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Short-term persistence precedes pathogenic infection:**Infection kinetics of Cricket Paralysis virus****in silkworm-derived Bm5 cells**

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Abstract

Next generation sequencing has revealed the widespread occurrence of persistent virus infections in insects but little is known regarding to what extent persistent infections can affect cellular physiology and how they might contribute to the development of disease. In contrast to the pathogenic infections occurring in *Drosophila* S2 cells, it was observed that Cricket Paralysis virus (CrPV; Dicistroviridae) causes persistent infections in 9 lepidopteran and 2 coleopteran cell lines. The status of the persistent infection was subsequently investigated in more detail using silkworm-derived Bm5 cells, where the infection eventually becomes pathogenic after 3-4 weeks. The short-term persistence period in Bm5 cells is characterized by low levels of viral replication and virion production as well as by the production of viral siRNAs. However, during this period cellular physiology also becomes altered since the cells become susceptible to infection by the nodavirus Flock House virus (FHV). Pathogenicity and widespread mortality at 4 weeks is preceded by a large increase in virion production and the transcriptional activation of immune-related genes encoding RNAi factors and transcription factors in the Toll, Imd and Jak-STAT pathways. During the infection of Bm5 cells, the infective properties of CrPV are not altered, indicating changes in the physiology of the host cells during the transition from short-term persistence to pathogenicity. The *in vitro* system of Bm5 cells persistently infected with CrPV can therefore be presented as an easily accessible model to study the nature of persistent virus infections and the processes that trigger the transition to pathogenicity, for instance through the application of different “omics” approaches (transcriptomics, proteomics, metabolomics). The different factors that can cause the transition from persistence to pathogenicity in the Bm5-CrPV infection model are discussed.

1. Introduction

Viral infections can generally be classified in two types, i.e. pathogenic and persistent. During pathogenic (acute) infections, interactions between virus and host become resolved during time and result in either the death of the host or the clearance of the viral infection by the immune response mechanisms. During persistent infections, on the other hand, an equilibrium is established between low replication of virus and depressed recognition by the immune system of the host that can be maintained over long periods of time (Goic and Saleh, 2012; Lidsky et al., 2017). Pathogenic infections have been studied extensively because of the clear outcomes that are obtained, for instance during baculovirus infections (Chen et al., 2013). However, deep sequencing efforts have revealed the ubiquity of persistent virus infections in living organisms, including insects (Roundy et al., 2017), which raises questions regarding their significance for the physiology of the hosts and the possible interactions with pathogenic infections when these occur. Maintenance of persistence can be important for the viral life cycle as illustrated by arbovirus infections in mosquito vectors during which it is essential that, after uptake by a blood meal, the virus can spread throughout the body and infect the salivary glands in the absence of adverse effects or fitness costs on the mosquito vector such that the transmission to the next vertebrate host is maximized (Cheng et al., 2016).

To establish persistence, a virus must employ strategies to avoid damage to the infected cells and to evade the immune response (reviewed by Randall and Griffin, 2017). The whole process is believed to entail repression of viral replication and translation, more specifically through control of the activity of the viral polymerase and the interaction with the RNAi response of the host (O'Neill et al., 2014; Kolliopoulou et al., 2017).

Infection of *Drosophila* cell lines with the RNA virus Flock house virus (FHV; Nodaviridae) has functioned as a model system to study the establishment of persistent viral infections (Jovel and Schneemann, 2011; Goic et al., 2013). After an initial period of acute (pathogenic)

infection by FHV, persistently infected cells are readily established in culture. Virions isolated from the persistently infected cells however retain their pathogenic properties when injected into *Drosophila* flies, indicating that changes in the physiology of the host cells are responsible for the state of persistence rather than changes in the virus. Several factors may contribute to dampen the intensity of viral replication and virion assembly during persistent infections such as the amplification of the RNAi response by the generation of viral DNA forms, the production of defective interfering RNAs and the altered (suboptimal) subcellular distribution of viral proteins (Jovel and Schneemann, 2011; Vodovar et al., 2012; Goic et al., 2013). Interestingly, the RNAi response in *Drosophila* S2 cells may also be functionally altered with respect to persistent infections with FHV since viral small RNAs do not associate with the Argonaute-2 (Ago-2) effector in the small interfering RNA (siRNA) pathway and are defective in RNAi sensor assays. Moreover, viral replication levels are more influenced by knockdown of Dicer-2 (Dcr-2) than Ago-2 leading to the proposal that direct dicing of viral replication complexes functions as a major antiviral strategy during persistent infection with FHV (Flynt et al., 2009).

Also during arbovirus infections of mosquitoes, the RNAi mechanism plays an essential role to maintain their persistent character. In this case, viral replication is kept at low levels by the RNAi mechanism of the mosquito vector which is possible since arboviruses in general are not believed to encode viral suppressors of RNAi (VSRs) (O'Neill et al., 2014). When Sindbis virus (*Alphavirus*, *Togaviridae*) genomes were engineered to contain a VSR gene, the persistent character of its infection was indeed transformed into a pathogenic one, characterized by increased viral replication and host mortality (Myles et al., 2008).

In contrast to research in dipteran insects (*Drosophila* and mosquitoes), studies of persistent viral infections are more limited in Lepidoptera. While acute infections of baculoviruses (large DNA viruses of the *Baculoviridae* family) in lepidopteran larvae are well studied and usually are very virulent (pathogenic), the occurrence of persistent infections is also well documented and has gained more interest recently (Kukan, 1999; Burden et al., 2003; Cory and Myers, 2003; Williams et al., 2017). Persistent infections of RNA viruses were also observed in larvae of the silkworm, *Bombyx mori* (Kolliopoulou et al., 2015) as well as in many lepidopteran cell lines (Swevers et al., 2016). Interestingly, in cell lines derived from *B. mori* (BmN4, VF) and the cabbage looper *Trichoplusia ni* (Hi5), RNAi-knockdown studies established a role for the siRNA pathway to control persistent infections of Macula-like latent virus (MLV; related to *Tymoviridae*) (Katsuma et al., 2018; Santos et al., 2018). Persistent infections of FHV in lepidopteran cell lines also interact with the RNAi machinery since the viruses encode an intact VSR and can be cleared from the cells by an RNAi mechanism (Swevers et al., 2016). On the other hand, production of viral siRNAs (vsiRNAs) is insufficient during persistent FHV infections of lepidopteran cell lines to affect RNAi reporter constructs as was also reported in *Drosophila* S2 cells (Flynt et al., 2009; Swevers et al., 2016).

Cricket paralysis virus (CrPV) is a positive-strand RNA virus (family: *Dicistroviridae*) that has a wide host range among insects and is known for its pathogenicity (Bonning and Miller, 2010; Zhao et al., 2018). In dipteran S2 and lepidopteran Hi5 cells, acute infections of CrPV cause high mortality. In *Drosophila* S2 cells, the high virulence of CrPV is caused by the 1A protein that acts as a VSR and inhibits slicer activity by Ago-2 (Nayak et al., 2010). Also in lepidopteran cells, RNAi can function as an antiviral mechanism since over-expression of the siRNA factors Dcr-2 and Ago-2 was observed to partially rescue cell viability in Hi5 cells acutely infected with CrPV (Santos et al., 2018).

In our experimental set-up, CrPV did not cause acute pathogenic infections in lepidopteran cell lines, in contrast to *Drosophila* S2 cells, which were used for the production of the virus. During follow-up studies in silkworm-derived Bm5 cells, however, it was observed that

cytopathic effects occurred with significant delay (3-4 weeks) after initial exposure to CrPV. In this study, the kinetics of the infection of Bm5 cells with CrPV is investigated in more detail with respect to the duration of the persistence period and the transition to pathogenesis. Through this analysis, an initial assessment is made of modifications in cellular physiology during the period of short-term persistence (low levels of virion and vSiRNA production, increased infectivity to other RNA viruses) and the changes that occur at the transition to pathogenesis (high levels of virion production, transcriptional activation of immune-related genes). Because of the time frame of 3-4 weeks and the easy accessibility of *in vitro* culture, the CrPV-Bm5 infection system can be presented as a model to study changes in cellular physiology and gene expression during the transition period from persistence to pathogenicity during infection by a potentially virulent RNA virus. As such, it could also function as a screening system for factors and compounds that modulate the kinetics of this transition period.

2. Materials and methods

2.1 Cells and viruses

Information of insect cell lines used in this study is presented in **Table 1**. All the cells were maintained at 27°C as described (Swevers et al., 2014). CrPV was produced using *Drosophila* S2 cells and purified by sucrose density ultracentrifugation as previously described (Cappelle et al., 2016). The final viral pellet was re-suspended in phosphate-buffered saline (PBS). The concentration of viral particles was estimated by transmission electron microscopy using a standard protocol for negative staining with Alcian blue and uranyl acetate (CODA-CERVA, Brussels, Belgium). The concentration of particles with morphology of picornaviruses (40 nm) was estimated at approximately 2×10^{10} particles/mL (**Fig. S1**). FHV was obtained from supernatant of S2 cells that were persistently infected with FHV (Swevers et al., 2016). Medium containing viral particles from S2-FHV cells was obtained after centrifugation (1,800 g; 5 min) of cell cultures at densities of approximately 3×10^6 cells/mL. Cell culture supernatants with FHV were stored at -80°C until usage.

2.2 Virus infection

Different cell lines (**Table 1**) grown to 80% confluency in 12-well plates were infected with CrPV at multiplicity of infection (m.o.i.) of 1. For extended experiments, Bm5 cells were seeded at 1×10^6 /mL in a 6-well plate followed by inoculation with CrPV at m.o.i. of 5. After incubation with CrPV for 2 hr, virus-containing medium was removed, and cells were washed twice and subsequently grown in fresh medium. At the end of every week, half the volume of the cell cultures from infected Bm5 cells was collected for analysis, and the remaining half volume was sub-cultured at 1:5 (v:v) with fresh medium. Cells from infected cultures were collected for analysis by centrifugation (1,800 g, 5 min) and supernatants were stored at -80°C until infectivity titers were determined by titration on S2 cells. In experiments of super-infection with FHV, Bm5 cells were seeded in 6-well plates and first pre-infected with CrPV (m.o.i. of 1, 5, or 10) for one week which was followed by addition of 1/10 volume of medium from S2-FHV cells. Addition of supernatant of S2 cells that are free of FHV (Taning et al., 2018) was used as control.

2.3 Cell viability assays

The viability of Bm5 cells after CrPV infection was quantified by their ability to reduce 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide (MTT) to formazan precipitate in quadruplicate wells (Soin et al., 2008). MTT (Sigma-Aldrich) at a final concentration of 5 mg/mL was added to each well for 3 hours. Formazan precipitate was dissolved by incubation in dimethyl sulfoxide (DMSO; Merck, Berlin, Germany), and its concentration was determined spectrophotometrically at 560 nm using Mustiskan EX microtiter plate reader (BioTek, Winooski, VT). Cell mortality was calculated as the percentage of dead cells

relative to control cells (treatment with PBS).

2.4 Virus titration by endpoint dilution assay

Since CrPV is lethal to S2 cells, measurements of the viable cell concentration using MTT can be correlated to the viral titer. Initially, a 1:10 dilution of the virus sample and eight further successive 10-fold dilutions were prepared and added to S2 cells in ten replicates. Plates were incubated for 72 hours after which microscopic observations and virus quantification were performed. Virus-induced mortality was measured by MTT assay. In each well of the titration plates, the cell supernatant was aspirated and the monolayer was washed with PBS three times. Twenty μL of MTT reagent was dissolved in 150 μL of serum-free medium and the mix was added into each well. The plates were then incubated for 4 hours after which the formazan was dissolved with 150 μL /well of DMSO for 10 min. The tissue culture infective dose (TCID_{50}) was calculated by linear interpolation between the infection rates observed at the tested dilutions as previously described (LaBarre and Lowy, 2001).

2.5 Strand-specific RT-PCR for negative RNA strand detection

Different protocols were used to detect CrPV by RT-PCR (summarized in **Table 2**). The protocols differ by the primers used in RT and PCR to detect more specifically the positive and the negative strand of the virus. The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to isolate total RNA according to the manufacturer's instructions, followed by DNase treatment (Turbo DNA-free kit; Life Technologies, Carlsbad, USA). For Tag-based primer detection, CrPV-F and CrPV-R primers were extended at their 5' ends by 21nt of non-CrPV sequence to generate CrPV-tagF and CrPV-tagR (Plaskon et al., 2009). RNA was reverse transcribed using CrPV-tagF or CrPV-tagR or in the absence of primer. The resulting synthesized cDNAs were incubated with 50 U of exonuclease I (USB) at 37°C for 30 min and purified (QIAquick PCR purification kit; Qiagen) to remove short fragments of oligonucleotides and residues of enzymatic reagents and to prevent amplification of non-strand specific products (Chatterjee et al., 2012). The PCR reactions were performed in a Labcycler (SensoQuest, Göttingen, Germany) with the following protocol: 2 min at 94°C , 35 cycles of 30 s at 94°C , 30 s at 56°C 30 s at 72°C , and finally 3 min at 72°C . One μL of cDNA was added to each 25 μL PCR reaction containing 2.5 μL of 10x PCR buffer, 1.5 mM MgCl_2 , 0.2 mM dNTPs, 0.5 μM primers and 1.25 U of Recombinant Taq DNA Polymerase (Life Technologies). An overview of the protocols and the primers for strand-specific detection of CrPV is presented in **Tables 2** and **3**. FHV RNA detection was also carried out by RT-PCR using oligo(dT) for the RT reaction (protocol I, **Table 2**) and specific primers for the PCR to amplify RNA2 (**Table 3**).

2.6 RT-qPCR

The RT-qPCR reactions were performed in CFX96 TM Real-Time PCR Detection System (Bio-Rad, Hercules, USA). Each reaction was done in duplicate and composed of 10 μL of GoTaq Master Mix (Promega, Madison, USA), 1 μL of each primer (10 μM), and 8 μL of cDNA (diluted 1:40 or 1:100). The amplification conditions were 95°C for 5 min followed by 40 cycles of 95°C for 30 s, and 60°C for 60 s. Translation initiation factor 4A (TIF4A) was used as the reference gene (Ioannidis et al., 2016). Primers used in RT-qPCR for detection of MLV virus and immune-related genes are presented in **Tables 3** and **4**, respectively. All qPCR experiments were performed as triplicates (three independent infections). The results were analyzed using qbase⁺ software (Biogazelle, Zwijnaarde, Belgium).

2.7 Statistical analysis

All experiments involved at least three biological replicates. For RT-qPCR, the relative gene expression data were analyzed using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001) and were further normalized based on the value of gene expression in control samples. The significance of difference between two groups was determined by the Independent Samples *t*-test. All statistical analyses were performed using SPSS 20.0 for Windows (IBM, Chicago,

IL) and graphs were plotted using GraphPad Prism v.400 for Windows (Graphpad Software, San Diego, CA).

2.8 Viral small RNA analysis

Small RNA libraries from CrPV-infected Bm5 cells were prepared using the TruSeq Small RNA Sample Preparation Kit (Illumina) as described (Kolliopoulou et al., 2015). After quality control, small RNAs were subsequently mapped to the MLV (accession number: AB186123) and CrPV (accession number: AF218039) genomes as described (Santos et al., 2018). Small RNA length distribution graphs were produced in R using the viRome package (Watson et al., 2013).

3. Results

3.1 CrPV can infect a variety of different lepidopteran and coleopteran cell lines

Twelve different cell lines (9 from Lepidoptera, 2 from Coleoptera and S2 from *Drosophila*) were treated with CrPV preparation at m.o.i. of 1 and subsequently observed for cytopathic effects. At 2 days post infection (p.i.), cytopathic effects were clearly observed in S2 cells while no cytopathic effects were detected in any of the other cell lines when followed over a period of 7 days. To check whether CrPV was present in the cells, RT-PCR was performed for detection of CrPV genomes or transcripts. Oligo-dT-based RT-PCR detection (protocol I, **Table 2; Fig. 1A**) confirmed the presence of CrPV sequences in all 12 cell lines. Because this protocol only indicates the presence of the virus in the cells, other protocols were applied to detect specifically the negative strand of the viral genome which would be indicative of viral replication. Using a specific primer in the RT reaction that is complementary to the negative strand of CrPV, clear amplification products were obtained for infections of S2, Hi5, Hi5-SF, GUTAW1 and CPB (protocol II, **Table 2; Fig. 1B**), which could reflect the synthesis of negative strand RNA in these infected cells. Recently, however, this method was criticized since it could not differentiate between cDNAs generated with virus-specific primers and those generated by false priming (Plaskon et al., 2009). An approach that uses an extended primer with a tag sequence during RT at higher temperature (55 °C), applies exonuclease I digestion to remove unincorporated primers in cDNAs and performs PCR with a tag-specific primer, was therefore employed to detect negative strand accumulation more correctly (protocol III, **Table 2**). Using this approach, a more valid demonstration of CrPV replication was obtained for S2, Bm5, Hi5 and Hi5-SF cells (**Fig. 1C**).

3.2 Time course of CrPV infection in Bm5 cells

Because no cytopathic effects were observed in any of the cell lines (with the exception of *Drosophila* S2 cells) following 1 week after treatment of CrPV, it was decided to follow CrPV-infected Bm5 cells for a more prolonged period (initial infection at m.o.i. of 5). Bm5 cells were selected because they were used as a model to study RNA virus infections and the RNAi mechanism in earlier work (Kolliopoulou and Swevers, 2013; Liu et al., 2014; Swevers et al., 2014, 2016).

It was observed that during the first three weeks after CrPV exposure, viability of Bm5 cells remained high (>60%; **Fig. 2B**). However, it was observed that viability was significantly lower by 5-10% in CrPV-infected cells at 2 weeks and 3 weeks p.i., while no differences were observed during the first week. On the other hand, growth of Bm5 cells did not differ between mock- and CrPV-infected cells for a period of three weeks (**Fig. S2**). By contrast, during the fourth week, widespread mortality was observed (<20% viability; **Fig. 2B**). The high mortality during the fourth week of infection was preceded by an approximately 1,000-fold increase in CrPV titers in the culture medium (**Fig. 2C**). The time course of CrPV infection in Bm5 cells contrasts strongly with the kinetics observed in S2 cells, where a significant decrease in mortality is already observed after 24 hours and viral titers start to increase at 36-48 hours (**Fig. S2 and S3**). However, despite the different kinetics, final titers

of CrPV obtained in media of infected Bm5 cells (after 4 weeks) were similar to those detected in supernatants of infected S2 cultures (after 3 days) (approximately $5\text{-}7 \times 10^8$ TCID₅₀/mL as assayed on S2 cells).

A highly stringent protocol of RT-PCR was used to detect both positive and negative RNA strands of CrPV during the course of infection of Bm5 cells (protocol IV which uses Superscript IV instead of Superscript II enzyme and columns for cDNA purification in addition to tag-primers and exonuclease digestion; Chatterjee et al., 2012; **Table 2**). Using this specific and accurate RT-PCR protocol, RNA strands of both polarities were detected in CrPV-infected Bm5 cells that increased in abundance during the second and third week after initial exposure to CrPV (**Fig. 2A**).

3.3 Production of viral siRNAs during CrPV infection

To investigate whether CrPV infection during the period of short-term persistence can activate the RNAi response, small RNA sequencing was performed on RNA isolated from Bm5 cells at one and two weeks p.i.. In both samples, viral siRNAs of 20 nt size corresponding to CrPV were clearly observed, together with reads < 15 nt, that are generated by an unknown mechanism and could be general degradation products (**Fig. 3**). Approximately double the amount of reads of CrPV-specific vsiRNAs was observed at 2 weeks than at 1 week p.i. (**Fig. 3; Fig. S4**). However, during this period the number of reads (~250-500) remained low and comparable to those of MLV that is persistently infecting Bm5 cells (Swevers et al., 2016). VsiRNAs mapped equally between the positive and negative strands of the CrPV genome indicating their origin from viral genome replication intermediates as was observed for other single-stranded RNA viruses (**Fig. S4**; Katsuma et al., 2018; Santos et al., 2018). No viral small RNAs of the size range 24-30 nt were observed, indicating the absence of the activation of the PIWI-associated RNA (piRNA) pathway (Senti and Brennecke, 2010).

3.4 Transcriptional activation of immune-related genes during CrPV infection of Bm5 cells

Infections with dicistroviruses such as *Drosophila* C virus (DCV) and CrPV are known to induce complex transcriptional responses during infection of *Drosophila* that include several immune-related genes (Dostert et al., 2005; Merklings et al., 2015b). It was therefore of interest to see whether a transcriptional induction of immune-related genes could also be observed in CrPV-infected Bm5 cells, especially during the period when short-term persistence evolves into pathogenicity (3-4 weeks after initial CrPV exposure; **Fig. 2**).

A striking increase in expression of key immune-related genes is predominantly observed at 3 weeks p.i., encoding the NF- κ B transcription factors Rel and Relish (approximately 5-10-fold; belonging to the Toll and Imd pathway, respectively), the RNAi factors Dcr-2 and Ago-2 (~ 10-25-fold) and the Stat transcription factor (> 50-fold; Jak/STAT pathway) (**Fig. 4**). Also at 2 weeks p.i., clear transcriptional responses but at lower intensity were observed (*dcr-2*, ~ 5-fold; *ago-2*; ~ 10-fold; *Stat*, ~ 30-fold). Other genes such as *Imd* (encoding an adaptor protein) and *domeless* (*dome*; encoding a transmembrane receptor in the Jak/STAT pathway) showed a more moderate transcriptional response (**Fig. 4**). Of three antimicrobial peptide (AMP) genes examined, only *Cecropin E* (*CecE*) was transiently increased at 2 weeks p.i..

3.5 Increased susceptibility to other RNA viruses during CrPV infection of Bm5 cells

Since Bm5 cells are persistently infected with MLV (Swevers et al., 2016), it was of interest to determine whether CrPV could modulate the replication levels of MLV when the infection evolves, especially when CrPV replicates to high levels and triggers a strong transcriptional response of immune-related genes at 3 weeks p.i. (**Fig. 2 and 4**). As is observed for CrPV, MLV RNA levels are increased when the infection becomes pathogenic (**Fig. 2 and 4**). However, compared to CrPV, which replicates to high levels (~1,000-fold increase; **Fig. 2**), the increase in MLV is much more moderate (~10-fold at 4 weeks p.i.; **Fig. 5A**).

On the other hand, in contrast to several other lepidopteran cell lines, Bm5 cells are refractory to infection with the nodavirus FHV (**Fig. S5**; Swevers et al., 2016). When Bm5 cells were pre-infected with CrPV, however, infection of FHV was observed by RT-PCR (**Fig. 5B**). The increase in susceptibility to FHV seems to occur in a dose-dependent manner since higher levels of FHV RNA could be detected in Bm5 cells infected with CrPV at higher m.o.i. (**Fig. 5B**).

3.6 Infection kinetics of CrPV are not altered after production in Bm5 cells

Since the preparation of CrPV stock occurred using *Drosophila* S2 cells as hosts, it is conceivable that selection of CrPV variants/mutants occurred for efficient infection of S2 cells and that such variants were probably not well adapted to cause pathogenic infections in Bm5 cells. In such scenario, it can also be expected that during the persistence period in Bm5 cells new variants/mutants become selected that can infect Bm5 cells with improved efficiency and increased pathogenicity. To test this possibility, Bm5 cells were infected with supernatants of either CrPV-infected S2 cells or CrPV-infected Bm5 cells that were collected at periods of terminal high pathogenicity and cell mortality (3 days and 4 weeks p.i. for S2 and Bm5 cells, respectively). During the pathogenic period of infection, titers of CrPV are similar between S2 and Bm5 cells, when assayed for infection of S2 cells (section 3.2; approximately $5-7 \times 10^8$ TCID₅₀/mL) (**Fig. 2** and **S3**).

When Bm5 cells were infected with either type of CrPV-containing cellular medium (administered at 1:100 (v:v)), infection kinetics with respect to persistence evolving into pathogenicity were similar regardless of the source of CrPV (S2 or Bm5 medium) (**Fig. 6**). This experiment indicates that during the evolution of CrPV infection in Bm5 cells, no particular variants or mutants were selected that would improve their infection efficiency in Bm5 cells. The differential infection kinetics between S2 and Bm5 cells with respect to CrPV therefore seems to be determined by differences in the physiology of the host cells.

4. Discussion

In this study, we describe an experimental system of infection of Bm5 cells by CrPV that remains stable for 2-3 weeks before evolving into a pathogenic infection. This period of virus infection without significant pathogenic effects has a duration that is relevant for the study of persistent infections in nature. With respect to the silkworm, from which Bm5 cells are derived, its life cycle has a duration of 6-8 weeks (Tazima, 1964) which is longer than the short-term persistence period of CrPV in Bm5 cells. However, Bm5 cells are derived from ovarian tissue (Grace, 1967) and other tissues in the silkworm such as hemocytes and fat body may trigger more robust immune reactions that may delay further transition to pathogenicity in silkworm larvae. Furthermore, if infections occur during the last larval stage, the persistence period may extend through the pupal stage and the laying of eggs, effectively realizing vertical transmission of the virus. Regarding other insects, the period of persistence in this study is comparable or exceeds the time during which arbovirus infections in mosquitoes acquire the competence for transmission to the next vertebrate hosts (Rückert et al., 2017). From this viewpoint, the *in vitro* system of Bm5 cells persistently infected with CrPV may emerge as an easily accessible model to study the nature of persistent virus infections and the processes that trigger the transition to pathogenicity, possibly providing insights in the regulation of the process of persistence/pathogenicity during virus-insect associations considered important for human health and agriculture.

The cause of the significant delay in cytopathic effects (short-term persistence of 2-3 weeks) during infection of Bm5 cells with CrPV, which is also observed in all other cell lines investigated, with the exception of the producer cell line S2 (**Fig. 1**), is currently unknown. The analysis indicates low levels of virion and vsiRNA production (**Fig. 2** and **3**) and increased sensitivity to infection by other RNA viruses (**Fig. 5**) during the short-term

persistence period. At the transition to pathogenesis, levels of virion production increase (**Fig. 3**) and transcriptional induction of immune-related genes occurs (**Fig. 5**). In the following, several features of the Bm5-CrPV infection model are discussed in more detail such as possible cell variability of infection, transcriptional activation of immune-related genes, RNAi response and reprogramming of the cellular environment by viral genes.

First, it is considered rather unlikely that infection of Bm5 cells with stock virus is not homogenous and that only a small number of cells become initially pathogenically infected, as is for instance observed for infection of *Helicoverpa zea* MG8 cells with the RNA virus Providence virus (PrV; Carmotetraviridae). Although PrV infection does not cause pathogenicity at the cell population level, a true persistent infection status nevertheless is not achieved in MG8 cells. It appears that PrV causes pathogenic infection of individual cells but the propagation rate to other cells is very low such that no significant pathogenicity is observed at the population level (Pringle et al., 2003; Short et al., 2013). By contrast, in CrPV-infected Bm5 cells eventually (after 4 weeks) high mortality is observed which indicates that most cells in the population became infected. The rather abrupt increase in virion production after three weeks may indicate that a large number of cells were already infected after two weeks while a sudden acceleration of virus infectivity across the population seems less likely. However, experiments of immunostaining of Bm5 cells during the course of CrPV infection, using antibodies specific for CrPV-encoded proteins or replication intermediates with dsRNA structure (Khong et al., 2017), are needed to clarify conclusively the issue of the spread of CrPV infection in Bm5 cells. In this context, it can also be mentioned that after application of CrPV at a much higher m.o.i of 25 to Hi5 cells, pathogenic infections are observed of which the kinetics are similar to those in S2 cells (Santos et al., 2018). Thus, infections may become pathogenic when the levels of viruses exceed a particular threshold value and their intracellular replication causes intracellular damage and activation of immune-related genes (see also discussion further below). The value of infection at low m.o.i. lies in the establishment of a short-term persistence period that can be employed for detailed analysis of the status of persistence and its eventual evolution to pathogenicity by high-throughput approaches (see also discussion further below).

A high (1,000-fold) increase in CrPV virion titer is observed in the cellular medium at 3 weeks p.i. (**Fig. 2**), which is accompanied with a major transcriptional response of several immune-related genes in the Toll/Imd pathways, encoding the NF- κ B transcription factors Rel and Relish and the adaptor protein Imd (**Fig. 4B**). Induction of expression of genes in the RNAi (*dcr-2* and *ago-2*) and the Jak/STAT pathways (*Stat*) is already observed after two weeks but is increased after three weeks (**Fig. 4A** and **4C**). In S2 cells and *Drosophila* flies, a complex transcriptional response is observed following infection with CrPV and the related (*Drosophila*-specific) dicistrovirus DCV that includes immune-related genes belonging mainly to the Jak-STAT pathway (Dostert et al., 2005; Merklings et al., 2015a,b). Furthermore, several studies have shown that flies that are mutant in RNAi, Imd/Toll and Jak-STAT pathways become more sensitive to dicistrovirus infection (Dostert et al., 2005; Costa et al., 2009; Kemp et al., 2013; Bronkhorst and van Rij, 2014; Ferreira et al., 2014; Gammon and Mello, 2015; see review by Swevers et al. (2018)). Thus, the induction of immune-related genes in Bm5 cells following CrPV infection may occur as an antiviral defense mechanism. However, dicistrovirus infections generally were not reported to induce expression of AMP genes (Dostert et al., 2005; Kemp et al., 2013), which is confirmed in our study since responses of three investigated AMP genes were either absent or transient (**Fig. 4**).

Transcriptional responses to virus infection in *Drosophila* can be partially explained as the consequence of direct detection of pathogen-associated molecular patterns (PAMPs) by viral sensors such as the helicase domain of Dicer-2 (Deddouche et al., 2008). On the other hand,

the occurrence of cellular damage and generation of stress signals caused by viral replication are thought to constitute a major contribution to the transcriptional response (Merkling et al., 2015a,b). Of relevance may also be the observation that excessive stimulation of the immune response can cause significant cellular damage, resulting in decreased tolerance to virus infections (Moreno-Garcia et al., 2014; Merkling et al., 2015a; Lamiabile et al., 2016). From this perspective, the high transcriptional response of immune-related genes at week 3 p.i. could also have contributed significantly to the widespread mortality at 4 weeks p.i. (**Fig. 4**) observed in our study.

During infection of Bm5 cells with CrPV, the RNAi mechanism, involving production of 20 nt vsiRNAs, is activated (**Fig. 3**). While low numbers of vsiRNAs are observed during the first two weeks of the persistence period, it is expected that much higher numbers are produced when high numbers of virions are produced (at 3 weeks) and severe pathogenicity (at 4 weeks) ensues (**Fig. 2**), as is observed in other experimental systems (Lan et al., 2015; Zografidis et al., 2015). In addition, a transcriptional response is observed for the key siRNA factors Dcr-2 and Ago-2 that is also observed during other virus infections (Kolliopoulou et al., 2015). Besides vsiRNAs of 20 nt size, other viral small RNAs of very short length (“peak” at the smallest size analyzed of 10 nt; **Fig. 3**) were also observed that may correspond to other RNA degradation pathways. During infection of mosquitoes with Dengue virus, the production of “unusually small” (or “ultrashort”) viral small RNAs was also reported (Hess et al., 2011; Wang et al., 2018). However, the viral small RNAs in this study (10-12 nt) are considerably shorter than the “ultrashort” species reported during arbovirus infections (13-19 nt) and cypovirus infections of the silkworm midgut (16-17 nt; Zografidis et al., 2015) and may be considered as products of non-specific RNA degradation.

By contrast, no viral small RNAs in the size range of 24-30 nt are observed (**Fig. 3**), indicating that the piRNA pathway was not activated (Senti and Brennecke, 2010). In contrast to the siRNA pathway, the role of the piRNA pathway in the antiviral defense mechanism remains uninvestigated in most insects (Lewis et al., 2018). In contrast to Bm5 cells, production of viral piRNAs was detected in BmN4 cells (derived from persistent MLV infection; Katsuma et al., 2018), another cell line derived from *B. mori*, that is considered to have a germline origin and has been used as a model to study the control of transposons by the piRNA mechanism (Sakakibara and Siomi, 2017). However, production of vpiRNAs may become more important when infection progresses and viral copies become produced in high numbers (Goic et al., 2016; Katsuma et al., 2018). Thus, it remains possible that vpiRNAs become produced during the pathogenic period of CrPV infection of Bm5 cells, which will require deep sequencing of small RNAs during this infection period.

The CrPV genome encodes a powerful VSR protein, CrPV-1A, that is considered an important virulence factor (Nayak et al., 2010). In *Drosophila* S2 cells, it was demonstrated that the 1A protein interacts with Ago-2 and inhibits its slicer activity. CrPV-1A may have a similar VSR function in Bm5 cells but it needs to be taken into account that the sequence conservation of Ago-2 protein between *Drosophila* and *Bombyx* is rather low (35% sequence identity and 53% similarity) and that *ago-2* underwent a period of accelerated evolution within the *Drosophila* genus specifically and in insects in general that is driven by the host-virus arms race (Obbard et al., 2006; Wynant et al., 2017). Thus, the VSR mechanism of 1A protein needs to be tested in Bm5 cells and it may be possible that comparable weak interactions of CrPV-1A with *Bombyx* Ago-2 could contribute to the short-term persistence in Bm5 cells.

On the other hand, other studies have established multiple functional roles for CrPV-1A that include the inhibition of stress granule assembly and host transcription in addition to its VSR activity (Khong et al., 2017). The 1A protein may be the “driver” of the pathogenicity of CrPV infection and its continuous cellular accumulation is expected to re-program the

cellular machinery for accommodation to virus production.

An important observation is that the host specificity of CrPV virions was not altered after pathogenic infection of Bm5 cells since it infected both S2 and Bm5 cells with similar kinetics as the original stock (**Fig. 6**; also note that titration of CrPV produced in Bm5 cells was carried out using S2 cells during which no altered kinetics were observed). Thus, the transition from short-term persistence to pathogenicity can be attributed to changes in the host cells rather than to selection of viral mutants displaying altered host cell preference.

As already pointed out, the short-term persistence period and the accessibility of an *in vitro* system consisting of a homogenous cell population constitute a useful model for the application of “omics” approaches (transcriptomics, proteomics, metabolomics) to gain new insights in the process of virus persistence and the triggers that promote its transition to pathogenesis. Besides the observation of low levels of viral replication and vsiRNA production, the short term persistence period can indeed be considered as a “black box” during which it can be hypothesized that the cellular environment becomes gradually modified by viral gene expression. Changes in cellular physiology are documented by the observation that during short-term persistence of CrPV, Bm5 cells become permissive to infections with FHV (**Fig. 5**). To better understand the nature of the persistence period, a metabolomics study was recently initiated that compared different time points of persistence and pathogenicity. Strikingly, metabolome profiles could be clearly separated at different time points during CrPV infection of Bm5 cells (in contrast to S2 cells) and they may provide further clues regarding the status of viral persistence and its evolution through time (L. Wang, L. Swevers and G. Smagghe, unpublished results).

Besides providing new insights into the process of viral persistence, the Bm5-CrPV infection model could also function as a screening system for factors and compounds that modulate the persistent character of the infection (stress factors such as temperature and heavy metals, hormonal compounds, xenobiotics, co-infections of other viruses). The time frame also allows the testing of the involvement of cellular genes that regulate the transition (gene silencing and over-expression experiments).

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Table 1: Insect cell lines used in this study.

Cell line	Origin	Maintenance medium	References
Lepidopteran cell lines			
Bm5	Ovary-derived from <i>Bombyx mori</i>	IPL-41 ^a +10 % FBS ^b	(Grace, 1967)
BmN4	Ovary-derived from <i>Bombyx mori</i>	IPL-41+10 % FBS	(Katsuma et al., 2005)
CF203	Midgut-derived from <i>Choristoneura fumiferana</i>	Insect-Xpress ^c +2.5% FBS	(Sohi et al., 1993)
GUTAW1	Midgut-derived from <i>Helicoverpa zea</i>	Ex-cell 420 ^d	(Goodman et al., 2004)
Hi5 (BTI-TN-5B1-4)	Embryo-derived from <i>Trichoplusia ni</i>	IPL-41+10 % FBS	(Granados et al., 1994)
Hi5-SF (serum-free)	Embryo-derived from <i>Trichoplusia ni</i>	IPL-41	(Granados et al., 1994)
Sf21	Ovary-derived from <i>Spodoptera frugiperda</i>	IPL-41+10 % FBS	(Vaughn et al., 1977)
Sf9-SF (serum-free)	Ovary-derived from <i>Spodoptera frugiperda</i>	Sf900 TM II SFM ^e	(Vaughn et al., 1977)
SL2	Ovary-derived from <i>Spodoptera littoralis</i>	IPL-41+10 % FBS	(Chejanovsky and Gershburg, 1995)
Dipteran cell lines			
S2-FHV	Hemocytes from <i>Drosophila melanogaster</i>	Insect-Xpress	(Schneider, 1972)
S2	Hemocytes from <i>Drosophila melanogaster</i>	Insect-Xpress+10% FBS	(Schneider, 1972)
Coleopteran cell lines			
TcA	Whole body-derived from <i>Tribolium castaneum</i>	Ex-cell 420+10 % FBS	(Goodman et al., 2012)
CPB	Pupae-derived from <i>Leptinotarsa decemlineata</i>	Ex-cell 420+10 % FBS	(Long et al., 2002)

a, IPL-41 Insect Medium (Sigma Aldrich, Overijse, Belgium);

b, FBS, fetal bovine serum (

c, Insect-Xpress Medium (VWR, Leuven, Belgium);

d, Ex-cell 420 Serum-Free Medium for Insect Cells (Sigma Aldrich, Overijse, Belgium);

e, Sf900TMII SFM Serum Free Medium (Life Technologies SA, Merelbeke, Belgium)

Table 2: Protocols used for detection of CrPV presence/replication.

Protocol	RT Primer	RT temperatures	Enzymes	/ Exonuclease I digestion	cDNA Purification	PCR Primers	Purpose	Specificity	Reference
I. (Fig. 1A)	Oligo-dT	Superscript II / 42°C		No	No	CrPV-F& CrPV-R	detection of CrPV (+)- and (-)-strand		
II. (Fig. 1B)	CrPV-F	Superscript II / 42°C		No	No	CrPV-F& CrPV-R	detection of CrPV (-)-strand	low	
III. (Fig. 1C)	CrPV-TagF	Superscript II / 55°C		Yes	No	Tag & CrPV-R	detection of CrPV (-)-strand	high	(Plaskon et al., 2009)
IV. (Fig. 2)	CrPV-TagF	Superscript IV/ 55°C		Yes	Yes	Tag & CrPV-R	improved detection of CrPV (-)-strand	highest	(Chatterjee et al., 2012)
	CrPV-TagR	Superscript IV/ 55°C		Yes	Yes	Tag & CrPV-F	improved detection of CrPV (+)-strand		

Table 3: Primers used in RT-PCR and qPCR detection/amplification of CrPV, MLV and FHV.

Application of Primers	Primer names	Sequence (5'-3')	Product size (bp)
Detection of CrPV	CrPV F	TGTCAACCCGACGCTTACA	406
	CrPV R	TGTATTCCTCTCCCCTCGCA	
	Tag	GGCCGTCATGGTGGCGAATAA	
	CrPV-TagF	<u>GGCCGTCATGGTGGCGAATAA</u> TGTCAACCCGACGCTTACA	
	CrPV-TagR	<u>GGCCGTCATGGTGGCGAATAA</u> TGTATTCCTCTCCCCTCGCA	
Detection of MLV (RdRp)	qBmMLV-RdRp-4809F	CGGTCTCTTCTGCGGCTATT	101
	qBmMLV-RdRp-4911R	AGGAAGGGTTCGGTCGTCTA	
Detection of FHV	FHV-RNA2-F	ATCCGATCAAGTGTCTCCT	500
	FHV-RNA2-R	ATCCAAACCAACAAGACCGT	

The underlined sequences in CrPV-tagF and CrPV-tagR represent the non-CrPV Tag sequences.

Table 4: Primers used in RT- qPCR for detection of the expression changes of immune-related genes in Bm5 cells during CrPV infection.

Genes	Accession number	Sequence (5'-3')	Product size (bp)
Rel	AB096087	F: CCAGCAAAGCCCTCAGA; R: GTTGTGCGGGTGCGGTT;	185
Dome	XM_012690822	F: TTAACTGGCATTGAACACC; R: TGAATCACTAAGCACATCGG	128
Stat	NM_001163916	F: GCCAGGTGTTGGACGATGA R: CACGATGGTCTTGCTGTTGAT	182
Imd	XM_004930229	F: AGCCGGAAGATGAGCATTTA R: GTATCAATTCTGCCTTCGGTGA	100
Relish	AB298441	F: TTCGGTGGAATGGGTATCAT R: GCTGAACTTCAAACGCACAA	165
Tak1	GQ426306	F: TAATGGCCGCTGAGGCGCAC R: ACGGGCGAGCTCGATGTCCT	169
CecE	D17394	F: CTTCTGCTTCGCGTTGGT R: AAGGATTTGCTTGCCCTAT	159
Def	NM_001043905	F: GGTGCTCGTGTGTTGTTG R: CAAATCGCAGTCTCTGTTGC	165
Hemo	FJ911550	F: GGGAGGAGACTGGTCATCAA R: GGAATTCCTGTCGCCTTACA	88
BmTIF4A	DQ443290	F: TTCGTAAGGCTCTTCTCGT R: CAAAGTTGATAGCAATTCCC	174
Ago-2	NM_001043530	F: CGAAGCTAATGAATACCGGTCG R: CGTGCAGCATACTCTGTGCA	105
Dicer-2	NM_001193614	F: AAATGACTGACCTGAGATCCGC	87

R: AGCAGAGGTACTTGTGCAATCC

Figure 1. Detection of CrPV by RT-PCR in different insect cell lines. (A) Oligo-dT-based RT-PCR detection of CrPV (protocol I). (B) Negative-strand-specific RT-PCR detection of CrPV negative-strand (protocol II). (C) Tag-based negative-strand-specific RT-PCR detection of CrPV negative-strand (protocol III). PCR was performed for 35 cycles. RT-PCR was performed on samples collected at 2 days (D2) and 7 days (D7) post infection. For each cell line and time point, two reactions are shown, i.e. CrPV-treated (+) and mock-infected (control) (-).

Figure 2. Time course of CrPV infection in Bm5 cells. (A) Specific detection of positive and negative RNA strands of CrPV at two and three weeks p.i. by RT-PCR (protocol IV). The number of cycles used in PCR is indicated. (B) Viability of mock- and CrPV-infected Bm5 cells at weekly intervals for a period of 4 weeks. Asterisks indicate significant survival difference compared between CrPV- and mock-infected Bm5 cells (** $P < 0.01$, *** $P < 0.001$, n.s, no significant difference observed). (C) CrPV titer in media of CrPV-infected Bm5 cells at weekly intervals for a period of 4 weeks.

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Figure 3. Size distribution of CrPV-derived viral small RNA reads. Small RNAs were mapped to the genomes of CrPV (left) and MLV (right). Shown are the results of the sample following CrPV infection of Bm5 cells collected at two weeks p.i..

Figure 4. Transcriptional response of selected immune-related genes during CrPV infection of Bm5 cells. Immune-related genes are grouped into sensors and effectors of the RNAi pathway (*dcr-2* and *ago-2*; panel A), signaling components of the Toll pathway (*Rel*; panel B), the Imd pathway (*Imd*, *Tak1* and *Relish*; panel B) and the JAK/STAT pathway (*dome*, *Stat*; panel C) and AMP effector genes (*Cecropin E* (*CecE*),

Hemolin (Hem) and *Defensin (Def)*; panel **D**). Statistically significant differences are indicated with distinct letters (Liu et al., 2014). W0, W2 and W3 refer to time after infection with CrPV (in weeks). N=3.

Figure 5. CrPV infection of Bm5 cells affects the infection properties of the RNA viruses MLV and FHV. (A) Measurement of replication levels of persistently infecting MLV by RT-qPCR at different times after administration of CrPV (m.o.i. of 5). W0, W2, W3 and W4 refer to time after infection with CrPV (in weeks). N=3. (B) Stimulation of infection of Bm5 cells by FHV following pre-infection with CrPV at different m.o.i.. FHV RNA genomes are detected by RT-PCR. FHV(-) and FHV(+) refer to the addition to Bm5 cells of cell culture supernatants of S2 cells that were either FHV-free or persistently infected with FHV (Taning et al., 2018), respectively, at 1:10 (v:v). Indicated is the m.o.i. of CrPV during pre-infection of Bm5 cells (FHV(-): one sample of FHV detection for m.o.i. of 1, 5 and 10 of CrPV: FHV(+): three repeats of FHV detection for each m.o.i. of CrPV).

Figure 6. Infection kinetics of Bm5 cells with CrPV that was produced either in S2 cells or Bm5 cells. Supernatants of pathogenically infected S2 cells or Bm5 cells with similar titer ($5-7 \times 10^8$ TCID₅₀/mL) were added to fresh Bm5 cells at 1:100 (v:v) and cell mortality (indicative of transition to pathogenicity) was assessed at different time points (1-4 weeks) by MTT assay. Mock-infected cells received supernatant from uninfected cells. Results were obtained from three biological repeats for each experimental condition.

Highlights:

- CrPV infection of Bm5 cells is characterized by a short-term persistence period.
- During short-term persistence, CrPV replication and viral siRNA production is low.
- During short-term persistence, increased infectivity of FHV is observed.
- Pathogenesis is preceded by transcriptional activation of immune genes.
- The Bm5/CrPV system is easily accessible to high-throughput approaches.