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RESEARCH REPORT

Transcriptomic analysis of strain-specific and gender-specific response of silkworm to BmNPV infection

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Abstract

Bombyx mori nucleopolyhedrovirus (BmNPV) is one of the main pathogens causing serious economic losses in sericulture. However, the molecular mechanism of silkworm resistance to BmNPV is still largely unclear, and the differences in the anti-BmNPV response between silkworms of different genders have been rarely studied. In this study, BmNPV resistant strain NB and BmNPV sensitive strain 306 of different genders were used as experimental materials to inoculate BmNPV, and their transcriptomes were sequenced to analyze their response to BmNPV. Eighteen genes specifically differentially expressed in NB after BmNPV inoculation were finally obtained through transcriptomic analysis, fourteen of which were up-regulated and four were down-regulated, suggesting that they might be related to BmNPV resistance. Among them, the expression abundance of eight genes were higher in males than in females, and one gene was in the contrary. These genes suggested that there were certain differences in the anti-BmNPV response between silkworms of different genders. This study provided a new understanding of the molecular mechanism of silkworm resistance to BmNPV and the differences in the anti-BmNPV response between silkworms of different genders, and laid a foundation for future prevention and control of BmNPV.

Key Words: *Bombyx mori*; BmNPV resistance; transcriptome sequence; strain-specific response; gender-specific response

Introduction

The silkworm, *Bombyx mori* has been domesticated for more than 5000 years and is an important economic insect in many developing countries (Goldsmith *et al*., 2005). It is also a good model *Lepidoptera* insect that is often used in the study of insect genetics and immunology (Tanaka *et al*., 2009; Guo *et al*., 2015). *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a baculovirus that infects silkworms. Although some silkworm strains with BmNPV resistance like NB and CVDAR have been cultivated (Chen *et al*., 1991; Qing *et al*., 2019), their cocoon yield and quality still do not meet the production requirements. So far, the main silkworm strains used for production like Yuke9 and Yuncan10 are BmNPV-sensitive strains (Liu *et al*., 2013; Yang *et al*., 2019). There is no effective method or drug to control this virus disease, which brings huge losses to silkworm industry every year. Therefore it is very urgent and important to identify the BmNPV resistant mechanism of silkworm. There have been many researches on silkworm resistance to BmNPV, and some resistance genes have been successively identified. For example, the increased expression of suppressor of cytokine signaling 2 (BmSOCS2) has been reported to be correlated with suppression of BmNPV replication in silkworm (Yuan *et al*., 2020). Arginine kinase (BmAK) and trypsin (Bmtryp) have also been reported to be involved in the antiviral process (Kang *et al*., 2011; Ponnuvel *et al*., 2012). However, the molecular mechanism of silkworm resistance to BmNPV has not been clearly interpreted yet.

In nature, male and female individuals of many species have great differences in many aspects (Matthews *et al*., 2019). For example, there is sexual dimorphism in genetic loci for anthropometric traits (Randall *et al*., 2013). And the fungal infection could induce sex-specific transcriptional changes in *Silene latifolia* (Zemp *et al*., 2015). Similarly, there are certain differences in the development, cocoon characters and disease resistance between silkworms of different genders (Qin *et al*., 2014; Chen *et al*., 2016). However, the differences in the anti-BmNPV response between silkworms of different genders have been rarely studied.

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Table 1 Primers used in RT-qPCR

GENE	Forward Primer	Reverse Primer	Product Length (bp)
BGIBMGA004955	5' ATCGAGATTCGGTCACGAGC 3'	5' TTCAGCTGATCGCGCCAATA 3'	169
BGIBMGA009799	5' ACCTGGCTAGGACTGAACGA 3'	5' GGTGGTGCCACACCTTTGTA 3'	220
BGIBMGA006251	5' GGGAGGTTGCAACGGTGAT 3'	5' CGGGGCTGATACATTTGCCT 3'	173
BGIBMGA004809	5' ACTCGAAGTGGCTCTCAACG 3'	5' AGCTTCAATAGCTGCCGTGT 3'	205
BGIBMGA012486	5' AGCGGTTGCAGTTTCTACGA 3'	5' CAGTTCGTGCATACCCCAGT 3'	168
BGIBMGA010514	5' TTGCAAGCCGTTTTTGCTGT 3'	5' TCTCCGGCCTCCAGTAGTAG 3'	188
BGIBMGA010863	5' TGCTGCATAAGATGCGGAGA 3'	5' TGTAGCACCTGGCTACTTGG 3'	219
BGIBMGA011868	5' TGCCAGGCTAACACAGACAG 3'	5' CTCTGAGCCTGCACTTCCAT 3'	150
BGIBMGA009091	5' CAGCCAGGGTTCGGTTGAAA 3'	5' TCAACGGGTACGCATTTTCC 3'	180
BmRPS3	5' CGATTCAACATTCCAGAGCA 3'	5' GAACACCATAGCAAGCACGAC 3'	142

The primers used in RT-qPCR ,and their product length were listed above. BmRPS3 was used as reference gene in this study

In this study, we conducted transcriptome sequencing of NB (BmNPV-resistant strain) and 306 (BmNPV-sensitive strain) in a gender specific manner. Through comparative transcriptomic analysis, differentially expressed genes in silkworms after BmNPV inoculation were obtained. And some candidate genes that may be related to BmNPV resistance were finally obtained according to Venn analysis. This study provides a new understanding of the molecular mechanism of silkworm resistance to BmNPV and the differences in the anti-BmNPV response between silkworms of different genders.

Materials and methods

Virus and silkworm

The BmNPV and silkworms were all preserved in our laboratory. The virus was obtained from the hemolymph of infected larvae and purified by repeated and differential centrifugation according to the method of Rahman *et al*. (2004), and the virus concentration (OB/mL) was measured by the hemocytometer. The half lethal concentration (LC50) of NB reached 6.39×10^8 OB/mL, which was nearly 1000 times higher than that of 306. The first four instar larvae were reared with fresh mulberry leaves. On the first day of fifth instar, the PBS solution containing BmNPV polyhedrons (1×10^7 OB/mL) was evenly smeared on the surface of fresh mulberry leaves and fed to the experimental group. After 24 hours feeding with virus, the experimental group was refed with normal mulberry leaves. And the control group was set simultaneously. On the third day of the fifth instar, the whole silkworms NB♂-V, NB♀-V, 306♂-V, 306♀-V of the experimental group and NB♂-C, NB♀-C, 306♂-C, 306♀-C of the control group were stored in the -80 °C refrigerator for later use.

RNA extraction and transcriptome sequencing

Trizol method was used to extract total RNA from the eight samples, with three biological replicates for each sample. The purity, concentration and integrity of RNA were determined by NanoDrop ONE/ONEC (Thermo, USA), Invitrogen Qubit 4 (Thermo, USA), Agilent 2100 BioAnalyzer (Agilent Technologies, USA) and agarose gel electrophoresis. After that, mRNA was enriched with Oligo(dT) magnetic beads to construct a cDNA library. After the library was qualified with Agilent 2100 BioAnalyzer (Agilent Technologies, USA), transcriptome sequencing was performed using Illumina Novaseq 6000 (Illumina, USA).

Transcriptome assembly, annotation and gene expression analysis

The raw reads were filtered by removing reads with adaptor, reads with a N ratio of more than 10 % (N denotes bases that cannot be identified), and reads containing more than 50 % bases with a Qphred ≤ 20 to obtain the clean reads. Then the clean reads were aligned with the reference genome (http://metazoa.ensembl.org/Bombyx_mori/Info/Index) by HISAT2 to obtain the mapped reads for subsequent transcriptome assembly and gene expression analysis. Stringtie software was used to assemble the mapped reads based on the reference genome. GO, KEGG, COG, NR, Swiss-Prot and Pfam databases were used to annotate the assembled transcriptome. The expression abundance of the annotated genes was analyzed using RSEM software. Read counts data of genes was obtained from the alignment result and annotation file. Standardized gene expression abundance was obtained by FPKM (Fragments per kilobase of transcript per million Fragments) transformation (Mortazavi *et al*., 2008).

Table 2 Gene annotation results statistics

Database	Expressed Gene number	percent	All Gene number	percent
GO	4388	32.54 %	4531	28.78 %
KEGG	7162	53.12 %	7501	47.64 %
COG	12652	93.84 %	13372	84.93 %
NR	13125	97.34 %	14097	89.53 %
Swiss-Prot	9403	69.74 %	9609	61.03 %
Pfam	10083	74.78 %	10311	65.49 %
Total-anno	13187	97.80 %	14219	90.31 %
Total	13483	100.00 %	15745	100.00 %

Expressed Gene number: Numbers of expressed genes in this study in each database
All Gene number: Numbers of all expressed genes of silkworm in each database
Total-anno: Total expressed genes that have been annotated in all databases
Total: Total expressed genes in all databases

Differentially expressed genes (DEGs) analysis

Gene expression abundance was compared between the experimental group and the control group using DEseq2 software to obtain up-regulated DEGs and down-regulated DEGs. The comparison schemes were as follows: NB♂-V vs NB♂-C, NB♀-V vs NB♀-C, 306♂-V vs 306♂-C, 306♀-V vs 306♀-C. P-adjust < 0.05 and |log2FC| ≥ 1 were set as standard to screen DEGs. Venn diagrams were constructed to obtain unique DEGs of NB♂-V and NB♀-V, as well as DEGs that were common to NB♂-V and NB♀-V but not to 306♂-V or 306♀-V. Further analysis was made on the DEGs that were common to NB♂-V and NB♀-V but not to 306♂-V or 306♀-V to select genes that differentially expressed between NB♂-V and 306♂-V, as well as between NB♀-V and 306♀-V. Genes with low expression abundance (FPKM<10) in all samples were not considered, and genes with a ratio ≥ 1.35 were considered to be up-regulated, genes with a ratio ≤0.74 were considered to be down-regulated. The genes differentially expressed in NB♂-V and NB♀-V were screened from these genes to analyze the differences in the anti-BmNPV response between NB of different genders.

The functions of DEGs and candidate genes were classified by GO analysis, including molecular functions, cellular components and biological processes.

Real-time quantitative PCR (RT-qPCR) analysis

Nine genes that might be related to BmNPV resistance were selected and their relative expression levels in NB-V, NB-C, 306-V and 306-C (mixed samples including male and female silkworms) were detected by RT-qPCR. And relative expression levels of 4 genes were detected in NB♂-V and NB♀-V as well. All the Primers were listed in Table 1. RT-qPCR reactions were prepared with the HiScript Q RT SuperMix Kit (Vazyme, Nanjing, China), following the manufacturer's instruction. Reactions were carried out in Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermo Fisher, Nanjing, China). All samples were performed in triplicate. *B.mori* ribosomal protein s3 (BmRPS3) gene was used as a reference gene. And the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak *et al*., 2001).

Results

Overview of transcriptome sequencing results

A total of 172.4 GB of clean reads were obtained for transcriptomic analysis. For all libraries, the GC content was about 46 %, and the Q30 was all higher than 92.12 %, indicating that the sequencing data were of sufficient quality and accuracy for further analysis. Most reads of all samples were mapped successfully with the reference genome, with a uniquely mapped ratio of about 80 % (S1 Table). There was no significant difference in the sequencing results among the biological replicates, indicating that the library construction and sequencing results were qualified.

Table 3 DEGs statistics

Experiment	Control	Up-regulated	Down-regulated	DEGs
306♀-V	306♀-C	741	1410	2151
306♂-V	306♂-C	251	363	614
NB♀-V	NB♀-C	328	222	550
NB♂-V	NB♂-C	644	279	923
Total				3205

Standard: P-adjust < 0.05 & |log2FC| ≥ 1

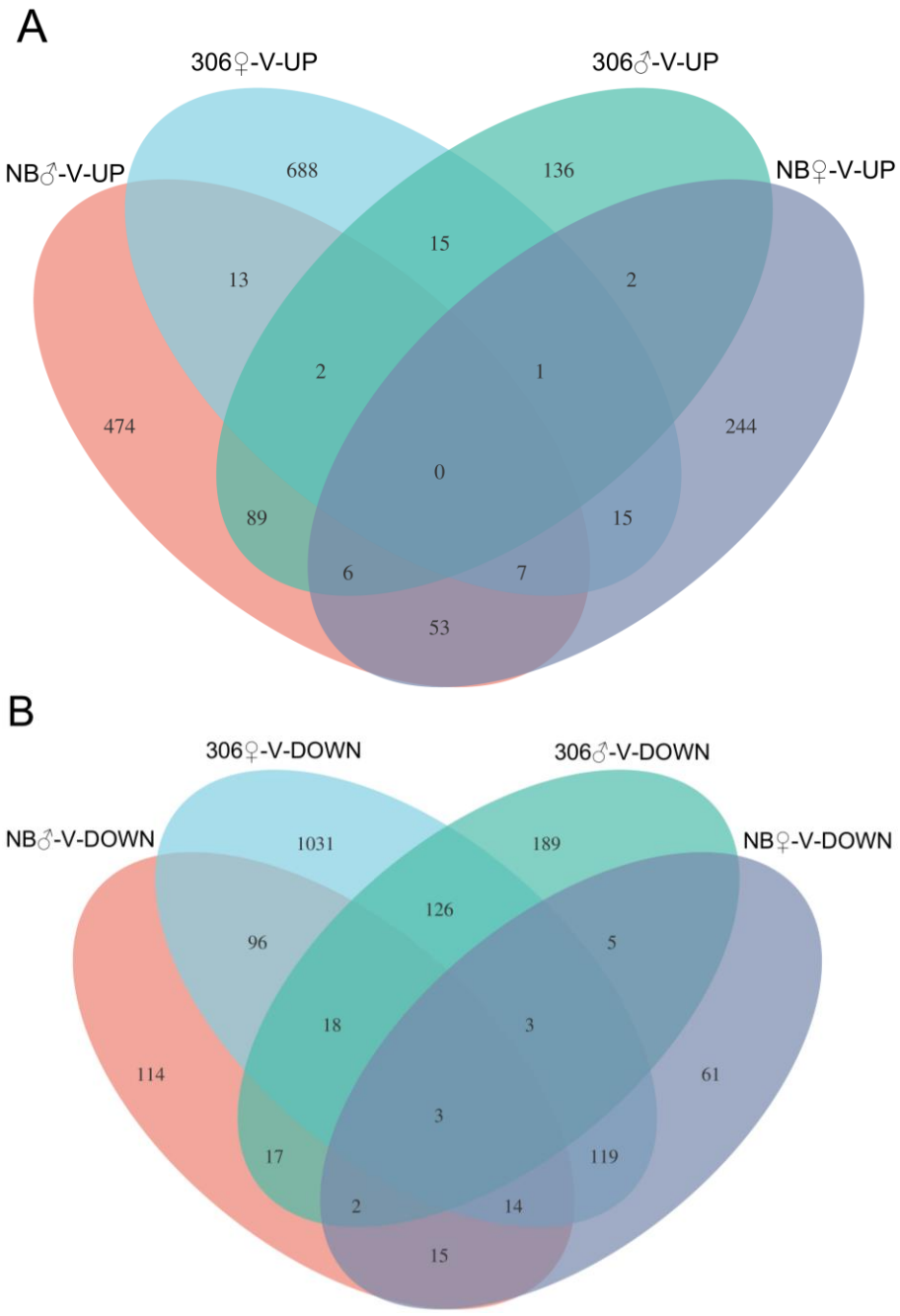

Fig. 1 Venn diagrams showing the DEGs of different samples. (A) Venn diagram of up-regulated DEGs; (B) Venn diagram of down-regulated DEGs

Gene annotation and expression analysis

Reference sequence alignment was performed in GO, KEGG, COG, NR, Swiss-Prot and Pfam databases, and 13,187 of the 13,483 expressed genes were successfully annotated (Table 2). And gene expression abundance was then analyzed.

Differentially expressed genes (DEGs) analysis

A total of 3205 DEGs were obtained by comparing the gene expression of the experimental group and the control group (Table 3). In both male and female, NB had more up-regulated DEGs than down-regulated DEGs after BmNPV inoculation, while 306 were on the contrary. And the number of DEGs was higher in NB male than in female, while that in 306 was higher in female than in male.

In order to further analyze which DEGs were related to BmNPV resistance, Venn diagrams were performed on the up-regulated and down-regulated DEGs respectively (Fig 1). According to the Venn diagrams, 588 specific DEGs were found in NB♂-V (S2 Table), of which 474 were up-regulated and 114 were down-regulated. While NB♀-V had 305 specific DEGs (S3 Table), of which 244 were up-regulated

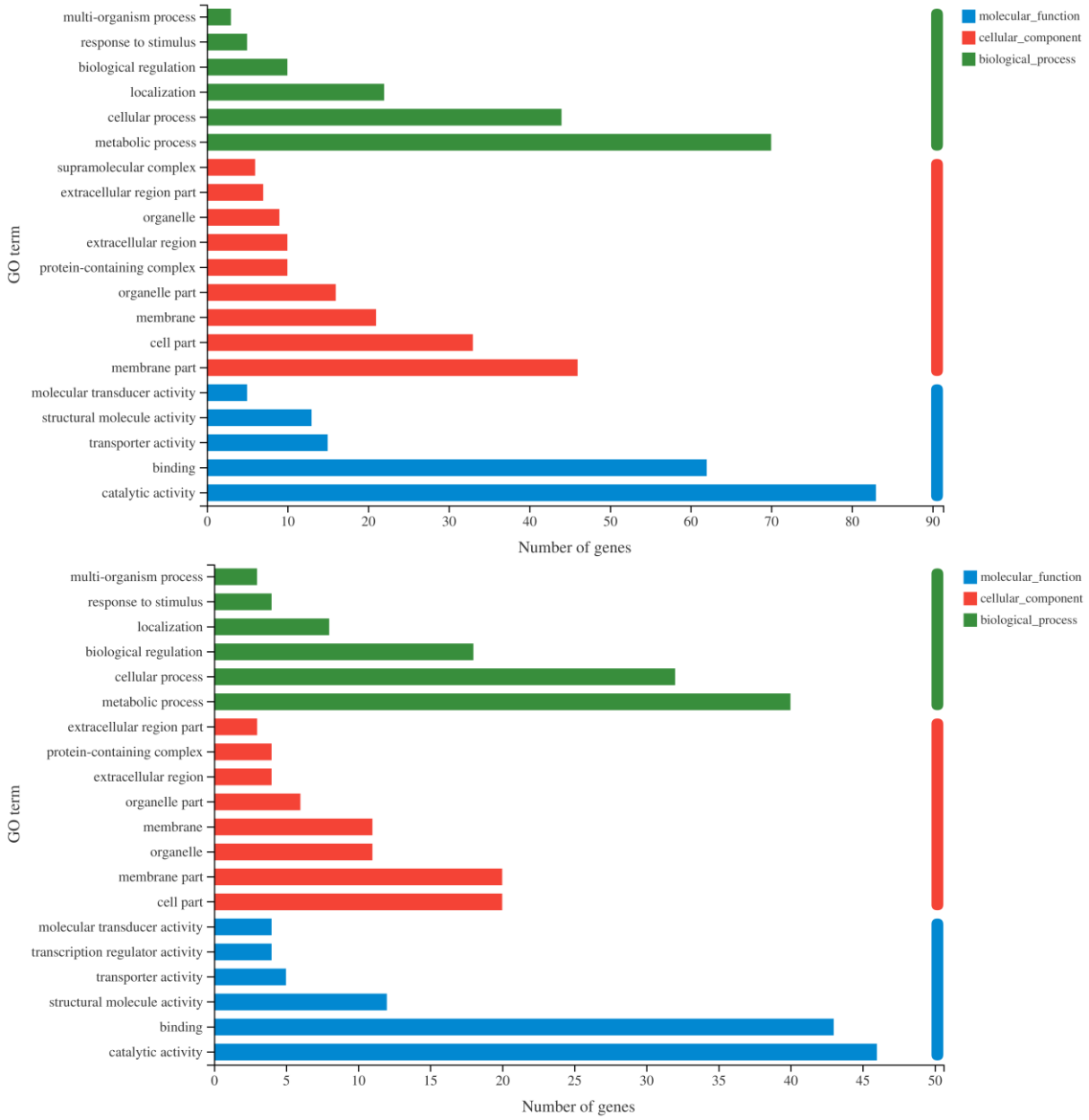

Fig. 2 Gene ontology (GO) functional annotation of specific DEGs. (A) GO functional annotation of specific DEGs of NB♂-V; (B) GO functional annotation of specific DEGs of NB♀-V. The X axis shows the number of genes, and the Y axis shows the categories of gene functions, including molecular functions, cellular components, and biological processes

and 61 were down-regulated. GO analysis showed that specific DEGs of NB♂-V and NB♀-V had similar functional categories, both of which were mainly involved in binding, metabolic process, membrane, catalytic activity and others (Fig 2). However, the number of specific DEGs involved in all functions of NB♂-V was about twice that of NB♀-V, which was consistent with the ratio of their total DEGs (NB♂-V : NB♀-V = 923 : 550).

Meanwhile, 68 DEGs (53 up-regulated and 15 down-regulated) that were common to NB♂-V and NB♀-V but not to 306♂-V and 306♀-V were also obtained according to the Venn diagrams (S4 Table). Go analysis revealed that 8 genes were related to metabolic process, 5 genes were related to membrane part, 8 genes were related to binding, and 12 genes were related to catalytic activity (Fig 3). By analyzing the expression abundance of these 68 genes, 18 genes that differentially expressed between NB♂-V and 306♂-V as well as between NB♀-V and 306♀-V were obtained. Among them, 4 genes were down-regulated and 14 were up-regulated in NB males and females after BmNPV inoculation (Table 4). These genes might be involved

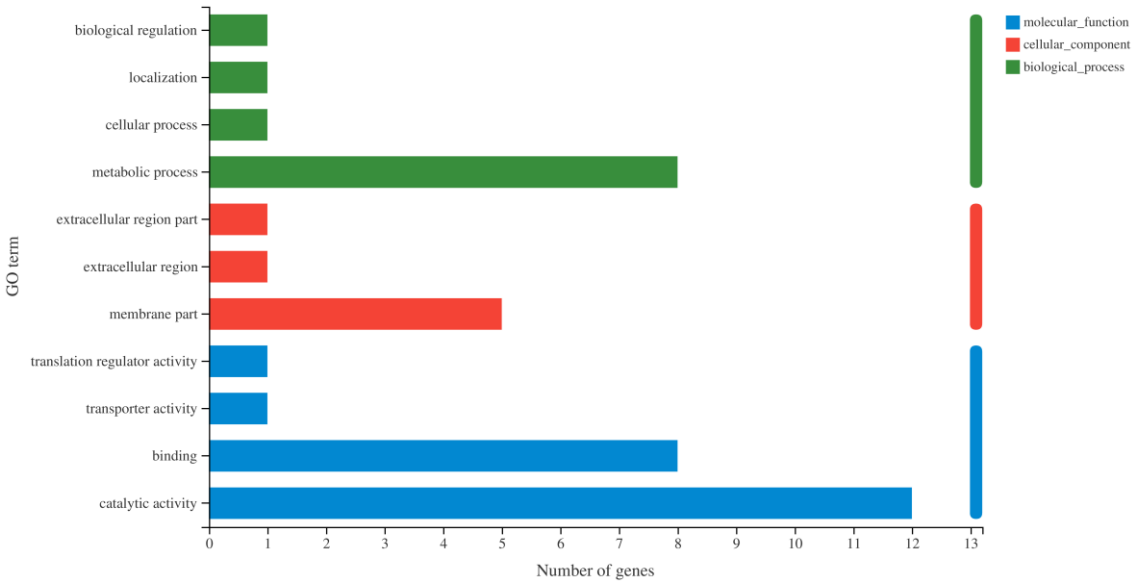

Fig. 3 Gene ontology (GO) functional annotation of 68 specific genes of NB males and females

Table 4 Expression abundance of 18 genes

Gene ID	NR annotation	306♂-V FPKM	306♀-V FPKM	NB♂-V FPKM	NB♀-V FPKM	NB♂-V vs 306♂-V ratio	NB♀-V vs 306♀-V ratio	
BGIBMGA010563	juvenile hormone acid O-methyltransferase-like	26.31	51.06	2.29	3.82	0.09	0.07	
BGIBMGA004955	serine protease inhibitor 13 precursor	17.48	13.3	4.84	5.25	0.28	0.39	Down-regulated
BGIBMGA002176	None	26.38	311.93	7.22	5.26	0.27	0.02	
BGIBMGA001147	facilitated trehalose transporter Tret1-like	20.3	17.44	1.19	1.2	0.06	0.07	
BGIBMGA014348	uncharacterized protein LOC106069352	3699.11	108.64	7868.94	3157.47	2.13	29.06	
BGIBMGA012002	sericin 3 precursor	5463.96	179.71	16584.36	6671.33	3.04	37.12	
BGIBMGA001213	None	108.52	2.26	1161.74	112.25	10.71	49.67	
BGIBMGA009261	uncharacterized protein LOC105842476	0.1	0	94.66	9.37	916.06		
BGIBMGA009799	aldo-keto reductase AKR2E4-like	112.76	93.66	288.16	182.82	2.56	1.95	
BGIBMGA006251	zonadhesin-like	8.47	3.61	16.05	8.16	1.89	2.26	
BGIBMGA004219	uncharacterized protein LOC101743399	7.81	8.29	48.88	47.61	6.26	5.74	
BGIBMGA004809	uncharacterized protein LOC105842986	70.93	66.23	121.04	90.48	1.71	1.37	Up-regulated
BGIBMGA012486	cytochrome b5-related protein	20.06	42.64	44.91	95.09	2.24	2.23	
BGIBMGA010285	uncharacterized protein LOC101738995	4.13	7.83	18.49	24.06	4.48	3.07	
BGIBMGA010514	glucose dehydrogenase [FAD, quinone] isoform X1	1.36	0.81	10.95	1.72	8.05	2.14	
BGIBMGA010863	aspartate--tRNA ligase, mitochondrial	6.5	6.33	53.93	40.12	8.29	6.34	
BGIBMGA011868	uncharacterized protein LOC101742906	1.53	1.67	17.83	17.47	11.63	10.48	
BGIBMGA009091	fungal protease inhibitor F-like	925.76	186.12	1814.67	1295.84	1.96	6.96	

The ratio represents the fold change of FPKM values: genes with a ratio ≥ 1.35 were considered to be up-regulated, genes with a ratio ≤ 0.74 were considered to be down-regulated

Table 5 Expression abundance of 9 genes

Gene ID	NR annotation	NB♂-V FPKM	NB♀-V FPKM	NB♂-V vs NB♀-V ratio	
BGIBMGA012486	cytochrome b5-related protein	44.91	95.09	0.47	Down-regulated
BGIBMGA014348	uncharacterized protein LOC106069352	7868.94	3157.47	2.49	Up-regulated
BGIBMGA012002	sericin 3 precursor	16584.36	6671.33	2.49	
BGIBMGA001213	None	1161.74	112.25	10.35	
BGIBMGA009261	uncharacterized protein	94.66	9.37	10.1	
BGIBMGA009799	aldo-keto reductase AKR2E4-like	288.16	182.82	1.58	
BGIBMGA006251	zonadhesin-like	16.05	8.16	1.97	
BGIBMGA010514	glucose dehydrogenase [FAD, quinone] isoform X1	10.95	1.72	4.61	
BGIBMGA009091	fungal protease inhibitor F-like	1814.67	1295.84	1.4	

in the anti-BmNPV response of silkworm.

By comparing the expression abundance of these 18 genes in NB♀-V and NB♂-V, nine genes were found to have different expression abundance, of which 8 genes had higher expression abundance in NB♂-V and 1 gene had higher expression abundance in NB♀-V (Table 5). These genes denote that NB of different genders have certain differences in the anti-BmNPV response.

RT-qPCR validation of DEGS

The relative expression levels of 9 genes in NB-V, NB-C, 306-V, and 306-C were detected by RT-qPCR (Fig 4A), as well as the relative expression levels of 4 genes in NB♀-V and NB♂-V (Fig 4B). The RT-qPCR results were consistent with the transcriptome data, confirming the reliability of transcriptome sequencing result in this study.

Discussion

In this study, we obtained some genes involved in the silkworm response to BmNPV infection through transcriptome sequencing, so as to study the mechanism of silkworm resistance to BmNPV and the differences in the anti-BmNPV response between silkworms of different genders. By comparing the gene expression between the experimental group and the control group, some differentially expressed genes that might be related to the BmNPV resistance were successfully identified. Among them, BmTret1 has already been reported to be involved in the anti-BmNPV response (Yang *et al*., 2016), but its resistance mechanism has not been clarified. In many non-mammals, Tret1 can transport exogenous trehalose into cells and induce autophagy (Sarkar *et al*., 2007), while the autophagy of silkworm cells is conducive to BmNPV infection (Wang *et al*., 2017). Therefore, we speculated that the down-regulation of BmTret1-like may play a role in the anti-BmNPV response by regulating autophagy. A variety of serine proteases have also been proved to have anti-BmNPV activity (Nakazawa *et al*., 2004; Li *et al*., 2017). Among the candidate genes obtained in this study, both serine protease inhibitor 13 and fungal protease inhibitor F-like were the inhibitors of serine proteases (Pham *et al*., 1996). And they might be involved in the anti-BmNPV process by interacting with serine proteases.

Viruses must rely on cellular proteins to complete replication in cells, so the protein metabolism of the host plays an important role in the game between host and virus (Emmett *et al*., 2005). In this study, some candidate genes related to protein metabolism were also identified. For example, aspartate-tRNA ligase could regulate protein translation (Ibba *et al*., 2000; Ribas *et al*., 2000). Cytochrome B5 could significantly regulate the function of Cytochrome P450, thus extensively affecting the metabolism of exogenous and endogenous compounds (Zhang *et al*., 2007; Im *et al*., 2011). GO analysis showed that LOC105842986 might have lyase activity and participate in the transport and metabolism of amino acids, and LOC101738995 might be involved in coenzyme binding and catalysis activity. The up-regulated expression of these genes suggested that they might influence the replication of BmNPV by regulating protein metabolism of silkworm.

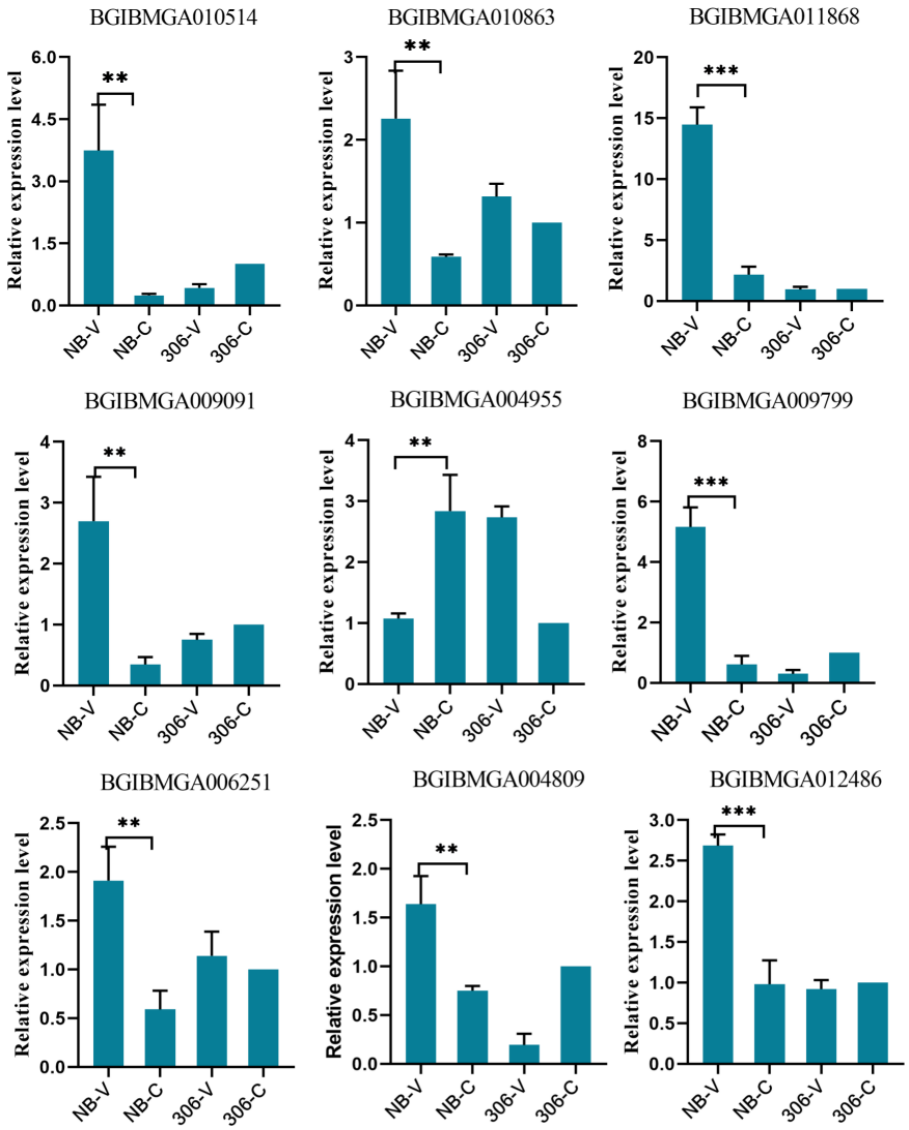

Fig. 4 A) RT-qPCR results of 9 genes. B) RT-qPCR results of 4 genes. The X axis represents different samples, and the Y axis shows relative gene expression levels. The significance of changes in different gene expression levels was also indicated. Samples used for qRT-PCR of nine genes were mixed samples of female and male silkworms, and samples used for qRT-PCR of four genes were female and male silkworm samples respectively

Ecdysone and juvenile hormone are the two most important hormones in insects, which can jointly regulate the growth, development and metamorphosis process of insects (Herboso *et al*., 2015; Riddiford *et al*., 2020). In this study, Aldo-keto reductase-like (AKR2E4-like) was up-regulated, and juvenile hormone acid O-methyltransferase-like (JHAMT-like) was down-regulated in NB after BmNPV inoculated. AKR2E4 could catalyze the production of ecdysone (Yamamoto *et al*., 2017), while JHAMT was the enzyme that catalyzes the last step of juvenile hormone biosynthesis in lepidopteran insects (Shinoda *et al*., 2003). Therefore, changes in their expression abundance may influence the biosynthesis of ecdysone and juvenile hormone. Interestingly, in this study, the expression abundance of Sericin 3 precursor and BGIBMGA009261 (contained Fibroin P25 domain) in NB were up-regulated after BmNPV inoculation, which might be caused by the changed titer of ecdysone and juvenile hormone. In drosophila, steroid hormone signaling played a key role in regulating innate immunity and fighting bacterial infection (Regan *et al*., 2013). Ecdysone could closely regulate innate immunity to recognize and defend against bacterial infection by controlling the expression of pattern recognition receptor （PGRP-LC）in drosophila (Rus *et al*., 2013). While juvenile hormone was an immunosuppressive factor that could strongly interfere with this ecdysone-dependent immune enhancement (Flatt *et al*., 2008). However, these two hormones have not

been found to be involved in the immune system of silkworm. In this study, the expression abundance of AKR2E4-like and JHAMT-like were changed in NB after BmNPV inoculation, suggesting that juvenile hormone and ecdysone might participate in the regulation of immune response and the anti-BmNPV process in silkworm.

The pupation time of NB males was about 24 hours earlier than that of females under normal rearing conditions, and this phenomenon still maintained in NB of different genders after BmNPV inoculation. Therefore, a number of genes must be differentially expressed between NB of different genders after BmNPV inoculation. However, it is not clear whether there are differences in the anti-BmNPV response between silkworms of different genders. In this study, NB of different genders produced different numbers of DEGs and different specific DEGs after BmNPV inoculation, indicating that they did have different responses to BmNPV. Although GO analysis showed that the functional classifications of their specific DEGs were similar, there were differences in the genes involved in the anti-BmNPV response and their expression abundance. We screened nine genes that differentially expressed in NB of different genders after BmNPV inoculation. Among them, The expression abundance of cytochrome b5-related protein was lower and fungal protease inhibitor F-like and AKR2E4-like was higher in NB♂-V than those in NB♀-V. These genes have been discussed above.,and they might participate in the anti-BmNPV response by regulating protein metabolism, protein interaction or other ways. While the higher expression abundance of Sericin 3 precursor, BGIBMGA009261 (contained Fibroin P25 domain), glucose dehydrogenase [FAD, quinone] isoform X1 and zonadhesin-like in NB♂-V might be related to the faster growth and development of NB males. These differences in genetic background between silkworms of different genders were still maintained after BmNPV inoculation, but whether they were involved in the anti-BmNPV response remains to be further studied.

In conclusion, this study provides some insights into the mechanism of silkworm resistance to BmNPV and the gender-specific differences in anti-BmNPV response of silkworm.

Acknowledgments

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