Transcriptional Responses of *Daphnis nerii* Larval Midgut to Oral Infection by 2 *Daphnis nerii* cypovirus-23

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Title:

Transcriptional Responses of *Daphnis nerii* Larval Midgut to Oral Infection by
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Abstract

Background: *Daphnis nerii* cypovirus-23 (DnCPV-23) is a new type of cypovirus and has lethal effect on the oleander hawk moth, *Daphnis nerii*, which feeds on leave of *Oleander* and *Catharanthus* et al. After DnCPV-23 infection, the change of *Daphnis nerii* responses has not been reported.

Methods: In order to better understand the pathogenic mechanism of DnCPV-23 infection, 3rdinstar *Daphnis nerii* larvae were orally infected with DnCPV-23 occlusion bodies and the transcriptional responses of the *Daphnis nerii* midgut were analyzed 72 h post-infection using RNA-seq.

Results: The results showed that 1,979 differentially expressed *Daphnis nerii* transcripts in the infected midgut had been identified. KEGG analysis showed that protein digestion and absorption, Toll and Imd signaling pathway were down-regulated. Based on the result, it was possible the function of food digestion and absorption in insect midgut was impaired after virus infection. In addition, the down-regulation of the immune response may be conducive to viral immune escape. Glycerophospholipid metabolism and xenobiotics metabolism were up-regulated. These two types of pathways may affect the viral replication and xenobiotic detoxification of insect, respectively.

Conclusion: These results may facilitate a better understanding of the changes in *Daphnis nerii* metabolism during cypovirus infection and serve as a basis for future research on the molecular mechanism of DnCPV-23 invasion.

Keywords: *Daphnis nerii* cypovirus-23; midgut; transcriptome analysis.

Introduction

The oleander hawk moth, *Daphnis nerii* (*D. nerii*), belongs to Lepidoptera, Sphingidae family, is a worldwide pest[1]. *D. nerii* larvae damages leave of *Oleander*, *Catharanthus*, *Vinca*, *Adenium*, *Vitis*, *Tabernaemontana*, *Gardenia*, *Trachelospermum*, *Amsonia*, *Asclepias*, *Carissa*, *Rhazya*, *Thevetia*, *Jasminum* and *Ipomoea* each year [2, 3], which affect the landscape and the medicinal value of these plants. At present, the chemical pesticide decamethrin is used to control *D. nerii* [2].

Cypovirus is a member of the *Reoviridae* family, and is characterized by its single layered capsid [4]. DnCPV-23 was isolated from naturally diseased *D. nerii* larvae. This was a new type of cypovirus based on different electrophoretic migration patterns and conserved terminal sequences [1, 5, 6]. In addition to *Daphnis nerii*, it has been found that DnCPV-23 can also induce infection and death in many species of Sphingidae insects, such as *Cephonodes hylas* Linnaeus, *Ampelophaga rubiginosa* Bremer & Grey, and *Agathia lycaenaria* Kollar. The genome of DnCPV-23 consists of ten segments of linear double-stranded RNA, referred to as Genomic Segments 1 (*SI*) to 10 (*SI0*), in accordance with the fragments from longest to shortest [7]. Our previous research and unpublished data demonstrated that the virus can successfully replicate on the Sf9 [8] and *Manduca sexta* cell lines QB-MS 2-2[9]. However, the molecular mechanism of the interactions between the new type cypovirus and its hosts remains unclear. It is necessary to clearly identify the interactions between the virus and its hosts in order to achieve an in-depth understanding, and reveal the exploitation potential of the virus for future insecticide development.

Recently, many studies in the field have generated large amounts of data using the aforementioned high-throughput approaches, from the silkworms or BmN cells infected with BmCPV, including: (1) The possible host's RNAi response against BmCPV challenge in persistent and pathogenic *Bombyx mori* model was compared. During the pathogenic infection, it was found that higher level RNAi responses against BmCPV was observed, which further demonstrated the importance of RNAi as an antiviral mechanism [10]. (2) Gene expression profiles [11-19], DNA methylation [20], and lipidomic profile [21] of silkworm midgut or BmN cells after BmCPV infection were analyzed. These results suggested that many genes (for example, genes expressing Calreticulin, FK506-binding protein, and protein kinase c inhibitor gene, microRNAs, and activated protein kinase C) may play important roles in BmCPV replication. In addition, epigenetic regulation may have influence on silkworm-virus interaction, and BmCPV may modulate the lipid metabolism of cells for their own self-interest. Until now, the molecular mechanism underlying the midgut infection of DnCPV-23 is not clearly understood. Furthermore, since transcriptome analyses regarding *D. nerii* or

DnCPV-23 have not yet been performed, this study aims to fill this gap about the new type cypovirus.

In this study, the transcriptomes of both uninfected control and DnCPV-23-infected *D. nerii* midgut tissues were compared. The *D. nerii* transcripts which were the most significantly up or down-regulated at the 72 hours post-infection were identified following oral infection by DnCPV-23. This study's differential expression analysis successfully resulted in the identification of a total of 1,979 transcripts with at least 2-fold changes (FC) in expression levels. The functional annotations of these differentially expressed (DE) transcripts revealed the overall trends in the important hosts' cellular processes affected by the virus infection. Among all of the DEGs, 298 DEGs had KEGG (Kyoto Encyclopedia of Genes and Genomes) annotations, of which 118 were up-regulated genes and 180 were down-regulated genes. Through KEGG analysis, we found many important signal pathways changed after DnCPV infection, which play an important role in insect reproduction, immunity, digestion and absorption, xenobiotic metabolism and viral replication[10, 13, 22, 23]. Therefore, the results obtained in this study provided a detailed characterization of midgut-specific cellular responses to cypovirus infection, which was also important for further understanding the complex virus-host interactions between DnCPV-23 and *D. nerii*.

Materials and methods

2.1 *Daphnis nerii* larval midgut and virus stock

Newly wild-caught second instar larvae with a similar mass were used in this research investigation for the virus infection. Before infection, the *D. nerii* were supplied with 12-hour day/night cycles under $50 \pm 5\%$ relative humidity conditions and were nurtured on oleander leaves at $27 \pm 1^\circ\text{C}$ for three days. The midgut tissues were collected from four pathogenically infected larvae at 72 hours[13, 15] after feeding with DnCPV-23. The same tissues were also collected from three uninfected control larvae at the same time point. DnCPV was originally isolated from the larvae of *D. nerii* and propagated in *D. nerii* larvae as well [1]. The virion solution of DnCPV-23 utilized for infecting the *D. nerii* was stored at 4°C in the dark.

2.2 Virus inoculation

In this study, the DnCPV-23 viral stock was suspended in distilled water at a concentration of 2×10^7 polyhedra/mL. Then, 100 μ L of the viral suspension was spread evenly on one piece of oleander leaf measuring approximately 4 cm $\times$ 1.5 cm each in size. The leaf was then fed to four *D. nerii* larvae. The dose of infection was calculated as 2×10^6 polyhedra per larva. In addition, three control larvae were fed the same quantity of leaves treated with only distilled water. After approximately 12 hours, fresh oleander leaves were used to feed the inoculated larvae after the DnCPV-23-inoculated leaves had been completely consumed.

2.3 Sample preparation

The midguts of both DnCPV-23-infected and control larvae were collected at 72 h post-inoculation by dissecting the larvae on ice. The isolated midgut was then quickly washed in 0.8% diethylpyrocarbonate (DEPC)-treated physiologic saline solution to remove the attached leaf pieces, and then frozen in liquid nitrogen[13] [24]. For transcriptomic analysis, the expression level of DnCPV-23 *S1*, *S10* genes were detected from CASE and CON samples using real-time PCR with the primer pair S1-RTPCR-F (5'-GTGCTGATGGTCTGCTAA-3'), S1-RTPCR-R (5'-TGATTGATGACGACATTGAG-3'), and S10-RTPCR-F (5'-GTCCGCCAATACTCTCAG-3'), S10-RTPCR-R (5'-CGTAGTCCATCGTCAATCA-3'). The *GAPDH* gene was used as the internal reference gene, as detailed in Table 1.

2.4 RNA sequencing

All of the RNA-seq procedures were conducted by the Oebiotech Company (Shanghai, China). The total RNA was extracted from the *D. nerii* midgut tissue using TRIzol reagent (Invitrogen, USA) according to the manufacture's protocols. The RNA integrity and concentrations were checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). In addition, seven RNA samples (including three uninfected samples and four infected samples) with RNA integrity were used to construct the libraries. The cDNA libraries were prepared using a TruSeq RNA Sample Preparation

Kit (Illumina, USA) according to the manufacturer's protocols. Thereafter, the obtained cDNA libraries were sequenced on the Illumina HiSeq2500 platform, which generated paired-end raw reads of 125 bp.

2.5 De novo assembly and functional annotation

The raw data was pretreated by discarding reads with adaptors and low quality (quality scores < 30). Then, the raw data was assembled using Trinity software with default parameters for de novo transcriptome assembly. Transcripts that were not shorter than 300 bp were used for subsequent analysis. In order to obtain the functional annotations of predicted protein-coding sequences, we searched against various databases, including the NCBI non-redundant (NR) protein, SwissProt, and euKaryotic Orthologous Groups (KOG) using Blastx with an E-value < 10^{-5} . The top hit was utilized to assign gene names. Whereafter, the Gene Ontology (GO) annotations of the transcripts were then analyzed based on SwissProt annotations, and functional classifications were assigned by WeGO software. In addition, for the purpose of determining the biological pathways involved, the KEGG pathway was annotated based on the KEGG Orthology (KO) identifiers.

2.6 Differential gene expression analysis

RNA sequencing results from the two groups were mapped to the assembled transcriptome using bowtie2 [25] and express [26]. The FPKM (fragments per kb per million reads) method [27] was utilized to calculate the expression levels of the unigenes, which eliminated the influencing effects of the different gene lengths and sequencing levels. The differences in the unigene expressions between the two groups were calculated with DESeq [28] and any significant differences were determined with $P < 0.05$ and an absolute value of log2 fold change > 1.

2.7 Real-Time Quantitative Reverse Transcription PCR (Real-Time qRT-PCR)

This study utilized qRT-PCR to verify the DEGs recognized by the RNA-seq. The total RNA was isolated from the samples of the transcriptomic analysis using TRIzol reagent (Life Technologies), and was then treated with DNase I (Fermentas, Glen Burnie, MD,

USA). We reversely transcribed 1 µg of the total RNA per sample into complementary DNA (cDNA) using a PrimeScript RT Reagent Kit (Takara). Then, qRT-PCR was performed using Talent qPCR PreMix SYBR Green (Tiangen, China) on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems™). One cycle was added for melting curve analysis for all the reactions in order to verify the product specificity. The expression level of each gene relative to that of the *GAPDH* gene was calculated using the $2^{-\Delta\Delta CT}$ method [29]. All of the primers for the aforementioned target genes were listed in Table 1.

Table 1

Primers used in the qRT-PCR for the validation of the RNA-seq.

Primer name	Primer sequence (5' to 3')	Target gene
B13-F1	GATTCACACCAGCATTACAG	<i>CYP6AB13</i>
B13-R1	CAGTCGTATATCTCGCCATA	
B6-F1	GGACTATTGTTGGCGAATC	<i>CYP6B6</i>
B6-R1	TTGTGGAAGAAGACGATGT	
B45-F2	GCGATACCGAACCAGAAC	<i>CYP6B45</i>
B45-R2	ATTGGCAGTAAGTGTGAGTT	
GAPDH-F1	TATGTTCGTTGTCGGAGTTA	<i>GAPDH</i>
GAPDH-R1	TAGCAGTAGTGGCGTGTA	

Results

3.1 Virus infection of the samples

Prior to the transcriptome analysis, qRT-PCR was used to detect the mRNA levels of the DnCPV-23 *S1* and *S10* genes in the infected and uninfected samples. The results showed that the infected group had been successfully infected based on the high relative expression of the viral gene mRNA compared with uninfected group (Fig. 1).

194 **Fig. 1.** Detection of the viral RNA in transcriptome samples at 72 hpi (hours post infection). After
195 feeding for 72 hours, the mRNA levels of DnCPV-23 *S1* (A) and *S10* (B) in the midgut of *D. nerii*
196 were detected. The asterisk (***) denotes the presence of a statistically significant difference (p <
197 0.001) by unpaired Student's t test.

198 3.2 Transcriptome sequencing and assembly

199 The RNA-Seq data from the DnCPV-23-infected and control groups contained 346.39
200 million reads, and 334.60 million clean reads after trimming, among which 96.17 to
201 97.39% per sample were determined to be useful. The acquired clean reads were
202 assembled into 31,696 unigenes (> 300 bp). The average length of these unigenes was
203 1,347.61 bp, and the N50 length was 2,348 bp, other information about these unigenes
204 were shown in Table 2. This study then assembled 31.696 unigenes ranging from 301
205 bp to 32,420 bp. The total unigene length was 42,713,980.

206 **Table 2**

207 Statistics of the assembly results.

Term	All	>=500bp	>=1000bp	N50	Total_Length	Max_Length	Min_Length	Average_Length
Unigene	31696	20703	12663	2348	42713980	32420	301	1347.61

208 3.3 Transcriptome annotation

209 A total of 31,696 assembled unigenes were searched against the public databases,
210 including the NR, Swissprot, KOG, GO, and KEGG databases, among which 16,820
211 (53.1%) (Fig. 2) unigenes were annotated. The distribution patterns of the unigenes in

the different databases were as follows: 16,615 unigenes in the NRdatabase, 11,152 unigenes in the Swissprot database, 10,374 unigenes in the KOG, 10,468 unigenes in the GO, and 5,501 unigenes in the KEGG databases (Table 3). Fig. 2 shows the degree of overlap between the unigenes annotated in the different databases. It was found that 4,353 (13.7%) unigenes overlapped in all five databases, while 12,390 (73.7%) unigenes overlapped in two or more databases.

Table 3

Annotation statistics for each database.

Anno_Database	Annotated_Number	300 <= length < 1000	Length >= 1000
NR	16615(52.42 %)	6217(19.61 %)	10398(32.81 %)
Swissprot	11152(35.18 %)	2921(9.22 %)	8231(25.97 %)
KEGG	5501(17.36 %)	1694(5.34 %)	3807(12.01 %)
KOG	10374(32.73 %)	2758(8.70 %)	7616(24.03 %)
eggNOG	15249(48.11 %)	5239(16.53 %)	10010(31.58 %)
GO	10468(33.03 %)	2670(8.42 %)	7798(24.60 %)
Pfam	10594(33.42 %)	2505(7.90 %)	8089(25.52 %)

Fig. 2. Venn diagram showing the degree of overlapping of the unigenes annotated based on different databases. Numbers in different colors represent the number of unigenes annotated through one or more annotation libraries.

3.4 Significant impacts of the viral infection on the hosts' transcriptome expressions

As shown in Fig. 3, the main component PCA1 had reached 41.56%, and the main component PCA2 had reached 27.23%. Therefore, the percentage total of the two was 68.79%, which accounted for a high proportion and represented the overall population to a large extent. This study's principal component analysis manifested a clear separation of the samples with the two treatments (Fig. 3A), which indicated that the samples had good repeatability. The heat map of the gene expressions is presented in Fig. 3B. The results suggested that the samples could be distinguished by these DEGs. The results revealed that the viral infection could exert obvious influences on the midgut gene expressions. In addition, the transcriptome results revealed that 1,166 genes were down-regulated (accounting for 3.68% of the total assembled unigenes) and 812 genes (accounting for 2.56% of the total assembled unigenes) were up-regulated as a response to the DnCPV-23 infection (Fig. 3C).

Fig. 3. Influence of DnCPV-23 infection on *D. nerii* transcriptome: A. Plot of the 1st and 2nd principal component of the sample variations using the principal component analysis, in which the red dots represent samples without DnCPV-23 infection, and the green dots denote infected samples. B. Heat map of 1,978 differently expressed genes (DEGs) in the infected samples and controls. C. After infection, 812 genes were up-regulated (red bars) and 1,166 genes were down-regulated (blue bars).

3.5 Analysis of the differently expressed genes

In this study, KEGG function enrichment analysis was performed on the differential genes expressed in the DnCPV-23-infected group and the uninfected control group in order to clarify the relevant biological pathways involved in the differential genes. Among all of the DEGs, 298 DEGs had KEGG annotations, of which 118 were up-regulated genes and 180 were down-regulated genes. According to the pValue of KEGG analysis of up-regulated and down-regulated signal pathways, we identified 20 most significant signal pathways each. These pathways play an important role in insect

253 reproduction, immunity, digestion and absorption, xenobiotic metabolism and so on.

254

255 **Fig. 4.** KEGG classifications of DEGs after DnCPV-23 infection (Top 20): A. Down-regulated

256 pathways; B. Up-regulated pathways. Horizontal axis of the figure is the enrichment score. The

257 larger the bubble, the more the number of DEGs. The bubble color changes from purple-blue-green-

258 red, indicating that the smaller the enrichment pValue and the greater the significance.

259 3.6 qRT-PCR validation of DEGs

260 In order to verify the reliability of the transcriptome data and the DEG results obtained

261 by RNA-seq, three *CYP450* genes were selected for qPCR analysis. As shown in Fig.

5, the fold-change values of DnCPV_1 sample vs Mock_1 sample obtained in the qPCR analysis results were consistent with the values obtained by the RNA-seq for all of the selected genes.

Fig. 5. Validation of RNA-seq profiles by real-time qPCR. To validate the RNA-seq data, the relative mRNA levels of three selected *CYP450* DEGs in the DnCPV_1 sample were examined by qPCR; The mRNA levels by qPCR are presented as the fold change compared with the Mock_1 sample after normalization against *GAPDH*. The relative expression levels from the RNA-seq analysis were calculated as RPKM values. Error bars show mean $\pm$ SEM.

Discussion

This study analyzed the transcriptome of both the uninfected *D. nerii* midgut and the DnCPV-23- infected *D. nerii* midgut, presented unique gene expression profiles induced by DnCPV-23 infection for the first time. In addition, KEGG function enrichment analysis was performed on the differential genes expressed after DnCPV-23 infection. Compared with uninfected *D. nerii* midgut, the transcriptome profiles of the infected samples displayed universally changed transcript abundances for many pathways.

Based on the pValue of KEGG analysis regarding up-regulated and down-regulated signal pathways, we identified 20 most significant signal pathways each. Among these signal pathways, the retinol metabolism pathway and vitamin digestion and absorption signal pathway were down-regulated, which was consistent with the

transcriptome study about BmCPV infected midgut vs non-infected midgut [13]. In addition, protein digestion and absorption pathway was down-regulated in accord with previous research[10]. DnCPV infection may destroy the functions of digestion and the absorption of midguts, which causes the disturbance of protein and amino acid metabolism in *D. nerii* [13, 30]. The gene *PGRP* (NR annotation: peptidoglycan recognition protein 2; Gene id: TRINITY_DN13195_c0_g1_i3_3) in Toll and Imd signaling pathway related to immunity was down-regulated as well, which was different from previous work [10], we speculate down-regulation of *PGRP* expression may cause the immune escape of the virus. The gene *CASP6* (NR annotation: caspase-6, Gene id: TRINITY_DN10280_c0_g1_i1_3) related to apoptosis in this study was down-regulated. Apoptosis process, one of the innate antiviral responses in many host organisms, is considered to be an antiviral response[31-33], and down-regulation of apoptosis process is conducive to the replication and spread of viruses. Our result conflicted with the work by Guo et al[11]. We speculated the contradiction may be related to the different stages of virus-host interaction or the heterogeneity of different species against viruses.

In this study, the up-regulation of glycerophospholipid metabolism was consistent with Zhang's research[21]. The up-regulation of this pathway may be related to the viral replication[22, 23]. In addition, Glycine, serine and threonine metabolism were up-regulated in this transcriptome analysis. In the study by Wu et al, two genes related to this signaling pathway were up-regulated and the other down-regulated. Expression of many *CYP450* genes was up-regulated, which was similar to the research of Wu et al [13]. These related signaling pathways may be conducive to the host's elimination of toxic and harmful substances[34, 35], which may essentially be a self-protection mechanism of the host.

Conclusion

This study revealed substantial differences in the transcriptions of the *D. nerii* genes related to digestion, immunity, glycerophospholipid metabolism and toxic substances metabolism induced by DnCPV-23 replication. Findings obtained in this research

further enriched the understanding of cypovirus-*Spodoptera* insect interactions in midgut and provided additional basic information for the future exploitation of DnCPV-23.

Abbreviations

DnCPV-23: *Daphnis nerii* cypovirus-23;
D. nerii: *Daphnis nerii*;
PGRP: Peptidoglycan Recognition Protein;
CASP-6: Caspase-6

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Author contributions

W. Kuang, C. Yan designed and performed the experiments and analysed the data. Z. Zhan was responsible for revising the manuscript. Z. Zhan, L. Guan and J. Chen collected *Daphnis nerii* larval. J. Wang and J. Li provided suggestions. W. Kuang, C. Yan and L. Jin wrote the manuscript. L. Jin, X. Zhou, and G. Ma supervised the project and revised the manuscript.

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Availability of data and materials

The original data of the transcriptome has not been released yet.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval and consent to participate

Not applicable.

Consent to publication

Not applicable.

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