1. 7. 1. Cleaved proteins

Cleaved protein strategy has been used to design several RNA virus-based vectors to express many proteins/antigens (Bramel-Verheije *et al.*, 2000; Mattion *et al.*, 1996; Mattion *et al.*, 1995). Foreign proteins are expressed as fusions with the viral polyprotein and are subsequently cleaved at an engineered 3C protease cleavage site (Mattion *et al.*, 1995). An extended polyprotein is produced in infected cells and proteolytically processed into the complete array of viral proteins plus the foreign peptide, which is excluded from mature virions (Andino *et al.*, 1994). Using this method, exogenous peptides up to 400 amino acids, have been successfully expressed by poliovirus recombinants (Andino *et al.*, 1994). It has been shown in another study, however, that polioviruses carrying smaller inserts had the tendency to grow better. Viable viruses were more readily obtained with insertions of less than 300 bases (Mattion *et al.*, 1995).

1. 7. 2. Presentation systems

In recent years there has been considerable interest in producing novel vaccines through the development of epitope presentation systems. In these systems, peptide sequence corresponding to an epitope from an animal pathogen is biochemically fused to a carrier molecule that is capable of assembly into a macromolecular structure (Porta *et al.*, 1994). Among the carrier molecules which have been used are the coat proteins of bacterial, animal and plant viruses (Porta *et al.*, 1994; Joelson *et al.*, 1997). However, there is a size limit for inserts that can be tolerated using this strategy. A 16-amino acid peptide fused to the C terminus of the capsid protein subunit of the tomato bushy stunt virus was successfully expressed, and its sequence was retained in the genome through six sequential passages in plants (Joelson *et al.*, 1997). Inserts as

long as 22 amino acids have also been shown to be tolerated by cowpea mosaic virus without any deleterious effect on virus growth (Porta *et al.*, 1994).

1. 7. 3. Self-replicating RNA systems

Systems for the expression of heterologous genes based on self-replicating RNAs (replicons) of a number of positive-strand RNA have been constructed. These viruses include poliovirus (Choi *et al.*, 1991; Porter *et al.*, 1995), sindbis virus (Xiong *et al.*, 1989; Kamrud *et al.*, 1995; Agapov *et al.*, 1998), kunjin virus (Khromykh & Westaway, 1997; Khromykh *et al.*, 1998), and semliki forest virus (Liljestrom & Garoff, 1991; Zhou *et al.*, 1994). In these systems, structural genes are replaced by foreign sequences of proteins of interest, leading to the production of replicons, which are capable of initiating replication and gene expression after RNA transfection but are not packaged into viruses (Agapov *et al.*, 1998). The packaging of replicons is possible by supplying the viral structural genes *in trans* (Agapov *et al.*, 1998).

The success of these expression systems is due to a high level of expression, easy construction of recombinants, and the ability to produce high-titer stocks of pseudo-infectious particles containing recombinant replicon RNA packaged by the structural proteins supplied from co-transfected helper RNA (Varnavski & Khromykh, 1999).

1. 8. MAIN AIMS OF THE PROJECTS AND OUTLINE OF THE THESIS

Increasing knowledge on the association of honeybee viruses with other honeybee parasites, primarily the ectoparasitic mite *Varroa destructor*, and their implication in the mass mortality of

honeybee colonies, has resulted in increasing awareness and interest in honeybee viruses. The role of viruses as honeybee pathogens seems to be more important than previously thought. Viral infections in honeybees occur frequently and may cause severe clinical symptoms and enormous losses.

Despite their economic and ecological impact, comprehensive molecular studies on honeybee viruses have only recently been initiated. This is surprising given the fact that the majority of these viruses are remarkably amenable to molecular analysis. To provide a foundation of molecular information on honeybee viruses, our laboratory has embarked on two projects aimed at determining the complete nucleotide sequence of the Black Queen Cell Virus and the Acute Bee Paralysis Virus. These two viruses have been shown to be implicated in increased honeybee mortality in African honeybee colonies infected with varroa mite in South Africa.

The identification, monitoring and prevention of spread of bee viruses is of considerable importance, particularly when considering the lack of information on the natural incidence of virus infections in honeybee populations worldwide. However, because virus particles are often present in small numbers and can only be detected by laborious infection experiments, viruses are not yet included in the health certification procedures for honeybee imports and exports (Allen and Ball, 1996). Developing a sensitive diagnostic technique would help to identify viruses present in bees under natural conditions, and could be used to screen virus preparations, employed in research, to ensure they are free of other contaminant viruses.

The availability of bee virus sequence data raised the possibility of using the RT-PCR technique for the identification and detection of BQCV and ABPV. Unique PCR primers were

developed against these two viruses. The results of testing these primers against purified virus particles and virus-infected bees are presented in **Chapter 2**.

In addition to its association with *Varroa destructor* in South Africa and in many parts of the world, BQCV is known for its universal association with the microsporidian parasite *Nosema apis*. Nosema disease is probably the most widespread disease of adult honeybees (Swart *et al.*, 2001). Because of its association with two of the most important and widespread parasites of the honeybee, BQCV is an ideal candidate among honeybee viruses for an in-depth molecular study. The aim of the study would be primarily to develop new molecular tools to study the molecular biology, persistence, pathogenesis, and more importantly interaction of honeybee viruses with other parasites. It will open opportunities for the design of new experiments aimed at understanding the bee parasitic mite syndrome.

Infectious clone technology has become an essential tool in the molecular genetic analysis of positive-strand RNA viruses. It allows direct manipulation of their viral genomes at the molecular level, thus resolving problems relating to virus replication, virulence, pathogenesis and more importantly host-virus interactions. The development of this technology for honeybee viruses will facilitate the creation of chimeric viruses or the introduction of specific mutations into their viral genomes. The first step in the development of this technology for BQCV or any other honeybee virus must be the demonstration of the infectivity of its naked RNA. Since there is no cell culture system available for honeybee viruses, it was necessary to demonstrate the ability of naked genomic RNA from BQCV to initiate an infection upon direct injection into live bee pupae (**Chapter 3**).

The development of a reverse genetics system for BQCV is described in **Chapter 4**. A system was established for the *in vitro* production of full-length infectious transcripts for BQCV. To show that the genome of this virus can be manipulated, a single base mutation abolishing an *EcoRI* restriction site was introduced by PCR-directed mutagenesis. The *EcoRI* restriction site was present in the wild type virus but not in the mutated virus.

The potential of BQCV as a vector was further explored by insertion of sequences to express foreign proteins. The sequence encoding for the Green Fluorescent Protein (GFP) was inserted into the BQCV genome. The results of these experiments are presented in **Chapter 5**.

CHAPTER 2

DETECTION OF BLACK QUEEN CELL VIRUS AND ACUTE BEE PARALYSIS VIRUS FROM HONEYBEES BY REVERSE TRANSCRIPTASE PCR*

2. 1. Abstract

A reverse transcriptase PCR (RT-PCR) assay was developed for the detection of black queen-cell virus (BQCV) and acute bee-paralysis virus (ABPV), two common honeybee viruses. Complete genome sequences were used to design unique PCR primers within a 1-kb region from the 3' end of both genomes to amplify a fragment of 700 bp from BQCV and 900 bp from ABPV. The combined guanidinium thiocyanate and silica membrane method was used to extract total RNA from samples of healthy and laboratory-infected bee pupae. In a blind test, RT-PCR successfully identified the samples containing BQCV and ABPV. Sensitivities were of the order of 130 genome equivalents of purified BQCV and 1600 genome equivalents of ABPV.

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2. 2. Introduction

The complete nucleotide sequence of the genome of two important viruses, BQCV and ABPV, has been determined in our laboratory (Govan *et al.*, 2000; Leat *et al.*, 2000). These viruses, together with another two unidentified viruses, have been shown to be implicated in increased mortality in South African honeybee colonies infected with *Varroa* mite (Swart *et al.*, 2001).

BQCV was originally found in dead honeybee queen larvae and pupae (Bailey & Woods, 1977) and has been shown to be the most common cause of death of queen larvae in Australia (Anderson, 1993). The virus has isometric particles and 30 nm in diameter and a single-stranded RNA genome of 8550 nucleotides excluding the poly(A) tail (Leat *et al.*, 2000). BQCV is often present in bees infested with the Microsporidian parasite, *Nosema apis* (Bailey *et al.*, 1983; Allen & Ball, 1996) and may be implicated in the mortality of bees infected with this parasite.

ABPV was originally discovered as an inapparent infection in laboratory experiments (Bailey *et al.*, 1963) and is widespread as a latent and inapparent infection (Allen & Ball, 1996; Hung *et al.*, 1996). This virus spreads by way of salivary gland secretions of adult bees and in food stores to which these secretions are added (Ball, 1985). It has a single-stranded RNA genome of 9470 nucleotides excluding the poly(A) tail (Govan *et al.*, 2000). ABPV has been identified as a major factor contributing to the mortality of honeybees in colonies infested by *Varroa jacobsoni* (Ball & Allen, 1988).

Several methods have been used to detect honeybee viruses including immunodiffusion, enzyme-linked immunosorbent assay (ELISA), enhanced chemiluminescent western blotting and

RT-PCR (Allen & Ball, 1996; Allen *et al.*, 1986; Stoltz *et al.*, 1995). The most commonly used among these methods is still the immunodiffusion test because it is rapid, inexpensive and specific (Allen & Ball, 1996). However, the serological methods have the drawbacks of limited availability of antisera and questions regarding the specificity of some antisera as a result of antiserum production from preparations containing virus mixtures. Raising antisera is also a time consuming process with a large amount of a given virus needed to raise the antiserum. Consequently, the use of the serological method would be limited to laboratories that can produce large amounts of pure virus to raise a library of suitable antisera. By contrast, a diagnostic technique using RT-PCR can be rapidly implemented in independent laboratories after the basic protocol and primer sequences are made available.

RT-PCR has been used to detect a variety of RNA viruses including the picorna-like insect viruses (Chungue *et al.*, 1993; van der Wilk *et al.*, 1994; Stoltz *et al.*, 1995; Canning *et al.*, 1996; Stevens *et al.*, 1997; Harris *et al.*, 1998; Johnson & Christian, 1999; Hung & Shimanuki, 1999). The technique is reliable, specific and sensitive. However, RT-PCR experiments on insects are usually hampered by the problem of inhibitory components, which compromise reverse transcription and PCR reactions (Chungue *et al.*, 1993). To overcome this problem, many RNA extraction methods have been developed or modified in order to remove these inhibitors (Singh, 1998).

In the method described here total RNA was extracted from infected and healthy bee pupae using Nucleospin RNAII total RNA isolation Kit of Macherey-Nagel. The kit uses the combined guanidinium thiocyanate and silica membrane methods. This method has been successfully used

in aphids, plants and mosquitoes (Stevens *et al.*, 1997; Naidu *et al.*, 1998; Chungue *et al.*, 1993; Harris *et al.*, 1998).

2. 3. Materials and methods

2. 3. 1. Virus stocks, propagation, and purification

BQCV was previously isolated from adult bees in South Africa and identified as BQCV using an antiserum raised against the original BQCV isolate (Leat *et al.*, 2000). The virus was propagated in apparently healthy white- to purple-eyed drones or worker bee pupae and subsequently purified as described by Leat *et al.* (2000). Aliquots of 100- μ l CsCl-purified virus were stored at -20°C. Inocula of ABPV, Kashmir Bee Virus (KBV) and Cloudy Wing Virus (CWV) were kindly supplied by B. Ball (Rothamsted Experimental Station, UK) and successfully propagated in our laboratory, except for CWV where a working stock was provided. To determine the concentration of the BQCV and ABPV virus stocks, viral RNA was phenol extracted, ethanol precipitated and quantified with an UV spectrophotometer (Sambrook *et al.*, 1989). Since the length and molecular weight of each genome is known, the number of genome copies could be calculated.

2. 3. 2. RNA extraction

Individual healthy or infected bee pupae were weighed, placed in 1.5ml Eppendorf tubes, an appropriate volume of grinding buffer (0.1M NaCl, 0.1M glycine, 10mM EDTA, pH 9.5) was added and then ground with a disposable pestle. The volume of grinding buffer used was about 200 μ l per 30mg of bee material. Total RNA was extracted from approximately 250 μ l of the

homogenate following the manufacturers' instructions. Pure RNA was finally eluted in 40µl of RNase-free water.

2. 3. 3. Reverse transcription and PCR amplification

Reverse transcription-polymerase chain reactions were performed using Titan™ RT-PCR system (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany). Each 20µl reaction mixture contained 9µl template, 0.2 mM of each dNTP, 0.5µM forward primer, 0.5µM reverse primer, 5mM DTT, 0.4 µl Titan polymerase mix and 4µl 5xRT-PCR buffer (1.5mM MgCl₂). Reverse transcription and amplification was performed in a Hybaid OMN-E thermocycler. The 9µl template contained either RNA extracted with Nucleospin RNAII total RNA isolation Kit or whole virus particles treated at 90 °C for 5 min prior to amplification. The RT-PCR profile used was as follows; a reverse transcription stage at 50 °C for 30 min, followed by an initial denaturation stage at 94 °C for 2 min. This was followed by 35 amplification cycles of 94 °C for 30s, 60 °C for 30s, and 72 °C for 30s, and a final extension step at 72 °C for 7min. The resulting PCR products were visualized by electrophoresis through 1-% agarose gel containing ethidium bromide.

2. 3. 4. Oligonucleotide primers

Oligonucleotides for detection of ABPV and BQCV were designed within a 1-kb region from the 3'end of their respective genomes (ABPV accession number AF150629, Govan *et al.*, 2000; BQCV accession number AF183905, Leat *et al.*, 2000). The ABPV primers were ABPV1, 5'-TAA TGT GTC CAG AGA CTG TAT CCA I-3' and ABPV2, 5'-GCT CCT ATT GCT CGG TTT TTC GGT I-3' corresponding to nucleotides 8460-8484 and 9336-9360 respectively. These

primers amplify a 900bp fragment. The BQCV primers were BQCV1, 5'-TGG TCA GCT CCC ACT ACC TTA AAC I-3' and BQCV2, 5'-GCA ACA AGA AGA AAC GTA AAC CAC I-3' corresponding to nucleotides 7850-7874 and 8526-8550 respectively. These primers amplify a 700bp fragment.

2. 4. Results and discussion

different strains of ABPV and BQCV may vary slightly in genome sequence, deoxyinosine residues (I) were incorporated into the 3' end of each primer to ensure its annealing to the template in case of a strain-specific mismatch at this position. Primers were compared to all honeybee virus sequences available in the databases. The maximum degree of sequence identity was 64%. This occurred between ABPV primer and BQCV, Cloudy Wing Virus (CWV) and Kashmir Bee Virus (KBV) and also between BQCV2 primer and ABPV. Primers were tested against four honeybee viruses available in our laboratory ABPV, BQCV, CWV and KBV. Purified virus stocks were used directly for RT-PCR as described above.

1 (lane 1-5) shows that ABPV primers amplified a fragment of the predicted molecular weight (900bp) from the ABPV genome. They failed to produce any PCR products when tested against BQCV, CWV and KBV. Similarly, BQCV primers were specific to BQCV and failed to amplify any fragments from ABPV, CWV or KBV (figure 1, lane 6-11). The BQCV PCR amplicon was 700bp as predicted.

Using these primers, the RT-PCR was able to detect ABPV and BQCV in laboratory-infected bee pupae. Figure 2 (lane -4) shows that RT-PCR was able to detect ABPV in laboratory-infected bee pupae. Figure 2 (lane 5-8) shows similar results with BQCV, where the predicted

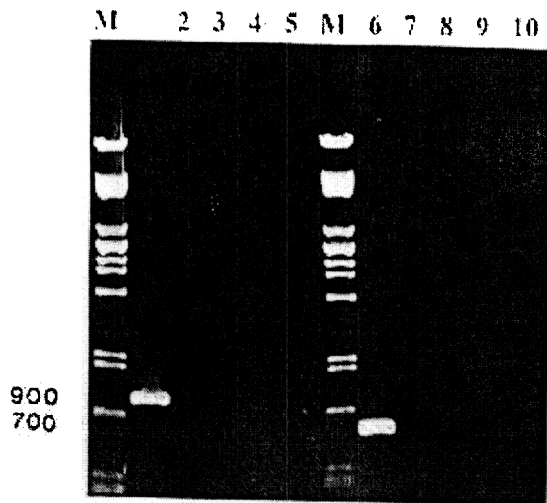


Fig. 2. 1. Test of the RT-PCR amplification specificity of the ABPV primers set (lane 1-5) and the BQCV primers set (lane 6-10). Each set was tested against four different honeybee viruses. M, *Pst* lambda DNA marker; lane 1, ABPV; lane 2, BQCV; lane 3, CWV; lane 4, KBV; lane 5, water (negative control); lane 6, BQCV; lane 7, ABPV; lane 8, CWV; lane 9, KBV; lane 10, water (negative control).

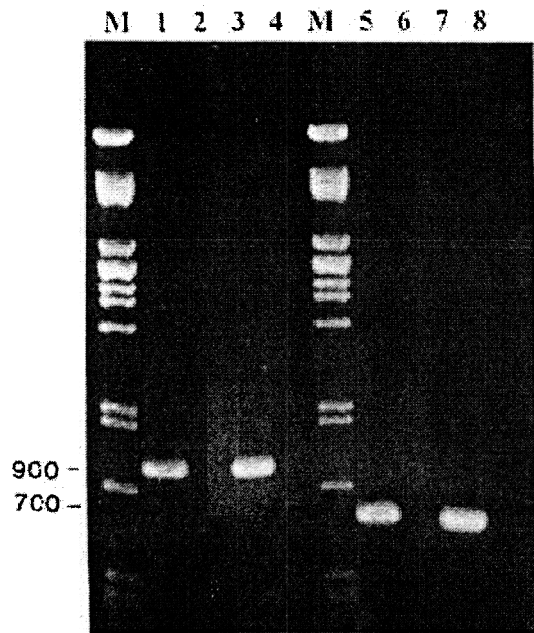


Fig. 2. 2. Detection of ABPV and BQCV in honeybee by RT-PCR. Extraction of the total RNA from healthy and laboratory-infected bee pupae and RT-PCR amplification were performed as described above. M, *Pst* lambda DNA marker; lane 1, ABPV-infected bee pupae; lane 2, healthy bee pupae; lane 3, ABPV virus stock (positive control); lane 4, water (negative control); lane 5, BQCV-infected bee pupae; lane 6, healthy bee pupae; lane 7, BQCV virus stock (positive control); lane 8, water (negative control).

700bp fragment from BQCV was amplified. In both cases, no PCR fragments were amplified from the healthy bee total RNA. In addition, a blind test was performed on 30 samples of healthy bees and bees infected with ABPV or BQCV. RT-PCR successfully identified the samples containing ABPV and BQCV.

To determine the sensitivity of the RT-PCR, stocks of ABPV and BQCV containing 1.6×10^7 and 1.3×10^8 genome equivalents/ μ l respectively were diluted and tested. The total reaction mixture of the RT-PCR (20 μ l) was loaded on an agarose gel, and samples were considered positive if a DNA band of the predicted molecular weight was visible. Figures 3 and 4 show the detection sensitivity of RT-PCR on these samples.

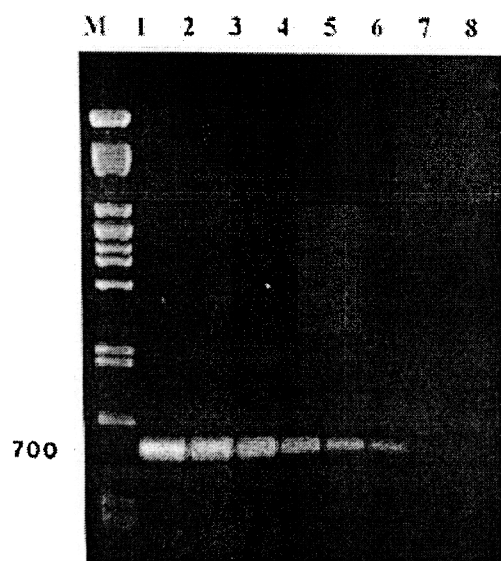


Fig. 2. 3. Determination of the sensitivity of amplification by RT-PCR of the BQCV. Serial 10-fold dilutions of BQCV virus stock containing 1.3×10^8 genome copies/ μ l. One microlitre of this stock was used in the RT-PCR mixture. M, *Pst* lambda DNA marker; lane 1, BQCV; lane 2, 10^{-1} BQCV dilution; lane 3, 10^{-2} BQCV dilution; lane 4, 10^{-3} BQCV dilution; lane 5, 10^{-4} BQCV, lane 6, 10^{-5} BQCV; lane 7, 10^{-6} BQCV dilution; lane 8, 10^{-7} BQCV dilution; lane 9, water (negative control).

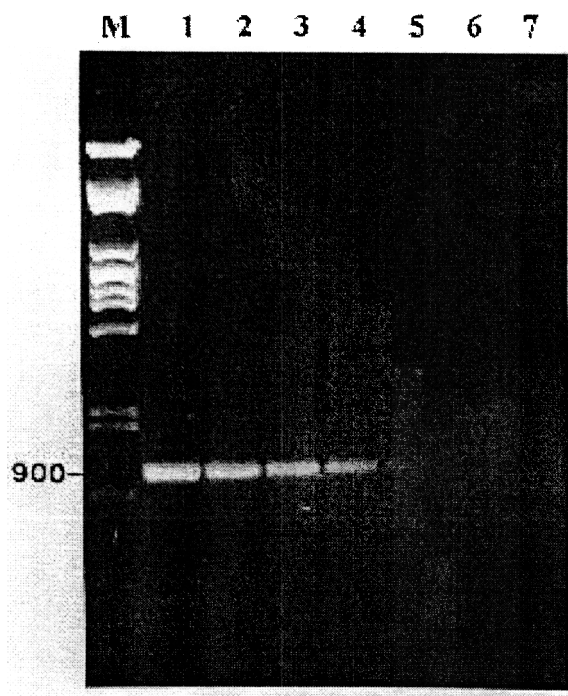


Fig. 2. 4. Determination of the sensitivity of amplification by RT-PCR of the ABPV. Serial 10-fold dilutions of ABPV virus stock containing 1.6×10^7 genome copies/ μ l. One microlitre of this stock was used in the RT-PCR mixture. M, *Pst* lambda DNA marker; lane 1, ABPV; lane 2, 10^{-1} ABPV dilution; lane 3, 10^{-2} ABPV dilution; lane 4, 10^{-3} ABPV dilution; lane 5, 10^{-4} ABPV; lane 6, 10^{-5} ABPV; lane 7, water (negative control).

The detection limit of ABPV and BQCV was estimated to be about 1600 and 130 genome equivalents respectively. These results are similar to other reports on RT-PCR detection limits. (Canning *et al.*, 1996) achieved a sensitivity of 1000 genome copies of the barley yellow dwarf virus. (van der Wilk *et al.*, 1994) reported a sensitivity of 300-400 viral particles of purified tobacco rattle virus. (Wilde *et al.*, 1990) also achieved a sensitivity of this order. They reported positive results from 500 genomic copies of rotavirus RNA. The 10-fold difference in sensitivity between ABPV and BQCV primers could be attributed to the difference in the size of the amplified fragments. Shorter fragments have been shown to be more sensitive than longer ones.

In potato Y virus, (Singh & Singh, 1997) achieved a 100-fold difference in sensitivity when amplifying two fragments of 1016 and 704 bp in size from the 3' end of the genome.

In this study, a sensitive RT-PCR assay was developed for the detection of purified ABPV and BQCV or the detection of these viruses in infected pupae. Similarly, RT-PCR assays for the identification of other bee viruses can be developed when the genome sequences become available.

2. 5. Acknowledgements

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We also thank B. Ball (Rothamsted Experimental Station, Harpenden, United Kingdom) for providing virus stock

CHAPTER 3

THE BLACK QUEEN CELL VIRUS GENOMIC RNA IS INFECTIOUS IN HONEYBEE PUPAE*

3. 1. Abstract

The ability of the BQCV naked genomic RNA to initiate a complete infectious cycle upon injection into honeybee pupae was tested. Purified BQCV particles, viral RNA, RNase A-treated BQCV particles, RNase A-treated viral RNA and phosphate buffer were injected into bee pupae. The bee pupae were incubated at 30-35°C for 8 days prior to the virus being purified. Virus particles were generated by injection of bee pupae with purified BQCV particles, viral RNA and RNase A-treated BQCV particles. No virus was recovered from bees injected with RNase A-treated viral RNA or with phosphate buffer. An amount of 66 ng of viral RNA per bee pupae was found to be sufficient to cause a regular infection.

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3. 2. Introduction

Evidence has been presented suggesting interactions between honeybee viruses and parasites. BQCV is often associated with a Microsporidian parasite, *Nosema apis* (Bailey *et al.* 1983; Allen & Ball, 1996) and may be implicated in the mortality of bees infested with this parasite. Of even more concern is the relationship between the honeybee viruses and the *Varroa destructor* mite. It has been suggested that viruses and mites acting together have a significant negative effect on the colonies. This has led to the use of the term “bee parasitic mite syndrome” to describe a disease complex of colonies simultaneously infested with mites and infected with viruses and accompanied with high mortality (Shimanuki *et al.*, 1994).

The use of infectious clone technology is likely to be very useful in understanding the relationship between viruses and mites. Infectious clone technology would allow direct manipulation of the genomes of honeybee viruses, which would make it possible for individual viruses to be labelled and tracked in colonies infested with mites. In addition, direct manipulation of the genomes of honeybee viruses at the molecular level, would facilitate studies on replication, virulence, pathogenesis and more importantly host-virus interactions. The first step in the development of this technology for BQCV or any positive-stranded RNA virus must be the demonstration of infectivity of its naked RNA. The RNA genomes of these viruses must function as mRNA and be able to initiate a complete infectious cycle upon introduction into appropriate host cells.

In this study, BQCV viral RNA was tested for infectivity in honeybee pupae. The optimum amount of viral RNA to give infection was also determined.

3. 3. Materials and Methods

Viral RNA was extracted with the Nucleospin RNA II total RNA isolation kit (Macherey-GmbH & Co., Düren, Germany). The RNA was then quantified with an UV spectrophotometer. Several preparations of viral RNA were made in order to examine its infectivity. The preparations were as follows: a positive control of purified BQCV particles, BQCV particles treated with RNase A, viral RNA, and viral RNA treated with RNase A. Phosphate buffer was used as a negative control. Different amounts of RNA were injected to determine the optimum amount of infectious RNA. RNase A was used at a concentration of 9 ng RNase A/ μ g RNA and incubated at 25°C for 1 hour. The complete degradation of RNA treated with RNaseA, was confirmed by running samples on a 1-% agarose gel (Figure 3. 1.).

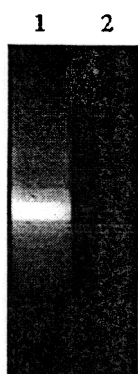


Fig. 3. 1. Confirmation of the complete degradation of RNA following treatment with RNase A. RNase A was used at a concentration of 9 ng RNase A/ μ g RNA and incubated at 25°C for 1 hour. Lane1, viral RNA; lane 2, RNase A-treated viral RNA.

Two microlitres of each preparation was injected into bee pupae. The bee pupae were incubated at 30-35°C for 8 days prior to the virus being purified as described by (Leat *et al.*,

The formation of virus particles was first confirmed by electron microscopy. Samples were negatively stained with 1-% uranyl acetate and examined under a transmission electron microscope.

3. 4. Results and discussion

BQCV particles generated by injection of bee pupae with naked genomic RNA were readily observed by electron microscopy (Figure 3. 2).

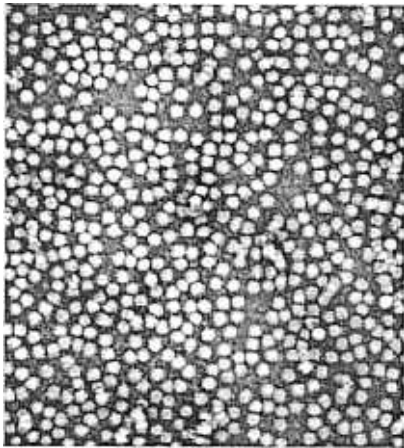


Fig. 3. 2. Electron micrograph of 30 nm purified RNA-generated BQCV particles stained with 1-% uranyl acetate.

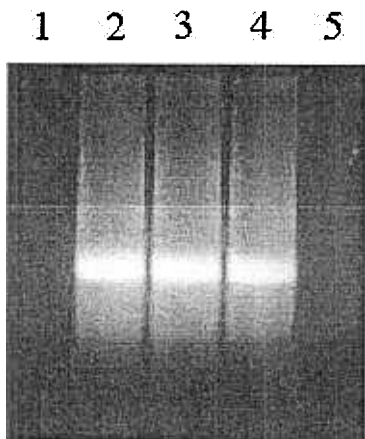


Fig. 3. 3. Confirmation of generation of virus particles from infection experiments, using the RNA extraction method. Virus extracts were prepared from infected bee pupae as described in the text. RNA was then extracted from samples using the Nucleospin RNA II total RNA isolation kit, and run on a 1-% agarose gel. Lane 1, extract from bee pupae injected with phosphate buffer (negative control); lane 2, extract from bee pupae injected with BQCV particles (positive control); lane 3, extract from bee pupae injected with RNase-treated BQCV particles; lane 4, extract from bee pupae injected with RNA; lane 5, extract from bee pupae injected with RNase-treated RNA.

Virus particles were also generated from injection with pure BQCV particles and BQCV particles treated with RNase A, but not from injection with RNase A-treated viral RNA or with

phosphate buffer. This shows clearly that RNase A abolishes the infectivity of naked RNA while having no influence on the infectivity of intact particles. The electron microscope results were further confirmed by extracting RNA from samples and running it on 1-% agarose gel (Figure 3. 3.).

Viral RNA was recovered from samples injected with viral RNA, pure BQCV particles and BQCV particles treated with RNase A, but not from those injected with RNase A-treated viral RNA or with phosphate buffer. Furthermore, viral particles obtained from the different bee samples were confirmed as BQCV, using the RT-PCR method described by Benjeddou *et al.* (2001) (Figure 3. 4.).

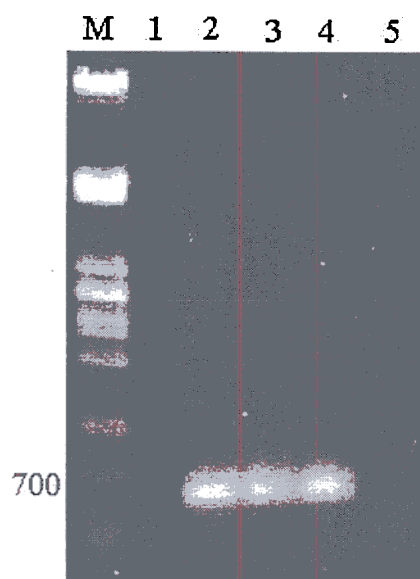


Fig. 3. 4. Confirmation of the authenticity of the recovered viral particles using the RT-PCR method described by Benjeddou *et al.* (2001). Lane 1, bee pupae injected with phosphate buffer (negative control); lane 2, bee pupae injected with BQCV particles (positive control); lane 3, bee pupae injected with RNase-treated BQCV particles; lane 4, bee pupae injected with RNA; lane 5, bee pupae injected with RNase-treated RNA.

The amount of 66 ng of viral RNA per bee pupae was found to be sufficient to generate a regular infection. This was simply confirmed by obtaining an even field of virus particles when samples were observed under electron microscope.

Demonstrating that BQCV naked RNA is infectious in bee pupae would make it possible to overcome the limitation of not having a cell culture system, and to design experiments aimed at developing a system for the production of infectious clones/transcripts for this virus.

3. 5. Acknowledgements

We thank Mr. Mike Allsopp, from the Agricultural Research Council, South Africa, for his assistance during this work. This work was funded by the National Research Foundation, South Africa.

CHAPTER 4

DEVELOPMENT OF INFECTIOUS TRANSCRIPTS AND GENOME MANIPULATION OF THE BLACK QUEEN-CELL VIRUS OF HONEYBEES

4. 1. Abstract

The South African isolate of the Black Queen-Cell Virus (BQCV), a honeybee virus, was previously found to have an 8550 nucleotide genome excluding the poly(A) tail. Its genome contained two ORFs, a 5'-proximal ORF encoding a putative replicase protein and a 3'-proximal ORF encoding a capsid polyprotein. Long Reverse Transcription-PCR was used to produce infectious transcripts for BQCV, and to manipulate its genome. Primers were designed for the amplification of the complete genome, the *in vitro* transcription of infectious RNA, and PCR-directed mutagenesis. An 18-mer antisense primer was designed for reverse transcription (RT) to produce full-length single-stranded cDNA (ss cDNA). Unpurified ss cDNA from the RT reaction mixture was used directly as a template to amplify the full genome using long high fidelity PCR. The SP6 promoter sequence was introduced into the sense primer to transcribe RNA directly from the amplicon. RNA was transcribed *in vitro* with and without the presence of a cap analog and injected directly into bee pupae and incubated for 8 days. *In vitro* transcripts were infectious but the presence of a cap analog did not increase the amount of virus recovered. A single base

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mutation abolishing an *EcoRI* restriction site was introduced by fusion-PCR, to distinguish viral particles recovered from infectious transcripts from the wild type virus (wtBQCV). The mutant virus (mutBQCV) and wtBQCV were indistinguishable using electron microscopy and western blot analysis. The *EcoRI* restriction site was present in wtBQCV and not in mutBQCV.

4. 2. Introduction

The 30 nm BQCV particles contain a single-stranded RNA genome and four capsid proteins, with molecular masses of 34, 32, 29 and 6 kDa. The South African isolate of BQCV was found to have an 8550 nucleotide genome, excluding the poly(A) tail. The genome contained two ORFs, a 5'-proximal ORF encoding a putative replicase protein and a 3'-proximal ORF encoding a capsid polyprotein (Leat *et al.*, 2000). It has been proposed that BQCV should be added to the *Cricket paralysis-like viruses* group (Leat *et al.*, 2000). This novel group of insect infecting RNA viruses includes *Cricket paralysis virus* (CrPV), *Drosophila C virus* (DCV), *Plautia stali intestine virus* (PSIV), *Rhopalosiphum padi virus* (RhPV), and *Himetobi P virus* (HiPV) (van Regenmortel *et al.*, 2000). The genomes of CrPV, DCV, PSIV, RhPV and HiPV are monopartite and bicistronic with replicase proteins encoded by a 5'-proximal ORF and capsid proteins by a 3'-proximal ORF. In the case of PSIV, translation initiation of the 3'-proximal ORF has been demonstrated to be dependent on an internal ribosome entry site (IRES), starting at a CUU codon (Sasaki & Nakashima, 1999). Similarly, it has been suggested that translation initiation of the 3'-proximal ORF of BQCV is facilitated by an IRES at a CCU codon (Leat *et al.*, 2000).

The study of viruses and their interactions with host cells and organisms has benefited greatly from the ability to engineer specific mutations into viral genomes, a technique known as reverse genetics (Pekosz *et al.*, 1999). Such reverse genetics systems have been developed for a number of positive-stranded RNA viruses, including *Picornaviruses*, *Caliciviruses*, *Alphaviruses*, *Flaviviruses*, and *Arteriviruses*, whose RNA genomes range in size from ~7 to 15 kb in length (Yount *et al.*, 2000). The production of cDNA clones and/or PCR-amplicons, from which infectious RNA can be transcribed *in vitro*, is an essential step in the development of reverse

genetics systems for these viruses. The availability of these clones/PCR-amplicons has facilitated the study of the genetic expression and replication of RNA viruses by use of mutagenesis, deletions, and insertions and by complementation experiments. It has also enhanced the understanding of the molecular mechanisms of natural or induced RNA recombination, and of plant-virus interactions such as cell-to-cell movement. This has resulted in the development of new viral vectors and vaccines (Boyer & Haenni, 1994).

The production of infectious RNA transcripts from PCR-amplicons has become a method of choice for many investigators because of the improvements in the PCR in terms of fidelity and length of amplification. The pioneering work of Gritsun and Gould (1995 and 1998) has also resulted in the improvement of this method by using a combination of primer sets and optimising their concentrations. This method is simple, rapid, and it overcomes the problem of instability of certain sequences in bacteria (Hayes & Buck, 1990; Gritsun & Gould, 1995; Tellier *et al.*, 1996; Gritsun & Gould, 1998; Campbell & Pletnev, 2000).

To our knowledge, no reverse genetics system have been described for BQCV, or any other member of the *Cricket paralysis-like virus* group. Here we describe the development of infectious transcripts and the manipulation of the BQCV genome using long reverse transcription-PCR, and adapting systems and strategies used in previous studies (Gritsun & Gould, 1995; Tellier *et al.*, 1996; Gritsun & Gould, 1998). The BQCV mutants produced from infectious transcripts have the potential to be used in new experiments aimed at understanding the bee parasitic mite syndrome.

4. 3. Materials and methods

4. 3. 1. Virus stocks, propagation, and purification

BQCV was previously isolated from adult bees in South Africa and identified as BQCV using an antiserum raised against the original BQCV isolate (Leat *et al.*, 2000). The virus was propagated in apparently healthy white- to purple-eyed drones or worker bee pupae and subsequently purified as described by (Leat *et al.*, 2000). Aliquots of 100- μ l CsCl-purified virus were stored at -20°C.

4. 3. 2. RNA purification

RNA was extracted from 100- μ l CsCl-purified virus or transcription reaction mixture using the Nucleospin RNA II total RNA isolation kit (Macherey-Nagel GmbH & Co., Düren, Germany), following the manufacturers' instructions. Pure RNA was finally eluted in 40- μ l RNase-free water, quantified with an UV spectrophotometer and used immediately.

4. 3. 3. Primers

Primers used in this work (Table 4. 1.) were designed on the basis of the nucleotide sequence of BQCV (BQCV (SA), accession no. AF183905). They were designed for the production of full-length ss cDNA of the BQCV genome, the PCR amplification of this genome, the *in vitro* transcription of infectious RNA, and PCR-directed mutagenesis. The SP6 promoter, *NotI*, and *KpnI* sequences were introduced into the relevant primers (Table 4.1.)

| Primer | Restriction site | Nucleotide sequence (*) | Position on the genome |
|--|--------------------------|---|----------------------------|
| <u>Reverse transcription and genome amplification primers</u> | | | |
| FCDNA | | 5'-TTTTTTTTTTTTTGCAAC-3' | 8546-8563 (polyA included) |
| INFECLONE1 | <i>NorI</i> | 5'-GGGTAT GCGGCCGC ATTAGGTGACACTATAGAATACGCAA AATTGCGTATAGTATATAAAAT-3' | 1-26 |
| RFCDNA | <i>KpnI</i> | 5'-GGGTAT GGTACC (T ₂₅)GCAACAAGAAGAAACGTAAACC-3' | 8529-8575 (polyA included) |
| 5RFCDNA | | 5'-AAGTACACGGAATATATATCATCTATAAGA-3' | 4296-4325 |
| 3FFCDNA | | 5'-AAAATATAGAAGCTGCTAGACTTGGAAAAGA-3' | 4221-4250 |
| <u>EcoR I (position 4066) mutagenesis primers</u> | | | |
| ECORIF1 | <i>EcoRI</i> (abolished) | 5'-GTGCTCTACCTAACCACCTTGGATGAATGCG AGTTC AT-3' | 4037-4073 |
| ECORIR1 | <i>EcoRI</i> (abolished) | 5'-GGAGGATCCTTTACCTTTCCCATCAAGATGA ACTCGC -3' | 4064-4100 |
| MUTF | | 5'-TGCAATCTGTTGGTTCTAC-3' | 3623-3641 |
| MUTR | | 5'-CATCCTTCCTTAACGAAAG-3' | 4448-4466 |

(*) Restriction site in bold and SP6 promoter in Italics.

Table 4. 1. Primers used for reverse transcription, full-length genome amplification and PCR-directed mutagenesis

4. 3. 4. Reverse transcription of viral RNA

Viral RNA, extracted as described above, was reverse transcribed using the Universal RiboClone cDNA Synthesis System (Promega Corporation, Madison, USA). The reverse transcription reaction was carried out in a volume of 25 µl and stopped after completion of the first strand cDNA synthesis. Two micrograms of viral RNA were used together with 2 µl of the primer FCDNA at a concentration of 0.5 mg/ml to synthesize the first cDNA strand. Manufacturer's recommendations were followed, except that the reaction was incubated at 42°C for 4 hours. One microlitre of RNase H (2-units/µl) was then added and the reaction was incubated for 20 min at 37°C.

4. 3. 5. Amplification of the full-length BQCV genome by long high fidelity PCR

Expand 20 kb^{PLUS} PCR System (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany) was used to amplify the full-length BQCV genome. This was achieved using a 50- μ l reaction, performed in 0.2-ml thin-wall PCR tubes using a Perkin Elmer 9600 thermocycler. Unpurified ss cDNA from the RT reaction mixture was used directly as template in the PCR reaction mixture. Each PCR reaction mixture contained 2- μ l of template, 0.5 mM of each deoxynucleotide triphosphate, 0.3 μ M forward primer (INFECLONE1), 0.3 μ M reverse primer (RFCDNA), 10 μ l 5xPCR buffer, 0.75 mM MgCl₂, 0.75 μ l enzyme mix, and dH₂O to a total volume of 50 μ l. The reaction was prepared in two separate master mixes as recommended by the manufacturer. The long PCR profile started with an initial denaturation stage at 94°C for 2 min followed by 3 cycles of 93°C for 15 s, 57°C for 30 s, and 68°C for 6 min. A third stage consisted of 7 cycles of 92°C for 10 s, 70°C for 30 s, and 68°C for 6 min. The final stage of 20 cycles consisted of 92°C for 10 s, 70°C for 30 s, and 68°C for 6 min with an increment of 10 s each cycle. The final extension was performed at 68°C for 20 min. After completion of the amplification, 2 units of RNase H were added to the PCR product and incubated at room temperature for 2 hours to avoid any possible RNA carry-over in the following steps. The PCR reaction product was then purified using the High Pure PCR purification Kit from Roche as instructed by the manufacturers. The elution volume was 50- μ l. The concentration of the purified amplicon was determined and the DNA was stored at -20°C until further use.

4. 3. 6. PCR-directed mutagenesis

Two overlapping genome halves (5'-half and 3'-half) were amplified using the mutagenic primers ECORIF and ECORIR1 (Table 4. 1.). This introduced a single base mutation at position 4068 abolishing an *EcoRI* site. The fragments were then joined using fusion-PCR. The reaction mixtures for these PCR reactions were identical to that of the full genome amplification except that primers INFECLONE1 and ECORIR1, and ECORIF1 and RFCDNA were used to amplify the 5'-half and the 3'-half of the genome respectively. The profile for the amplification of the 5'-half was as follows: a denaturation step at 94°C for 2 min followed by one cycle of 93°C for 15 s, 57°C for 30 s, and 68°C for 5 min. This was then followed by 29 cycles of 92°C for 10 s, 70°C for 30 s, and 68°C for 5 min and a final extension step at 68°C for 10 min. The profile for the amplification of the 3'-half was identical to that of the 5'-half except that the annealing temperature was 63°C in the first cycle and 71°C in the following 29 cycles. Both PCR products were gel-purified to avoid carry-over.

The fusion-PCR reaction mixture was identical to that of the full-genome amplification except that a mixture of 45 ng of the 5'-half and 50 ng of the 3'-half were used as templates. The reaction mixture lacking the enzyme mix and the primers (INFECLONE1 and RFCDNA) was heated at 94°C for 2 min. The enzyme mix was added when the temperature reached 94°C. One cycle of 92°C for 10 s, 80°C for 30s, and 68°C for 9 min was used to extend the templates to cover the whole genome. Primers (INFECLONE1 and RFCDNA) were then added at 92°C at the beginning of the third stage, which consisted of 29 cycles of 92°C for 10 s, 70°C for 30 s, and

68°C for 9 min. The extension was performed at 68°C for 20 min. The PCR product obtained was purified and used for *in vitro* transcription reactions as described below

4. 3. 7. *In vitro* transcription of the full-length BQCV genome

Purified PCR products were run on a 0.8 % agarose gel, the correct band was excised and gel-purified using Nucleotrap gel purification kit (Macherey-Nagel). The *in vitro* transcription was performed using the Riboprobe[®] *in vitro* Transcription System (Promega). The PCR product containing the SP6 promoter and a full-length copy of the BQCV genome was used as a template in the *in vitro* transcription reactions. Five micrograms of the PCR product was added to a 100- μ l *in vitro* transcription reaction mix with or without the presence of a cap analog (Roche). The final concentration of the cap analog in the reaction mixture was 0.5 mM. The reaction mixture contained 20- μ l of transcription optimized 5xbuffer, 10 mM of DTT, 100 units of RNase inhibitor, 0.75 mM of each the rNTPs, 45 units of SP6 RNA polymerase, and RNase-free dH₂O to the final reaction volume. The reaction was incubated at 37°C for 2 hours and 30 min. Five units of RQ1 RNase-free DNase was then added to the reaction mix and incubated for further 15 min at 37°C to remove the template DNA. The transcribed RNA was extracted from the reaction mixture with the Nucleospin RNA II total RNA isolation kit (Macherey-Nagel), eluted in 40 μ l RNase-free water, and quantified with an UV spectrophotometer. RNA was used immediately in the infection experiments.

4. 3. 8. Infectivity test of transcripts and progeny virions

Four batches of apparently healthy white- to purple-eyed drones or worker bee pupae were injected simultaneously with viral RNA, phosphate buffer, authentic *in vitro* transcribed RNA

(wild type sequence) and mutated *in vitro* transcribed RNA. Every batch consisted of 50 bee pupae. The amount of RNA injected was 66 ng and 660ng of viral RNA and *in vitro* transcribed RNA respectively. The bee pupae were incubated at 30-35°C for 8 days then the virus was purified as described by Leat *et al.* (2000). For every treatment, virus was purified from 5 lots of 10 bee pupae homogenized together (Leat *et al.*, 2000). Recovered virus was 10-fold diluted and re-propagated as above. The amount of virus recovered was estimated in genome equivalents and as a mean value of the 5 lots for each treatment, as described by Benjeddou *et al.* (2001). In this method, viral RNA is phenol extracted, ethanol precipitated and quantified with an UV spectrophotometer. Since the molecular weight of the virus genome is known, the number of genome equivalents could be calculated.

4. 3. 9. Electron microscopy

The formation of particles was first confirmed by electron microscopy. Purified virus samples were negatively stained with 1-% uranyl acetate and examined under a transmission electron microscope.

4. 3. 10. Western blot

Capsid proteins were resolved on 12-% SDS-PAGE gels using standard protocols (Sambrook *et al.*, 1989). Proteins were blotted onto PVDF membrane and detected using rabbit polyclonal antibodies against purified BQCV and the BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim, Mannheim, Germany) following the manufacturers' instructions.

4. 3. 11. Detection of the mutated virus by RT-PCR and restriction digestion

MUTF and MUTR primers were used to amplify a fragment of 844 bp from the virus genome by RT-PCR. The amplified fragment extends from nucleotide 3623 to nucleotide 4466 in the BQCV genome. It covers two *Eco*RI sites at positions 3884 and 4066. The first site was left unaltered, the second was mutated eliminating the *Eco*RI site. The strategy for the detection of the mutation is summarized in figure 4

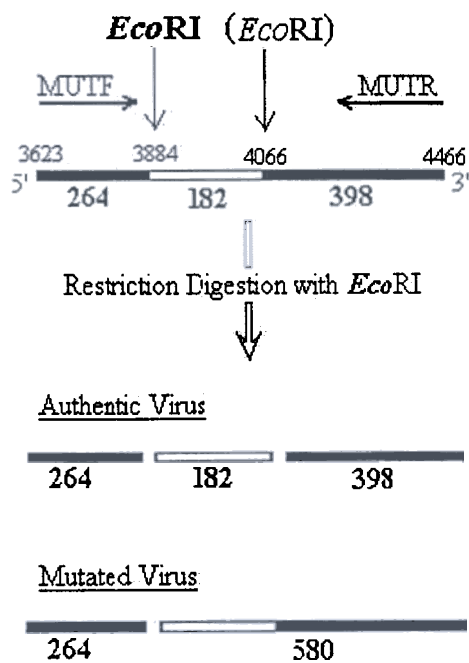


Fig. 4. 1. Strategy for the identification of mutated virus by RT-PCR and restriction digestion. An 844 bp fragment is amplified by RT-PCR, purified with a commercial PCR purification Kit, and digested with the *Eco*RI restriction enzyme. Three fragments of 398 bp, 264 bp and 182 bp will result from this digestion in the case of authentic virus, and 2 fragments of 580 bp and 264 bp in the case of mutated virus.

RT-PCRs were performed using the Titan RT-PCR system (Roche). Each 20- μ l of reaction mixture contained 9- μ l of template, 0.2 mM of each deoxynucleoside triphosphate, 0.5 μ M

MUTF primer, 0.5 μ M MUTR primer, 5 mM dithiothreitol, 0.4 μ l of Titan polymerase mix, and 4 μ l of 5xRT-PCR buffer (1.5 mM MgCl_2). The 9- μ l template contained either RNA extracted with Nucleospin RNAII total RNA isolation Kit or whole virus particles treated at 90°C for 5 min prior to amplification. The RT-PCR profile used was as follows: a reverse transcription stage at 50°C for 30 min, followed by an initial denaturation stage at 94°C for 2 min. This was followed by 35 amplification cycles of 94°C for 30s, 52°C for 30s, and 72°C for 1 min, and a final extension step at 72°C for 7min. The PCR reaction product was then purified using the High Pure PCR purification Kit (Roche), as instructed by the manufacturers, and eluted in 50- μ l elution buffer. Four hundred nanograms of amplified DNA was digested with *Eco*RI and run on a 2 % agarose gel.

4. 4. Results

4. 4. 1. Construction of full-length cDNA of BQCV

Primers were designed for the amplification of the BQCV full genome by long high fidelity PCR. Viral RNA was extracted from CsCl-purified BQCV samples using a commercial RNA extraction kit. The RNA concentration was determined and run on a 1-% agarose gel to confirm its integrity (data not shown). The primer FCDNA, an 18-mer primer with an annealing temperature of 42°C, was used for the reverse-transcription reaction. The reaction was incubated at 42°C for 4 hours to ensure the synthesis of full-length ss cDNA. RNase H was then added to the reaction mixture to degrade the RNA. Two microlitres of the reverse-transcription reaction were used directly in the 50- μ l PCR reaction mixture to amplify the entire genome of BQCV. The INFECLONE1, used as forward primer, contained the SP6-promoter sequence in addition to a *Not*I restriction site to facilitate the cloning of the PCR product when needed. The reverse

primer RFCDNA contained a poly(T) stretch of 25 bases and a *Kpn* restriction site. The PCR product was purified and run on a 0.8% agarose gel (figure 2, lane 1). To confirm that the whole BQCV genome was amplified, the primer 5RFCDNA was used together with INFECLONE1 and the primer 3FFCDNA together with RFCDNA to amplify the 5' and 3' halves of the genome respectively and run on a 0.8% agarose gel (data not shown). The full-length cDNA band was gel purified to ensure that it was free from shorter DNA and again treated with RNase H to prevent any RNA carry-over.

4. 4. 2. Introduction of a genetic marker mutation by PCR-directed mutagenesis

Two overlapping fragments covering the 5' and the 3' halves of the genome and containing the same single base mutation from A to G at position 4068 were successfully amplified (figure 4. 2., lanes 2 and 3). The mutation abolished an *EcoRI* site at position 4066, which is 182 bases apart from another *EcoRI* site that was left unaltered. The 2 amplicons were gel-purified to prevent ss cDNA carry-over and used together as template for the fusion PCR (figure 4. 2., lane 4). In order to confirm the success of the mutation, 9-ng of the purified PCR product was used in another PCR reaction to amplify an 844 bp fragment which was then digested with *EcoRI* as described above (data not shown).

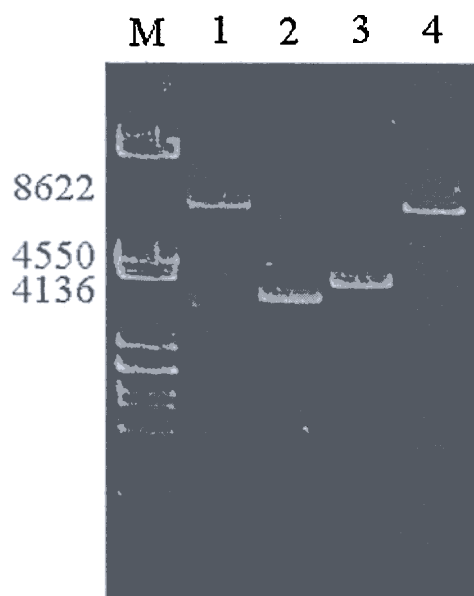


Fig. 4. 2. Amplification of the BQCV genome, and introduction of a mutation by fusion-PCR, using long high fidelity PCR. The silent mutation A-4068 to G, abolishing the *EcoRI*⁴⁰⁶⁶ site, was introduced into the overlap of the 5'-half and 3'-half amplicons using mutagenic primers (table 4.1.) as described in Materials and Methods. The 2 amplicons were joined together with fusion-PCR. M, *Pst* lambda DNA marker; lane 1, full-length genome amplification (8622 bp); lane 2, amplification of the 5'-half (4136 bp) with introduction of a single point mutation; lane 3, amplification of the 3'-half (4550 bp) with introduction of the same single point mutation; lane 4, fusion of the two genome halves by fusion-PCR.

4. 4. 3. *In vitro* transcription and infectivity test of transcripts

PCR products were used as templates for the *in vitro* synthesis of transcripts corresponding to the authentic and mutated genome sequences. The synthesized RNAs were purified and their concentrations were measured. After incubation at 37°C for 2 hour and 30 min, the reaction yield was in the order of 5.7-7.8 µg of RNA/µg of template DNA. Transcripts were injected directly into bee pupae, incubated at 30-35°C for 8 days and virus was finally extracted. The infectivity of viral RNA was estimated to be 1.3×10^{11} genome equivalents per ng of RNA injected, and that of *in vitro* transcribed RNA was 3.7×10^8 genome equivalents per ng of RNA injected. The

addition of a cap analog to the transcription reaction did not improve the infectivity of the transcribed RNA molecules. However, the virus recovered from infection with *in vitro* transcribed RNA was as infectious as the wild type virus.

4. 4. 4. Authentication of the virus recovered from bees used in the infectivity test

Viral particles generated from injection of bee pupae with wtBQCV viral RNA and mutated RNA transcripts (mutBQCV), were physically indistinguishable when examined using transmission electron microscopy (figure 4. 3.). Western blot analysis of the capsid proteins of the various viruses indicated that they are indeed BQCV particles (figure 4. 4.). RNA was extracted from virus particles and run on a 1-% agarose gel (figure 4. 5.). This confirmed that the RNA had been packaged into the observed viral particles. The same RNA was used in the RT-PCR reaction to amplify an 844 bp fragment that covered the region where the *EcoRI* site was abolished in the mutated RNA transcripts. The fragment also covered an *EcoRI* site that was left unaltered. The purified PCR product was digested with *EcoRI*. Distinction between wild type and mutant virus was made possible by the digestion pattern of the amplified fragment (figure 4. 6.).

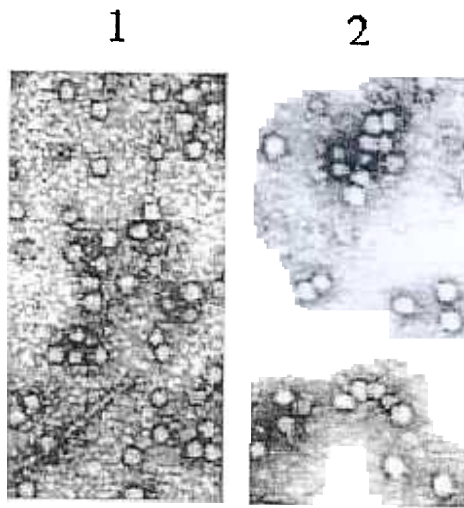


Fig. 4. 3. Electron micrographs of BQCV virus particles extracted from bee pupae injected with viral or mutated *in vitro* transcribed RNA, as described in Materials and Methods except that the process was stopped at the sucrose gradient step. (1) Wild type virus (wtBQCV) recovered from bee pupae injected with viral RNA. (2) Mutant virus (mutBQCV) recovered from bee pupae injected with mutated *in vitro* transcribed RNA.

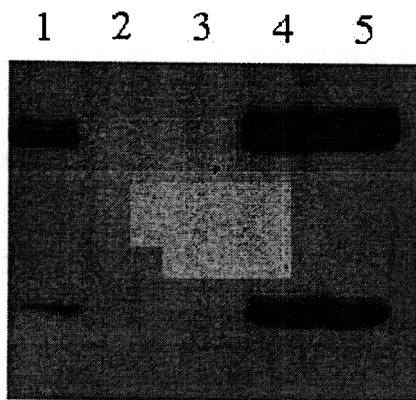


Fig. 4. 4. Western blot analysis of the capsid proteins of virus particles. Wild type virus and virus particles extracted from bee pupae infected with authentic and mutated *in vitro* transcribed RNA were examined. Virus extracts were prepared from bee pupae as described in Materials and Methods, except that the process was stopped at the sucrose gradient step. Lane 1, CsCl-purified wtBQCV particles (positive control); lane 2, SDS gel-loading buffer (negative control); lane 3, extract from bee pupae injected with phosphate buffer (negative control); lane, 4 extract from bee pupae injected with authentic *in vitro* transcribed RNA; lane 5, extract from bee pupae injected with mutated *in vitro* transcribed RNA.

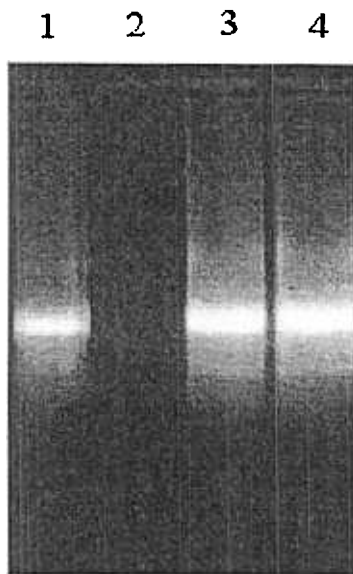


Fig. 4. 5. Confirmation of generation of virus particles by using the RNA extraction method. Virus extracts were prepared from bee pupae as described in Materials and Methods, except that the process was stopped at the sucrose gradient step. RNA was then extracted from samples using the Nucleospin RNA II total RNA isolation kit, and run on a 1% agarose gel. Lane 1, CsCl-purified wtBQCV particles (positive control); lane 2, extract from bee pupae injected with phosphate buffer (negative control); lane 3, extract from bee pupae injected with authentic *in vitro* transcribed RNA; lane 4, extract from bee pupae injected with mutated *in vitro* transcribed RNA.

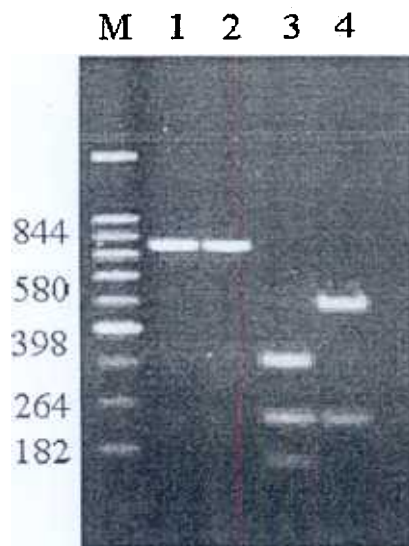


Fig. 4. 6. Detection of the mutant virus with RT-PCR and restriction digestion of the amplicon with *EcoRI*. An 844 bp fragment was amplified by RT-PCR, purified using the High Pure PCR purification Kit (Roche), and digested with the *EcoRI* restriction enzyme. M, 100 bp molecular weight marker (Promega); lane 1, undigested PCR product from the wild type BQCV virus (wtBQCV); lane 2, undigested PCR product from the mutant virus (mutBQCV); lane 3, digested PCR product from the wtBQCV; lane 4, digested PCR product from the mutBQCV.

4. 5. Discussion

Attempts to construct a full-length clone of BQCV, following a multistep strategy based on the genomic cDNA library available from a previous sequencing project (Leat *et al.*, 2000), was not successful. Genome-length ss cDNA was synthesized by reverse-transcription using a specific primer, with an annealing temperature of 42 degrees, and an extended incubation time of 4 hours. The incubation period was extended to increase the number of full-length cDNA copies. The entire genome was amplified with long high fidelity PCR using 2- μ l of the unpurified RT reaction mixture, and a second reverse primer carrying a polyA tail and a *Kpn* site. To avoid interference from the initial RT primer the second reverse primer was designed to have a higher melting temperature (Gritsun & Gould, 1998). The purified amplicon was used directly for the *in vitro* synthesis of transcripts covering the entire genome (Gritsun & Gould, 1995; Tellier *et al.*, 1996; Gritsun & Gould, 1998). When injected into bee pupae, these transcripts were infectious. This simple and rapid approach was previously used (Hayes & Buck, 1990; Gritsun & Gould, 1995; Tellier *et al.*, 1996; Gritsun & Gould, 1998; Campbell & Pletnev, 2000). This approach overcomes the high instability of full-length cDNA clones in bacteria (Rice *et al.*, 1989; Lai *et al.*, 1991; Sumiyoshi *et al.*, 1992; Skotnicki *et al.*, 1993; Khromykh & Westaway, 1994; Campbell & Pletnev, 2000). This instability was possibly due to toxicity of viral sequences in bacteria or to recombination and mutation events during propagation (Boyer & Haenni, 1994). Attempts made to generate an infectious clone from the amplified genomic DNA were not successful. However, efforts are still being made to clone this full-length amplicon because of the significant advantages that infectious clones offer.

Relative infectivity of infectious clones from previous studies is very difficult to compare since the results obtained were expressed differently by various authors, depending on the system studied and the method used (Boyer & Haenni, 1994). However, results obtained from various studies showed that the infectivity of RNA transcripts was lower than that of authentic virion RNA (Lai *et al.*, 1991; Sit & AbouHaidar, 1993; Khromykh & Westaway, 1994; Gritsun & Gould, 1995; Iwamoto *et al.*, 2001). These studies showed that viral particles generated from infectious transcripts are as infectious as wild type particles. In this study, infectivity was expressed as the number of genome equivalents recovered per ng of injected RNA (Benjeddou *et al.*, 2001). The infectivity of RNA transcripts was about 350-fold lower than the infection efficiency of the wild type viral RNA. The recovered virus was also as infectious as the wild type. The reduced infection efficiency could be explained by the presence of non-infectious DNA templates (amplicons) as a result of errors introduced in the RT and/or PCR reactions. The presence of extra nucleotides at the 5' and 3' ends of the *in vitro*-transcribed RNAs could have also negatively affected their infectivity (Lai *et al.*, 1991; Sit & AbouHaidar, 1993; Khromykh & Westaway, 1994; Boyer & Haenni, 1994). The extraneous nucleotides could have been precisely trimmed or excluded by the expressed RNA-dependent RNA polymerase during replication (Khromykh & Westaway, 1994; Yao & Vakharia, 1998), and the progeny virus acquired an infection efficiency as high as the parent virus. Increasing the poly(A) tail at the 3' end was not explored, but capping of the RNA transcripts did not improve infectivity. It is widely accepted that a cap structure (m⁷GpppG) is required at the 5' end of the transcripts for optimum infectivity, possibly because it enhances translation initiation or it improves their stability by conferring a greater resistance to host cell nucleases (Boyer & Haenni, 1994). In a few cases, however, both capped and uncapped transcripts have been proved to be highly infectious (Boyer & Haenni,

1994). In contrast, uncapped transcripts were shown to be more infectious than capped ones for tomato bushy stunt virus (Hearne *et al.*, 1990). It is not known if BQCV has a viral-encoded protein (VPg) or a cap structure at the 5'-extremity of its genome. Therefore, it is not clear if adding the cap structure would have compensated for the lack of a VPg as has been suggested for other viruses (Boyer & Haenni, 1994). Since the capping of the RNA transcripts was not experimentally confirmed, the possibility that lower infectivity was due to poor capping efficiency can not be excluded (Sit & AbouHaidar, 1993).

Progeny virus recovered from bee pupae infected with *in vitro* transcribed RNA molecules and the wild type virus could not be distinguished using electron microscopy and western blot analysis. Distinction between the two viruses was made possible by introducing a mutation, using fusion-PCR. The fusion-PCR method, used for mutagenesis, was a combination of the method used by Gritsun & Gould (1995) with that of Rebel *et al.* (2000). The mutation abolished one of two *EcoRI* sites, 182 bases apart. This genetic marker mutation was used to clearly demonstrate that viral particles, recovered from these experiments, originated from infectious transcripts, and were not simply the product of an activated inapparent infection.

To our knowledge, the development of this reverse genetics system for BQCV is the first for honeybee viruses. This development will open new opportunities for studies directed at understanding the molecular biology, persistence, pathogenesis, and interaction of these viruses with other parasites. Given that the BQCV genome can be manipulated, the potential of this virus as a vector will be explored by insertion of sequences to express foreign proteins.

4. 6. Acknowledgements

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CHAPTER 5

ENGINEERING THE BLACK QUEEN-CELL VIRUS AS A VECTOR FOR THE EXPRESSION OF THE GREEN FLUORESCENT PROTEIN

5. 1. Abstract

Genome-length ss cDNA was synthesized by reverse-transcription, and used directly for the amplification of two fragments, 5'-BQCV and 3'-BQCV, extending from the 5'end to base number 7600 and from base number 7526 to the 3' end respectively, using long high fidelity PCR. The sequence encoding for GFP was also amplified using high fidelity PCR. The three fragments were successfully joined together by fusion-PCR following a stepwise strategy and using the overlaps between fragments that were created by relevant primers. The obtained amplicon (gfpBQCV) was used as template in the *in vitro* transcription reactions. The sequence encoding the green fluorescent protein was confirmed by RT-PCR to have been integrated in the *in vitro* transcribed RNA of gfpBQCV. The gfpBQCV transcripts were infectious, but it was not possible to detect any fluorescence from whole bees or electrophoresed bee extracts. An RT-PCR analysis of the progeny virus recovered showed that no GFP-encoding sequence was present in the viral genome, but the virus was authenticated as being BQCV.

5. 2. Introduction

The South African isolate of BQCV was found to have an 8550 nucleotide genome, excluding the poly(A) tail. The genome contained two ORFs, a 5'-proximal ORF encoding a putative replicase protein and a 3'-proximal ORF encoding a capsid polyprotein (Leat *et al.*, 2000). The 3'-proximal ORF (ORF 2) was shown to encode the BQCV capsid polyprotein, which is proteolytically processed into 4 mature structural proteins, CP1, CP2, CP3 and CP4 of a molecular weight of 29, 6, 32 and 34 kDa respectively (Leat *et al.*, 2000).

A reverse genetics system was previously established for BQCV using long high fidelity PCR (**Chapter 4**). It was also shown possible to introduce a single base mutation abolishing an *EcoRI* restriction site using fusion-PCR. This mutation makes it possible to distinguish between mutant virus (mutBQCV) and wild-type virus (wtBQCV). The BQCV mutants produced from infectious transcripts have the potential to be used in new experiments aimed at understanding the bee parasitic mite syndrome. However, the detection of the mutation is laborious and the virus needs to be purified first. An alternative to this system is to develop a BQCV-based vector to express a marker gene.

The gene encoding the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been demonstrated to function as a marker gene for transformation studies in several different biological systems (Baulcombe *et al.*, 1995; Shivprasad *et al.*, 1999; Li *et al.*, 2000). The GFP is an ideal marker gene because it allows for the nondestructive visualization of the spread of infection and relative quantification of levels of gene expression (Shivprasad *et al.* 1999).

In the present study, a method successfully used to generate poliovirus recombinants for the expression of exogenous peptides up to 400 amino acids (Andino *et al.*, 1994), was adopted with modification to construct BQCV recombinants to express the green fluorescent protein.

5. 3. Materials and methods

5. 3. 1. Generation of recombinant virus

Synthesis of ss cDNA from the wtBQCV genome, the construction of a full-length amplicon carrying the SP6 promoter, the production of *in vitro* transcripts from this amplicon, the infection of bee pupae, and subsequent purification of virus was performed as described previously in **Chapter 4**. In this study, All PCR reactions were performed in 0.2-ml thin-wall PCR tubes using a Perkin Elmer 9600 thermocycler. A reaction volume of 50- μ l was used throughout the project. PCR reaction products were then purified using the High Pure PCR purification Kit from Roche as instructed by the manufacturers. The elution volume was 50- μ l. The concentration of the purified amplicons was determined and the DNA was stored at -20°C until further use. Alternatively, PCR products were run on a 0.8-1% agarose gel, the correct band was excised and gel-purified using Nucleotrap gel purification kit (Macherey-Nagel).

5. 3. 2. Primers

Primers used in this work (Table 5.) were designed on the basis of the nucleotide sequence of BQCV (BQCV (SA), accession no. AF183905) and the Enhanced Green Fluorescent Protein (eGFP, accession no. AF323988). They were designed for the production of full-length ss cDNA of the BQCV genome, the PCR amplification of this genome, the *in vitro* transcription of infectious RNA, and the fusion PCR for the insertion of the GFP sequence into the BQCV

genome. The SP6 promoter, *NotI*, and *KpnI* sequences were introduced into the relevant primers (Table 5.1.).

| Primer (Restriction site) | Nucleotide sequence (*) | Position on the wtBQCV genome |
|------------------------------|---|----------------------------------|
| FCDNA | 5'-TTTTTTTTTTTTGCAAC-3' | 8546-8563 (polyA included) |
| INFECLONE1 (<i>NotI</i>) | 5'-GGGTAT GCGGCCGC <i>ATTAGGTGACTATA</i> <i>GAATACGCAAAATTGCGTATAGTATATAAAT</i> -3' | 1-26 |
| CP3EGFP(1)R | 5'-GTGAACAGCTCCTCGCCCTTGCTCACCATTAAAC AAAGATGATCCTCTGGGGTTTTGTTG-3' | 7571-7600 |
| EGFP(1)F | 5'-GGATCATCTTTGTTAATGGTGAGCAAGGGCGAG GAGCTGTTAC-3' | |
| EGFP(1)R | 5'-CTTCCTCCCTGGCTTGACAGCTCGTCCATGCC-3' | |
| EGFPCP4(1)F | 5'-CGGCATGGACGAGCTGTACAAGCCAGGGAGGA AGGGGATGG-3' | 7526-7545 |
| RFCDNA40 (<i>KpnI</i>) | 5'-GGGTAT GGTACC (T ₄₀)GCAACAAGAAGAAAC GTAAACC-3' | 8529-8614 (polyA included) |

(*) Restriction site in bold and SP6 promoter in Italics.

TABLE 5. 1. Primers used for reverse transcription, full-length genome amplification and insertion of a sequence encoding the green fluorescent protein (GFP).

5. 3. 3. Construction of the gfpBQCV amplicon

The construct made to insert the GFP encoding sequence into the BQCV genome was named gfpBQCV. A long fragment from wtBQCV genome extending from the 5'end to base number 7600 and covering CP3/CP4 cleavage site was amplified by PCR and named 5'-BQCV (figure 5 1.). A second fragment also covering CP3/CP4 cleavage site, and extending from base number 7526 to the 3' end of the genome, was amplified by PCR and named 3'-BQCV (figure 5.-1.)

The sequence encoding GFP was inserted between sequences encoding CP3 and CP4 of the capsid polyprotein, and the CP3/CP4 cleavage site incorporated between CP3 and GFP as well as between GFP and CP4 (figures 5. 1. and 5. 2.).

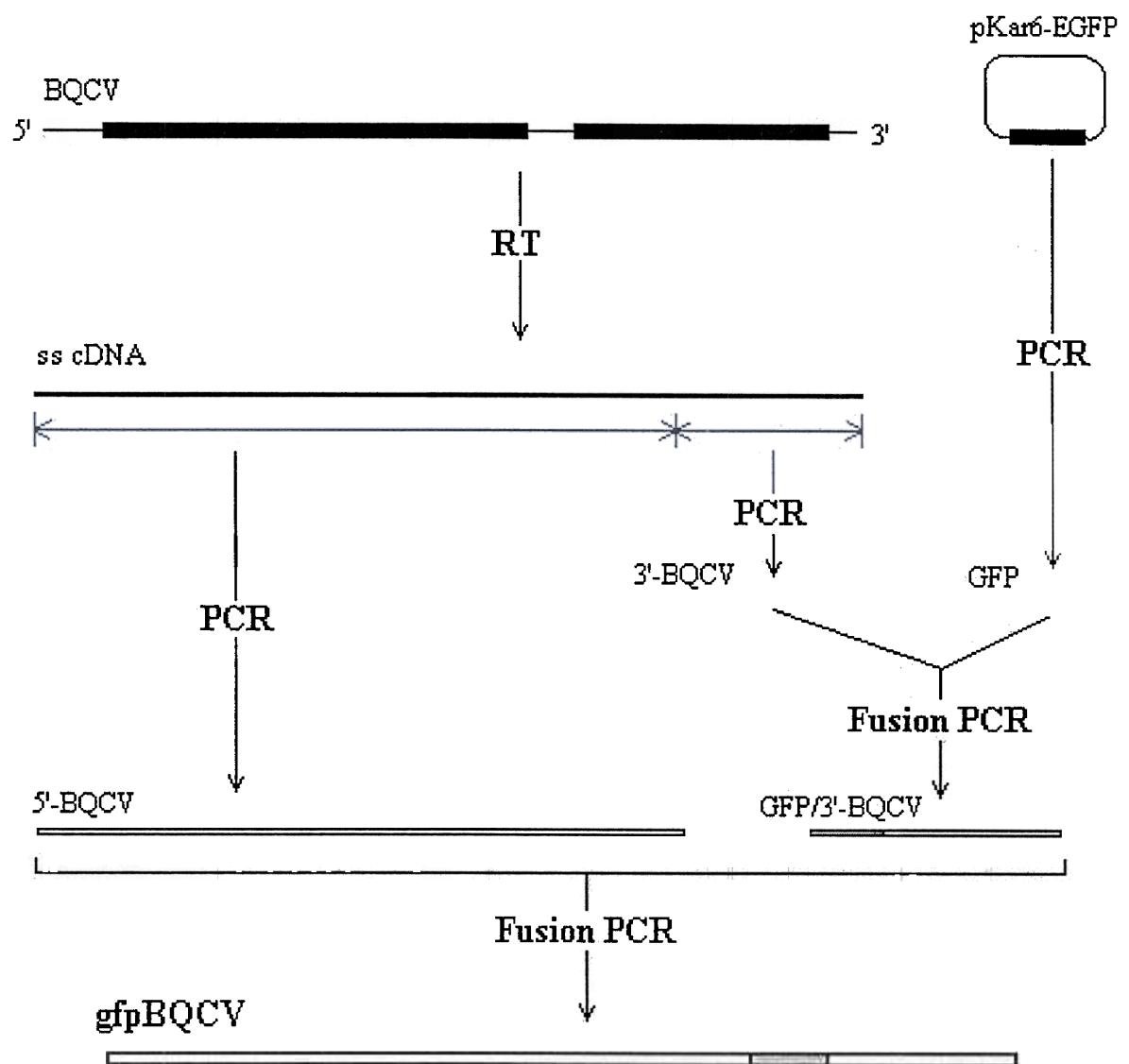


Fig. 5. 1. Construction of the gfpBQCV amplicon. The 3 fragments 5'-BQCV, GFP, and 3'-BQCV were amplified by high fidelity PCR, and were then joined together by fusion-PCR to obtain the gfpBQCV amplicon.

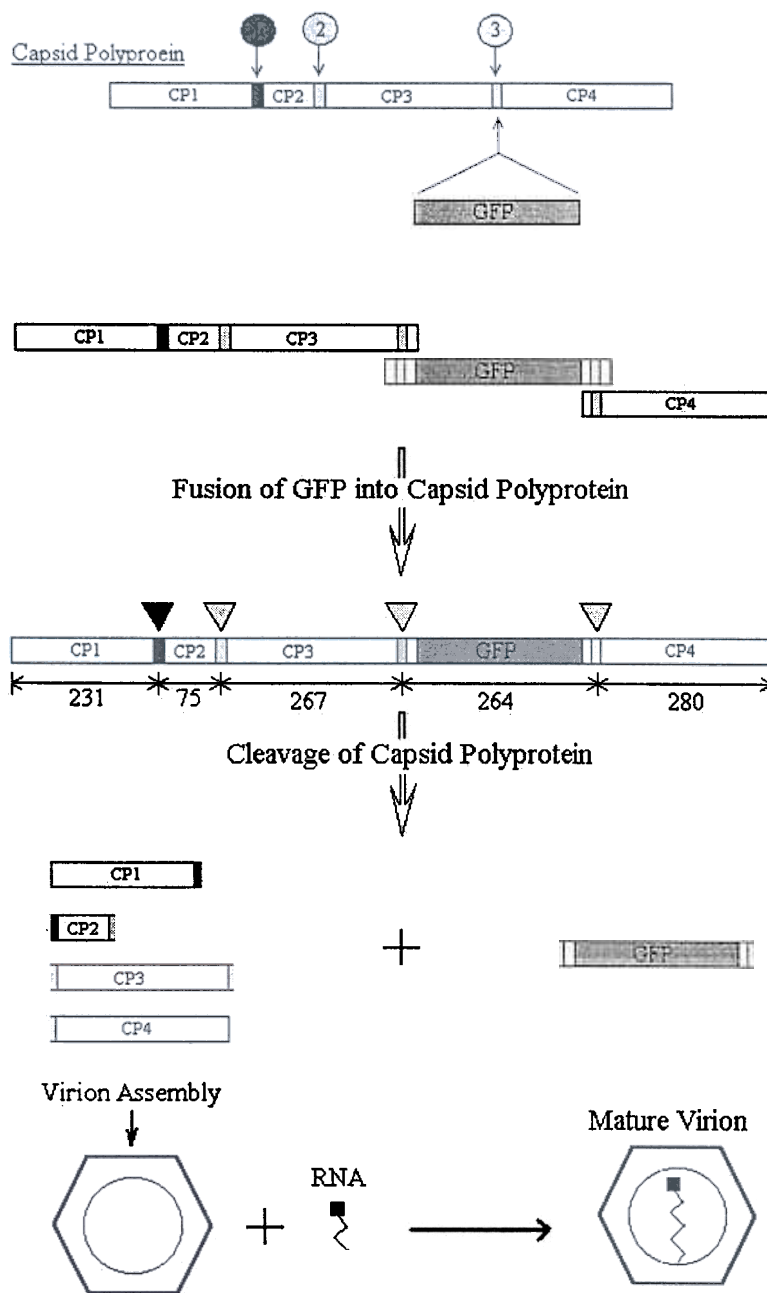


Fig. 5. 2. Strategy used to insert the GFP-encoding sequence into the capsid polyprotein between CP3 and CP4. The proposed cleavage pattern of the new recombinant polyprotein is also presented.

The sequence encoding the GFP was amplified from plasmid pKar6-EGFP (kindly donated by Robert Blanvillain, University of Perpignan, France). Titan system, an RT-PCR system but using a PCR enzyme blend from the Expand High Fidelity PCR System (Roche), was used to amplify the GFP sequence. Each PCR reaction mixture contained 10 ng of template DNA, 0.2 mM of each deoxynucleotide triphosphate, 0.5 μ M forward primer (EGFP(1)F), 0.5 μ M reverse primer (EGFP(1)R), 10 μ l 5xPCR buffer (1.5 mM MgCl₂), 5 mM dithiothreitol, 1 μ l enzyme mix, and dH₂O to a total volume of 50 μ l. The PCR profile started with an initial denaturation stage at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min. The final extension was performed at 72°C for 7 min.

The 5'-BQCV fragment was amplified using Expand 20 kb^{PLUS} PCR System (Roche). Each PCR reaction mixture contained 2- μ l of wtBQCV ss cDNA template, 0.5 mM of each deoxynucleotide triphosphate, 0.3 μ M forward primer (INFECLONE1), 0.3 μ M reverse primer (CP3EGFP(1)R), 10 μ l 5xPCR buffer, 0.75 mM MgCl₂, 0.75 μ l enzyme mix, and dH₂O to a total volume of 50 μ l. The long PCR profile started with an initial denaturation stage at 94°C for 2 min followed by 2 cycles of 93°C for 15 s, 57°C for 30 s, and 68°C for 10 min. A third stage consisted of 28 cycles of 92°C for 10 s, 70°C for 30 s, and 68°C for 10 min. The final extension was performed at 68°C for 20 min.

The 3'-BQCV fragment was amplified using the Titan System. The conditions and the profile were identical to those used for the amplification of sequence encoding the GFP, except that 2- μ l of wtBQCV ss cDNA was used as template, and the annealing temperature was 60°C. The forward and reverse primers used were EGFP4(1)F and RFCDNA40 respectively.

The 3'-BQCV and GFP fragments were joined by fusion PCR using the Titan System. The obtained fragment was named GFP/3'-BQCV (figure 5. 1). The fusion-PCR reaction mixture was identical to those of GFP and 3'-BQCV amplifications except that a mixture of 9 ng of the GFP amplicon and 12 ng of the 3'-BQCV amplicon were used as template. The reaction mixture lacking the enzyme mix and the primers (EGFP(1)F and RFCDNA40) was heated at 94°C for 2 min. The enzyme mix was added when the temperature reached 94°C. One cycle of 94°C for 30 s, 78°C for 30s, and 68°C for 3 min was used to extend the templates to cover the whole fused fragment. Primers (EGFP(1)F and RFCDNA40) were then added at 94°C at the beginning of the third stage, which consisted of 29 cycles of 94°C for 30 s, 70°C for 30 s, and 68°C for 2 min. The extension was performed at 68°C for 15 min. The PCR product obtained was purified and fused to 5'-BQCV

Expand 20 kb^{PLUS} PCR System was used for the fusion reaction of 5'-BQCV and GFP/3'-BQCV to obtain the gfpBQCV amplicon (figure 5. 1). The reaction mixture was identical to the one used in the amplification of 5'-BQCV except for the template and the set of primers. A mixture of 83 ng of the 5'-BQCV amplicon and 20 ng of the GFP/3'-BQCV amplicon were used as template. The forward and the reverse primers were INFECLONE1 and RFCDNA40 respectively. The procedure for the fusion reaction was similar to the one described for the joining of 3'BQCV and GFP fragments. The fusion-PCR profile was as follows: an initial denaturation stage at 94°C for 2 min, followed by one cycle of 92°C for 10 s, 78°C for 30 s, and 68°C for 12 min. This was then followed by 29 cycles of 92°C for 10 s, 70°C for 30 s, and 68°C for 1 min. The final extension was performed at 68°C for 25 min. The PCR product obtained

was purified and used directly for the *in vitro* transcription reaction to produce gfpBQCV infectious transcripts.

5. 3. 4. *In vitro* transcription and infection experiments

The *in vitro* transcription reactions were performed essentially as described in **Chapter 4**. The cap analog was excluded from these reactions, but the poly(A) stretch was extended from 25 to 40 bases by using the primer RFCDNA40 in the PCR reactions. The reaction yield was in the order of 6.1-7.5 µg of RNA/µg of template DNA.

Two batches of apparently healthy white- to purple-eyed worker bee pupae were injected simultaneously with phosphate buffer, and gfpBQCV *in vitro* transcribed RNA. Every batch consisted of 50 bee pupae. The amount of *in vitro* transcribed RNA injected was 660 ng. The bee pupae were incubated at 30-35°C for 8 days then the virus was purified as described by Leat *et al.* (2000). For every treatment, virus was purified from 5 lots of 10 bee pupae homogenized together (Leat *et al.*, 2000). Recovered virus was 10-fold diluted and re-propagated as above. The amount of virus recovered was estimated in genome equivalents and as a mean value of the 5 lots for each treatment, as described in **Chapter 4**.

5. 3. 5. Detection of the GFP

Whole bees and SDS-PAGE gel-electrophoresed bee samples were illuminated with 100 W hand-held UV lamp (UV Products, Upland, CA 91786, Black Ray model B 100AP). Bee pupae were ground with a pestle and mortar in two volumes of grinding buffer (0.1 M NaCl, 0.1 M glycine, 10 mM EDTA (pH 9.5)). Samples were then centrifuged to pellet debris and collect

supernatant. Thirty microlitres of the collected supernatants were electrophoresed, without prior heat treatment, on a 12-% SDS-PAGE gel

5. 4. Results and discussion

The development of a reverse genetics system for BQCV (**Chapter 4**), and especially the fact that its genome can be manipulated, has opened new opportunities for studies aimed at exploring the potential of this virus as a vector to express foreign proteins. The insertion of a sequence encoding the green fluorescent protein (GFP) is of a particular interest, because of the significant advantages that this gene offers over other marker genes such as the *Escherichia coli* *UidA* gene (GUS) (Baulcombe *et al.*, 1995). Among many other advantages, the fluorescence of GFP is an intrinsic property and does not depend on exogenous substrates or co-factors, the simplicity and ease of detecting the fluorescence, and more importantly the GFP is non-toxic and appears not to interfere with cell growth and function (Baulcombe *et al.*, 1995). In addition, the production of new virus recombinants carrying the GFP-encoding sequence will replace/improve the previously developed system (**Chapter 4**). BQCV mutants (mutBQCV) produced in the previous system carried a marker mutation, and were proposed for use in new experiments aimed at understanding the bee parasitic mite syndrome. The large size of the GFP-encoding sequence (717 bp) will also offer a challenge regarding the BQCV's uptake capacity for foreign sequences.

The 3'-proximal ORF (ORF 2) was previously shown to encode the BQCV capsid polyprotein, which is proteolytically processed into 4 mature structural proteins (Leat *et al.*, 2000). These proteins will be referred to as CP1, CP2, CP3 and CP4 based on their proximity to the N terminus of the capsid polyprotein (figure 5. 2.). In contrast with CP1 and CP2, the molecular masses of CP3 and CP4 were fairly consistent, when determined by SDS-PAGE or

calculated from the conceptual translation of ORF 2 (Leat *et al.*, 2000). In addition, the cleavage position between CP3 and CP4, referred to as ③ in figure 5. 2., was proposed to correspond to a 3C-like protease cleavage site (Leat *et al.*, 2000). Therefore, it was decided to insert the GFP-encoding sequence between the sequences encoding for CP3 and CP4, and to duplicate the sequence encoding the cleavage site ③. This site will be positioned between CP3 and GFP, as well as between GFP and CP4 (figure 5. 2.). The duplicated sequence was extended beyond the exact cleavage point in both directions to cover certain residues that might be necessary for efficient cleavage, as well as to conserve the tertiary structure that has been speculated to be important (Stanway, 1990). The extended polyprotein would be produced in infected bee pupae, and proteolytically processed into mature capsid proteins plus the GFP, which would be excluded from the mature virions (figure 5. 2.).

Genome-length ss cDNA was synthesized by reverse-transcription as described in **Chapter 4**, and used directly for the amplification of 5'-BQCV and 3'-BQCV fragments by long high fidelity PCR. The sequence encoding for GFP was also amplified using high fidelity PCR. The three fragments were successfully joined together by fusion-PCR following a stepwise strategy and using the overlaps between fragments that were created by the relevant primers (figure 5. 3.).

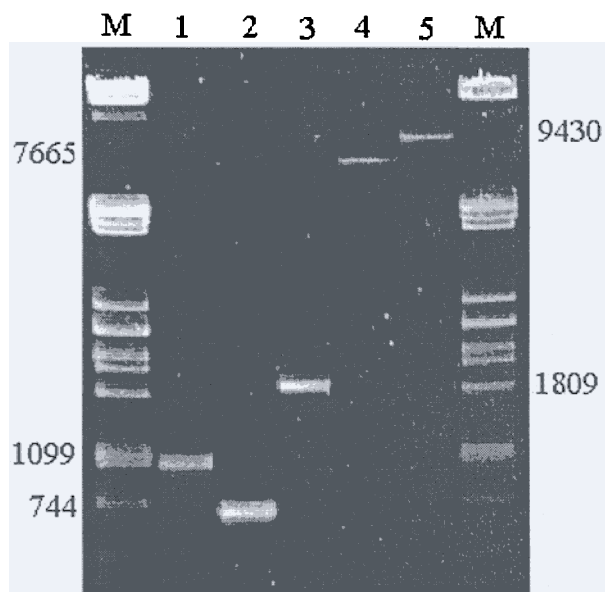


Fig. 5. 3. Amplification of the fragments used for the construction gfpBQCV. M, *Pst* lambda DNA marker; lane 1, 3'-BQCV amplicon; lane 2, GFP amplicon; lane 3, GFP/3'-BQCV amplicon; lane 4, 5'-BQCV amplicon; lane 5, gfpBQCV amplicon.

The obtained amplicon (gfpBQCV) was used as template in the *in vitro* transcription reactions. Using the same primers and profile for the initial amplification of GFP, the sequence encoding the green fluorescent protein was confirmed by RT-PCR to have been integrated in the *in vitro* transcribed RNA of gfpBQCV (figure 5. 4.).

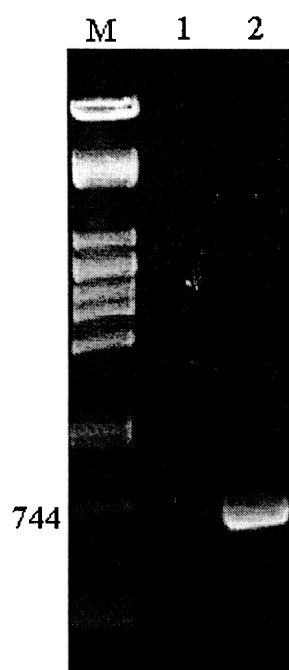


Fig. 5. 4. Detection of the GFP-encoding sequence from gfpBQCV transcripts by RT-PCR. M, *Pst* lambda DNA marker; lane 1, dH₂O (negative control); lane 2, GFP amplicon.

In the infection experiments, wtBQCV was not injected to avoid any possible contamination, and the incubation periods were extended beyond 8 days to increase virus yield, depending on the conditions of the bee pupae. The gfpBQCV transcripts were infectious and the yield was of the order of 6.2×10^7 genome equivalents per ng of transcribed RNA injected. It was not possible to detect any fluorescence from whole bees or electrophoresed bee extracts. Given the low infectivity of transcripts, it was believed that GFP was indeed produced in the bee but below detectable levels. The recovered virus was then 10-fold diluted and re-propagated along with the extract recovered from bee pupae injected with phosphate buffer, to ensure that any virus recovered in the second propagation was not just a result of activation of an inapparent infection. Although a regular infection was obtained in the second propagation, once again it was not

possible to detect any fluorescence. An RT-PCR analysis of the virus recovered from the two propagations showed that no GFP-encoding sequence was present in the viral genome, but the virus was authenticated as BQCV using the RT-PCR method described by Benjeddou *et al.* (2001) (figure 5. 5.).

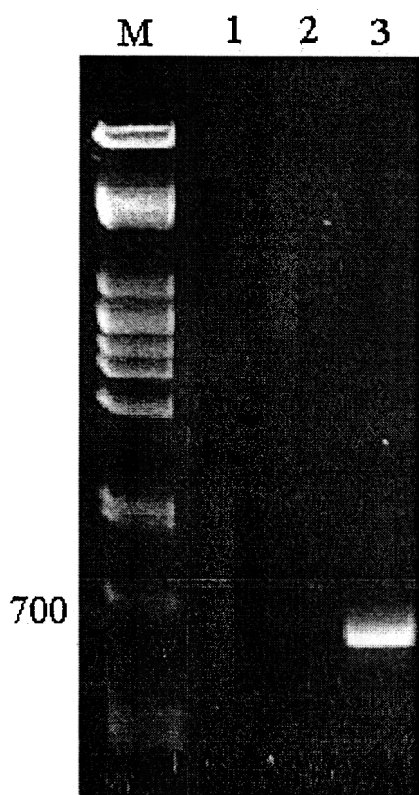


Fig. 5. 5. Authentication of the BQCV virus using the RT-PCR method described by Benjeddou *et al.* (2001). M, *Pst* lambda DNA marker; lane 1, dH₂O (negative control); lane 2, phosphate buffer extract; lane 3, BQCV amplicon.

The failure to obtain recombinant gfpBQCV and the harvest of wild-type virus only from the infection experiments could be explained by an homology-driven instability of recombinants. Homologous recombination could have deleted the region between the repeated sequences, which included the GFP-encoding sequence, regenerating the original wild-type virus as it was suggested for some constructs of tobacco mosaic virus-based vectors (Shivprasad *et al.*, 1999). In the case of cowpea mosaic virus (CPMV), a construct carrying just an height-base direct repeat sequence was shown to revert to wild-type CPMV by a single-step process (Porta *et al.*, 1994).

Although the construction of a BQCV-based vector was not successful, it was demonstrated that the previously developed reverse genetics system for BQCV is a robust system that will allow the development of new constructs using new designs and strategies. One of the options that could be used to avoid a direct sequence-repeat would be to insert the sequence encoding for the self-cleaving 2A protease of foot-and-mouth disease virus, as it was used in the case of the porcine reproductive and respiratory syndrome virus (Bramel-Verheij *et al.*, 2000).

5. 5. Acknowledgements

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CHAPTER 6

CONCLUSIONS

Studies on honeybee viruses started as early as the 1960s. In addition, the chronic paralysis, acute paralysis and sacbrood, the first three viruses of bees to be recognized, were also the first small non-occluded viruses to be identified in insects. However, these studies essentially focussed on the identification and physical characterization of honeybee viruses, and comprehensive molecular studies on these viruses have only recently been initiated.

To date, the complete genome sequences of three honeybee viruses, namely the acute bee paralysis virus (ABPV), the black queen cell virus (BQCV) and sacbrood virus, have been determined. Among these three viruses, only sacbrood causes symptoms that can confidently be attributed to viral infection. The availability of sequence data has prompted the initiation of the project aimed at developing a molecular detection method for two important honeybee viruses in South Africa, ABPV and BQCV. Unique PCR primers and a basic protocol were developed for the detection of these two viruses using a reverse transcriptase PCR (RT-PCR) assay. Primers were tested against purified virus particles and virus-infected bees, and sensitivities were of the order of 130 genome equivalents of purified BQCV and 1600 genome equivalents of ABPV.

The RT-PCR assay developed in this study could become a standard method in the health certification for honeybee (and honeybee products) imports and exports, and in the screening of virus preparations used in research. However, it is necessary to improve this system in terms of sensitivity and the number of viruses included. This assay must be tested against field samples, to find out whether it will help to identify viruses present in bees under natural conditions. It is

also important to continue the genome sequencing of other honeybee viruses, to enable the development of similar assays for these viruses, and finally to develop a multiplex PCR system in which multiple viruses could be identified simultaneously using a single reaction.

The range of molecular tools adapted for investigations on honeybee viruses is rather limited, and the development of new systems, such as reverse genetics, is likely to open new opportunities in honeybee virus research. This development will facilitate studies directed at understanding the molecular biology, persistence, pathogenesis, and more importantly the interaction of honeybee viruses with other parasites.

To our knowledge, the development of this reverse genetics system for BQCV is the first for honeybee viruses. The development of this technology will facilitate the creation of chimeric viruses, or the introduction of specific mutations into their viral genomes. Such mutant viruses produced have the potential to be used in new experiments aimed at understanding the bee parasitic mite syndrome.

Although the method used is simple, rapid, and overcomes the problem of instability of certain sequences in bacteria, the system developed can be refined and explored in many ways. Efforts to clone the full-length "infectious" amplicon must be continued, because of the advantages that infectious clones offer in terms of cost and infectivity. Once this amplicon is cloned, attempts can be made to develop a system by which infectious cDNAs (*in vivo*-transcribed RNA) are produced. The potential of this virus as a vector could then be explored, by insertion of sequences to express foreign proteins.

Despite its few limitations, an attempt was made to use the reverse genetics system developed for BQCV to explore the potential of this virus as a vector to express foreign proteins by insertion of a sequence encoding the green fluorescent protein (GFP). The use of GFP offers many advantages over other marker genes. Among many other advantages, GFP is simple and easy to detect, and is non-toxic and appears not to interfere with cell growth and function. In addition, the large size of the GFP-encoding sequence (717 bp) will also offer a challenge regarding the BQCV's uptake capacity for foreign sequences.

Although the construction of a BQCV-based vector to express GFP was not successful, it was demonstrated that the previously developed reverse genetics system for BQCV is a robust system that will allow the development of new constructs using new designs and strategies. The production of new virus recombinants carrying the GFP-encoding sequence would replace/improve the system developed earlier, in which BQCV mutants (mutBQCV) are proposed to be used in new bee parasitic mite syndrome experiments. Another application of viral constructs with GFP could be in screening honeybee races/populations for susceptibility/resistance to viral infections. It would be possible to inspect the honeybees directly to diagnose virus infection without relying on symptoms, which are most of the time absent.

Efforts should also be made to construct BQCV virus-based vectors to express other proteins, including antigens of viruses, bacteria and parasites of the honeybee. In the future, success may be achieved in making single constructs that will express multiple foreign proteins for use against multiple honeybee disease agents.

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