Induction of host defence responses by \textit{Drosophila} C virus

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Host–pathogen interactions are driven by constant competition between the host defence responses and the pathogen’s ability to control the host to enable completion of its replication cycle. Knowledge concerning both of these components is required to understand the complex interactions involved. \textit{Drosophila} C virus (DCV) is a well-studied and relatively simple virus which is a natural pathogen of the widely used model organism \textit{Drosophila melanogaster}, making it an ideal model system for studying invertebrate host–virus interactions. While previous studies have focused on the \textit{Drosophila} response to virus infection (Cherry & Perrimon, 2004; Dostert \textit{et al}., 2005; Roxstrom-Lindquist \textit{et al}., 2004; Sabatier \textit{et al}., 2003), none have focused on the equally important contribution of DCV to these interactions. In the DCV replication cycle, DCV components have many points of interaction with the \textit{Drosophila} cell and there are differences in the susceptibility between \textit{Drosophila} strains and in the pathogenicity of the DCV isolates, suggesting that there is some specificity in the balance of the interaction between host and virus (Johnson & Christian, 1999; Thomas-Orillard \textit{et al}., 1995).

DCV is a member of the genus \textit{Cripavirus} of the family \textit{Dicistroviridae}. DCV particles are non-enveloped and 30 nm in diameter with a T=3 icosahedral structure (Plus \textit{et al}., 1975; Tate \textit{et al}., 1999). The positive-sense RNA genome includes 9264 nt and contains two large open reading frames (ORFs) which encode the non-structural and structural polyproteins (Johnson & Christian, 1998). Non-structural proteins encoded by ORF1 include helicase, protease and RNA-dependent RNA polymerase (Johnson & Christian, 1998). The structural proteins encoded by ORF2 are VP0, VP1, VP2, VP3 and VP4, with VP0 a precursor of VP3 and VP4; these proteins assemble to form the DCV capsid (Reavy & Moore, 1983; Tate \textit{et al}., 1999).

Insect responses that are specific for virus infection have been investigated using the genetically tractable \textit{Drosophila melanogaster}. Most studies focus on interactions with \textit{Drosophila} C virus (DCV), which is a member of the family \textit{Dicistroviridae}. DCV is a non-enveloped, T=3 icosahedral virus with a positive-sense RNA genome. It was demonstrated recently that several genes controlled by the Jak-STAT pathway are specifically upregulated upon DCV infection. To investigate the virus factors that induce these responses, we used the Jak-STAT regulated genes as reporter genes. Challenge of flies with non-infectious DCV particles or double-stranded RNA did not stimulate significant upregulation of the antiviral response genes. In addition, there was no difference in reporter gene upregulation between \textit{Drosophila} challenged with three different strains of DCV. This suggests that upregulation of these \textit{Drosophila} genes may require virus replication and may involve the non-structural proteins of DCV.

Relatively little is known about innate immune responses of \textit{Drosophila} that are specific for viruses (Cherry & Silverman, 2006). RNA interference is the most studied and is a general antiviral defence. However, a number of viruses, including DCV, encode suppressors which inhibit this response (van Rij \textit{et al}., 2006). Microarrays and proteomics have been used to identify genes and proteins that are upregulated in response to DCV infection in \textit{Drosophila} (Dostert \textit{et al}., 2005; Roxstrom-Lindquist \textit{et al}., 2004; Sabatier \textit{et al}., 2003). A number of genes have been found to be upregulated in response to DCV infection and one protein has been identified as being induced in the haemolymph of DCV-infected \textit{Drosophila} (Sabatier \textit{et al}., 2003).

Dostert \textit{et al}., 2005 identified 140 genes that were upregulated in adult \textit{Drosophila} 24 and 48 h post injection with DCV. Two thirds of these genes were not upregulated in response to bacterial or fungal infections (Dostert \textit{et al}., 2005). Among the most upregulated of these virus-specific genes were \textit{CG12780}, \textit{vir-1} and \textit{CG9080} (Dostert \textit{et al}., 2005). Each of these three genes contain putative STAT-binding sequences in their promoter regions and loss of function \textit{hop}^{M88} / \textit{hop}^{nsvl} mutant \textit{Drosophila} supported the implied role of the Jak-STAT regulation of these genes, with the mutant \textit{hop} flies having a greatly reduced ability to induce these genes (Dostert \textit{et al}., 2005). In addition, \textit{vir-1}
was not upregulated in response to bacterial or fungal infection or stresses including heat shock, cold shock, mechanical pressure, dehydration and UV irradiation (Dostert et al., 2005). For the present study, CG12780, vir-1 and CG9080 were used as reporters of Drosophila response to DCV infection. Throughout our experiments we used the Drosophila strain Champetieres (DGRC number 103403), which was obtained from the Drosophila genetic resource centre. The flies were maintained at 25 °C with a 12 h day/night cycle on standard Drosophila medium. DCV isolates EB, C and Z were originally purified from wild and laboratory stocks of Drosophila from Australia, France and Morocco, respectively (Christian, 1992; Jousset et al., 1972; Plus et al., 1975). DCV EB was plaque-purified and all three isolates were confirmed to be free of cricket paralysis virus contamination. DCV isolates were propagated in DL2 cells and purified by differential centrifugation through 10–40 % sucrose gradients, essentially as described previously (Johnson & Christian, 1998; Johnson et al., 2000). Each virus was resuspended in 50 mM Tris, aliquoted and stored at −20 °C.

The general approach for our experiments involved micro-injecting 4–5 days old, male Drosophila with either DCV, DCV component or buffer control into the upper lateral part of the fly abdomen, essentially as described previously (L’Héritier, 1952). The mortality of one group of Drosophila was monitored for survival curves. Forty-eight hours post injection, five Drosophila were collected and pooled from each treatment group and five Drosophila were collected from buffer-injected control flies (3–5 replicates per treatment for all experiments). The Drosophila samples were then homogenized with TRI reagent (Molecular Research Center) using a bead beater with 3 mm glass beads and total RNA was extracted as per manufacturer’s instructions. Extracted RNA was DNase-treated to avoid genome contamination and reverse transcribed using random primers and SuperScript III reverse transcriptase (Invitrogen), following the manufacturer’s instructions, with a 30 min incubation at 50 °C. To control for within-group variation all samples were reverse-transcribed in parallel.

Quantitative PCR was carried out using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), as per the manufacturer’s instructions, using primers listed in Supplementary Table S1 (available with the online version of this paper). PCR was carried out using 2 μl cDNA template. The PCR temperature profile was 95 °C for 2 min, 50 °C for 2 min and 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 20 s, and was performed with a Rotor-Gene 6000. A control without reverse transcriptase was used to confirm that all genomic DNA had been removed. RT-qPCR data were analysed using REST software (Pfaffl et al., 2002). Expression of CG12780, vir-1 and CG9080 was normalized using house-keeping genes actin 79B (GenBank accession no. NM_079486) and rpl32.

Experiments were undertaken to delineate the DCV components or processes that induce upregulation of the Drosophila reporter genes. The DCV isolates are estimated to differ by up to 10 % at the nucleotide level (Johnson & Christian, 1999), so the Drosophila response to three DCV isolates, DCV C, DCVEB and DCV Z, was compared in order to determine whether virus genetic variation affected upregulation of the reporter genes. Drosophila were injected with approximately 100 nl of either 1.2 × 10^8 IU DCVEB ml⁻¹, an equivalent particle concentration of DCVC or DCVZ or buffer, and the relative expression of CG12780, vir-1 and CG9080 was determined as described above.

Compared with the negative control Drosophila, Drosophila infected with each DCV isolate showed statistically significant upregulation of all three genes (Fig. 1, P<0.05 in each case). This confirmed that the response was not restricted to the DCV–Drosophila strain combinations used in the previous study (Dostert et al., 2005). However, direct comparison between isolates found no difference in the level of upregulation of these genes, except for the statistically significant but small (1.5-fold, P=0.03) difference in the upregulation of vir-1 between DCVEB and DCVZ. Therefore, there was no correlation between the genetic differences of the virus isolates and the level of gene upregulation of the reporter genes. This is consistent with the previous finding that vir-1 is upregulated in response to infection by flock house virus (FHV), which like DCV is a non-enveloped, single-stranded, positive-sense RNA virus (Dostert et al., 2005), suggesting that infection with positive-sense RNA viruses may stimulate a common response pathway in Drosophila. This being the case, it is possible that the reporter genes would be stimulated by

![Fig. 1. Drosophila reporter genes are significantly upregulated in response to infection by three DCV isolates compared with mock-infected Drosophila. The horizontal line indicates a twofold increase in transcript levels and the vertical lines represent standard error.](image-url)
processes in the replication cycle that are common across virus groups.

One of the early points of interaction between the host and virus is between the host cell and the viral capsid. The DCV capsid attaches to receptors on the Drosophila host cell and is endocytosed into the cytoplasm; this step has been shown to be rate-limiting for DCV pathogenesis (Cherry & Perrimon, 2004). To test whether non-infectious virus is endocytosed into the cytoplasm; this step has been confirmed by an infectivity assay in DL2 cells (Scotti, 1977). Untreated DCV was prepared from the same virus stock and processed as for inactivated virus, except there was no exposure to UV light. Drosophila were injected with approximately 100 nl of either 1.2 × 10^8 IU ml^-1 of DCV, UV-inactivated DCV of the same dilution or buffer control. The relative abundance of CG12780, vir-1 and CG9080 transcripts in response to UV-inactivated DCV was compared with buffer-injected Drosophila, with DCV-injected Drosophila as a positive control.

UV-inactivated DCV did not induce any significant differences in the levels of reporter gene transcript expression compared with the control Drosophila (P>0.05 in each case), whereas the DCV-injected Drosophila showed significant (P<0.05) upregulation of all three genes (Fig. 2a). Mortality of DCV-infected flies was similar to previous experiments at 100% mortality by 4 days post infection, whereas 0% mortality was observed in the UV-inactivated DCV-injected group (Fig. 2b). This suggests that the reporter genes are not induced in flies challenged with non-infectious virus.

Another process that is common during positive-sense RNA virus replication is production of dsRNA. RNA silencing, which is activated by dsRNA, is a well-understood antiviral response in invertebrates, and DCV is known to induce this pathway (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006). While the DCV reporter genes are not involved in this pathway, it is possible that the DCV dsRNA is inducing Drosophila responses that are independent of RNA silencing. It has previously been shown that dsRNA is taken up into cells of adult Drosophila following injection of dsRNA into the abdominal cavity (Dziotyeva et al., 2001; Goto et al., 2003); here, we introduced dsRNA into adult Drosophila using a similar approach. Briefly, a region of the DCV genome was amplified using the primers DCV-ORF2-Fw and DCV-ORF2-Rv (Supplementary Table S1) and purified using a MinElute PCR purification kit (Qiagen). RNA was transcribed using a MEGAscript kit (Ambion) and purified using the MEGAClear protocol (Ambion).

DCV was UV-inactivated by exposure to a total of 12 000 mJ UV light (4 × 3 min bursts). Inactivation was confirmed by an infectivity assay in DL2 cells (Scotti, 1977). Untreated DCV was prepared from the same virus stock and processed as for inactivated virus, except there was no exposure to UV light. Drosophila were injected with approximately 100 nl of either 1.2 × 10^8 IU ml^-1 of DCV, UV-inactivated DCV of the same dilution or buffer control. The relative abundance of CG12780, vir-1 and CG9080 transcripts in response to UV-inactivated DCV was compared with buffer-injected Drosophila, with DCV-injected Drosophila as a positive control.

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Drosophila were injected with 400 ng µl^-1 DCV-ORF2 dsRNA, 1.2 × 10^8 IU ml^-1 DCV_EB, buffer control or 200 ng µl^-1 non-specific dsRNA (kindly provided by Darren Underwood, University of Queensland, Australia) and the relative expression of CG12780, vir-1 and CG9080 was determined.

The DCV-ORF2 dsRNA did not induce vir-1 transcription, although CG12780 and CG9080 were induced 1.9 and 1.7-fold, respectively (P=0.04, Fig. 3a). The Drosophila response to non-specific dsRNA was not significantly different from the buffer-injected controls (P>0.05 in each case), and positive control DCV infection caused significant upregulation of each of the three reporter genes (P<0.05 in each case). DCV-infected Drosophila died by day four, whereas the mortality of flies injected with DCV dsRNA or non-specific dsRNA was similar to the buffer-injected control Drosophila (Fig. 3b). Thus, while induction was observed following injection of DCV-dsRNA for two of the reporter genes, CG12780 and CG9080, the biological significance is not clear as it was below twofold difference, and no induction was observed following injection of non-specific dsRNA. Taken together, this suggests that dsRNA

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**Fig. 2.** UV-inactivated DCV (UV-DCV) does not induce upregulation of Drosophila reporter genes. (a) The positive control, DCV-injected Drosophila (DCV), showed significant upregulation of all three genes. The horizontal line indicates a twofold increase in transcript levels and vertical lines represent standard error. (b) Survival assay of UV-inactivated DCV-, DCV- and buffer-injected Drosophila. Note the buffer and UV-DCV data points are completely overlaid.
is not sufficient to stimulate upregulation of those three *Drosophila* genes.

Other possible candidates for DCV components and processes that upregulate these virus-induced reporter genes include the non-structural proteins, other stages of RNA replication, virion assembly and release of virus from the host cell. These interactions would be challenging to study in the whole-fly model, but a similar experimental approach could be undertaken in cultured *Drosophila* cells. Protein expression plasmids could be used to express specific components under the control of constitutive or inducible promoters in transfected cells. In addition, if infectious virus can be recovered from a DCV infectious clone, mutational and deletion mapping could identify components of the virus that are important in inducing the *Drosophila* response. A similar experimental design to the previous experiments could then be carried out to identify components of DCV involved in inducing the *Drosophila* response.

Studies of the complex interactions underlying the *Drosophila*–virus system have largely ignored the contribution of the virus to these interactions. The replication cycle of DCV involves a number of interactions with *Drosophila* host cells which could be responsible for the upregulation of the DCV-infection-induced genes CG12780, vir-1 and CG9080. This study found that neither dsRNA nor non-infectious DCV particles were sufficient to stimulate upregulation of the three reporter genes. The upregulation of these genes seems to be a general response, with little or no difference observed in the *Drosophila* response to three different DCV isolates.

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### References


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