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RESEARCH REPORT

Discovery and functional analysis of a new gene (Bm123) in silkworm (*Bombyx mori*)

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Abstract

Previously our research group used the microarray analysis and suppression subtractive hybridization technologies to find a *Bombyx mori* resistance related gene (NCBI ID: NP_001153678.1) to *B. mori* nucleopolyhedrovirus (BmNPV) and the gene was named *Bm123*. But there are no more confirmatory studies about Bm123. In this study, BmNPV resistant strain NB, susceptible strain 306, hybrid group 306♀×NB♂ (resistant strain) and NB♀×306♂ (resistant strain) were analyzed by transcriptomic sequencing and Weighted Gene Co-expression Network Work Analysis (WGCNA) to verify the new gene *Bm123* function. Correlation analysis between the WGCNA data and phenotype showed that *Bm123* is a gene in ME Turquoise module. This module has a strong correlation with disease resistance phenotype (correlation coefficient is 0.753, P value is 0.0047), indicating that *Bm123* is a correlated gene with anti-BmNPV. The full length of *Bm123* gene was 691 bp, which is not similar with any sequences of other species in NCBI database. But the Bm123 protein contained the transcriptional activator (multiprotein bridge factor 2, MBF2) domain in the 34 to 122 amino acid sequence, closely to *Tribolium castaneum* by the evolutionary relationship analysis. The BmNPV resistance function, developmental expression pattern and tissue expression pattern of Bm123 were analyzed by using silkworm resistant strain BC_{10} (screened by eight backcross and two generation of NB and 306 through hybridization and selfing method, each generation is constructed from the feed by adding BmNPV), NB and sensitive strain 306. It was found that after infection with orally BmNPV, the mRNA and protein levels of Bm123 were up-regulated in the midgut of BC_{10} and NB, and almost not expressed in 306, indicating that *Bm123* was a gene associated with resistance to BmNPV. Bm123 protein expression in various tissues of silkworm (fat body, hemolymph, midgut, epidermis, testis, ovary, malpighian tubule and silk gland) was analyzed. It was found that Bm123 was highly expressed in the midgut and malpighian tubule, while the expression in other tissues was lower. Analysis of Bm123 expression in different development stages of silkworm (eggs, 1st to 5th instar larvae, pupae and moth) found that the expression level of Bm123 increased in the 3rd, 4th and 5th instar. The expression level of Bm123 decreased during the pupae and moth stages. It was speculated that the expression of Bm123 was related to the evolution of resistance genes in silkworm. *In situ hybridization* showed that the *Bm123* gene of BC_{10} was localized in the nucleus of columnar epithelial cells of the midgut, suggesting that Bm123 protein interacts with BmNPV in the silkworm cell nucleus.

Key Words: *Bombyx mori*; Transcriptomic analysis; WGCNA; Bm123; MBF2

Introduction

Silkworm (*Bombyx mori*) is one of the most important economic insects in China. However, the silkworm diseases seriously threaten sericulture development. *B. mori* nucleopolyhedrovirus (BmNPV) in particular has a more serious impact on silkworm production, accounting for about 70 % of the loss of cocoons caused by the whole silkworm disease (Singh *et al*., 2019). Sericulture researchers have reported pathogenesis, transmission routes, and prevention and treatment methods of the disease, especially the screening of disease resistance genes has been the key research direction. By investigating the 344 silkworms sources, our research team found a unique resistant strain (Chen *et al*., 1991), and proved that the resistance of silkworms to BmNPV was controlled by a dominant single gene (Feng *et al*., 2012). Red fluorescent protein (RFP) in the anterior midgut of

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Table 1 The reaction process of *Bm123* gene expression by Northern blot

Composition	Dosage
Bm123 PCR amplified fragment	50 ng
Random primer (0.2 μg/μL)	4 μL
Sterilized water	Add to 10 μL
Incubate at 70 °C for 5 min, quickly place on ice	
dNTP mix (2.5 mM each)	2 μL
Bovine serum albumin (BSA) (10 mg/mL)	2 μL
10×buffer	2 μL
Klenow enzyme (5 u/μL)	1 μL
α-32P-dCTP	1 μL
Sterilized water	Add to 20 μL
Incubation was done at 37 °C for 1 h.	

silkworm can inactivate BmNPV (Hayashiya, 1978). The *B. mori* lipase-1 (BmLipas-1) isolated from the digestive juice of silkworm has a strong anti-BmNPV activity (Ponnuvel *et al*., 2003). The *Bms3a* gene expression level was changed in resistant strains of silkworm, indicating Bms3a was related to resistance of silkworm to BmNPV (Xu *et al*., 2008). Different protein expression between resistant and susceptible strains of silkworms revealed that β-N-acetylglucosaminidase and aminoacylase (Liu *et al*., 2010a), serine protease-4 and caspase-1 (Qin *et al.*, 2012) were related to the resistance of silkworm to BmNPV. It is also reported that arginine kinase and polyprotein are related to silkworm resistance to viruses in the fat body proteome of three silkworm strains (NB, 306 and BC_9) (Liu *et al*., 2010b). *B. mori* serine protease 142 (BmSP-142), isolated from the digestive juice of silkworm, has anti-BmNPV activity (Li *et al*., 2017). Phosphoenolpyruvate carboxykinase mitochondrial subunit (PEPCK-M) can enhance the expression of autophagy genes (ATGs) to inhibit BmNPV proliferation (Guo *et al.*, 2019). However, the nuclear hormone receptor (BmNHR96) of silkworm promotes BmNPV entering into the body of silkworm, which is a gene promoting virus infection (Yang *et al*., 2017). Autophagy gene (*BmATG13*) can promote proliferation and replication of BmNPV (Xiao *et al*., 2019).

There have been many reports on the resistance related genes of silkworm to BmNPV, but there is no evidence to prove the main gene, and its disease resistance mechanism has been unclear. Previously, our research group used microarray analysis (Zhou *et al*., 2013) and suppression subtractive hybridization technology (Gao *et al*., 2018) screened different expression gene in midgut infected by BmNPV of silkworm resistant strain near-isogenic line BC_{10} (screened by eight backcross and two generation of NB and 306 through hybridization and selfing method, each generation is constructed from the feed by adding BmNPV), resistant strain NB, and susceptible strain 306. This research found that Bm123 (NCBI ID: FJ770215) was up-regulated in the two resistant strains as a resistance related gene. However, there is no study reported the BmNPV antiviral characteristics of Bm123. In this study, first, BmNPV resistant strain NB, susceptible strain 306, hybrid group 306♀×NB♂ (resistant strain) and NB♀×306♂ (resistant strain) were analyzed by transcriptomic sequencing to verify the new gene *Bm123* function. Further, resistant functional analysis of Bm123 were executed using BC_{10}, NB and 306 infected by BmNPV.

Materials and Methods

Silkworm strains and virus

Silkworm highly resistant strain BC_{10}, NB, NB♀×306♂, 306♀×NB♂ and susceptible strain 306 of BmNPV were used in this study. BC_{10} is screening by eight backcross and two generation of NB and 306 through hybridization and selfing method, each generation is constructed from the feed by adding BmNPV screening. The NB♀×306♂ strain was obtained by breeding male NB and female 306, and the 306♀×NB♂ strain was obtained by breeding male 306 and female NB. The BmNPV T3 viral strain was used.

Virus feeding and tissue collection

The silkworms were reared in the clean silkworm room, and samples were taken at the egg, 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} instars, pupae and moth stages, and ten samples were collected at each stage. When the silkworm reached the 5^{th} instar, each silkworm was fed with 1×10^6 BmNPV per os. After 6, 12, 24, 36, 48, and 72 h of virus feeding, the silkworms were dissected to collect fat body, hemolymph, midgut, epidermis, testis, ovary, malpighian tubule and silk gland tissues of silkworms, and stored at -70 °C.

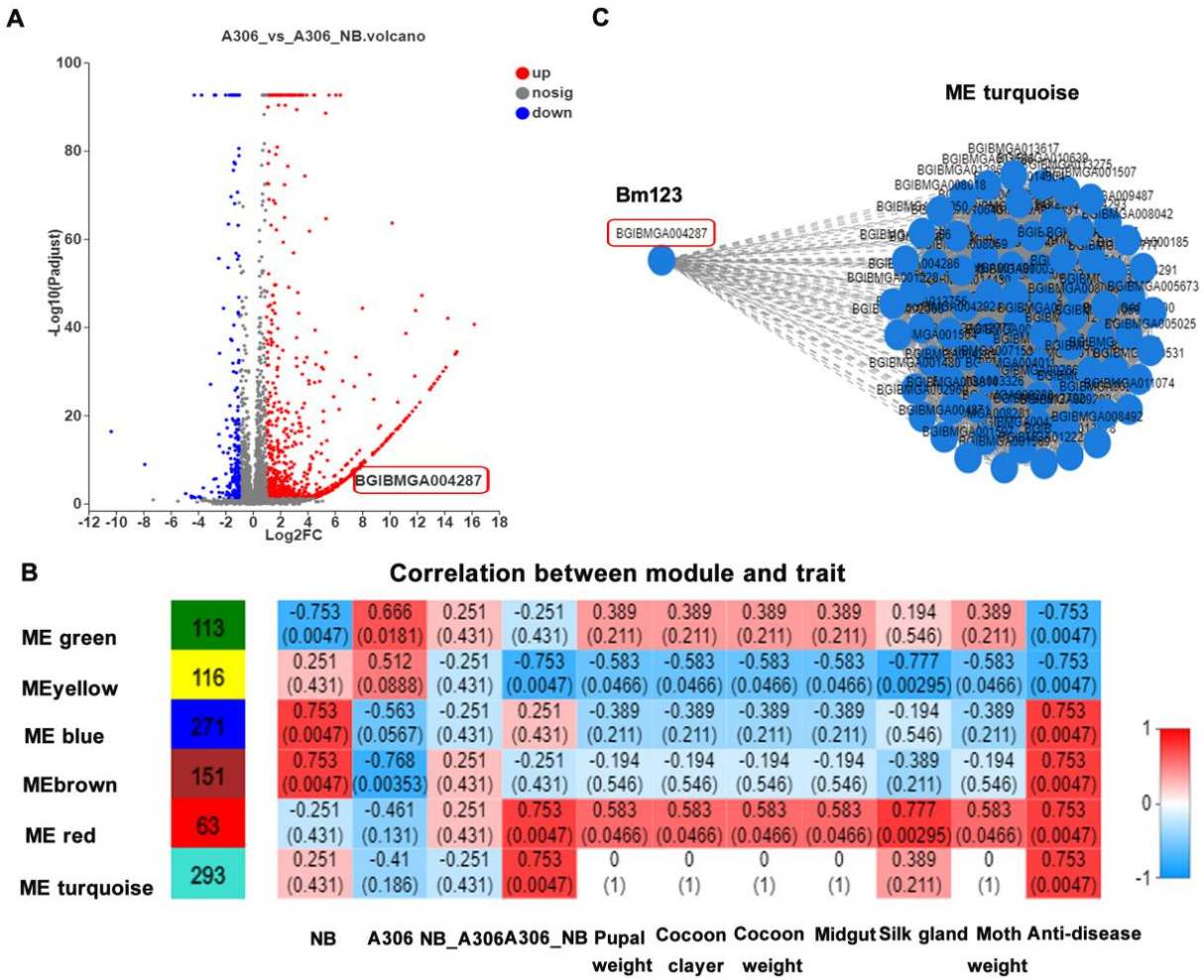

Fig. 1 (A) volcano plot showing 1007 DEGs between 306♀×NB♂ and 306; among which Bm123 (transcriptome ID: BGIBMGA004287) was an up-regulated gene. (B) WGCNA module analyzes the correlation between differential genes and phenotypic traits; (C) Visualization analysis of Bm123 in ME turquoise

Transcriptomic sequencing and Weighted Gene Co-expression Network Work Analysis (WGCNA) to screen resistant genes

1) Sequencing reagents and instruments

The cDNA library construction kit (SuperScript II Reverse Transcriptase) was purchased from Invitrogen.The TruSeq RNA Sample Prep and TruSeq PE Cluster were purchased from Illumina company.

Instruments: Nucleic acid protein analyzer (NanoDrop, Thermo); Bioanalyzer (Agilent 2100 Bioanalyzer, Inc.); Genome Analyzer II (Illumina).

2) Transcriptomic sequencing process

Nanodrop, Qubit 2.0 and Aglient 2100 were used to detect the purity, concentration and integrity of RNA samples to ensure the quality of samples for transcriptomic sequencing. After the sample was qualified, the library was constructed. Eukaryotic mRNAs were first enriched with magnetic beads containing Oligo (dT), mRNA was randomLy interrupted by Fragmentation Buffer. Secondly, the first cDNA strand was synthesized using mRNA as template and random Hexamers. Then buffer, dNTPs, RNase H and DNA Polymerase I were added to synthesize the second cDNA strand. AMPure XP Beads were used to purify cDNA. Purified double stranded cDNA were then repaired, added A tail and connected for sequencing. Then AMPure XP Beads were used to select the fragment size. Finally, cDNA library was obtained by PCR enrichment. After the library was constructed, Qubit 2.0 and Agilent 2100 were used to detect the library concentration and Insert Size, respectively. Q-PCR method was used to accurately quantify the effective concentration of the library to ensure the quality. HiSeq 2500 was used for high-throughput sequencing and the sequencing reading length was PE125. The raw data were obtained. Joint sequences and low quality reads of raw data were filtered and removed to obtain high quality clean data. Clean Data were sequentially assembled to obtain the unigene library of the species. Based on unigene library, the expression quantity analysis and gene structure analysis are carried out, and the

differential expression analysis was carried out according to the gene expression quantity in different samples or different sample groups.

3) Screening and analysis of differentially expressed genes (DEGs)

In the process of analysis of differentially expressed genes(DEGs), the accepted effective Benjamini-Hochberg method have been used to correct the original significance p values (p-value), and finally the adjusted p values (False Discovery Rate, FDR) were used to screen significantly DEGs. In the screening process, FDR ≤ 0.01 and FC (Fold change) ≤ 2 were used as screening criteria. FC represented the ratio of the expression quantity between two samples groups.

4) Correlation analysis between phenotypes and genotypes of DEGs

Weighted correlation network analysis (WGCNA) was used to analyze the relationship between biological characteristics and DEGs to detect BmNPV resistant genes. WGCNA (v1.47) was used to construct an unsigned co-expression network on the transcription expression matrix (Langfelder and Horvath, 2008). The nodes were defined as genes and grouped into different gene networks with similar expression levels. The WGCNA algorithm first assumes that the gene network follows a scale-free distribution and defines the relevant time of gene co-expression (Wang *et al*., 2017a). The correlation module diagram of genotype and phenotype was constructed through WGCNA analysis. CutreeDynamic and mergeCloseModules were used as network construction and module detection methods. The relationship between modules and specific phenotype was analyzed. If the module correlation coefficient is close to 1, most of the modules are related to the corresponding characteristics. In this study, the relationship between transcripts and sample characteristics was investigated, and important modules related to the characteristics were identified. Cytoscape (V3.5.0) was used to visualize the co-expression network (Shannon *et al*., 2003).

Sequence characteristics and evolutionary analysis of Bm123

The sequence of silkworm Bm123 was obtained by transcriptomic analysis. Bm123 amino acid and protein analysis used NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast). Protein blast in the NCBI database revealed that Bm123 contained multiprotein bridge factor 2 (MBF2) domain. MBF2 gene of several species was downloaded from NCBI, and the protein sequences of Bm123 and other species were compared. The 13 sequence names and sequence numbers were as follows: *Culex tarsalis* (JAV33098.1); *Drosophila persimilis* (EDW32147.1); *Drosophila grimshawi* (EDW01064.1); *Drosophila mojavensis* (EDW09458.1); *Drosophila willistoni* (EDW85625.2); *Drosophila sechellia* (EDW56946.1); *Drosophila pseudoobscura* (Q290W7); *Samia cynthia* (BAA34219.1); *Danaus plexippus plexippus* (OWR45174.1); *Drosophila virilis* (EDW62015.1); *Tribolium castaneum* (XP 001811691.1); *Sergentomyia schwetzi* (QHO60785.1); *Nyssomyia neivai* (JAV05291.1). Multiple sequence alignment of Bm123 was performed using DANMAN 8.0, and evolutionary tree analysis was performed using NJ method of MEGA 5.2.

Identification of Bm123 *gene expression using Northern blot*

A random primer method was used to synthesize the silkworm *Bm123* specific probe and α-32P-dCTP purchased from Beijing Furui Corporation, China. The reaction process is as in Table 1.

After oral administration of BmNPV 48 h, the midgut of silkworm strains, including NB, 306 and BC_{10} were collected. Total RNA was extracted using the Trizol method. Non-deformable Polyacrylamide gel electrophoresis gel was used to separate RNA and transfer to the membrane, and prehybridize. After prehybridization, the probe α-32P-dCTP-*Bm123* was added to continue hybridization for 12 h to 16 h. And then the membrane was wrapped with plastic film and placed in a cassette. Then, the X-ray film was pressed tightly. The cassette was placed at -70°C for 3 d to develop autoradiographs.

Identification and quantification of Bm123 *by Reverse transcription PCR (RT-PCR) and quantitative real-time PCR (QRT-PCR)*

The eggs, larvae, pupae, moth and various tissues of silkworm 5th instar (fat body, hemolymph, midgut, epidermis, testis, ovary, malpighian tubule and silk glands) of silkworm BC_{10}, NB and 306 strain were collected. RT-PCR and QRT-PCR were performed with *Bm123* specific primers, including 5'CCCCAGTTCCACTAACAGAGC'3, 5'GGTGAGTTTATGAACCGAAGAGT'3. The housekeeping gene was ribosomal protein L8 (*BMRPL-8*), the primers: 5'TTCCGCGATCCATACAAGTTC'3, 5'CGACCTCTATCACCCATTTTCTCT'3.

Bm123 expression analysis using Western blot

The silkworm BC_{10} and 306 strains were collected, and the midgut of eggs, larvae, pupae, moth and 5th instar tissues (fat body, hemolymph, midgut, epidermis, testis, ovary, malpighian tubule and silk gland) were cut and placed. The sample were added liquid nitrogen and ground into powder. 5 mL RIPA lysate was added and ground for 10 min. The homogenate was transferred to an EP tube and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a new EP tube as WB samples. SDS-PAGE electrophoresis was performed, and the membrane was transferred. After transmembrane, samples were incubated at room temperature for 1 h by the primary antibody of Bm123 (anti-rabbit antibody, self-made by our research group). After washing three times with PBST (phosphate-buffered saline with tween), anti-rabbit secondary antibody were added and incubated at room temperature for 1 h. After washing the membrane three times with PBST, the color was developed, and the picture was taken.

In situ *hybridization analysis of silkworm midgut*

After 48 h of BmNPV infection, fresh midgut of silkworm NB and 306 strains were collected and fixed

Table 2 Partial transcriptomic differential expression genes between 306♀×NB♂ and 306

Gene_ID	Gene description	FC(306_NB/306)	Log2FC(306_NB/306)	Pvalue	Significant	Regulate
BGIBMGA003323	Putative membrane protein	3827.188	11.90206916	4.37888E-46	yes	up
BGIBMGA014524	Serine protease	9968.006	13.28308917	2.1291E-29	yes	up
BGIBMGA010640	Lipase	2125.396	11.05351565	9.29749E-21	yes	up
BGIBMGA004287	Hypothetical midgut protein Bm123	95.534	6.577938594	1.6705E-06	yes	up
BGIBMGA002905	Acyl-CoA binding protein	35.65	5.155814899	6.29306E-23	yes	up
BGIBMGA005781	Heat shock protein 25.4	3.229	1.691120945	0.004663434	yes	up
BGIBMGA012031	Carboxylesterase	0.419	-1.255001364	1.08571E-16	yes	down
BGIBMGA000029	Nucleolar protein family A member 2	0.284	-1.815229193	3.41449E-13	yes	down
BGIBMGA009073	Serine protease inhibitor	0.344	-1.539012289	5.66688E-09	yes	down
BGIBMGA008116	CTP synthase	0.434	-1.204447268	2.48365E-07	yes	down
BGIBMGA007371	Bm8 interacting protein 2d-2	0.35	-1.516173143	0.003090896	yes	down

with 4 % paraformaldehyde. An *in situ hybridization* analysis was performed. The overnight fixed samples were embedded, sectioned and incubated with 100% methanol containing 3 % peroxidation hydrogen at room temperature for 10 min to block endogenous peroxidation activity. After washing with phosphate-buffered saline (PBS), the slides were incubated in citric acid buffer (1.8 mM citric acid and 8.2 mM sodium citrate) at 95 ℃ for 10 min. And then the slides were incubated with blocking buffer (10 % bovine serum albumin and PBS) at room temperature for 1 h. The sections were incubated with α-32P-dCTP-*Bm123* for 1 h. After washing with PBS, the slides were incubated with anti-rabbit IgG-HRP for 30 min followed by diaminobenzidine (DAB) substrate solution. Then the picture were got.

Statistical analysis

All data were analyzed using OriginPro 8.5.1 and GraphPad prism 6. The T-test between the two groups was used to determine the statistically significant of different sample by SPSS. The data were triplicate. Statistical significance was defined as $*p < 0.05$, $**p < 0.01$, n = 3.

Results

Transcriptomic sequencing and WGCNA analysis

According to the transcriptomic analysis of NB, 306 and their positive and negative hybrid strains, 1007 DEGs between 306♀×NB♂ and 306 were purchased, including up-regulated 783 genes and down-regulated 224 genes. *Bm123* belonged to the up-regulated genes database and its ID was BGIBMGA004287 in transcriptomic data (Fig 1A). Partial DEGs data in Table 2 showed that FC (306_NB/306) and Log2FC (306_NB/306) of *Bm123* were 95.53 and 6.578, indicating it was a significantly up-regulated gene. In order to screen BmNPV resistant genes, the relationship between DEGs and biological phenotypic characteristics was analyzed through WGCNA and the relationship correlation module was established. The results showed that there were six modules represented by six colors, namely ME green, ME yellow, ME blue, ME brown, ME red and ME turquoise (Fig 1B). Among them, the genes of ME green and ME yellow were negatively correlated with phenotypic trait of resistance, and these of ME blue, ME brown, ME red and ME turquoise were positively correlated with resistance. The correlation coefficient with BmNPV resistance phenotype in ME turquoise modules is 0.753 (P value 0.0047), followed with silk gland phenotype (coefficient 0.389, P value 0.211). However the coefficients with silkworm pupae weight, cocoon layer, cocoon weight, midgut and moth weight were 0 in this module (Fig 1B). These results indicated this module is most closely correlated with BmNPV resistance phenotype, yet not correlated with other phenotypes. Visualization analysis of DEGs in ME turquoise found *Bm123* (BGIBMGA004287) existed in this module (Fig 1C), indicating *Bm123* is a BmNPV resistant related gene.

Bm123 *gene sequence analysis*

Bm123 gene obtained from transcriptome analysis data was compared in NCBI-BLAST (NCBI ID FJ770215), but no gene with high indentity consistency was found. The full length of this gene is 691bp, including open read frame (ORF) 372bp, 5 'non-translated region 45bp, 3' non-translated region 274bp.The ORF encoded a polypeptide chain of 123 amino acids (Fig 2A). The translated amino acid sequences were analyzed by BLASTP similarity comparison in NCBI to obtain other insect proteins with similar sequences. The amino acid sequence similarity between these proteins and Bm123 protein is 40 % ~ 50 %, respectively. There is no high similarity between silkworm and other insects both in the gene sequence and the protein sequence, indicating that Bm123 is a newly discovered gene. But

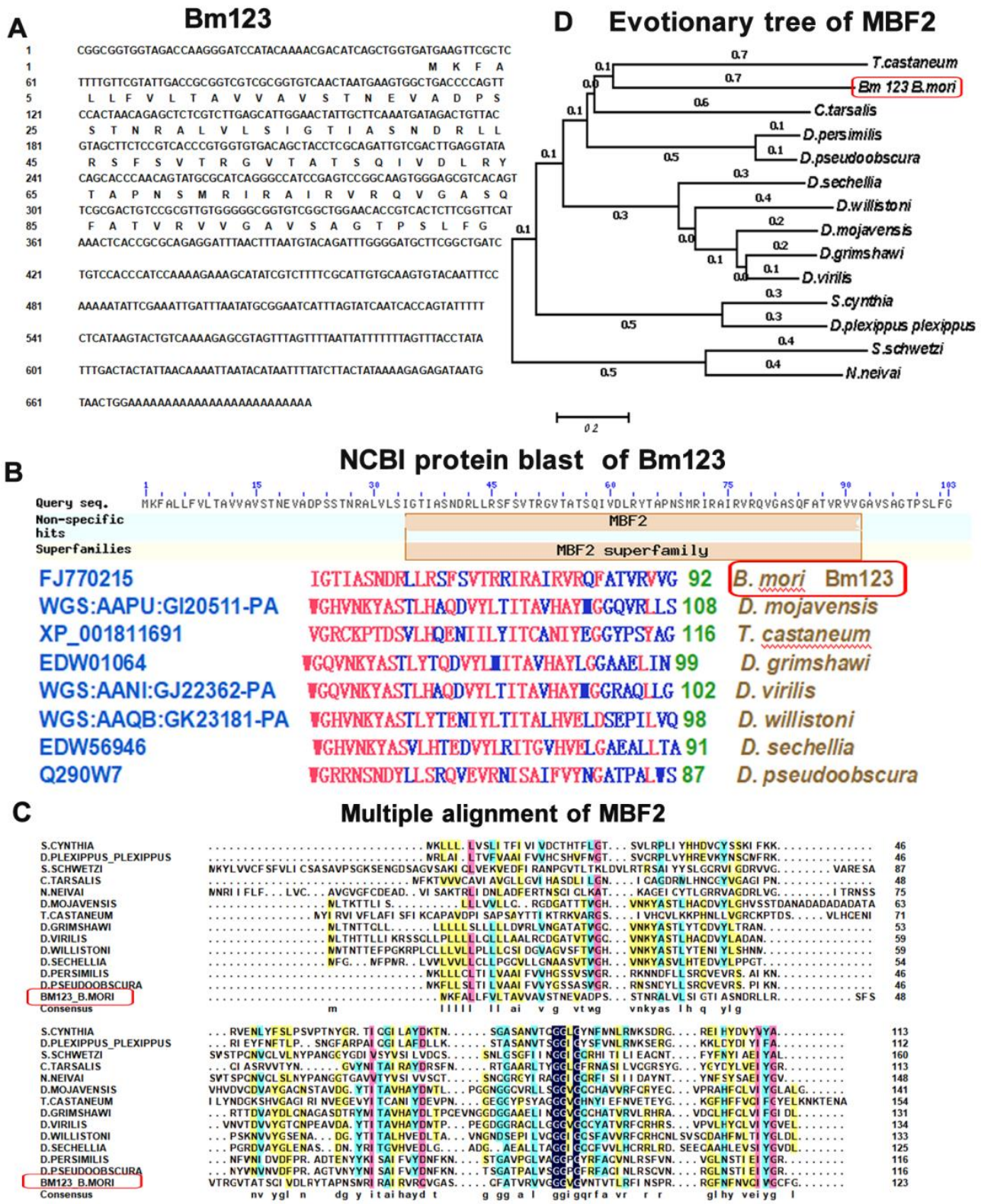

Fig. 2 (A) Full length of Bm123 gene of silkworm; (B) Bm123 protein domain prediction and analysis; (C) Multi-sequence alignment analysis of Bm123 protein sequences with MBF2 proteins of 13 species; (D) Evolutionary tree analysis of Bm123 protein sequence and MBF2 protein of these species,*Culex tarsalis* (JAV33098.1); *Drosophila persimilis* (EDW32147.1); *Drosophila grimshawi* (EDW01064.1); *Drosophila mojavensis* (EDW09458.1); *Drosophila willistoni* (EDW85625.2); *Drosophila sechellia* (EDW56946.1); *Drosophila pseudoobscura* (Q290W7); *Samia cynthia* (BAA34219.1); *Danaus plexippus plexippus* (OWR45174.1); *Drosophila virilis* (EDW62015.1); *Tribolium castaneum* (XP 001811691.1); *Sergentomyia schwetzi* (QHO60785.1); *Nyssomyia neivai* (JAV05291.1)

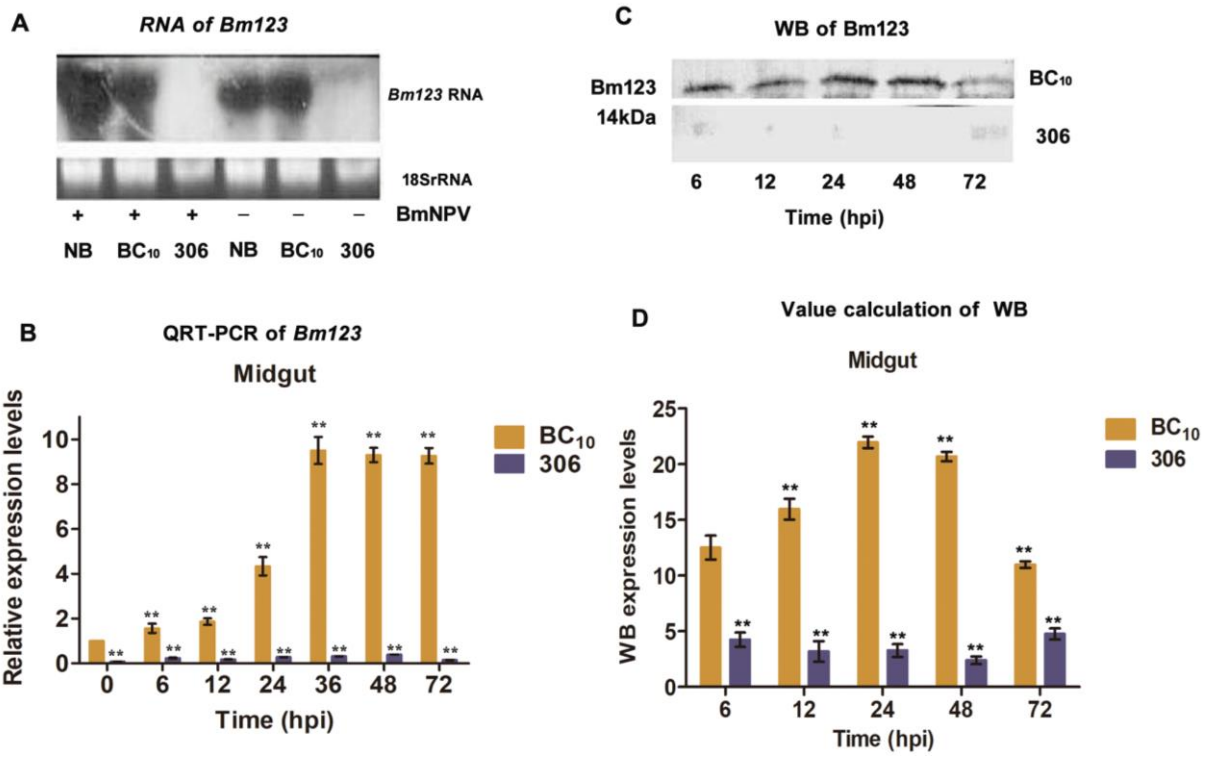

Fig. 3 Bm123 levels analysis in the midgut of silkworm infected by BmNPV. (A) Northern blot verification of *Bm123* gene differentially expressed in the midgut of silkworm BC_{10}, NB and 306 infected by BmNPV 48 h; (B) QRT-PCR analysis of different time expression patterns of *Bm123* gene in BC_{10} and 306 midgut of silkworm infected by BmNPV; (C and D) WB detected the different time protein expression of Bm123 in BC_{10} and 306 of silkworm midgut infected by BmNPV. $p^* < 0.1$, $p^{**} < 0.05$, n = 3

the amino acid sequence of Bm123 protein 34 ~ 120 contains a transcription activator MBF2 domain. Comparing of seven species on NCBI, *Drosophila Mojavensis* (EDW09458.1); *Tribolium Castaneum* (XP 001811691.1); *Drosophila Virilis* (EDW62015.1); *Drosophila Grimshawi* (EDW01064.1); *Drosophila Willistoni* (EDW85625.2); *Drosophila Sechellia* (EDW56946.1); *Drosophila Pseudoobscura* (Q290W7), the MBF2 domain was found to be conserved (Fig 2B), suggesting that Bm123 protein was related to transcriptional activation. Multiple sequence alignment analysis between Bm123 and 13 MBF2 related genes of other species found that the domain of Bm123 protein was consistent with that of MBF2 related proteins of other species (Fig 2C). Then, the MBF2 related protein sequences of these species were selected for the evolutionary tree analysis with the Bm123 ORF region (Fig 2D). It was found that the Bm123 protein of silkworm was most similar to that of *Tribolium castaneum* in terms of its evolutionary relationship and was subsequently classified into a branch with *Culex tarsalis*.

Identification of the Bm123 differential expression by Northern blot

To further identify the differential expression of the *Bm123* gene, total RNA from the midgut tissue of silkworm resistant strains (NB and BC_{10}) and susceptible strain (306) were extracted before and after feeding virus, and Northern blot analysis was performed. *Bm123* specific cDNA labeled with α-32P-dCTP was used as a probe named α-32P-dCTP-*Bm123*, and 18srRNA was used as an internal reference. As shown in Fig 3A, the *Bm123* hybridization band of BC_{10} and NB strains was slightly stronger than that of the strains without virus feeding, and the hybridization signal was not observed in 306 strain. These results indicated *Bm123* levels were higher in NB and BC_{10} strains than that in 306.

Analysis of Bm123 *mRNA level by QRT-PCR*

The expression phase of *Bm123* in midgut tissues was detected by QRT-PCR. The expression level of *Bm123* was low at 0 h of feeding virus, while increased significantly from 6 h to 36 h infected by BmNPV, and the mRNA expression level reached and remained the highest level after 36 h. However, no change in the *Bm123* expression level was observed in the midgut of 306 strain, and the expression level was very low (Fig 3B). The above results indicated that BmNPV up-regulated the mRNA expression of *Bm123* gene in resistant silkworm strain.

Analysis of Bm123 protein expression level by Western blot

Western blot analysis of Bm123 protein in midgut tissue showed that the expression level of Bm123 protein of BC_{10} strain was significantly higher

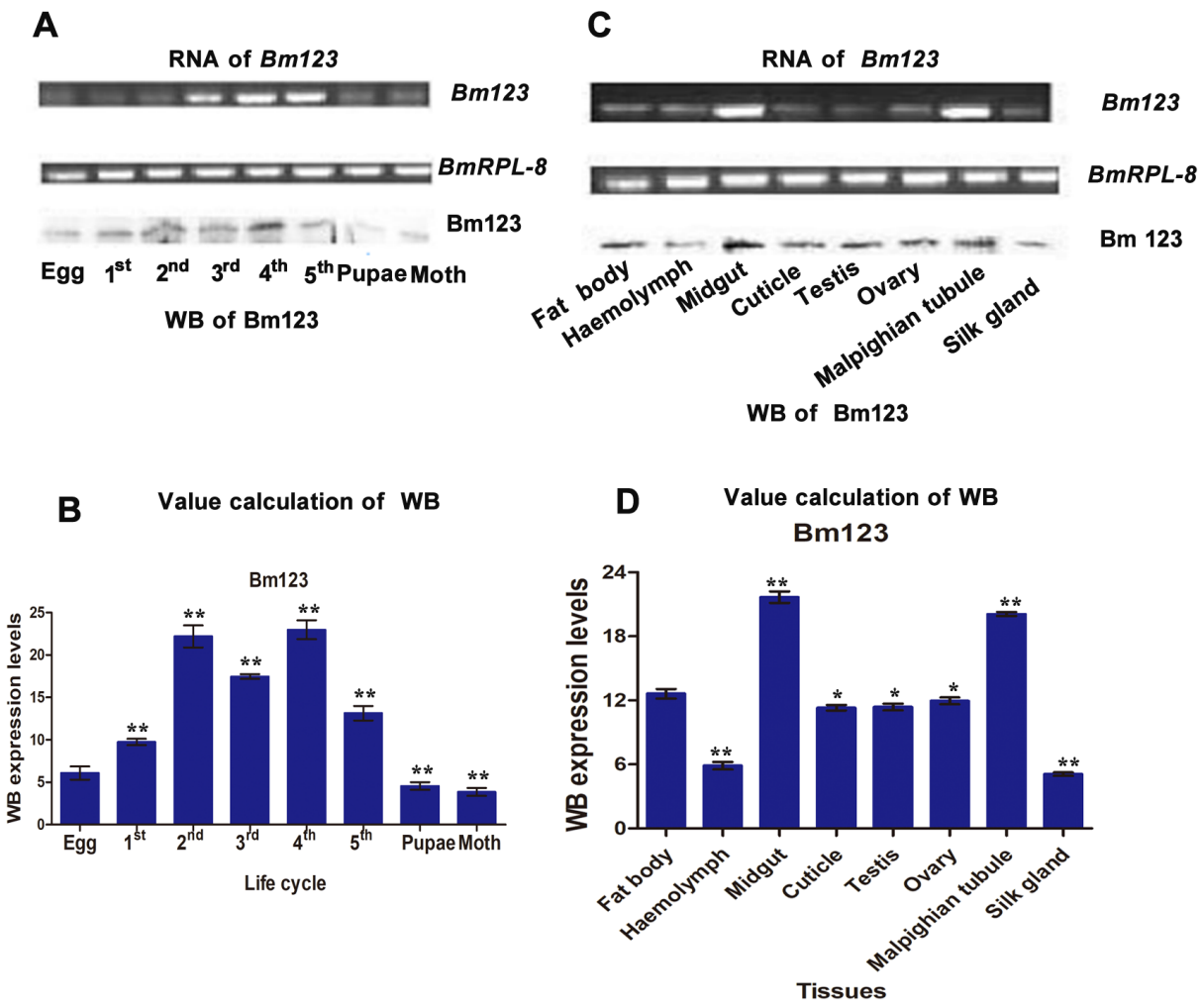

Fig. 4 RT-PCR and Western blot analysis of Bm123 gene expression in BC_{10} tissues and at various developmental stages.(A and B) Expression level of Bm123 at various developmental stages (egg, 1st, 2nd, 3rd, 4th and 5th instars, pupae and moth stages) of midgut; (C and D) Expression level of Bm123 in different tissues (fat body, hemolymph, midgut, epidermis, testis, ovary, malpighian tubule and silk gland). $p^* < 0.05$, $p^{**} < 0.01$, n = 3

than that of 306 strain. The protein expression level reached the highest at 24 h after feeding BmNPV, and the expression level decreased slightly after 72 h. There was no obvious expression of Bm123 protein in the midgut of 306 strain (Fig 3C and D). This result was consistent with the analysis of RNA expression analysis (Fig 3A and B), indicating that the difference in Bm123 protein expression in the midgut of BC_{10} and 306 strains was very obvious.

Expression levels of Bm123 protein at various development stages and tissues of silkworms

To further analyze the expression of Bm123 gene in silkworms, different development stages of BC_{10} and different tissues of BC_{10} 5th instar larvae were collected, Bm123 expression levels were detected from mRNA and protein levels. During the development of silkworm from eggs, 1st, 2nd, 3rd, 4th, 5th instar larvae, pupae to moth, Bm123 mRNA and protein expression levels were different. From eggs to 2nd instar larvae, the expression level of Bm123 was low. From 3rd instar, the expression level of Bm123 rose, and the expression level of Bm123 reached the highest in the 5th instar. The expression level of Bm123 decreased during the pupae and moth stages (Fig 4A and B).

Bm123 was expressed in all tissues of the silkworm, but the expression level was different in different parts. The expression of Bm123 was the highest in the midgut and malpighian tubule, and the expression in other tissues was lower (Fig 4C and D). The results of Western blot were similar with RT-PCR results, which indicates that there are tissue differences in Bm123 expression at both the mRNA and the protein levels, and Bm123 expression level is controlled by development regulation.

Positioning of Bm123 *gene in midgut*

Bm123 gene was localized by *in situ hybridization* in BC_{10} and 306 midgut. The midgut tissues of BC_{10} and 306 strains were selected after

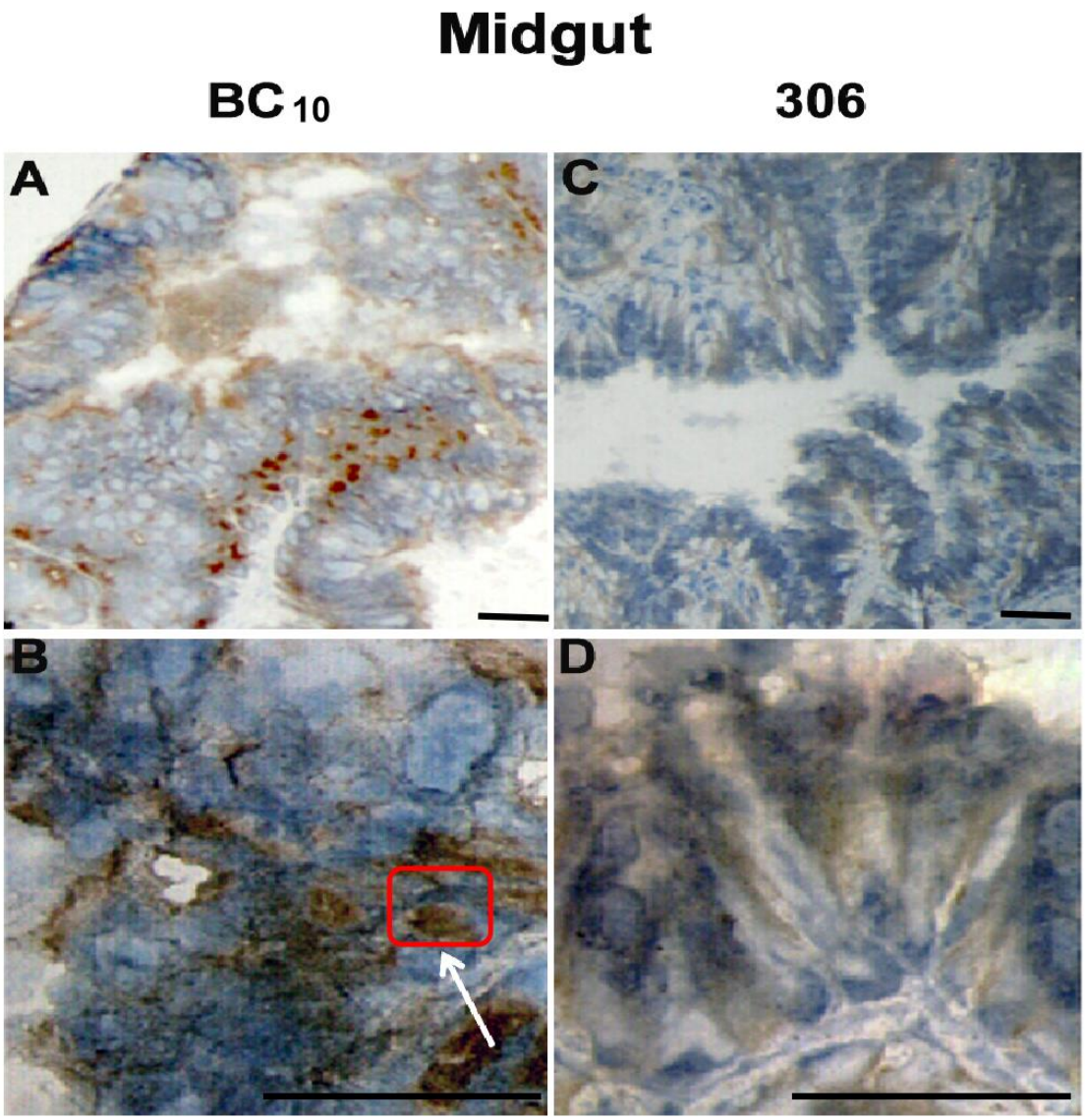

Fig. 5 *In situ* hybridization detection of *Bm123* gene localization in the midgut. A and B were BC_{10} midgut tissue;C and D were 306 midgut tissues.The red box shows the nuclei, and the arrows shows positive signal.The Bar = 50 μm

BmNPV infection. The color reaction generated by immunological binding of α-32P-dCTP-*Bm123* specific label determined the expression position of *Bm123* gene in midgut cells. At low magnification, there was a clear positive hybridization signal in the midgut tissue of BC_{10} strain (Fig 5A). Under high magnification, *Bm123* was mainly located in the nucleus of columnar epithelial cells in the midgut (Fig 5B). No positive signals were observed in 306 strain (Fig 5C and D).

Discussion

The resistance gene of silkworm strains to BmNPV is controlled by major dominant genes (Watanabe, 1986). Comparative genomics, transcriptomics and proteomics analyses revealed that many genes and proteins might be involved in BmNPV resistance (Bao *et al*., 2010; Zhou *et al*., 2013; Wang *et al*., 2017b; Yu *et al*., 2017; Wu *et al*., 2019), such as V-ATPase (Lü *et al*., 2013) and Bm-SP142 (Li *et al*., 2017). However, are these genes associated with resistance to BmNPV the dominant genes? It's still hard to determine. In this study, transcriptomic sequencing was used to identify the DEGs of four silkworms strains, including NB, 306, NB♀×306♂, and 306♀×NB♂. The correlation between BmNPV resistance phenotype and DEGs of 306♀×NB♂ and 306 was analyze by WGCNA to further identify the BmNPV resistance characteristics of Bm123. WGCNA is the most widely used method for disease studies and genes related to traits Identification (Chen *et al*., 2017; Huang *et al*., 2017; Liu *et al*., 2017a; Liu *et al*., 2017b;

Wang *et al*., 2017a). Through WGCNA analysis, ME turquoise module represented the high correlation of genotype and BmNPV resistance phenotype was found (Fig 1B). Bm123 in this module, meant a BmNPV resistance related gene (Fig 1C), and Bm123 was significantly up-regulated (Table 2). Both microarray analysis technology (Zhou *et al*., 2013) and suppression subtractive hybridization technologies (Gao *et al*., 2018) showed that Bm123 was an significantly up-regulated BmNPV resistance related gene. These results were consistent with our research.

In order to study the function of Bm123, the full length of Bm123 protein was amplified and analyzed, and its protein domain was predicted. Through protein comparison analysis, Bm123 sequence contained the MBF2 conserved domain. MBF2 is a transcription activated positive co-factor isolated from silkworm, combined with FTZ-F1 (fushi tarazu factor-1) to jointly activate gene transcription (Li *et al*., 1997; Liu *et al*., 1998). Evolutionary analysis shows that MBF2 gene is insect-specific and can resist bacterial invasion (Zhou *et al*., 2016a). *In situ hybridization* of this study showed that Bm123 gene of BC_{10} was localized in the midgut columnar epithelial nucleus, and Bm123 was not present in 306 (Fig 5). As known, FTZ-F1 is a nuclear hormone receptor transcription factor, mostly present in the nucleus (Sultan *et al*., 2014). Therefore, we hypothesized that MBF2 binds to FTZ-F1 in the nucleus. In addition, MBF2, BMFTZ-F1 and TATA Binding protein (TBP) form complexes (Li *et al*., 1994), and TBP was also found to interact with viral proteins (Lin and Green, 1991; Scholer *et al*., 1991). It is known that BmNPV enters the host nucleus and begins to replicate and proliferate.Therefore, we speculated that Bm123 protein could be involved in transcriptional regulation and virus interaction by binding FTZ-F1 and TBP in the nucleus, accelerating the transformation of silkworm larva into pupae (Cruz and Martin, 2007; Konopova *et al*., 2011), to escape from the infection of BmNPV, which may also be the original cause of the evolution of resistance of silkworm.

The near-isogenic lines BC_{10}, showed the same BmNPV resistance feature with NB strain, but its genetic background was 99.99 % similar to the susceptible strain 306, indicating the expression differences gene between BC_{10} and 306 may be mostly related with resistant phenotype. So next, BC_{10}, NB and 306, were used for Bm123 resistance performance analysis. The increasing expression of Bm123 DNA, mRNA and protein levels were detected by Northern blot, QRT-PCR and Western blot, indicating that BmNPV improved Bm123 activity in midgut in NB and BC_{10} strains. However, virus has no effect on the Bm123 activity in the midgut of 306 strain. Further study on the expression pattern of BC_{10} strain Bm123 at developmental stage found that the protein expression of Bm123 reached its peak in the third to fifth stages of the larva, interestingly the gene expression increased with the increase amount of eating mulberry leaves (Fig 4A and B). It is a meaningful result. BmNPV usually exists on mulberry leaves, which are eaten by silkworm, so the silkworm can get easily infected. In the egg and pupal stage, silkworm does not eat mulberry leaves, but eat a lot of mulberry leaves in the larva stage, which is the main stage of silkworm oral infection of BmNPV. Therefore, we supposed that the high expression of resistance-related genes in the larval stage may be a major defense mode formed in the long-term evolution of silkworm. Previous studies have shown that mulberry leaves containing BmNPV can activate the disease resistance gene of silkworm (Aikawa, 1962; Feng *et al*., 2013). But resistance to the virus of silkworm is weaker in later larval stages (Teakle *et al*., 1986; Kirkpatrick *et al*., 1998). Recent studies on the silkworm infected with *Nosema bombycis* also showed that long-term species evolution would lead to differences in disease-resistant genes among silkworm in different regions (Hassan *et al*., 2020), and this evolutionary nature of species supported our suppose. In addition, the expression level of MBF2 gene in silkworm was the highest before the 4th instar molting and then gradually decreased (Zhou *et al*., 2016b), and MBF2 was detected on the second and third days of the 3rd, 4th and 5th instars (Liu *et al*., 2000). These results indirectly confirm our previous speculation that Bm123 can activate transcription and participate in hormone regulation, promote the growth of silkworm larvae, and regulate virus infection, but how to regulate is still unknown. The Bm123 tissue expression pattern of BC_{10} strain found that the expression level was relatively higher in the midgut and malpighian tubule. Silkworm midgut is an important barrier for resisting BmNPV infection (Hath-Stapleton *et al*., 2003). Up-regulation of several resistant genes (BMLIPpase-1, BmNox and Bmserine Protease-2) was also detected in the midgut of four BmNPV resistant strains (P50, A35, A40 and A53) (Cheng *et al*., 2014). Hence, disease-resistant genes of silkworm strain should be highly expressed in the midgut, which is consistent with the results of this experiment. Malpighian tubule of *Lymantria dispar* has certain immune characteristics (Pannabecker *et al*., 1995). In addition, from the perspective of embryonic development, both the malpighian tubule and the midgut originate from the endoderm, so the malpighian tubule also has the characteristic of high expression of resistance genes. This study also found that Bm123 was highly expressed in the malpighian tubule of resistant silkworm strain. By detecting the gene expression pattern of BmNPV, some studies have found that the virus has obvious viral tissue tendency in host larvae (Hikida *et al*., 2018). The conclusion of this study can also explain the tissue tendency of gene expression.

This research verified the BmNPV resistance related function of the new gene Bm123 based on transcriptional sequencing and WGCNA of silkworm. The function and expression characteristics of Bm123 gene associated with anti-BMNPV revealed that Bm123 is a gene with transcription activator (MBF2) domain which located in cell nucleus of silkworm midgut. Further analysis of the resistance function of Bm123 as well as the development expression pattern and tissue expression pattern showed that Bm123 had obvious specific period and tissue expression characteristics as a BmNPV resistance gene.

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References

Aikawa M. Antiviral substance in the gut-juice of the silkworm. J. Insect Pathol. 4: 72-76, 1962.

Bao YY, Lv, ZY, Liu, ZB, Xue J, Xu YP, Zhang CX. Comparative analysis of *Bombyx mori* nucleopolyhedrovirus responsive genes in fat body and haemocyte of *B. mori* resistant and susceptible strains. Insect Mol Biol. 19, 347-358, 2010.

Chen KP, Lin CQ, Wu DX. Resistance of the conserved silkworm strains to nuclear polyhedrosis virus disease. Acta Sericol Sin. 17: 45-46, 1991.

Chen L, Yuan L, Wang Y, Wang G, Zhu Y, Cao R, *et al*. Co-expression network analysis identified FCER1G in association with progression and prognosis in human clear cell renal cell carcinoma. Int J Biol Sci. 13: 1361-1372, 2017.

Cheng Y, Wang XY, Du C, Gao J, Xu JP. Expression analysis of several antiviral related genes to BmNPV in different resistant strains of silkworm, *Bombyx mori*. J Insect Sci. 14: 76, 2014.

Cruz J, Martin D. Redundant ecdysis regulatory functions of three nuclear receptor HR3 isoforms in the direct-developing insect Blattella germanica. Mech Develop. 124: 180-189, 2007.

Feng F, Fu JG, Hu P. Genetic analysis of baculovirus resistance in lepidopteran model insect *Bombyx mori* L. Afr J Biotechnol. 11: 14417-14421, 2012.

Feng F, Hu P, Chen KP. Progress of antiviral mechanisms in the mulberry silkworm: A review. Afr J Microbiol Res. 7: 1173-1178, 2013.

Gao L, Yang Y, Yao Q, Chen K. Differentially expressed genes in the midguts of BmNPV-susceptible and resistant silkworm strains determined using suppression subtractive hybridization. ISJ-Invertebr Survival J. 15: 256-264, 2018.

Guo HZ, Xu GW, Wang BB, Xia F, Sun Q, Wang YM, *et al*. Phosphoenolpyruvate carboxykinase is involved in antiviral immunity against *Bombyx mori* nucleopolyhedrovirus. Dev Comp Immunol. 92: 193-198, 2019.

Haas-Stapleton EJ, Washburn JO, Volkman LE. Pathogenesis of *Autographa californica M* nucleopolyhedrovirus in fifth instar *Spodoptera frugiperda*. J Gen Virol. 84: 2033-2040, 2003.

Hassan W, Nath B, Ponnuvel K, Mishra R, Pradeep A. Evolutionary diversity in the intracellular microsporidian parasite nosema sp. infecting wild silkworm revealed by IGS nucleotide sequence diversity. J Mol Evol. 1-16, 2020.

Hayashiya K. Red fluorescent protein in the digestive juice of the silkworm larvae fed on host-plant mulberry leaves. Entomol Exp Appl. 24: 428-436, 1978.

Hikida H, Kokusho R, Kobayashi J, Shimada T, Katsuma S. Inhibitory role of the Bm8 protein in the propagation of *Bombyx mori* nucleopolyhedrovirus. Virus Res. 249: 124-131, 2018.

Huang X, Zhou Y, Liu W, Li H, Liang X, Jin R, *et al*. Identification of hub genes related to silicone-induced immune response in rats. Oncotarget. 8: 99772-99783, 2017.

Kirkpatrick BA, Washburn JO, Volkman LE. AcMNPV pathogenesis and developmental resistance in fifth instar Heliothis virescens. J Invertebr Pathol. 72: 63-72, 1998.

Konopova B, Smykal V, Jindra M. Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. Plos One. 6: e28728, 2011.

Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics. 9: 559, 2008.

Li FQ, Takemaru KI, Goto M, Ueda H, Handa H, Hirose S, *et al*. Transcriptional activation through interaction of MBF2 with TFIIA. Genes Cells. 2: 143-153, 1997.

Li FQ, Ueda H, Hirose S. Mediators of activation of fushi tarazu gene transcription by BmFTZ-F1. Mol Cell Biol. 14: 3013-3021, 1994.

Li G, Zhou Q, Qiu L, Yao Q, Chen KP, Tang Q, *et al*. Serine protease Bm-SP142 was differentially expressed in resistant and susceptible *Bombyx mori* strains, involving in the defence response to viral infection. Plos One. 12: e0175518, 2017.

Lin YS and Green MR. Mechanism of action of an acidic transcriptional activator in vitro. Cell 64:971-981, 1991.

Liu QX, Ueda H, Hirose S. Comparison of sequences of a transcriptional coactivator MBF2 from three Lepidopteran species *Bombyx mori*, Bombyx mandarina and Samia cynthia. Gene. 220: 55-59, 1998.

Liu QX, Ueda H, Hirose S. MBF2 Is a Tissue- and Stage-Specific Coactivator That Is Regulated at the Step of Nuclear Transport in the Silkworm *Bombyx mori*. Dev Biol. 225: 437-446, 2000.

Liu R, Zhang W, Liu ZQ, Zhou HH. Associating transcriptional modules with colon cancer survival through weighted gene co-expression network analysis. BMC Genomics. 18: 361, 2017a.

Liu X, Hu AX, Zhao JL, Chen FL. Identification of key gene modules in human osteosarcoma by co-expression analysis weighted gene co-expression network analysis (WGCNA). J Cell Biochem. 118: 3953-3959, 2017b.

Liu XY, Chen KP, Yao Q, Xia HC. Proteomic analysis of differentially expressed proteins involved in BmNPV resistance in the fat body of silkworm, *Bombyx mori*. Z Natmforsch C. 65: 713-718, 2010b.

Liu XY, Yao Q, Wang Y, Chen KP. Proteomic analysis of nucleopolyhedrovirus infection resistance in the silkworm *Bombyx mori* (*Lepidoptera: Bombycidae*). J Invertebr Pathol. 105: 84-90, 2010a.

Lü P, Xia HC, Lu G, Pan Y, Wang Y, Chen X, Lu HG, *et al*. V-ATPase Is Involved in Silkworm Defense Response against *Bombyx mori* Nucleopolyhedrovirus. Plos One, 8:e64962, 2013.

Pannabecker TL, Smith CA, Beyenbach K W, Wasserman RH, *et al*. Immunocytochemicallocalization of a plasma membrane calcium pump in the insect (*Lymantria dispar*) Malpighian tubule. J Insect Physiol. 41: 1105-1112, 1995.

Ponnuvel K, Nakazawa H, Furukawa S, Asaoka A, Ishibashi, J, Tanaka H, *et al*. A lipase isolated from the silkworm *Bombyx mori* shows antiviral activity against nucleopolyhedrovirus. J Virol. 77: 10725-10729, 2003.

Qin LG, Xia HC, Shi HF, Zhou YJ, Chen L, Yao Q, *et al*. Comparative proteomic analysis reveals that caspase-1 and serine protease may be involved in silkworm resistance to *Bombyx mori* nuclear polyhedrosis virus. J Proteomics. 75: 3630-3638, 2012.

Scholer HR, Ciesiolka T, Gruss P. A nexus between Oct-4 and EIA: implications for gene regulation in embryonic stem cells. Cell. 66: 291-304,1991.

Shannon P, Markiel A, Ozier O, Baliga N, Wang JT, Ramage D, *et al*. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13: 2498-2504, 2003.

Singh C, Singh J, Arunkumar K. Role of Toll-receptors in the inhibition of BmNPV proliferation and their interplay with antimicrobial seroin proteins in *Bombyx mori*. J Asia-Pac Entomol. 22: 897-902, 2019.

Sultan ARS, Oish Y, Ueda H. Function of the nuclear receptor FTZ-F1 during the pupal stage in *Drosophila melanogaster*. Dev Growth Differ. 56: 245-253, 2014.

Teakle RE, Jensen JM, Giles JE. Age-related susceptibility of Heliothis punctiger to a commercial formulation of nuclear polyhedrosis virus. J invertebr Pathol. 47: 82-92, 1986.

Wang WJ, Jiang WJ, Hou L, Duan HP, Wu YL, Xu CS, *et al*. Weighted gene co-expression network analysis of expression data of monozygotic twins identifies specific modules and hub genes related to BMI. BMC Genomics. 18: 872, 2017a.

Wang XY, Yu HZ, Xu JP, Zhang SZ, Yu D, Liu MH, *et al*. Comparative subcellular proteomics analysis of susceptible and near-isogenic resistant *Bombyx mori* (Lepidoptera) larval midgut response to BmNPV infection. Sci Rep-UK. 7: 45690, 2017b.

Watanabe H. Resistance of the silkworm, *Bombyx mori*, to viral infections. Agr Ecosyst Environ. 15: 131-139, 1986.

Wu P, Shang Q, Huang HL, Zhang SL, Zhong JB, Hou QR, *et al*. Quantitative proteomics analysis provides insight into the biological role of Hsp90 in BmNPV infection in *Bombyx mori*, J Proteomics. 203: 897-902, 2019.

Xiao Q, Wang L, Zhou XL, Zhu Y, Dong ZQ, Chen P, *et al*. BmAtg13 promotes the replication and proliferation of *Bombyx mori* nucleopolyhedrovirus. Pesti Biochem Phys. 157: 143-151, 2019.

Xu JP, Chen KP, Liu MH, Yao Q, Gao GT, Yuan Z, *et al*. Identification and characterization of Bms3a in *Bombyx mori* L. Afr J Biotechnol, 7: 3424-3430, 2008.

Yang JG, Liu TH, Dong XL, Wu YF, Zhang Q, Zhou L, *et al*. In vivo, RNA interference of, BmNHR96, enhances the resistance of transgenic silkworm to BmNPV. Biochem Bioph Res C. 493: 332-339, 2017.

Yu HZ, Wang XY, Xu JP, Ma Y, Zhang SZ, Yu D, *et al*. iTRAQ-based quantitative proteomics analysis of molecular mechanisms associated with *Bombyx mori* (Lepidoptera) larval midgut response to BmNPV in susceptible and nearisogenic strains. J Proteomics. 165: 35-50, 2017.

Zhou CY, Zha XF, Liu C, Han MJ, Zhang LY, Shi PP, *et al*. Identification of MBF2 family genes in *Bombyx mori* and their expression in different tissues and stages and in response to *Bacillus bombysepticus* infection and starvation. Insect Sci. 4: 502-512, 2016a.

Zhou CY, Zha XF, Shi PP, Wei S, Wang H, Zheng RW, *et al*. Multiprotein bridging factor 2 regulates the expression of the fibroin heavy chain gene by interacting with Bmdimmed in the silkworm *Bombyx mori*. Insect Mol Biol. 25: 509-518, 2016b.

Zhou Y, Gao L, Shi H, Xia H, Gao L, Lian CQ, *et al*. Microarray analysis of gene expression profile in resistant and susceptible *Bombyx mori* strains reveals resistance-related genes to nucleopolyhedrovirus. Genomics. 101, 256-262, 2013.