

GENE EXPRESSIONS RESPONSE TO *Bombyx mori* NUCLEOPOLYHEDROVIRUS (*BmNPV*) RESISTANCE/ SUSCEPTIBILITY IN THAI SILKWORM STRAINS

Saowani Khattamart and Monthira Monthatong*

Received: November 04, 2019; Revised: April 16, 2020; Accepted: May 26, 2020

Abstract

Grasserie disease in silkworm (*Bombyx mori*) caused by *Bombyx mori* nuclear polyhedrosis virus (*BmNPV*) is one of the important epidemic diseases affecting the sericulture in Thailand. This study aimed to evaluate the expression levels of *Bmlipase-1*, *Bmsp-2* and *Bmsop-2* genes responded to *BmNPV* infection in eight Thai silkworm strains which were Sumrong, Nanglai, Nangsiw, Nangtui, Nangnoi, Kaki, Pangpuay and NNK8 by Real-time quantitative PCR (qRT-PCR). The results demonstrated that the relative mRNA expression levels of *Bmlipase-1* and *Bmsp-2* were significantly and highly increased in the midguts of *BmNPV* infected larvae of Sumrong and Nanglai strains compared with those of non-infected larvae. Furthermore, the expression of *Bmsop-2* was dramatically up-regulated in the infected Pangpuay strain. These preliminarily reveals that Sumrong and Nanglai are the candidate strains for *BmNPV* resistance while Pangpuay may be the most *BmNPV* susceptible strain.

Keywords: *Bombyx mori*, *BmNPV*, *Bmlipase-1*, *Bmsp-2*, *Bmsop-2*

Introduction

In sericulture, the major problem of rearing mulberry silkworms, *Bombyx mori*, is the incidence of pathogenic diseases, causing the cocoon crop loss (Saksung and Tawonanukulkit, 1997). One of the serious silkworm infectious diseases is “grasserie disease” induced by *Bombyx mori* nucleopolyhedrovirus (*BmNPV*) infection. *BmNPV* invades silkworms through oral ingestion and the infected silkworms do not show any symptoms and feeds as usual in earlier age (Miao *et al.*, 2005). The occurrence of the disease appears during the late larval stages, the fourth and the fifth instar larvae, causing higher cocoon crop loss. *BmNPV* is classified in genus Alphabaculovirus, family

Baculoviridae. This genus composes of all nucleopolyhedroviruses, which are infected insects in Lepidoptera (Au *et al.*, 2013). Although the infection and invasion of *BmNPV* into silkworm can occur in any races, the proliferation of *BmNPV* is lower in the resistant silkworm strains compared to the susceptible ones (Bao *et al.*, 2010). In insects, the gut barrier has been considered as a primary significant disease defense mechanism. Subsequently, a number of proteins involving in antiviral activities in silkworm have been studied. Formerly, *Bmlipase-1*, *Bmserine protease-2* (*Bmsp*- and alkaline trypsin proteins was isolated from silkworm digestive juice and showed strong antiviral

Department of Biology, Faculty of Science, Khon Kaen University, Muang, Khon Kaen, 40002, Thailand. Tel. 043-202531; E-mail: monmon@kku.ac.th

* Corresponding author

activity against *BmNPV* (Ponnuvel *et al.*, 2003; Nakazawa *et al.*, 2004; Ponnuvel *et al.*, 2012). *B. mori* NADPH-oxidoreductase-like protein (*BmNox*) and *B. mori* suppressor of profilin2 (*Bmsop-2*) were also verified their abilities to against *BmNPV* (Xu *et al.*, 2005, 2008; Selot *et al.*, 2007). Later, *B. mori* arginine kinase (*BmAK*) gene has been found high expression in mid-gut of the resistant silkworm strain, indicating that *BmAK* is also a potential antiviral agent in the *BmNPV* resistant silkworm strain (Kang *et al.*, 2011). In 2004, Nakazawa *et al.* reported that *Bm-serine protease-2* (*Bmsp-2*) gene expression was tissue specificity. The gene was remarkably expressed in the midgut and no expression was presented in fat body, hemolymph, malpighian tube, silk gland and trachea (Nakazawa *et al.*, 2004). On contrary, Cheng *et al.* (2014) found that *Bmsp-2* was also expressed in larval haemolymph with much lower amount than that in midgut. *B. mori-serine protease142* was another protease enzymes that was recently reported high expression in the middle part of silkworm midgut having a potential role in immune responses against *BmNPV* infection (Li *et al.*, 2017).

In Thailand, sericulture was produced from both Thai and foreign silkworm strains. Thai native silkworm strains are more resistant to pathogens than foreign silkworm strains but produce less silk yarn. However, the level of *BmNPV* susceptibility or resistance in Thai silkworm strains has not been yet observed and recorded. In current study, the relative expression levels of *BmNPV* defense genes in the midguts of eight Thai domestic silkworm strains were observed using Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) in order to preliminary compare their levels of viral resistance.

Materials and Methods

BmNPV Preparation

BmNPV was collected from infected silkworm kindly provided from the Queen Sirikit Sericulture Center (Khon Kaen). *BmNPV* was purified by the method of Kaewwises *et al.* (2004). Briefly, *BmNPV* was homogenated in distilled water and filtered through four layers of tiny white cloth. The viral suspension was then centrifuged at 5,000 rpm for 15 min and repeated 3 to 4 times until the viral suspension is clear. The final sediment of *BmNPV* was suspended in distilled water and stored at 4°C for further experiment. The concentration of ODVs of *BmNPV* was investigated by using haemocytometer at 400X magnification under light microscope.

Collection and Rearing Silkworms

Eight strains of healthy *B. mori* third instar larvae, including Sumrong, Nanglai, Nangsiw, Nangtui, Nangnoi, Kaki, Pangpuay, and NNK8, were collected from the Silk Innovation Center, Mahasarakham University (Figure 1). The number of 3 larvae per each strain of the fourth instar larva was divided into two groups: an experimental and a control group. The larvae of experimental were fed with 2 cm² fresh mulberry leaves coated with 10⁵ OB/ml *BmNPV* ODVs. While, the larvae of control group were fed on fresh mulberry leaves treated with sterile distilled water. Then, both groups were reared with fresh mulberry leaves without virus at room temperature until the final stage of fifth instar larva. This experimental work was approved by the Animal ethics committee of Khon Kaen University (IACUC-KKU-85/61).



Figure 1. The characteristics of the fifth instar larvae of eight silkworm strains. a = Sumrong, b = Nanglai, c = Nangsiw, d = Nangtui, e = Nangnoi, f = Kaki, g = Pangpuay, h = NNK8

Midgut RNA Extraction

The midgut from individual fifth instar larva of both experimental and control groups was dissected in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄ prepared with DEPC treated H₂O) and transferred into 1.5 ml microcentrifuge tube to initiate homogenization. Total RNA was extracted by TRIzol reagent according to the manufacture instruction (Bio Basic, Canada). The concentration and purity of RNA was determined using a NanoDrop spectrophotometer (DeNovix, USA). To determine the concentration of total extracted RNA, the absorbance at 260 nm was measured and calculated as OD₂₆₀ × dilution factor × 40 ng/μl. The purification of total extracted RNA was estimated by OD₂₆₀/OD₂₃₀ ratio indicating phenol contamination and OD₂₆₀/OD₂₈₀ ratio indicating protein contamination.

cDNA Synthesis

The cDNA synthesis was performed by using the 2-steps RT-PCR Kit (Vivantis®, Malaysia) following manufacturer instruction. The first step, RNA-primer mixture was conducted in a final volume of 10 µl containing 1 µl of 1 µg RNA template. The mixture was then incubated at 65°C for 5 min and chilled on ice for 2 min. The second step, the first strand cDNA was synthesized within 2 µl of 10X Buffer, 0.5 µl of 200 unit/µl of reverse transcriptase and up to 20 µl of nuclease-free water. The solution mixture was incubated at 42°C for 60 min, followed by incubating at 85°C for 5 min.

PCR Primer Design

The PCR primers were designed by the Primer 3 software (version 0.4.0) available at (<http://bioinfo.ut.ee/primer3-0.4.0/>) based on the nucleotide sequences of three target genes related to *BmNPV* resistance/susceptibility which are *B. mori lipase-1* gene (GenBank accession no. AY945209), *B. mori serine protease-2* (*Bmsp-2*) gene (GenBank accession no. AY945211.1) and *B. mori suppressor of profiling-2* (*Bmsop-2*) gene (GenBank accession no. AY763110). *B. mori ribosomal protein SA* (*BmRpSA*) gene was used as the internal reference (GenBank accession no. NM_001112673). The designed primers and expected product sizes are shown in Table 1

Real-time Quantitative Reverse Transcription PCR (qRT-PCR)

RNAs of all eight silkworm strains were amplified in PCR reactions for testing the contamination of the genomic DNAs. The expression level of *Bmlipase-1*, *Bmsp-2* and *Bmsop2* in experimental and control silkworms of all eight strains were investigated through real-time quantitative RT-PCR (qRT-PCR) and using *B. mori ribosomal protein SA* (*BmRpSA*) gene as the internal reference gen. The qRT-PCR reaction of each gene was conducted under appropriate condition using the LightCycler® 480 Instrument II. The total volume of 20 µl PCR reaction was mixed with 10 µl of 2X

LightCycler® 480 SYBR Green I Master, 1 µl cDNA template (1:5), 0.4 µl of 10 µM specific primers and nuclease-free water up to 20 µl. PCR reactions were dropped down to duplicate wells in each sample within 96-well plates. PCR amplification was performed under the following conditions: one cycle of pre-incubation 95°C for 10 min, followed by 45 to 50 cycles of 95°C for 10 s, 50°C for 10 s, and 72°C for 20 s, with one cycle of melting curves of 95°C for 5 s, 65°C for 1 s, and 72°C for final extension. The one cycle of cooling 40°C for 30 s. The result of gene expression analysis was showed as the relative expression comparing with the *BmRpSA* gene. The relative expressions were calculated following the $2^{-\Delta\Delta Ct}$ method, which is $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{reference}}$ (Livak and Schmittgen, 2001) and were then analyzed by One-way ANOVA with IBM SPSS Statistics 25. Mean values were statistically compared by Duncan's multiple range test (DMRT) at $P < 0.05\%$ level.

Results and Discussion

Three target genes related to *BmNPV* antiviral response, including *Bmlipase-1*, *Bmsp-2* and *Bmsop-2* genes were examined from midguts of *BmNPV* infected 5th instar larvae. The relative gene expressions were then calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) compared to *BmRpSA* expression as an internal control. The results represented that *Bmlipase-1* relative expression level was remarkably increased in infected Sumrong and Nanglai strain which were 3.95 and 3.67, respectively and significantly different from control group ($p < 0.05$) (Table 2 and Figure 2). Apart from NNK8 strain, *Bmlipase-1* expressions in other five strains were also up-regulated but not significantly different comparing to control group. Similar to *Bmlipase-1*, the relative expression level of *Bmsp-2* was also significantly induced in Sumrong and Nanglai strains which were significantly different from control group (Table 2 and Figure 3). For the last gene that we investigated,

Table 1. The specific primers designed for qRT-PCR

Genes (Accession no.)		Primer (5'—3')	Product size (bp)
<i>Bmlipase-1</i> (AY945209)	Forward	5'GGTTCAGGGCTTGGTTGTTA3'	263
	Reverse	5'TGTAGACTGGCGTGGTCTTG3'	
<i>Bmsp-2</i> (AY945211.1)	Forward	5'CGTGATATGGACACTGGAG3'	203
	Reverse	5'TCTTGCTGGACTTGTGATCG3'	
<i>Bmsop-2</i> (AY763110)	Forward	5'CAGCGACGAGCAAAATGTTA3'	181
	Reverse	5'ACTTGCGTGAAATGGTCTCC3'	
<i>BmRpSA</i> (NM001112673)	Forward	5'GCCTGTTCTTGGCTTGTG3'	144
	Reverse	5'GATGTGGTGGTTGTTGGCAC3'	

Bmsop-2, the expressions were slightly declined in *BmNPV* infected Sumrong, Nanglai and Nangsiw strains. Meanwhile, infected Pangpuay strain showed the most dramatically increase of *Bmsop-2* expression level following with Nangnoi, NNK8, Nangtui and Kaki strains (Table 2 and Figure 4).

Discussion

In Thailand, breeding silkworm strains is under the responsibility of the Queen Sirikit department of sericulture, department of agriculture, Thailand. Thai local silkworm strains are more resistant to pathogens than foreign strains but the abilities of *BmNPV* resistance in Thai silkworms have never been characterized. Since 2003, *Bmlipase* and *Bmserrine* protease 2 proteins purified from *B. mori* larval digestive juice were reported as the antiviral activities against *BmNPV* (Ponnuvel *et al.*, 2003; Nakazawa, *et al.*, 2004). Subsequently, the levels of these gene expressions by qRT-PCR have been investigated for *BmNPV* resistance level of silkworm strains in many countries (Cheng *et al.*,

2014; Li *et al.*, 2017). On the contrary, *Bmsop-2* was identified in 2005 using FDD and found the highest expression in highly *BmNPV* susceptible strains using Northern blotting and real time PCR techniques (Xu *et al.*, 2005). In our current study, we investigated the levels of *BmNPV* resistances/susceptibilities of eight Thai silkworm strains that are mostly introduced to Northeastern Thai farmers via the relative expression levels of *BmNPV* defense related genes. *Bmlipase-1*, *Bmsp-2* and *Bmsop-2* transcription levels were compared in infected and non-infected (control) silkworm 5th instar larval midguts. The results represented that infected Sumrong and Nanglai strains relatively expressed the highest levels of *Bmlipase-1* and *Bmsp-2* comparing to the other strains. The observation was similar to the experiment of Cheng and coworkers who reported the increasing of relative expression level of *Bmlipase-1* from larval midgut in Chinese *BmNPV* resistant strains (A35, A53 and A40) compared to Chinese susceptible strain (P50) (Cheng *et al.*, 2014). The function of

Table 2. The relative gene expression including *Bmlipase-1*, *Bmsp-2* and *Bmsop-2* in the eight silkworm strains

Treatment	n	<i>Bmlipase-1</i>	<i>Bmsp-2</i>	<i>Bmsop-2</i>
Control	3	1.00±0.00	1.00±0.00	1.00±0.00
Sumrong	3	3.95±1.25*	4.20±1.42	0.74±0.39
Nanglai	3	3.67±0.20*	4.27±0.72	0.76±0.10
Nangtui	3	2.65±0.10	2.85±0.53	2.19±0.10
Kaki	3	2.43±0.46	2.78±0.69	1.82±0.39
Nangsiw	3	1.76±0.13	2.75±0.30	0.82±0.41
Nangnoi	3	1.25±0.44	1.33±0.28	3.03±1.47
Pangpuay	3	1.28±0.34*	1.22±0.20	5.05±1.54
NNK8	3	0.89±0.06	0.85±0.26	2.92±0.67

*Data are mean of relative expressions ± SE (n=3). Different letters indicatesignificant difference (p<0.05).

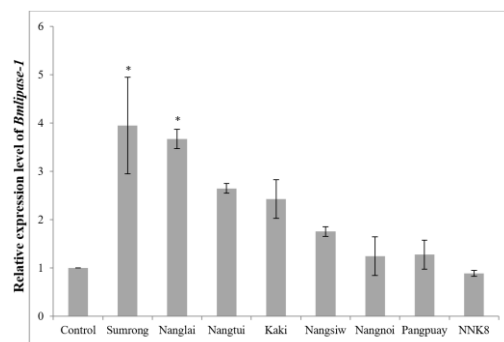


Figure 2. The characteristics of the fifth instar larvae of eight silkworm strains. a = Sumrong, b = Nanglai, c = Nangsiw, d = Nangtui, e = Nangnoi, f = Kaki, g = Pangpuay, h = NNK8

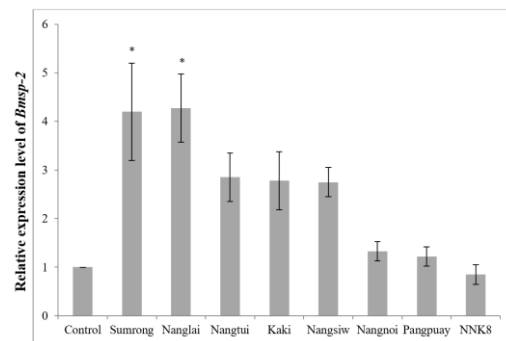


Figure 3. The transcription level of *Bmsp-2* in the larval midgut of infected eight *B. mori* strains. The asterisk (*) indicated significant difference from control (p<0.05)

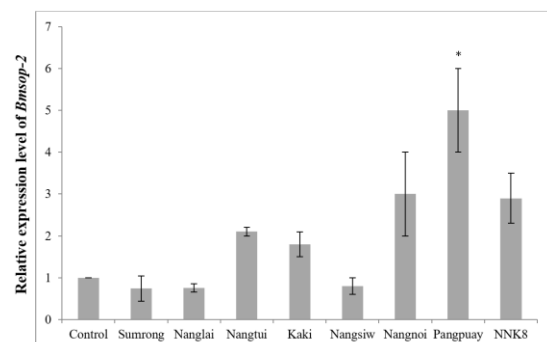


Figure 4. The transcription level of *Bmsop-2* in the larval midgut of infected eight *B. mori* strains. The asterisk (*) indicated significant difference from control (p<0.05)

Bmlipase-1 is to protect silkworm midgut epithelial cells from ODV invasion (Sagisaka *et al.*, 2010). *Bmsp-2* is the midgut specific protease and highly expressed in *BmNPV* resistant silkworms (Qin *et al.*, 2012). Conversely, *Bmsop-2* expressions were up-regulated in infected Pangpuay, Nangnoi and NNK8 stains. In agreement with Xu *et al.* (2005) the expression of *Bmsop-2* was the highest in *BmNPV* treated strain 306 which is highly susceptible strain from China.

Conclusions

In Thailand, breeding silkworm strains is under the responsibility of the Queen In conclusion, *Bmlipase-1* and *Bmsp-2* were up-regulated in the infected in silkworm midgut related to the ability to defend *BmNPV* infection. Our results, suggested that Sumrong and Nanglai silkworm strains seem to have high levels of viral resistance according to the high levels of *Bmlipase-1* and *Bmsp-2* expressions compared to those of the other strains. Meanwhile, Pangpuay may be that of *BmNPV* susceptible strains due to the strongly up-regulated expression of *Bmsop-2*. However, the precise study in resistance level bioassays should be further performed to confirm the correspondence.

Acknowledgement

This work was partially financial supported by Science Achievement Scholarship of Thailand (SAST). We are thankful for silkworm strain and *BmNPV* providing from the development of silk innovation center, Mahasarakham University and Queen Sirikit Sericulture Center (Khon Kaen), respectively.

References

- Au, S., Wu, W., and Pante, N. (2013). Baculovirus nuclear import: open, nuclear pore complex (NPC) sesame. *J. Viruses.*, 5:1,885-1,900.
- Bao, Y.Y., Lv, Z.Y., Liu, Z.B., Xue, J., Xu, Y.P., and Zhang, C.X. (2010). Comparative analysis of *Bombyx mori* nucleopolyhedrovirus responsive genes in fat body and haemocyte of *B. mori* resistant and susceptible strains. *Insect Mo Biol.*, 19:347- 358.
- Cheng, Y., Wang, X.Y., Du, C., Gao, J., and Xu, J.P. (2014). Expression analysis of several antiviral related genes to *BmNPV* in different resistant strains of silkworm, *Bombyx mori*. *J. Insect. Sci.*, 14:1-9.
- Kang, L., Shi, H., Liu, X., Zhang, C., Yao, Q., Wang, Y., Chang, C., Shi, J., Cao, J., Kong, J., and Chen, K. (2011). Arginine kinase is highly expressed in a resistant strain of silkworm (*Bombyx mori*, Lepidoptera): Implication of its role in resistance to *Bombyx mori* nucleopolyhedrovirus. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.*, 158:230-234.
- Kaewwises, M., Attathom, T., Chaeychomsri, S., and Chowpongpan, S. (2004). Detection of nucleopolyhedrovirus of mulberry silkworm, *Bombyx mori* by PCR technique. Proceedings of the 42nd Kasetsart University Annual Conference, 2004, Kasetsart, Thailand, p. 174-182.
- Li, G., Zhou, Q., Qiu, L., Yao, Q., Chen, K., Tang, Q., and Hu, Z. (2017). Serine protease *Bm-SP142* was differentially expressed in resistant and susceptible *Bombyx mori* trains, involving in the defense response to viral infection. *PLoS One.*, 12:1-15.
- Miao, X.X., Xu, S.J., Li, M.H., Li, M.W., Huang, J.H., Dai, F.Y., Marino, S.W., Mills, D.R., Zeng, P.Y., Mita, K., Jia, S.H., Zhang, Y., Liu, W.B., Xiang, H., Guo, Q.H., Xu, A.Y., Kong, X.Y., Lin, H.X., Shi, Y.Z., Lu, G., Zhang, X.L., Huang, W., Yasukochi, Y., Sugasaki, T., Shimada, T., Nagaraju, J., Xiang, Z.H., Wang, S.Y., Goldsmith, M.R., Lu, C., Zhao, G.P., and Huang, Y.P. (2005). Simple sequence repeat-based consensus linkage map of *Bombyx mori*. Proceedings of the National Academy of Sciences; November 8, 2005; Fudan University, Shanghai, China, p.102(45):16,303-16,308.
- Nakazawa, H., Tsuneishi, E., Ponnuvel, K.M., Furukawa, S., Asaoka, A., Tanaka, H., Ishibashi, J., and Yamakawa, M. (2004). Antiviral activity of a serine protease from the digestive juice of *Bombyx mori* larvae against nucleopolyhedrovirus. *J. Virol.*, 321:154-162.
- Ponnuvel, K.M., Nakazawa, H., Furukawa, S., Asaoka, A., Ishibashi, J., Tanaka, H., and Yamakawa, M. (2003). A lipase isolated from the silkworm *Bombyx mori* shows antiviral activity against nucleopolyhedrovirus. *J. Virol. Methods.*, 77:10,725-10,729.
- Ponnuvel, K.M., Nithya, K., Sirigineedi, S., Awasthi, A.K., and Yamakawa, M. (2012). In vitro antiviral activity of an alkaline trypsin from the digestive juice of *Bombyx mori* larvae against nucleopolyhedrovirus. *Arch. Insect Biochem. Physiol.*, 81:90-104.
- Qin, L.G., Xia, H.C., Shi, H.F., Zhou, Y.J., Chen, L., Yao, Q., Liu, X.Y., Feng, F., Yuan, Y., and Chen, K.P. (2012). 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(12):3,630-3,638.
- Selot, R., Kumar, V., Shukla, S., Chandrakuntal, K., Brahmaraju, M., Dandin, S.B., Laloraya, M., and Kumar, P.G. (2007). Identification of a soluble NADPH oxidoreductase (*BmNOX*) with antiviral activities in the gut juice of *Bombyx mori*. *Biosci. Biotechnol. Biochem.*, 71:200-205.
- Saksung, P. and Tawonanukulkit, C. (1997). Analysis and Obstacle to the Development Mulberry Silk. National Center for Genetic Engineering and Biotechnology. Bangkok.
- Xu, I.P., Chen, K.P., Liu, M.H., Yao, Q., Gao, G.T., and Zhao, Y. (2008). Identification and characterization of *Bms3a* in *Bombyx mori* L. *Afr. J. Biotechnol.*, 7:3,424-3,430.
- Xu, J.P., Chen, K.P., Yao, Q., Liu, M.H., Gao, G.T., and Zhao, Y. (2005). Identification and characterization of an NPV infection-related gene *Bmsop2* in *Bombyx mori* L. *J. Appl. Entomol.*, 129:425-431.