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Development of infectious transcripts and genome manipulation of *Black queen-cell virus* of honey bees

Mongi Benjeddou, Neil Leat, Mike Allsopp† and Sean Davison

Department of Biotechnology, University of the Western Cape, Private Bag X17, Bellville 7535, Cape Town, South Africa

The South African isolate of *Black queen-cell virus* (BQCV), a honey bee 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 (RT)–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 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 by 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 analogue and injected directly into bee pupae, which were then incubated for 8 days. *In vitro* transcripts were infectious but the presence of a cap analogue did not increase the amount of virus recovered. A single base mutation abolishing an *Eco*RI restriction site was introduced by fusion-PCR, to distinguish viral particles recovered from infectious transcripts from wild-type virus (wtBQCV). Mutant virus (mutBQCV) and wtBQCV were indistinguishable by electron microscopy and Western blot analysis. The *Eco*RI restriction site was present in wtBQCV and not in mutBQCV.

Introduction

Black queen-cell virus (BQCV) was first isolated from queen larvae and pupae of honey bees 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 interest in viruses as honey bee pathogens has often been academic, with many viruses persisting as inapparent infections. More recently, increasing knowledge of the interactions between honey bee viruses and parasites, notably the mite *Varroa destructor*, has led to suggestions that they may be involved in honey bee mortality (Bailey *et al.*, 1983; Ball & Allen, 1988; Allen & Ball, 1996; Brødsgaard *et al.*, 2000). This relationship between mite infestation and virus infection is not clearly understood. 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 have a high mortality (Shimanuki *et al.*, 1994). 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, contradictory results from different studies have caused confusion about the importance and the extent of the damage caused by some honey bee viruses. Bailey & Ball (1991) reported that *Acute bee paralysis virus* (ABPV) had never been associated with disease and mortality in nature. In contrast, ABPV was detected in large

Author for correspondence: Sean Davison.
Fax +27 21 959 3505. e-mail sdavison@uwc.ac.za

†**Present address:** Plant Protection Research Institute, Agricultural Research Council, Private Bag X5017, Stellenbosch 7599, South Africa.

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amounts in dead adult bees and in 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).

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 the 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 virus 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 PCR in terms of fidelity and length of amplification. The pioneering work of Gritsun and Gould (1995, 1998) has also resulted in the improvement of this method by using a combination of primer sets and optimizing their concentrations. This method is simple, rapid and it overcomes the problem of the instability of certain sequences in bacteria (Hayes & Buck, 1990; Gritsun & Gould, 1995, 1998; Tellier *et al*., 1996; Campbell & Pletnev, 2000).

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

Methods

■ **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 µl CsCl-purified virus were stored at −20 °C.

■ **RNA purification.** RNA was extracted from 100 µl CsCl-purified virus or transcription reaction mixture using the Nucleospin RNA II total RNA isolation kit (Macherey-Nagel GmbH & Co.), following the manufacturer's instructions. Pure RNA was finally eluted in 40 µl RNase-free water, quantified with a UV spectrophotometer and used immediately.

■ **Primers.** Primers used in this work (Table 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 single-stranded (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, *Not*I and *Kpn*I sequences were introduced into the relevant primers (see Table 1).

■ **Reverse transcription of viral RNA.** Viral RNA, extracted as described above, was reverse transcribed using the Universal RiboClone cDNA Synthesis System (Promega). The RT reaction was carried out in a volume of 25 µl and stopped after completion of first strand cDNA synthesis. Viral RNA (2 µg) was 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 h; 1 µl of RNase H (2 units/µl) was then added and the reaction was incubated for 20 min at 37 °C.

■ **Amplification of the full-length BQCV genome by long high-fidelity PCR.** Expand 20 kb^{PLUS} PCR System (Roche Diagnostics) was used to amplify the full-length BQCV genome. PCR was 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 µl template, 0·5 mM of each deoxynucleotide triphosphate, 0·3 µM forward primer (INFECLONE1), 0·3 µM reverse primer

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

Primer	Restriction site	Nucleotide sequence*	Genome position
Reverse transcription and genome amplification primers			
FCDNA		5′ TTTTTTTTTTTTTGCAAC 3′	8546–8563†
INFECLONE1	*Not*I	5′ GGGTAT**GCGGCCGC***ATTTAGGTGACACTATAGAATA* CGCAAAATTGCGTATAGTATATAAAT 3′	1–26
RFCDNA	*Kpn*I	5′ GGGTAT**GGTACC**(T_{25})GCAACAAGAAGAAACGTAAACC 3′	8529–8575†
5RFCDNA		5′ AAGTACACGGAATATATATCATCTATAAGA 3′	4296–4325
3FFCDNA		5′ AAAATATAGAACTGCTAGACTTGGAAAAGA 3′	4221–4250
***Eco*RI (position 4066) mutagenesis primers**			
ECORIF1	*Eco*RI‡	5′ GTGCTCTACCTAACCACTTGGATGAATGC**GAGTTC**AT 3′	4037–4073
ECORIR1	*Eco*RI‡	5′ GGAGGATCCTTTACCTTTCCCATCAAGAT**GAACTC**GC 3′	4064–4100
MUTF		5′ TGCAATCTGTTGGTTCTAC 3′	3623–3641
MUTR		5′ CATCCTTCCTTAACGAAAG 3′	4448–4466

* Restriction site in bold type and SP6 promoter in italic type.
† Poly(A) included.
‡ Restriction enzyme site abolished.

(RFCDNA), 10 μl 5 × PCR buffer, 0·75 mM $MgCl_2$ and dH_2O to make a total volume of 50 μl. 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 three cycles of 93 °C for 15 s, 57 °C for 30 s and 68 °C for 6 min. The third stage consisted of seven 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, two units of RNase H were added to the PCR product and incubated at room temperature for 2 h 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 manufacturer. The elution volume was 50 μl. The concentration of the purified amplicon was determined and the DNA was stored at −20 °C until further use.

■ **PCR-directed mutagenesis.** Two overlapping genome halves (5′ half and 3′ half) were amplified using the mutagenic primers ECORIF1 and ECORIR1 (see Table 1). This introduced a single base mutation at position 4068 abolishing an *Eco*RI 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 was used as a template. 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.

■ ***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 a 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. PCR product (5 μg) was added to a 100 μl *in vitro* transcription reaction mix with or without the presence of a cap analogue (Roche). The final concentration of the cap analogue in the reaction mixture was 0·5 mM. The reaction mixture contained 20 μl of transcription-optimized 5 × buffer, 10 mM DTT, 100 units RNase inhibitor, 0·75 mM of each rNTP, 45 units of SP6 RNA polymerase and RNase-free dH_2O to the final reaction volume. The reaction was incubated at 37 °C for 2 h 30 min. RQ1 RNase-free DNase (5 units) 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 μl RNase-free water and quantified with an UV spectrophotometer. RNA was used immediately in the infection experiments.

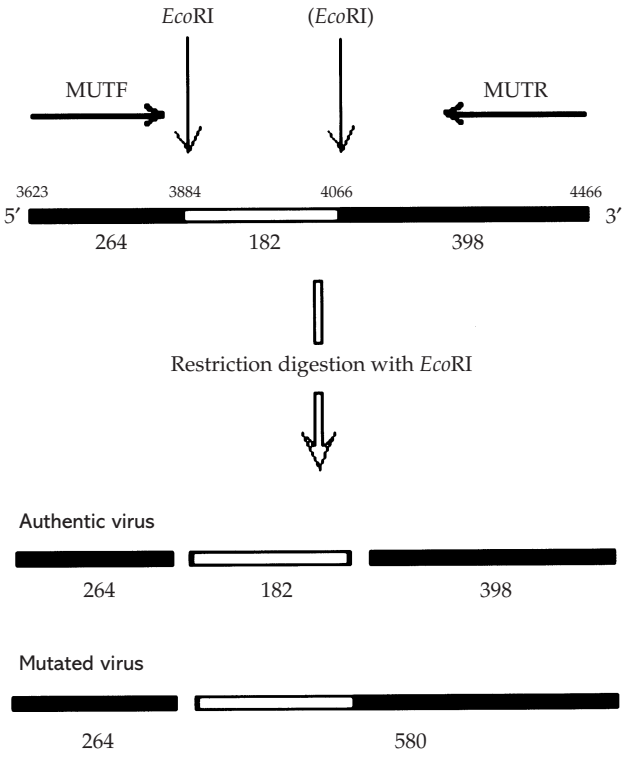

Fig. 1. Strategy for the identification of mutated virus by RT–PCR and restriction digestion. An 844 bp fragment is amplified by RT–PCR, purified with a commercial PCR purification kit, and digested with *Eco*RI. Three fragments of 398, 264 and 182 bp will result from this digestion in the case of authentic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

■ **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 and 660 ng of viral RNA and *in vitro*-transcribed RNA respectively. The bee pupae were incubated at 30–35 °C for 8 days and then the virus was purified as described by Leat *et al*. (2000). For every treatment, virus was purified from five lots of ten 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 five lots for each treatment as described by Benjeddou *et al*. (2001). In this method, viral RNA is phenol-extracted, ethanol-precipitated and quantified with a UV spectrophotometer. Since the molecular mass of the virus genome is known, the number of genome equivalents could be calculated.

■ **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.

■ **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) following the manufacturer's instructions.

■ **Detection of the mutated virus by RT–PCR and restriction enzyme 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 fist site was left unaltered; the second was mutated, eliminating the *Eco*RI site. The strategy for the detection of the mutation is summarized in Fig. 1. RT–PCRs were performed using the Titan RT–PCR system (Roche). Each 20 µl of reaction mixture contained 9 µl template, 0·2 mM each deoxynucleoside triphosphate, 0·5 µM MUTF primer, 0·5 µM MUTR primer, 5 mM dithiothreitol, 0·4 µl Titan polymerase mix and 4 µl 5 × RT–PCR buffer (1·5 mM $MgCl_2$). The template was 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: an RT 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 30 s, 52 °C for 30 s and 72 °C for 1 min and a final extension step at 72 °C for 7 min. The PCR reaction product was then purified using the High Pure PCR Purification Kit (Roche), according to the kit instructions, and eluted in 50 µl elution buffer. Amplified DNA (400 ng) was digested with *Eco*RI and run on a 2% agarose gel.

Results

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 a sample of RNA was 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 h to ensure the synthesis of full-length ss cDNA. RNase H was then added to the reaction mixture to degrade the RNA. A 2 µl sample of the reverse-transcription reaction was used directly in the 50 µl PCR reaction mixture to amplify the entire genome of BQCV. 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*I restriction site. This primer was designed to have a high annealing temperature to avoid interference from the initial RT primer, FCDNA. This strategy was first used by Gritsun & Gould (1998). The PCR product was purified and run on a 0·8% agarose gel (Fig. 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 the PCR products were run on a 0·8% agarose gel (data not shown). The full-length cDNA

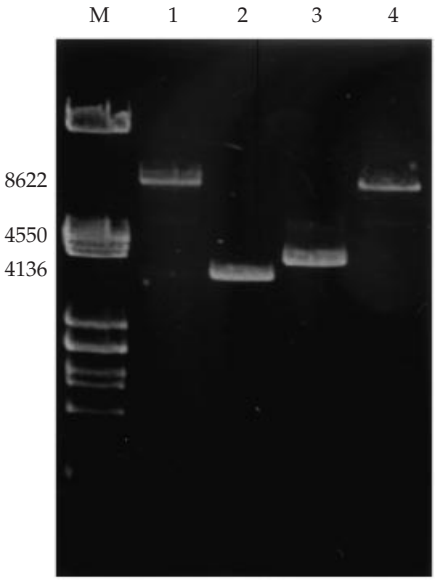

Fig. 2. Amplification of the BQCV genome, and introduction of a mutation by fusion-PCR, using long high-fidelity PCR. The silent mutation A (position 4068) to G, abolishing the *Eco*RI[4066] site, was introduced into the overlap of the 5′ half and 3′ half amplicons using mutagenic primers (see Table 1) as described in Methods. The two amplicons were joined together by 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.

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.

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 (Fig. 2, lanes 2 and 3). The mutation abolished an *Eco*RI site at position 4066, which is 182 bases apart from another *Eco*RI site that was left unaltered. The two amplicons were gel-purified to prevent ss cDNA carry-over and used together as template for the fusion PCR (Fig. 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 *Eco*RI as described above (data not shown).

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

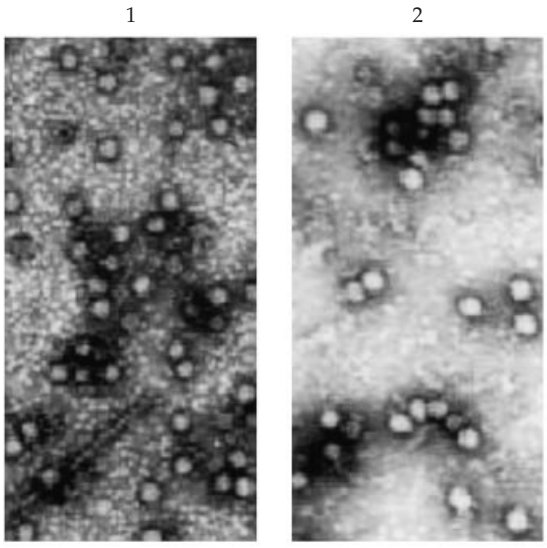

Fig. 3. Electron micrographs of BQCV virus particles extracted from bee pupae injected with viral or mutated *in vitro*-transcribed RNA as described in Methods, except that the process was stopped at the sucrose gradient step. (1) wtBQCV recovered from bee pupae injected with viral RNA. (2) MutBQCV recovered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

purified and their concentrations were measured. After incubation at 37 °C for 2 h 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 which were incubated at 30–35 °C for 8 days and virus was finally extracted. The infectivity of viral RNA was estimated to be $1{\cdot}3 \times 10^{11}$ genome equivalents per ng of injected RNA, and that of *in vitro*-transcribed RNA was $3{\cdot}7 \times 10^{8}$ genome equivalents per ng of injected RNA. The addition of a cap analogue 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.

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

Viral particles generated from injection of bee pupae with wild-type BQCV viral RNA and mutated RNA transcripts (mutBQCV) were physically indistinguishable when examined using transmission electron microscopy (Fig. 3). Western blot analysis of the capsid proteins of the viruses indicated that they are indeed BQCV particles (Fig. 4). RNA was extracted from virus particles and run on a 1% agarose gel (Fig. 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 *Eco*RI site was abolished in the mutated RNA transcripts. The fragment also covered an *Eco*RI site that was left unaltered. The purified PCR product was digested with

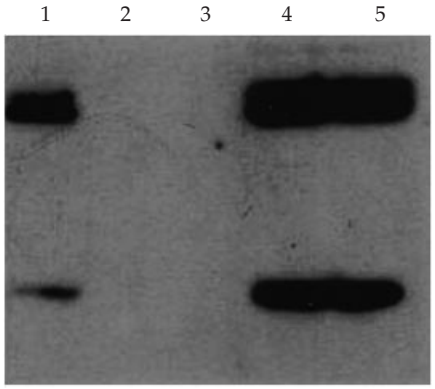

Fig. 4. Western blot analysis of the capsid proteins of virus particles. Wild-type virus and virus particles extracted from bee pupae infected with authentic and mutated *in vitro*-transcribed RNA were examined. Virus extracts were prepared from bee pupae as described in Methods, except that the process 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. 5. Confirmation of generation of virus particles by using the RNA extraction method. Virus extracts were prepared from bee pupae as described in 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.

*Eco*RI. Distinction between wild-type and mutant virus was made possible by the digestion pattern of the amplified fragment (Fig. 6).

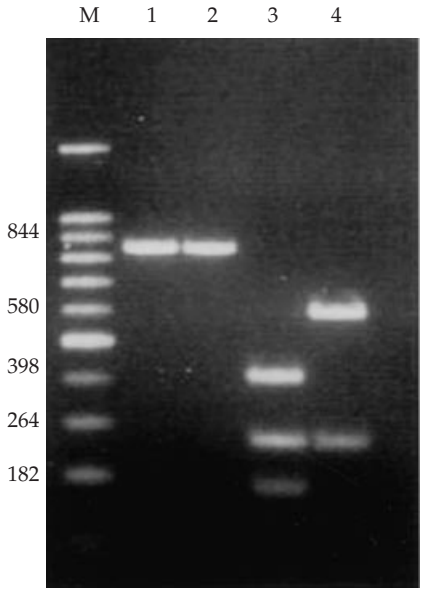

Fig. 6. Detection of the mutant virus with RT–PCR and restriction digestion of the amplicon with *Eco*RI. An 844 bp fragment was amplified by RT–PCR, purified using the High Pure PCR purification Kit (Roche), and digested with *Eco*RI. M, 100 bp molecular mass marker (Promega); lane 1, undigested PCR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

Discussion

Naked genomic RNA of many positive-strand RNA viruses has been shown to be infectious and the construction of infectious cDNA clones from these viruses has been described. However, because of the lack of a cell culture system in this study, preliminary experiments were necessary to establish whether the BQCV genomic RNA is infectious in bee pupae. Here we describe the development of full-length infectious RNA transcripts of BQCV.

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), were not successful. Genome-length ss cDNA was synthesized by RT using a specific primer, with an annealing temperature of 42 °C, and an extended incubation time of 4 h. The incubation period was extended to increase the number of full-length cDNA copies. The entire genome was amplified by long high-fidelity PCR using 2 µl of the unpurified RT reaction mixture, and a second reverse primer carrying a poly(A) tail and a *Kpn*I 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, 1998; Tellier *et al.*, 1996). When injected into bee pupae, these transcripts were infectious. This simple and rapid approach had been previously used (Hayes & Buck,

1990; Gritsun & Gould, 1995, 1998; Tellier *et al.*, 1996; 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 is 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 (m^7GpppG) 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 virus-encoded protein (VPg) or a cap structure at the 5′ end 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 cannot 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 *Eco*RI 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 honey bee 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.

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