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RESEARCH REPORT

# Cloning and subcellular localization of silkworm suppressor of cytokine signaling 6 and its expressional changes in response to the infection of *Bombyx mori nucleopolyhedrovirus*

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## Abstract

The role of suppressor of cytokine signaling (SOCS) 6 in the silkworm growth and development and in the immune response to *Bombyx mori nucleopolyhedrovirus* (BmNPV) infection remains largely unclear. In this study, we cloned the ORF of silkworm SOCS6, named as BmSOCS6. We found that BmSOCS6 locates mainly in cytoplasmic space, and expresses at the highest level in embryogenesis. BmSOCS6 expresses relatively highly in the fat body of BmNPV-susceptible silkworm 306 but lowly in BmNPV-resistant silkworm NB and BC8, and BmNPV inoculation further reduces its expression with more pronounced effect in NB and BC8 than 306. However, BmSOCS6 expression in the midgut and hemolymph decreases without BmNPV inoculation but recovers with BmNPV inoculation, both of which are more pronounced in NB and BC8 than 306. BmNPV inoculation also induces a general down-regulation of BmSOCS6 expression in BmN cells. SOCS6 has previously been shown to positively regulate apoptosis and negatively regulate the production of antimicrobial peptides (AMPs). Therefore, compared to susceptible silkworms, BmSOCS6 may promote apoptosis in the midgut and hemolymph with increased expression and enhance the production of AMPs in the fat body with decreased expression, contributing to the resistance to BmNPV infection in resistant silkworms.

**Key Words**: *Bombyx mori*; Suppressors of cytokine signaling 6; *Bombyx mori nucleopolyhedrovirus*; gene cloning; protein expression

## Introduction

Suppressor of cytokine signaling 6 (SOCS6) is an important member of the SOCS protein family that negatively regulates the signaling of various cytokines (Croker *et al.,* 2008; Yoshimura *et al.,* 2018). The SOCS family consists of at least eight members in vertebrates, including SOCS1-SOCS7 and the cytokine-inducible Src homology 2 (SH2)-containing protein (CIS). They usually contain a variable N-terminal sequence, a conserved SH2 domain and a conserved C-terminal SOCS box. The SOCS box can interact with Elongin B and C, Cullin-5 (or Cullin-2) and Rbx-1 to form an E3 ubiquitin ligase to mediate the ubiquitination and degradation of protein substrates recruited via the SH2 domain. These SOCS proteins regulate the classic Janus kinase-signal transducer and activators of the transcription (JAK-STAT) pathway through either direct inhibition of JAK activity, blockage of STAT binding to activated receptors, or promotion of the degradation of signaling receptors and kinases. These flexible and multiple ways of action of SOCSs enable a timely control of cytokine signaling to avoid hyper-activation of cytokine production and secreation, which may cause cytokine storm to increase morbidity and/or mortality (Yoshimura *et al.,* 2018). The JAK-STAT pathway is critically required for cell proliferation and all SOCS proteins may act as tumor suppressors due to their negative regulation of the pathway, however, SOCS6 has a unique function to promote apoptosis via induction of mitochondrial fission and fragmentation (Lin *et al.,* 2013; Zhang *et al.,* 2022). Consistently, SOCS6 expression is often generally downregulated in many kinds of cancer cells, such as the hepatocellular carcinoma (Zhang *et al.,* 2022), gastric cancer (Li *et al.,* 2021), colorectal cancer (Su *et al.,* 2018), Kaposi's sarcoma (Zhang *et al.,* 2019) and non-small cell lung cancer (Ye *et al.,* 2021).

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**Table 1** Primers used in this study

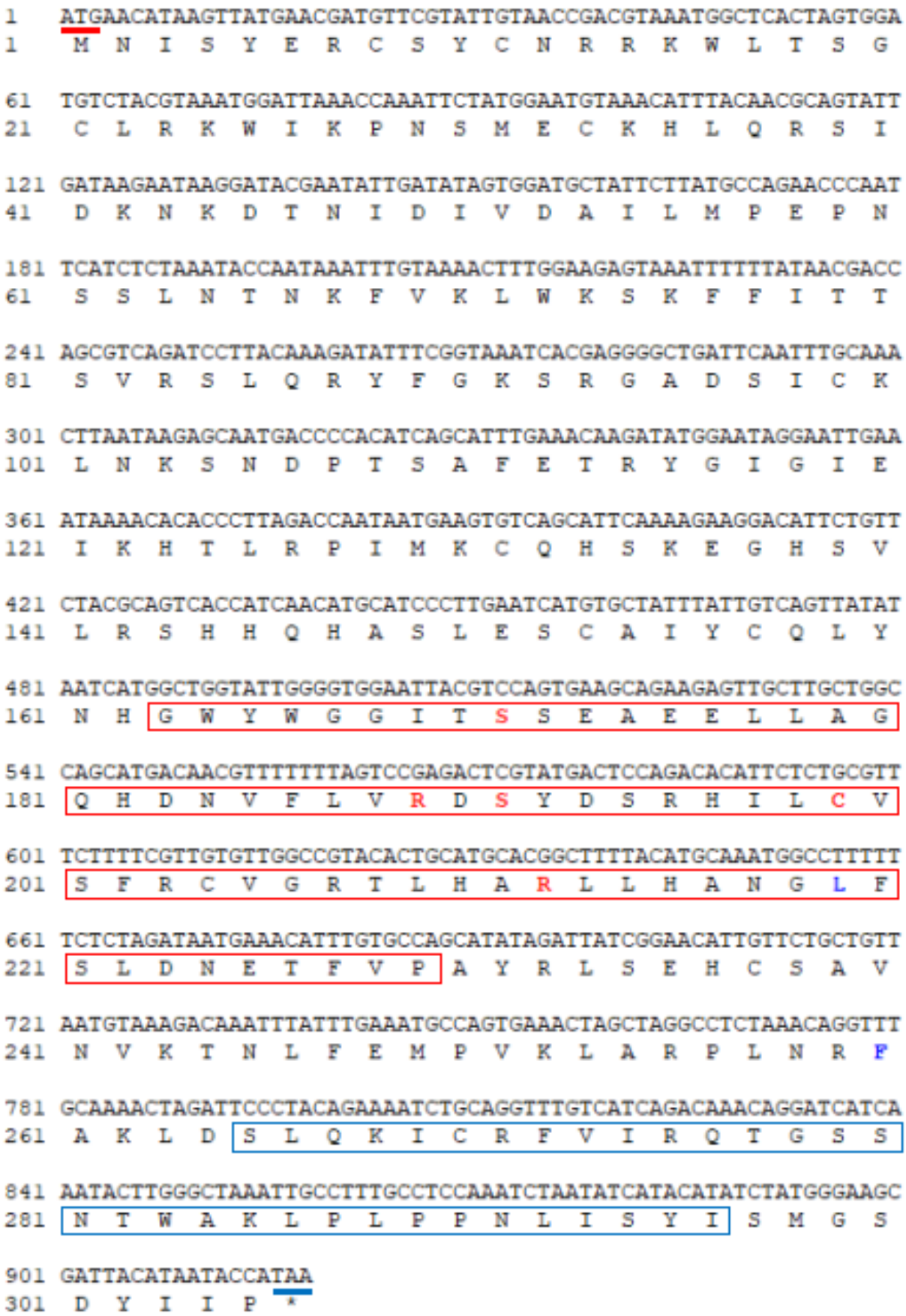
| Primer Name | Sequences (5' →3') |
|---|---|
| BmSOCS6, Forward | ATGAACATAAGTTATGAACGATGTT |
| BmSOCS6, Reverse | TTATGGTATTATGTAATCGCTTCC |
| EGFP-BmSOCS6, Forward | CGCGGATCCATGAACATAAGTTATGAACGATG |
| EGFP-BmSOCS6, Reverse | CCGCTCGAGTTATGGTATTATGTAATCGCTTCCC |
| BmSOCS6, RT-fqPCR, Forward | ACGCAGTCACCATCAACA |
| BmSOCS6, RT-fqPCR, Reverse | GGCCAGCAAGCAACTCTTCT |
| α-tubulin, RT-fqPCR, Forward | CTCCCTCCTCCATACCCT |
| α-tubulin, RT-fqPCR, Reverse | ATCAACTACCAGCCACCC |

Note: The underlined bases indicate sites for restriction endonucleases

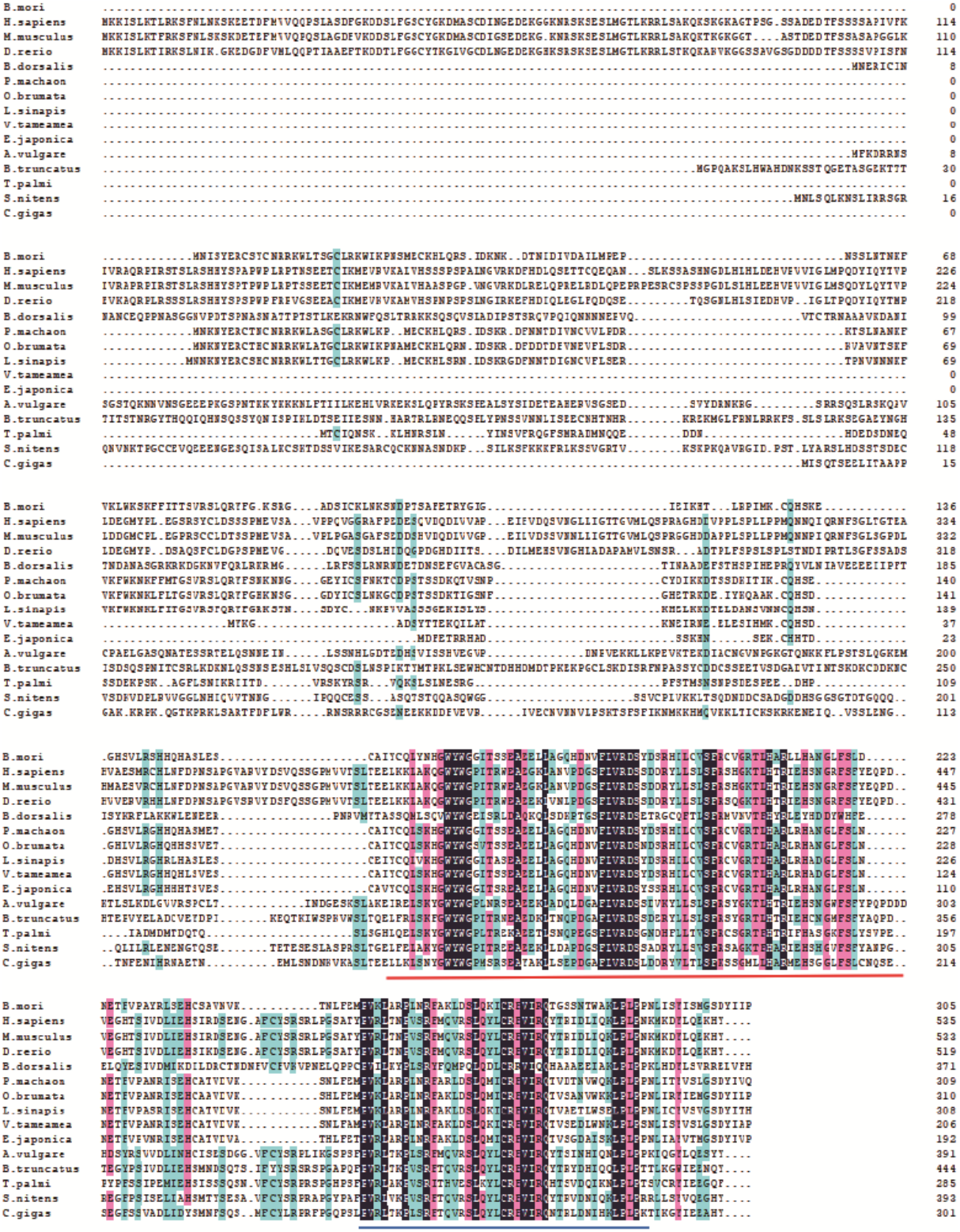
SOCS proteins negatively regulate interferon production and signaling through the JAK-STAT pathway, and are implicated in the infection processes of various viruses and in the host antiviral innate immune responses (Zhang *et al.,* 2023). Beside the SOCS1 and SOCS3, recent studies also begin to reveal the involvement of SOCS6 in these important processes. For example, the infection of transmissible gastroenteritis virus (TGEV) reduces the expression of an antiviral microRNA (miR-27b-3p), which targets SOCS6, in porcine testicular cells and piglets, leading to increased expression of SOCS6 to evade host antiviral immune response (Wang *et al.,* 2022). The infection of infectious bursal disease virus (IBDV) decreases the expression of gga-miR-454, an antiviral miRNA targeting the IBDV genomic segment and SOCS6, to promote IBDV replication which is partly enhanced by the increased expression of SOCS6 (Fu *et al.,* 2018). Interestingly, the down-regulation of SOCS6 expression is also required to promote the replication of certain viruses via mechanisms unrelated to the JAK-STAT pathway, such as the human papillomavirus (HPV) with a requirement of YAP, a transcription factor, for replication (Nieva *et al.,* 2020). YAP is a major component of the Hippo signaling pathway and an important enhancer for HPV replication. SOCS6 acts as the substrate recognition factor of a SCF ubiquitin ligase to target YAP for degradation in proteasome, while the HPV proteins E6 and E7 can promote the stability and activation of YAP through reducing the expression of SOCS6 (Hong *et al.,* 2014).

Compared to other SOCSs, the physiological and pathological functions of SOCS6 remain much less explored, especially in invertebrates including insects. Nonetheless, current research in invertebrates reveal distinctive functions of SOCS6 and its important roles in innate immunity. For example, instead of the inhibitory effects commonly observed in mammalian cells, the *Drosophila* SOCS6 (SOCS44A) and the *Bombyx mori* (*B.mori*) SOCS6 seem to enhance the EGFR signaling pathway via unknown mechanisms (Rawlings *et al.,* 2004; Abbas *et al.,* 2018). SOCS6 expression, alone or together with other SOCSs, can be significantly induced by challenges including bacteria, virus or fungus, such as in the crab *Eriocheir sinensis* (Qu *et al.,* 2018), mealworm beetle *Tenebrio molitor* (Patnaik *et al.,* 2019), and the Chinese oak silkworm (ApSOCS6) (Abbas *et al.,* 2021). ApSOCS6 has been shown to negatively regulate the production of antimicrobial peptides (AMPs) as knockdown of its expression increases the expression of several AMPs in fat body with enhanced bacteria clearance in hemolymph (Abbas *et al.,* 2021). It seems possible that SOCS6 may somehow inhibit invertebrate immunity to favor the infection and/or proliferation of pathogens, indicating it may regulate the susceptibility or resistance of invertebrates to various pathogens. Indeed, SOCS6, together with SOCS2, has been shown to be down-regulated in silkworm BmN cells treated with geldanamycin to inhibit Hsp90 to reduce the replication of *B. mori nucleopolyhedrovirus* (BmNPV) (Shang *et al.,* 2020). Silkworm is an economic insect and a research model for *Lepidoptera* insects. Silkworm is generally susceptible to the infection of many pathogens including especially the BmNPV, a serious silkworm virus with the capability to cause huge damage in sericulture (Hu *et al.,* 2023). During the past decade, our research group has identified a silkworm variety named as NB that is highly resistant to BmNPV infection. We also constructed a near isogenic variety named as BC8 that is resistant to BmNPV infection and is near isogenic with 306, a BmNPV-susceptible variety, through a series of cross of NB with 306 and backcross with the parent 306 (Hu *et al.,* 2023). The silkworm SOCS2 expresses at higher level in the NB than in the 306, and the over-expression of SOCS2 may inhibit the replication of BmNPV, indicating a possible relationship of SOCS proteins with BmNPV infection and silkworm resistance to BmNPV (Yuan *et al.,* 2020). In the present study, we cloned the silkworm SOCS6, examined its subcellular localization, and

A


1 ATGAACATAAGTTATGAACGATGTTCGTATTGTAACCGACGTAAATGGCTCACTAGTGGA
1 M N I S Y E R C S Y C N R R K W L T S G
61 TGTCTACGTAAATGGATTAAACCAAATTCTATGGAATGTAAACATTTACAACGCAGTATT
21 C L R K W I K P N S M E C K H L Q R S I
121 GATAAGAATAAGGATACGAATATTGATATAGTGGATGCTATTCTTATGCCAGAACCCAAT
41 D K N K D T N I D I V D A I L M P E P N
181 TCATCTCTAAATACCAATAAATTTGTAAAACTTTGGAAGAGTAAATTTTTTATAACGACC
61 S S L N T N K F V K L W K S K F F I T T
241 AGCGTCAGATCCTTACAAAGATATTTCGGTAAATCACGAGGGGCTGATTCAATTTGCAAA
81 S V R S L Q R Y F G K S R G A D S I C K
301 CTTAATAAGAGCAATGACCCCACATCAGCATTTGAAACAAGATATGGAATAGGAATTGAA
101 L N K S N D P T S A F E T R Y G I G I E
361 ATAAAACACACCCTTAGACCAATAATGAAGTGTCAGCATTCAAAAGAAGGACATTCTGTT
121 I K H T L R P I M K C Q H S K E G H S V
421 CTACGCAGTCACCATCAACATGCATCCCTTGAATCATGTGCTATTTATTGTCAGTTATAT
141 L R S H H Q H A S L E S C A I Y C Q L Y
481 AATCATGGCTGGTATTGGGGTGGAATTACGTCCAGTGAAGCAGAAGAGTTGCTTGCTGGC
161 N H G W Y W G G I T S S E A E E L L A G
541 CAGCATGACAACGTTTTTTTAGTCCGAGACTCGTATGACTCCAGACACATTCTCTGCGTT
181 Q H D N V F L V R D S Y D S R H I L C V
601 TCTTTTCGTTGTGTTGGCCGTACACTGCATGCACGGCTTTTACATGCAAATGGCCTTTTT
201 S F R C V G R T L H A R L L H A N G L F
661 TCTCTAGATAATGAAACATTTGTGCCAGCATATAGATTATCGGAACATTGTTCTGCTGTT
221 S L D N E T F V P A Y R L S E H C S A V
721 AATGTAAAGACAAATTTATTTGAAATGCCAGTGAAACTAGCTAGGCCTCTAAACAGGTTT
241 N V K T N L F E M P V K L A R P L N R F
781 GCAAAACTAGATTCCCTACAGAAAATCTGCAGGTTTGTCATCAGACAAACAGGATCATCA
261 A K L D S L Q K I C R F V I R Q T G S S
841 AATACTTGGGCTAAATTGCCTTTGCCTCCAAATCTAATATCATACATATCTATGGGAAGC
281 N T W A K L P L P P N L I S Y I S M G S
901 GATTACATAATACCATAA
301 D Y I I P *

B


B.mori
H.sapiens
M.musculus
D.rerio
B.dorsalis
P.machaon
O.brumata
L.sinapis
V.tameamea
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S.nitens
C.gigas

C

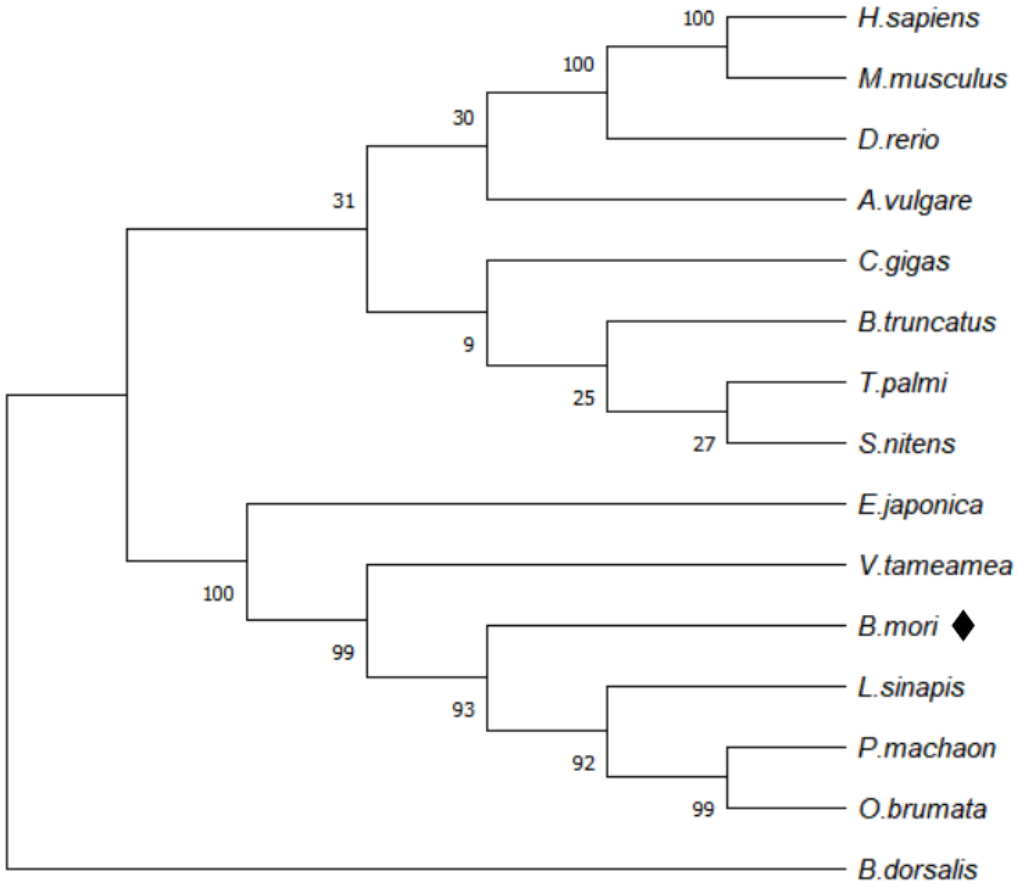


**Fig. 1** Sequence analysis of BmSOCS6. (A) Nucleotide and deduced amino acid sequence of cloned BmSOCS6. Red box, SH2 domain; blue box, SOCS box. The red colored amino acids represent binding sites for phosphorylated tyrosine. The two blue colored amino acids are mutated in this cloned BmSOCS6. The red underline, start codon (ATG); blue underline, stop codon (TAA). (B) Multiple sequence alignment. The red underline, SH2 domain; blue underline, SOCS box. The protein sequences of SOCS6 are from the following species: *Bombyx mori* (*B.mori*, Bm-SOCS6, NP_001185652.1), *Homo sapiens* (*H.sapiens,* Hs-SOCS6, NP_004223.2), *Mus musculus* (*M.musculus,* Mm-SOCS6, NP_061291.2), *Danio rerio* (*D.rerio,* Dr-SOCS6, XP_687041.2), *Bactrocera dorsalis* (*B.dorsalis,* Bd-SOCS6, JAC44579.1), *Papilio machaon* (*P.machaon,* Pm-SOCS6, KPJ14291.1), *Operophtera brumata* (*O.brumata,* Ob-SOCS6, KOB74914.1), *Leptidea sinapis* (*L.sinapis,* Ls-SOCS6, XP_050671799.1), *Vanessa tameamea* (*V.tameamea,* Vt-SOCS6, XP_026486802.1), *Eumeta japonica* (*E.japonica,* Ej-SOCS6, GBP66459.1), *Armadillidium vulgare* (*A.vulgare,* Av-SOCS6, RXG51611.1), *Bulinus truncatus* (*B.truncatus,* Bt-SOCS6, KAH9490972.1); *Thrips palmi* (*T.palmi,* Tp-SOCS6, XP_034249358.1), *Schistocerca nitens* (*S.nitens,* Sn-SOCS6, XP_049809680.1), *Colletes gigas* (*C.gigas,* Cg-SOCS6, XP_043248789.1). (C) Phylogenetic analysis of SOCS6

characterized its expression profiles in different developmental stages and in different tissues and organs. We also performed time-course analysis of its expressional changes in response to BmNPV infection in silkworms and in BmN cells. The greater down-regulation of BmSOCS6 expression in the fat body of NB and BC8 than in 306 induced by BmNPV inoculation may indicate that, the production of AMPs may be more greatly induced in the resistant silkworms to contribute to the BmNPV resistance. Moreover, the up-regulation of BmSOCS6 expression in the midgut or hemolymph of NB and BC8 as compared to 306 may indicate the enhancement of apoptosis to contribute to the BmNPV resistance in these resistant silkworms. Our data reveal the possible relationship of BmSOCS6 with BmNPV infection and the silkworm anti-BmNPV mechanisms, laying a good foundation for detailed investigation of these mechanisms in future studies.

## Materials and Methods

*Insects*

The domesticated silkworm (*Bombyx mori*), including NB, 306 and BC8, was maintained in our laboratory. The silkworm larvae were fed routinely with fresh mulberry leaves at room temperature (26 °C) with a day/night photoperiod of 12h/12h and a relative humidity of 70%.

*RNA extraction, gene cloning and sequence analysis*

Total RNA samples of silkworms were prepared using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was prepared using a reverse transcription kit (Vazyme, Jiangsu, China). PCR primers were designed to amplify the ORF of BmSOCS6 (Table 1), according to the sequence in GenBank (GenBank number: NM_001198723.1). PCR condition was as following: 5 min at 94 °C, followed by 30 cycles of 30s at 94 °C, 30s at 55 °C, 90s at 72 °C, 10 min at 72 °C for the final extension step. 1% agarose gel electrophoresis was used to analyze PCR products, and a PCR purification kit (Vazyme, Jiangsu, China) was used to recover the PCR fragment. The PCR fragment was cloned into pMD-19T, transformed into *E. coli* (DH5α) and the positive clones were verified by PCR and DNA sequencing (Sangon Biotech, Shanghai, China). To analyze the subcellular location of BmSOCS6 using the commonly employed eGFP fusion strategy, the ORF of BmSOCS6 was sub-cloned into the insect expression vector pIB/V5-EGFP to construct the pIB-EGFP-BmSOCS6 for transient expression in BmN cells (Table 1 for primers). The online software (http:‖web.Expasy.org/protparam/) was used to predict the protein sequence of BmSOCS6, as well as its physical-chemical properties. Search for homologous genes of BmSOCS6 was carried out by Blast P (http:‖www.ncbi.nlm.nih.gov). Multiple sequence alignment and phylogenetic analysis were also performed using protein sequences of BmSOCS6 and of other SOCS6 from varied species.

*Subcellular localization analysis of BmSOCS6*

The pIB-EGFP-BmSOCS6 was constructed and transfected into BmN cells to examine the subcellular localization of SOCS6 by fluorescence microscopy. BmN cells were cultured at 27 °C with TC100 medium containing 10% fetal bovine serum (FBS) and antibiotics. The cells were plated into a 24-well culture plate and incubated with freshly formulated medium containing 1.5% FBS. To prepare a transfection mixture of plasmid with cellfectin, 2 μL cellfectin II was mixed with 98 μL of serum-free medium and incubated at room temperature for 45 min to make solution A. Then plasmid (1-5 μg) was mixed with 100 μL of serum-free medium to make solution B, and finally 100 μL solution A was mixed with 100 μL solution B and incubated at room temperature for 45 min to obtain solution C. The culture medium was removed, and the cells were incubated with solution C for 6 hour at 27 °C, then incubated with fresh medium containing 10% FBS and antibiotics for 72 h. The cells were washed three times with PBS, treated with 4% paraformaldehyde for 15 min, washed and treated with 0.1% Triton X-100 for 15 min, washed and treated with DAPI solution for 10 min, and examined using fluorescence microscopy.

*Analysis of BmSOCS6 expression profiles*

The real-time fluorescence quantitative PCR (RT-fqPCR) was performed to determine the relative expression of BmSOCS6 as described previously (Xia *et al.,* 2019). The whole body samples included the oosperm (first day, right after oviposition), the larvae (first day of each instar), prepupa, pupa (first day) and moth (first day), and at least three silkworms were collected as one sample. The silkworms on the fourth day of the fifth instar were randomly selected and dissected to collect tissue and organ samples, including midgut, fat body, hemolymph, silk gland, head, and Malpighian tube.

To analyze the *in vivo* BmNPV infection, silkworms on the first day of the fifth instar were firstly starved and then orally inoculated with ODV of BmNPV (10 μL, $1\times10^9$ polyhedra / mL, in PBS) loaded in a microsyringe. The silkworms were divided into experimental group (with ODV) and control group (without ODV) with eighty silkworms per group. At each time point of 0, 3, 6, 12, 24, 48 hour after viral inoculation, ten silkworms were randomly selected from each group for dissection to collect samples, including midgut, fat body and hemolymph. The samples were washed with cold PBS, frozen in liquid nitrogen, and stored in freezer at -80 °C.

To analyze the *in vitro* BmNPV infection, BmN cells ($5\times10^5$ cells per well) were cultured in 12-well plates at 27 °C, and inoculated with the BV of BmNPV (MOI=5). The time points were set at 0 min, 5 min, 10 min, 30 min, 60 min, 90 min, 3 hour, 6 hour, 12 hour, 24 hour after viral inoculation. At each time point, culture medium was discarded, and cells were washed with sterilized PBS, lysed with 1 mL TRIZOL, transferred to a centrifuge tube for RNA extraction.

PCR primers for BmSOCS6 and the internal control tubulin (NCBI Reference Sequence: XM_021348632.1) were designed using Primer 5.0 software (Table 1). Reaction system comprised of SYBR Green Master Mix (10.0 μL), primer pairs (0.4 μL each), Rox Reference Dye2 (0.4 μL), template cDNA (2.0 μL) ddH2O (6.8 μL). Reaction conditions are as following: 30 s at 95 °C, 39 cycles of 15 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. The melting curve analysis was performed with PCR reaction at 95 °C for 15s, 60 °C for 1 min, and an increase of temperature from 60 °C to 95 °C at a rate of 0.5 °C per 10s. Data were processed using the classic $2^{-\Delta\Delta Ct}$ method. The amplification of tubulin was used as an internal control. Biological triplicate experiments were performed, and the P value was calculated with one way ANOVA (SPSS) to evaluate the significance of difference.

**Results**

*Gene cloning and bioinformatic analysis*

As shown in Fig. 1A, the cloned ORF of BmSOCS6 gene is of 918 bp, encoding a peptide of 305 amino acids. The predicted molecular weight of BmSOCS6 is of 35 kDa and the isoelectric point is of 9.45. Compared to the reported sequence of silkworm (Dazao) SOCS6 (GenBank: ADO51636.1), we found two amino acid mutations in our cloned BmSOCS6 sequence: F219L, S260F (colored in blue in Fig. 1A). The PCR template was the total cDNA prepared from the fat body of NB. We further optimized PCR reactions, tested high-fidelity DNA polymerases from different producers, performed PCR using cDNA samples from midgut, fat body and hemolymph of 306, NB and BC8. However, we always found the occurrence of the two amino acid

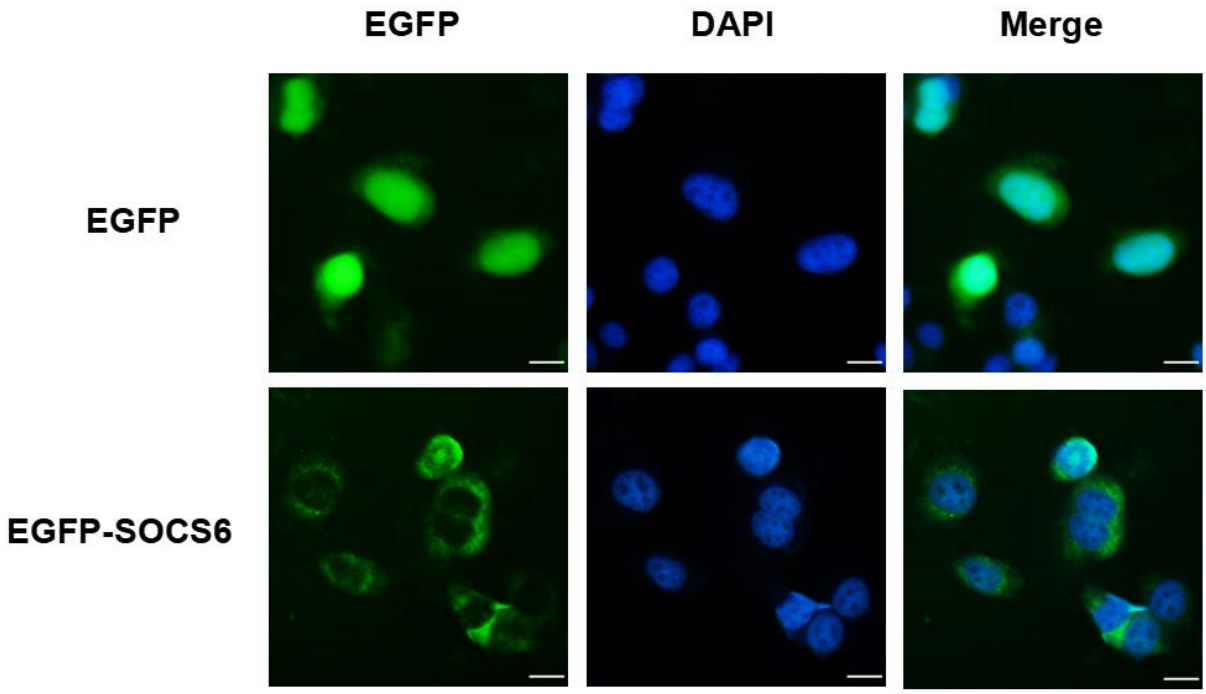


**Fig. 2** Sub-cellular localization of BmSOCS6. The EGFP alone or the EGFP-BmSOCS6 fused protein was transiently expressed in BmN cells as described in Materials and Methods. DAPI staining, blue (nucleus). EGFP or EGFP-BmSOCS6, green (cytoplasm). Scale bar = 100 μm

mutations but with no other mutations. Moreover, the site (F219L) may actually be a leucine as it is a leucine in SOCS6 sequences from all *Lepidoptera* insects included in the sequence alignment. The site (S260F) seems to be more like a phenylalanine rather than a serine as it is a phenylalanine in many other SOCS6 sequences including those from *Lepidoptera* insects. Therefore, we think they are naturally occurring mutations, which may be related to regional difference of the silkworm varieties as the silkworm Dazao is from Japan while the NB and 306 are from China. The F219L mutation is located in the conserved SH2 domain, but does not seem to affect the substrate binding function, and essential residues involved in the binding of substrate protein are red colored in Fig. 1A. The S260F mutation is located in the linker region between SH2 domain and SOCS box, which may also suggest non-essential role of it in the function of SOCS6. Therefore, we think the BmSOCS6 may still function normally in silkworms even with these mutations.

As shown in Fig. 1B, BmSOCS6 sequence has the highest similarity (71%) with the SOCS6 sequence of *Papilio machaon*, followed by the SOCS6 sequence of *Operophtera brumata* (69%), but with much lower similarity to SOCS6 sequences from human and mice. It is obvious that the SOCS6 proteins from human, mice and zebrafish are large in size and all have an additional and quite similar N terminal sequence of about 100 amino acids. As the N terminal region is usually involved in substrate protein recognition, the verterbrate SOCS6 protein may thus have additional functions and regulations as compared to invertebrate SOCS6. Nonetheless, all SOCS6 proteins contain the highly conserved SH2 domain (red underline) and SOCS box (blue underline), indicating their mechanism of action as being an essential component of ubiquitination ligases is still highly conserved. The phylogenetic tree analysis (Fig. 1C) showed that BmSOCS6 is highly conserved and is closely related to SOCS6 proteins from *Lepidoptera* insects.

*Subcellular localization of BmSOCS6*

The subcellular localization is informative about the cellular functions of target proteins. The cellular location of silkworm SOCS6 has not been reported, as far as we know. Thus, in this study we performed transient expression experiment using EGFP fusion strategy, a commonly used method, to examine the subcellular localization of BmSOCS6. The usage of EGFP fusion does not change the subcellular localization of target proteins as has been demonstrated in many studies including ours (Xia *et al.,* 2017), and is thus commonly applied in recent years for various purposes including subcellular localization analysis.

The transient expression construct pIB-EGFP-BmSOCS6 was prepared, verified by DNA sequencing, and transfected into BmN cells. The transfected cells were examined using fluorescence microscopy to investigate the location of the EGFP-BmSOCS6. As shown in Fig. 2, the EGFP expressed alone is distributed widely in the

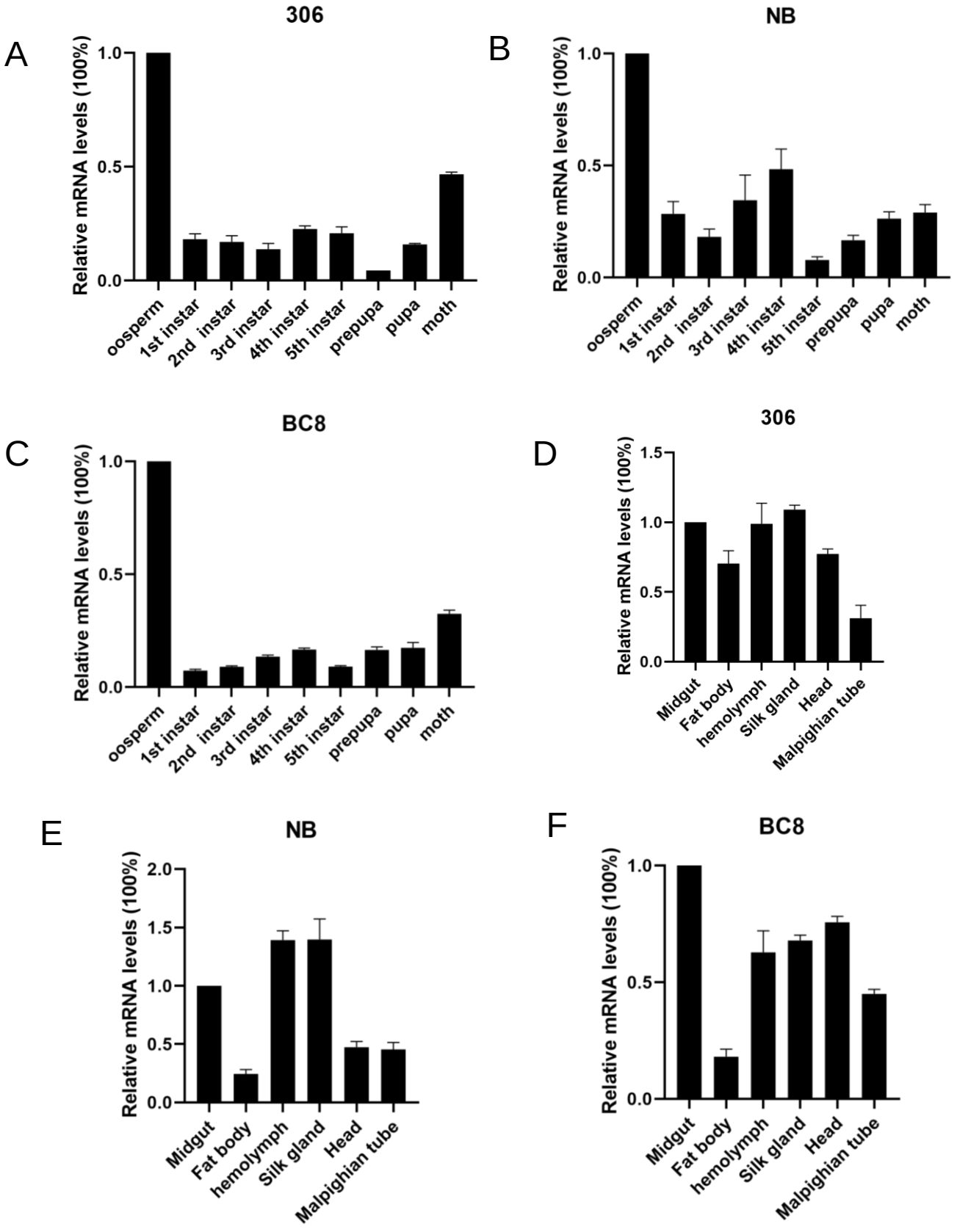


**Fig. 3** BmSOCS6 expression in silkworm. The expression of BmSOCS6 was examined in different developmental stages (A, B, C) and in different tissues and organs (D, E, F). (A) and (D), 306; (B) and (E), NB; (C) and (F), BC8. 306 is susceptible to BmNPV infection, while NB and BC8 are resistant to BmNPV infection. The RT-fqPCR was performed as described in Materials and Methods, and the expression level of BmSOCS6 is normalized against its level in oosperm or in midgut. Values are the mean of three independent measurements (mean±S.E., n = 3)

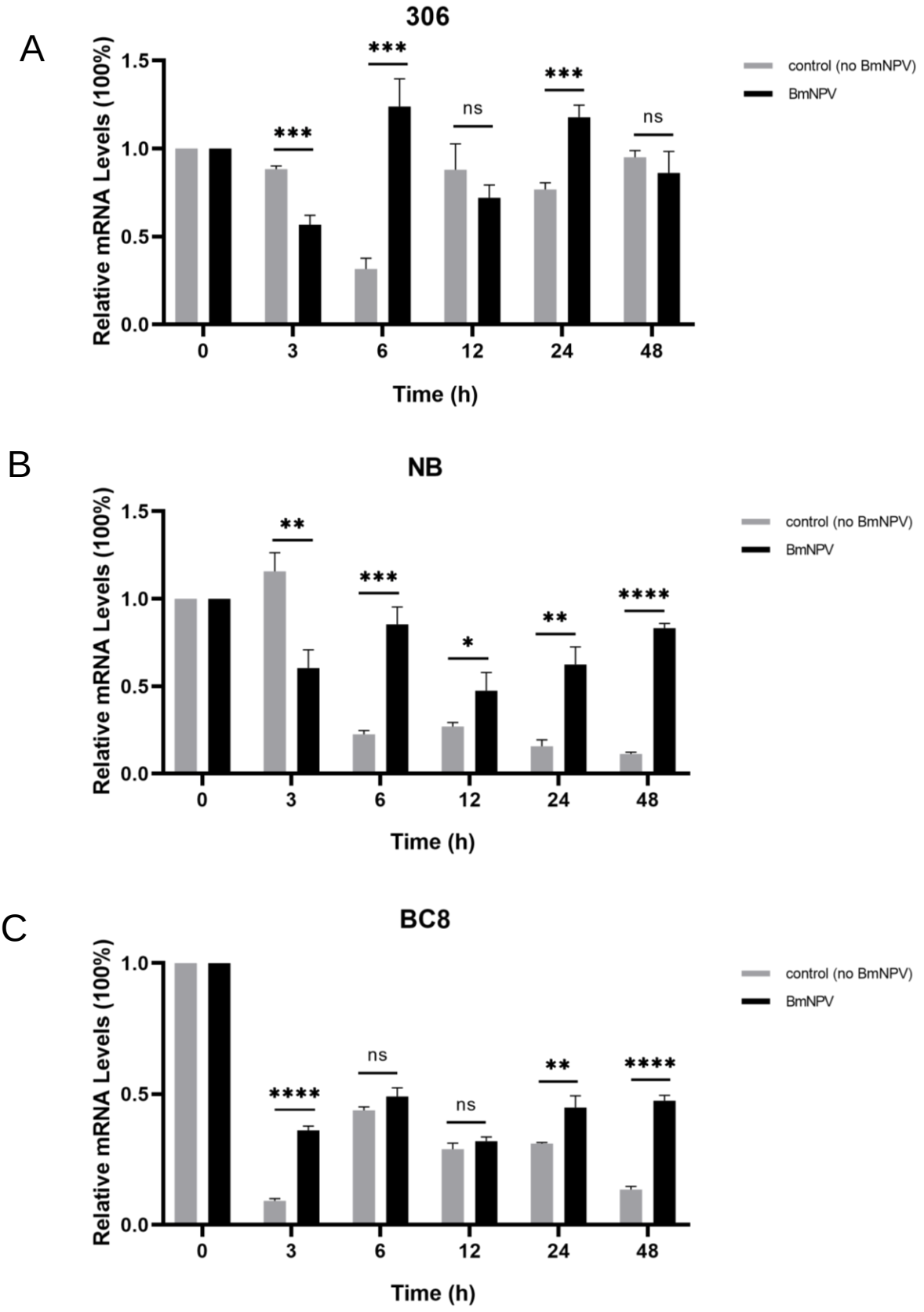


**Fig. 4** Time course analysis of BmSOCS6 expression in silkworm midgut with or without BmNPV inoculation. Silkworms include 306 (A), susceptible to BmNPV infection, and NB (B) and BC8 (C), resistant to BmNPV infection. The RT-fqPCR was performed as described in Materials and Methods, and the expression level of BmSOCS6 is normalized against its level at the time point of 0 hour. Values are the mean of three independent measurements (mean±S.E., n = 3). ****: $P < 0.0001$; ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$

cells with both cytoplasmic and nuclear locations, consistent with the common observation of EGFP behavior. However, the EGFP-BmSOCS6 fusion protein is mainly distributed in cytoplasmic space, indicating it is mainly located in the cytoplasm. Consistently, we did not find the existence of any nuclear localization sequence (NLS) in the BmSOCS6 sequence (http://www.moseslab.csb.utoronto.ca/NLStradamus/).

*The spatial and temporal expression of BmSOCS6*

As shown in Fig. 3 (A, B, C), BmSOCS6 expresses at the highest level in the oosperm of silkworm 306, NB and BC8 as compared to other developmental stages. Indicating it may play important function in the embryonic development. Compared to other stages, it expresses with the second highest level in moth of both 306 and BC8, the lowest level in prepupa of 306 and in the first instar larvae of BC8, the roughly similar levels in other stages in 306 or BC8. However, it is of the second largest level in the fourth instar larvae and the lowest level in the fifth instar larvae of NB. Therefore, it seems that the expression of BmSOCS6 in BC8 may be more affected by the allele gene from 306 in terms of whole body samples.

As shown in Fig. 3 (D, E, F), compared to other tissues and organs, BmSOCS6 expression is of much higher level in the silk gland, hemolymph and midgut of 306 than in other tissues with the lowest level in the Malpighian tube. Consistently, it is of the highest expression in silk gland and hemolymph of NB, and is of the highest level in midgut of BC8. However, it is of the lowest level in the fat body of both NB and BC8, which is opposite to its relatively high expression in the fat body of 306, indicating BmSOCS6 may have opposite regulations and functions in fat body between silkworm varieties resistant or susceptible to BmNPV infection.

*The expression of BmSOCS6 in silkworm midgut, fat body and hemolymph in response to BmNPV infection*

As shown in Fig. 4, the expression of BmSOCS6 in the midgut of 306 in the absence of BmNPV inoculation (control) firstly decreased from 0 hour to 6 hour then recovered to the basal level at the 12 hour, and maintained its level to 48 hour. It was of similar levels in the presence of BmNPV inoculation as compared to the control except for the time point of 6 hour, when its expression significantly increased with BmNPV inoculation. However, its expression greatly decreased in the midgut of NB and also decreased in the midgut of BC8 in the absence of BmNPV inoculation, while BmNPV inoculation increased its expression to reach basal levels in the midgut of NB and to maintain roughly 50% of its basal levels in the midgut of BC8. Together, it seems that BmSOCS6 expression is up-regulated by BmNPV inoculation in the midgut of these silkworms, which is the most prominent in NB, less prominent in BC8 and the least prominent in 306.

As shown in Fig. 5, BmSOCS6 expression remained at basal level for most time points but increased greatly at 48 h in the fat body of 306 without BmNPV inoculation, while its expression decreased from 6 h to 48 h with the BmNPV inoculation, indicating the infection of BmNPV induces the down-regulation of BmSOCS6 in the fat body of 306. However, its expression in the fat body of NB was of roughly basal levels with a weakly but clearly decreasing trend in the time course without BmNPV inoculation, but greatly and continuously decreased with BmNPV inoculation. In addition, its expression in the fat body of BC8 was also of basal levels except for a significant decrease at 48 h without BmNPV inoculation, while its expression also displayed a continuously decreasing trend from 3 h to 24 h with BmNPV inoculation. Together, it seems that BmSOCS6 expression is down-regulated in the fat body of these silkworms by BmnPV inoculation, which is the most prominent in NB, less prominent in BC8 and the least prominent in 306.

As shown in Fig. 6, the expression of BmSOCS6 in the hemolymph of 306 firstly increased then decreased slightly without BmNPV inoculation, while its expression firstly increased but then continuously and more prominently decreased with BmNPV inoculation except for a sharp increase at 48 h. By contrast, BmSOCS6 expression in the hemolymph of NB greatly decreased without BmNPV inoculation, however, in the presence of BmNPV inoculation, it greatly increased at 3 h and then slowly decreased to the level similar to the time point of 0 h. In addition, BmSOCS6 expression in the hemolymph of BC8 also decreased without BmNPV inoculation but with weaker effect as compared to NB except for a sharp increase at 24 h, while its expression decreased in a more consistent trend with BmNPV inoculation. Together, it seems that BmNPV inoculation induces up-regulation of BmSOCS6 expression in the hemolymph of resistant silkworms with more prominent effect in NB than in BC8, but induces down-regulation of BmSOCS6 in the hemolymph of susceptible silkworm 306.

*The expression of BmSOCS6 in silkworm BmN cells in response to BmNPV infection*

As shown in Fig. 7, the basal expression of BmSOCS6 in BmN cells remains stable. However, it decreased significantly 30 min after BmNPV infection, recovered to the basal level at 1 h, then decreased significantly from 90 min to 24 h with a weakly increasing trend from 6 h to 24 h. Therefore, it seems that the BmNPV infection induces a general down-regulation of BmSOCS6 in BmN cells.

**Discussion**

In present study we successfully cloned a SOCS6 ORF from a Chinese silkworm variety, which encodes a protein named as BmSOCS6. Its protein sequence contains two mutations (F219L and S260F) as compared to the reported sequence of SOCS6 identified from the Japanese silkworm Dazao. Multiple sequence alignment analysis suggested that the No. 219 seems more like a leucine and the No. 260 seems more like a phenylalanine, and the same amino acids also exist in the sequence of ApSOCS6 identified from the Chines oak silkworm (Abbas *et al.,* 2021). Therefore,

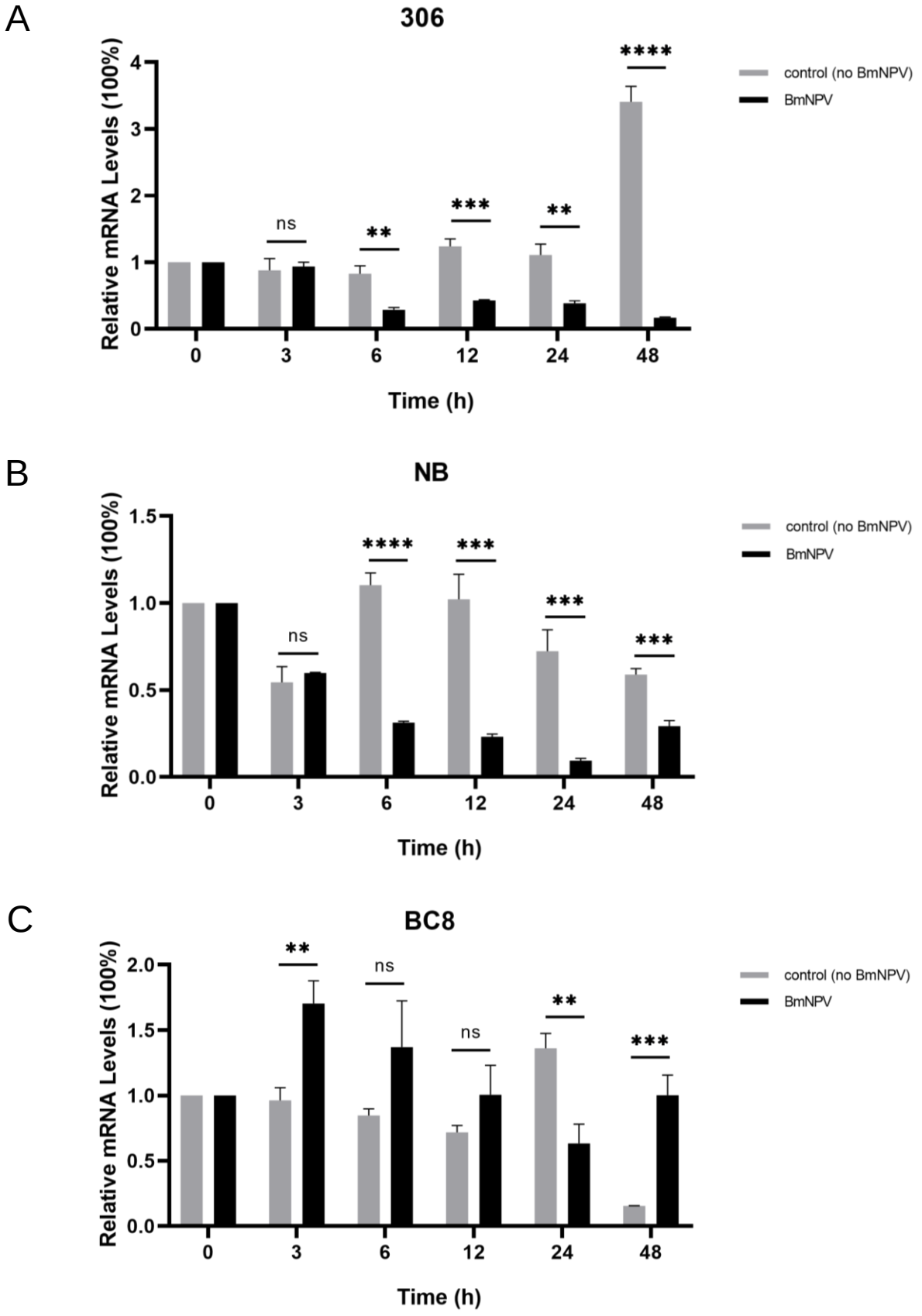


**Fig. 5** Time course analysis of BmSOCS6 expression in silkworm fat body with or without BmNPV inoculation. Silkworms include 306 (A), susceptible to BmNPV infection, and NB (B) and BC8 (C), resistant to BmNPV infection. The RT-fqPCR was performed as described in Materials and Methods, and the expression level of BmSOCS6 is normalized against its level at the time point of 0 hour. Values are the mean of three independent measurements (mean±S.E., n = 3). ****: $P < 0.0001$; ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$

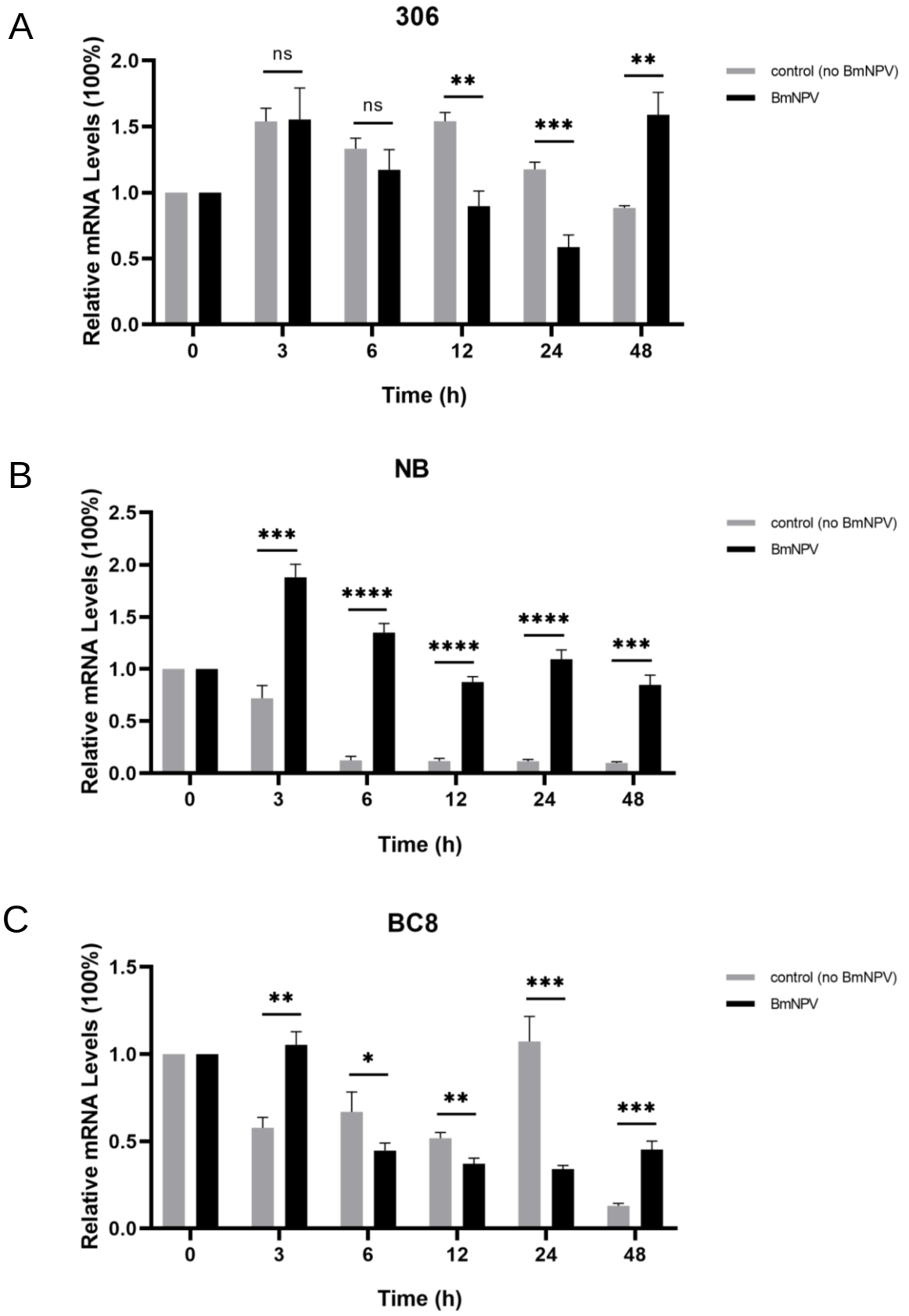


**Fig. 6** Time course analysis of BmSOCS6 expression in silkworm hemolymph with or without BmNPV inoculation. Silkworms include 306 (A), susceptible to BmNPV infection, and NB (B) and BC8 (C), resistant to BmNPV infection. The RT-fqPCR was performed as described in Materials and Methods, and the expression level of BmSOCS6 is normalized against its level at the time point of 0 hour. Values are the mean of three independent measurements (mean±S.E., n = 3). ****: $P < 0.0001$; ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$

it seems that these mutations may be naturally occurring and may be related to regional difference of silkworm resources. Nonetheless, as these mutations are not located in the essential sites in SH2 domain or SOCS box, it is reasonable to expect a similar function of these SOCS6 varieties in silkworms.

We found for the first time that BmSOCS6 is mainly located in the cytoplasmic space. By contrast, mammalian SOCS6 is located in both the cytoplasm and nucleus because it contains an N terminal NLS (Hwang *et al.,* 2007). We performed a through NLS search and did not find any NLS existing in the protein sequences of BmSOCS6 and other SOCS6 from many *Lepidoptera* insects, such as *Papilio Machaon*, and *Leptopilina heterotoma*, a *Hymenoptera* insect. However, NLS does exist in SOCS6 sequences from *Bactrocera dorsalis*, a *Diptera* insect, and *Schistocerca serialis cubense*, an *Orthoptera* insect. Therefore, not does each of the insect SOCS6 proteins contain NLS and locate in nucleus. As the N terminal sequence of BmSOCS6 is much shorter than that of mammalian SOCS6 (Fig. 1B), it is reasonable to expect the existence of different protein-protein interaction and consequently different function and regulation of BmSOCS6 as compared to mammalian SOCS6. We also found that BmSOCS6 expresses with the highest level in the embryogenesis during silkworm development. Consistently, the mealworm beetle TmSOCS6, identified from *Tenebrio molitor*, expresses at amazingly high level in the egg sample as compared to larva, pupa or adult samples (Patnaik *et al.,* 2019). The fruit fly SOCS6 (SOCS44A) also expresses in the late stage of embryogenesis (Rawlings *et al.,* 2004). These findings all suggest that SOCS6 may play important roles in the insect embryogenesis.

Recent studies begin to reveal the close relationship of SOCS6 with invertebrate innate immunity. The fat body and hemolymph represent two major immunocompetent organs. The fat body is mainly responsible for humoral immunity via production and secretion of AMPs, and the hemolymph is mainly responsible for cellular immunity via phagocytosis (Eleftherianos *et al.,* 2021; Vaibhvi *et al.,* 2022). Like mammalian liver and adipose tissue, insect fat body plays essential roles in nutrient storage and energy metabolism, detoxification, hormone and nutritional signaling, and the innate immune responses (Li *et al.,* 2019). Besides adipocytes, the cell clusters derived from the fat body of silkworm larvae include hemocyte, epithelial cell, muscle cell, and glial cell, and BmNPV infection can induce a strong antiviral response in the adipocytes and hemocyte-like cells (Feng *et al.,* 2023). AMPs expression increases in response to the infection of a NPV and can suppress the NPV replication in the Asian gypsy moth (Liu and Wang, 2023), confirming the important roles of AMPs in antiviral immune response. Silkworm hemocytes mainly include granulocytes, spherulocytes, prohemocytes, plasmatocytes and oenocytoids, and all of them except the spherulocytes have the activity of phagocytosis to destroy various pathogens (Ling *et al.,* 2006; Nakahara *et al.,* 2009; Tan *et al.,* 2013). Interestingly, the macrophage infectivity potentiator (MIP) protein, a virulence factor of *Legionella pneumophila*, can reduce the phagocytosis but increase the chemotaxis of macrophages via up-regulation of SOCS6 expression (Shen *et al.,* 2022), indicating the important role of SOCS6 in regulation of phagocytosis and/or chemotaxis.

The fruit fly contains a SOCS6 homologue, SOCS44A, but its relative expression in different tissues and organs during larva stages are not examined (Rawlings *et al.,* 2004). The mealworm beetle SOCS6, TmSOCS6, expresses at much higher level in the hemocytes than in the fat body of larvae, which, however, becomes only slightly higher in adults, and its expression increases in the fat body, gut and hemocytes of larvae following challenges with bacteria and fungus (Patnaik *et al.,* 2019). Besides insects, there is only one report on invertebrate SOCS6, which is EsSOCS6 identified from the Chinese mitten crab *Eriocheir sinensis* (Qu *et al.,* 2018). EsSOCS6 expresses highly in the hemocyte and hepatopancreas, which is corresponding to insect fat body. Its expression also increases in the hemocytes when treated with lipopolysaccharide (LPS), a bacteria component and a pathogen-associated molecular pattern (PAMP), *Aeromonas hydrophila* or polyinosinic-polycytidylic acid (poly (I:C), a synthetic dsRNA mimicking viral infection and a potent inducer of innate immunity.

In silkworms, SOCS6 from the silkworm Dazao (Abbas *et al.,* 2018) and ApSOCS6 from the Chinese oak silkworm (Abbas *et al.,* 2021), both of which are susceptible to NPV infection, expresses at high levels in both hemolymph and fat body, and increases further after challenges with bacteria, NPV, or fungus. Notably, knockdown of the expression of ApSOCS6 can increase the expression of AMPs in fat body and enhance the bacteria clearance in hemolymph, indicating it may inhibit the production of AMPs in the Chinese oak silkworm (Abbas *et al.,* 2021). In the present study, we found that, compared to other tissues and organs, the expression of BmSOCS6 in fat body is relatively higher in 306 but is very lower in NB and BC8. It is thus possible the production of AMPs may be at higher level in NB and BC8 than in 306. Moreover, in the presence of BmNPV inoculation, BmSOCS6 expression in the fat body decreases most prominently in NB, less prominently in BC8 and the least in 306, which may indicate the highest improvement of AMP production in NB, the less effect in BC8 and the least in 306. Although both NB and BC8 are resistant to BmNPV infection, our recent experiments suggest that NB seems to be more resistant than BC8 when tested with the highest dose of viruses (unpublished data). It is also conceivable as BC8 is derived from the crosses of NB and 306, and the genes from 306 may inevitably affect the immune responses of BC8. In addition, the 306 retains very low level of resistance with a LD50 (about $5\times10^4$ polyhedra / mL) being at least 10,000 fold lower than those resistant silkworms. Thus, it may still be able to produce AMPs but with very low levels. Therefore, our data plus others may indicate that the regulation of AMP production by BmSOCS6 may contribute to the silkworm resistance to BmNPV infection, which is worth further studies.

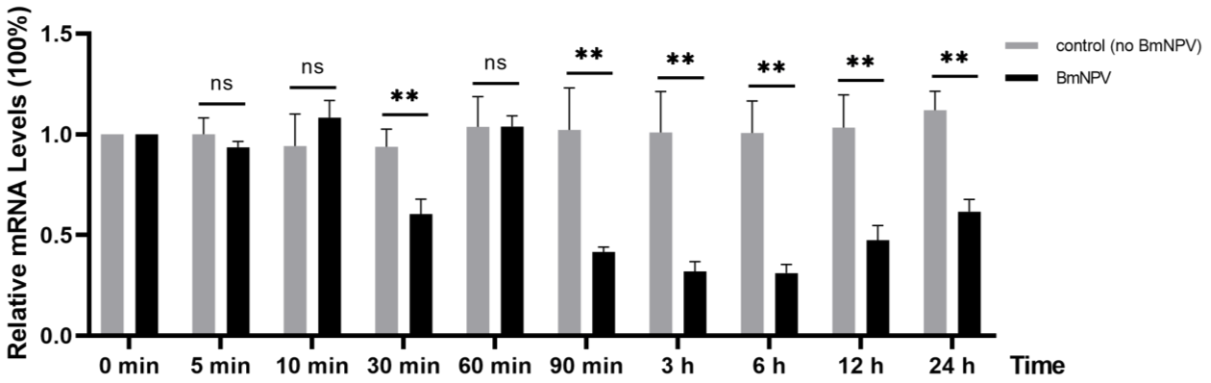


**Fig. 7** Time course analysis of BmSOCS6 expression in silkworm BmN cells with or without BmNPV infection. The RT-fqPCR was performed as described in Materials and Methods, and the expression level of BmSOCS6 is normalized against its level at the time point of 0 min. Values are the mean of three independent measurements (mean±S.E., n = 3). **: P < 0.01; *: P < 0.05

Apoptosis is a well-known host antiviral strategy, while BmNPV encodes inhibitor of apoptosis (IAP) to block apoptosis induced by viral infection (Chen *et al.,* 2020). The polyhedra of BmNPV is orally taken by silkworms and then is dissolved in the alkaline solution of the midgut to release the ODV to initiate infection. Thus the midgut becomes the first frontline for BmNPV infection to take place and for host to fight against BmNPV. Recent studies have revealed that component proteins of apoptosis pathway, such as caspase 1 (Qin *et al.,* 2012; Wang, Zhao *et al.,* 2021), and regulators of apoptosis, such as Bmapaf-1 (Wang *et al.,* 2020) and BmbetaGRP4 (Wang *et al.,* 2021), are either up-regulated or down-regulated in the midgut of resistant silkworms to collectively promote apoptosis to block BmNPV replication and transmission. Interestingly, SOCS6 can promote apoptosis via enhancement of mitochondrial fission and fragmentation (Lin *et al.,* 2013; Zhang *et al.,* 2022), which may confer antiviral function in silkworm midgut. In the present study, BmSOCS6 expression in the midgut and hemolymph decreases without BmNPV inoculation but recovers with BmNPV inoculation, both of which are most prominent in NB, less prominent in BC8 and the least in 306. The molecular mechanism underlying this unexpected finding seems unrelated to the effect of SOCS6 on the phagocytosis and/or chemotaxis as introduced before (Shen *et al.,* 2022), but may be related to the regulation of apoptosis by SOCS6. On the one hand, both the midgut cells and hemocytes undergo intensive proliferation in the fifth instar larvae, which may be facilitated by intrinsic inhibition of apoptosis. Consistently, compared to other tissues and organs, BmSOCS6 expresses at relatively higher levels in midgut and hemolymph of all these silkworms. On the other hand, the recovery of apoptosis may help silkworm fight the battle with BmNPV invasion and transmission, such as in NB and BC8. The up-regulation of BmSOCS6 expression in the hemolymph of NB is very prominent, yet is much less and fairly stable in BC8. However, its expresssion decreases at most of time points in the hemolymph of 306. In addition, BmNPV infection also induces a general down-regulation of BmSOCS6 expression in BmN cells, which are also susceptible to BmNPV infection and are commonly used for amplication of BmNPV. Thus, it seems possible that, in the presence of BmNPV inoculation, the up-regulation of BmSOCS6 expression may promote apoptosis in the midgut and hemolymph of NB and BC8 to help resist BmNPV infection, while the down-regulation of it may inhibit apoptosis in the midgut and hemolymph of 306 and in BmN cells to promote BmNPV replication. Therefore, the regulation of apoptosis by BmSOCS6 may also contribute to silkworm resistance to BmNPV infection, which is worth further studies.

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