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# Nucleopolyhedrovirus Induces Suppressor of Cytokine Signaling in the Beet Armyworm, *Spodoptera exigua*

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**Suppressor of cytokine signaling (SOCS) is known to play a key role as a negative feedback regulator in JAK/STAT signaling cascade in innate immunity. Our laboratory has recently been interested in elucidating the interactions between *Spodoptera exigua* (Se) and SeNPV. This context leads us to clone and characterize *SeSOCS* that may have important functions in response to SeNPV infection. Using the RT-PCR and TA cloning approach, we found a partial fragment (416 bp) of *SeSOCS*. Blast search and multiple alignment data showed that it has a homology to various insects such as *Anopheles gambiae* (78%), *Aedes aegypti* (75%), *Drosophila melanogastar* (77%), *Mus musculus* (69%), and *Homo sapiens* (69%). Temporal induction patterns of *SeSOCS* were analysed after being immune-challenged with either NPV or laminarin. It showed that the level of *SeSOCS* mRNA was strongly induced in a biphasic manner in response to SeNPV and laminarin, respectively. It seems that SOCS, a negative regulator of JAK/STAT signaling system is also present in *S. exigua* and may play a role in innate immunity albeit its precise role should be further elucidated at the molecular and cellular level in the early phase of SeNPV infection in larvae.**

**Key words**: *Spodoptera exigua*, Nucleopolyhedrovirus (NPV), SOCS

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## Introduction

Insect innate immunity has been extensively investigated using major experimental models such as *Drosophila melanogaster*, *Anopheles gambiae*, and *Manduca sexta*. Experimental evidences derived from these models indicate that when microorganisms invade an insect host, several pattern-recognition receptors in the innate immune system recognize unique patterns such as lipopolysaccharides (LPS), lipoteichoic acid (LTA) and beta-1, 3 glucan on the surface of microorganisms. In the Drosophila model, the Toll pathway (activated by fungi and gram-positive bacteria) and the Imd pathway (activated by gram-negative bacteria) lead to the synthesis of antimicrobial peptides. According to Wang and his colleagues (Wang *et al.*, 2006), an RNA interference pathway protects adult flies from infection by viruses. In addition, Dr. Dostert argues that the JAK/STAT signaling pathway is required but not sufficient for the antiviral response of drosophila (Dostert *et al.*, 2005). In a human malaria vector model, special attention has been putting towards studying mosquito innate immunity, focusing on the interactions between *Plasmodium berghei* and midgut/salivary

gland cells (Vlachou and Kafatos, 2005; Barillas-Mury *et al.*, 1999, 2000; Han *et al.*, 2000; Han and Barillas-Mury, 2002). Finally, the tobacco hornworm, *M. sexta* has been extensively used as a biochemical research model to study diverse cellular and humoral immune components present in hemolymph (Kanost *et al.*, 2004).

Although much progress has been made in the analysis of humoral and cellular immune responses against bacteria, fungi, and macroparasites using the key models, at present our knowledge of immune responses in agriculturally important insects such as *S. exigua* is still quite limited. The beet armyworm, *S. exigua* has been a serious agricultural pest and the larvae feed on many vegetable and important field crops. The *S. exigua* larvae are susceptible to SeNPV which has long been considered as a promising alternative candidate to chemicals for controlling agricultural insect pests. Although there is considerable data regarding the potential usage of SeNPV as a biopesticide candidate, little is known about the innate signaling mechanisms within the SeNPV-infected midgut cells, and their role in host-cell antiviral defense. Research paper works indicate that there was one report on the presence of STAT-like DNA-Binding Activity in *S. frugiperda* cells (Sliva and Haldosén, 1996). In addition, SOCS has been known to play a pivotal role innate immune response (Alexander and Hilton, 2004). Accordingly, we have come to be interested in the immune responses of *S. exigua* larvae in response to SeNPV, and specifically in JAK/STAT and SOCS signaling system in the context of the interactions between the virus and the midgut. Therefore, in this report, we seek to test the hypothesis that the activation of JAK/STAT signaling in *S. exigua* is critical for antiviral defense and that SOCS would be up-regulated after being immune-challenged with SeNPV and laminarin.

## Materials and Methods

### Insects

*Spodoptera exigua* larvae were obtained from the Department of Environment-friendly, Jonnam Agricultural Research Institute, and the colonies were maintained on cabbage in 31-ml (one ounce) plastic cups with paper lids in our insectary at 25°C and 60% RH with a photoperidod (16 light : 8 dark).

### Cloning of *SeSOCS*

The *SeSOCS* gene was isolated using PCR method with degenerate primers based on two protein regions well conserved in *Drosophila* SOCS. cDNA from the larvae of *S. exigua* immune-challenged with *E. coli* was used as a template. SOCS box (540 bp) obtained from PCR reaction (94°C 3 min, 94°C 40 sec, 50°C 40 sec, 72°C 40 sec, 72°C 10 min) was cloned into a TA cloning vector and the sequence was confirmed using BLAST search.

### Subcloning cDNA of *SeSOCS* box motif in pRSET expression vector

The subcloning of the *SeSOCS* box is outlined in Fig. 4 A. cDNA encoding *SeSOCS* box was excised from the TA cloning vector with *KpnI* and *EcoRI*, and ligated to the same restriction enzyme sites of the pRSET-A expression vector (Invitrogen). Ten colonies were randomly selected and grown in Luria-Bertani (LB) medium. Colony PCR-based positive colonies were identified, and sequence analysis indicates that the *SeSOCS* insert in pRSET vector was in-framed.

### Expression and induction of recombinant of *SeSOCS*

Following transformation, the expression of the recombinant *SeSOCS* was monitored. Expression was induced with 1 mM IPTG, resulting in the synthesis of an approximately 20 kDa protein as evidenced by 16.5% tricine SDS-PAGE of cell pellet lysates.

### *In vivo* SeNPV infection experiments

For SeNPV-infected samples, two hundred microliters of semi-purified original $10^8$ PIBs/ml were spread on the surface of the chinese cabbage leaves, and the third instar *S. exigua* larvae were fed on it and incubated at 25°C. Infected larvae were collected into 1.5 ml of eppendorf tube containing RNAlater solution after 4 hrs, 8 hrs and 12 hrs, respectively. On the other hand, healthy larvae were collected as SeNPV-uninfected control group.

### Laminarin injection experiments

1 μg of laminarin per *S. exigua* 3rd larva were injected into the integument using a microcapillary needle. RNA from the following times was examined: 0, 4 hrs, 8 hrs, 12 hrs and 24 hrs post injection.

### RNA isolation, cDNA synthesis and RT-PCR of *SeSOCS*

Total RNA was isolated from the 3rd instar larvae of *S. exigua* using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was also isolated from the samples stored at −70°C, and first-strand cDNA was synthesized from 2 μg of total RNA using *Accu-Power* RT PreMix (Bioneer, Korea) and the oligo $(dT)_{12-18}$ primer as described in the manufacturer's protocol. The actin gene sequence was used as an internal loading control. PCR cycle numbers were constant for a particular sequence in the multiple samples analyzed in a given experiment and chosen empirically to attain comparable

band intensities for the different markers in each experiment while avoiding saturation. The primers used were as follows: *actin* forward, 5'-GGCGATCATCATCTACGT-3' *actin* reverse, 5'-GTAGCTGCTGCAAACTTCGG-3', *S. exigua* SOCS foward, 5'-CGACAATAAGCCCGAAGGTA-3' *S. exigua* SOCS reverse, 5'-GTAGCTAGTGTGGGAGACGATGAC-3'. IAP2 forward, 5'-ATCTCGCCGG GGGTATCT-3'; IAP2 reverse, 5'-ATTAGGAGCGGAAG GTGCGTTGAA-3'.

## Results and Discussion

### Cloning and phylogenetic analysis of *SeSOCS* motif

PCR-based cloning approach was used to clone a highly conserved SOCS box domain using degenerate primer, and a 416 bp partial fragment of *SeSOCS* was cloned into TA vector. Sequence analysis indicates that it has high identies (69~77%) to SOCS box motif from various organisms such as *A. gambiae, Ae. aegypti, D. melanogaster*, *Mus musculus*, and *Homo sapiens* (Fig. 1A). This strongly indicates that SOCS box motif is highly conserved ranging from insects to human. In addition, a phylogenetic tree using CLUSTAL X (Thompson *et al.*, 1997) showed similar data (Fig. 1B). Finally, the matrix of amino acid identity percentage in the aligned central region between SOCS showed that *SeSOCS* box has highest homology to *A. gambiae* SOCS (Fig. 1C). However, to fully characterize the sequence of *SeSOCS*, the *SeSOCS* motif will be used to screen cDNA library of *S. exigua* larvae immune-challenged with SeNPV. Thus, we will get an idea how many different types of SOCS family are present in *S. exigua* in the near future.

### Biphasic expression of *SeSOCS* in response to both SeNPV and laminarin

To investigate the induction patterns of *SeSOCS* in the early phase after SeNPV infection, short time course experiments were conducted (Fig. 2). During the first twelve-hour period, the level of *SeSOCS* mRNA fluctuated much more than expected. Generally, the level of *SeSOCS* transcripts displays a biphasic expression pattern

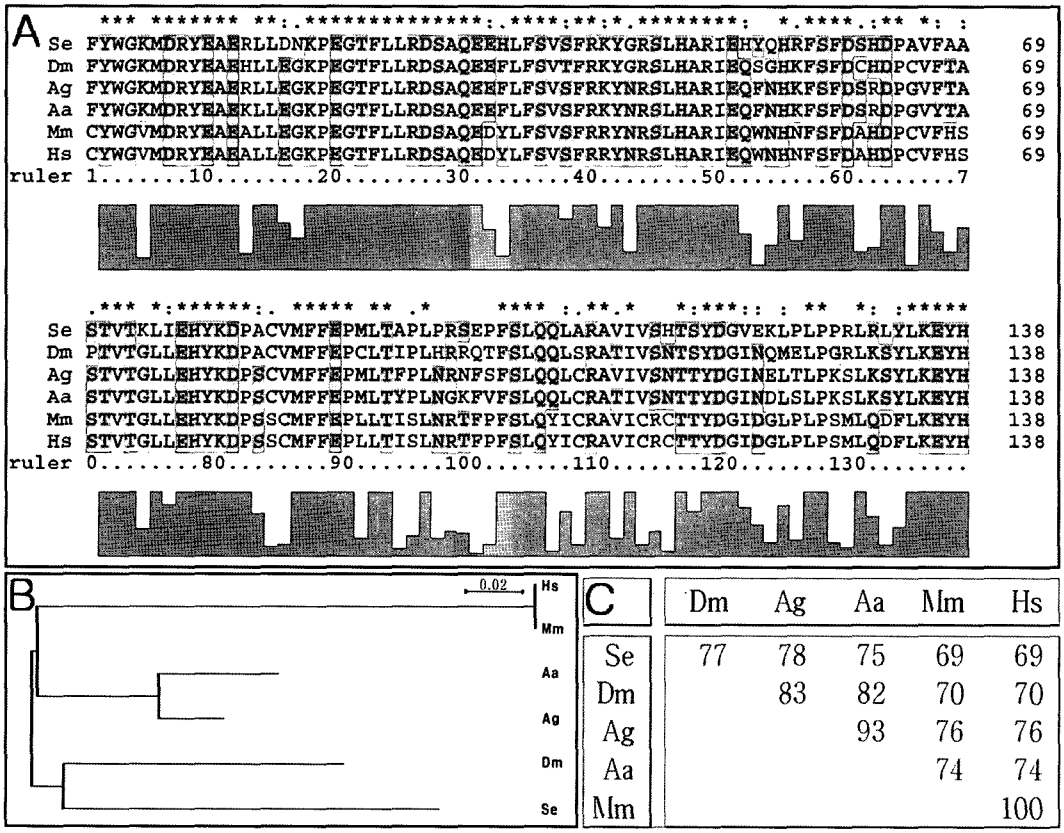


**Fig. 1.** Relationships of *SeSOCS* (se) to other SOCS. (A) Aligned sequences of the conserved domains of *SeSOCS* and five other SOCS. (B) Dendrogram based on the alignment of the SOCS sequence, constructed using CLUSTAL X (Thompson *et al.*, 1997). (C) Matrix of amino acid identity percentage in the aligned central region between SOCS. *Drosophila melanogastar* (Dm), *Anopheles gambiae* (Ag), *Mus musculus* (Mm), and *Homo sapiens* (Hs).

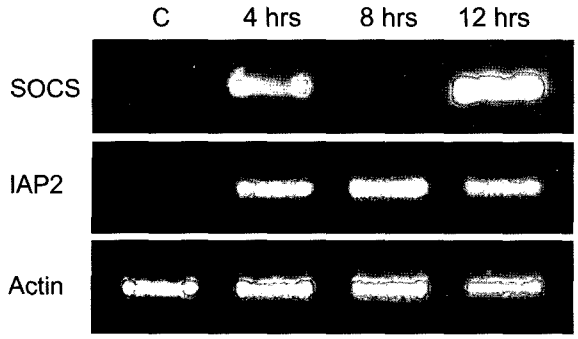


**Fig. 2.** Biphasic expression patterns of *SeSOCS* mRNA after SeNPV infection. SeNPV-inhibitor of apoptosis2 (IAP2) was used as a positive control of SeNPV infection. The internal loading control was verified by actin.

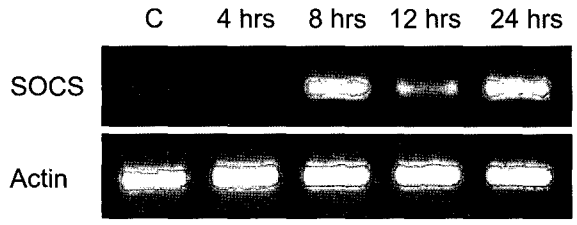


**Fig. 3.** Induction patterns of *SeSOCS* after laminarin injection. The internal loading control was verified by actin.

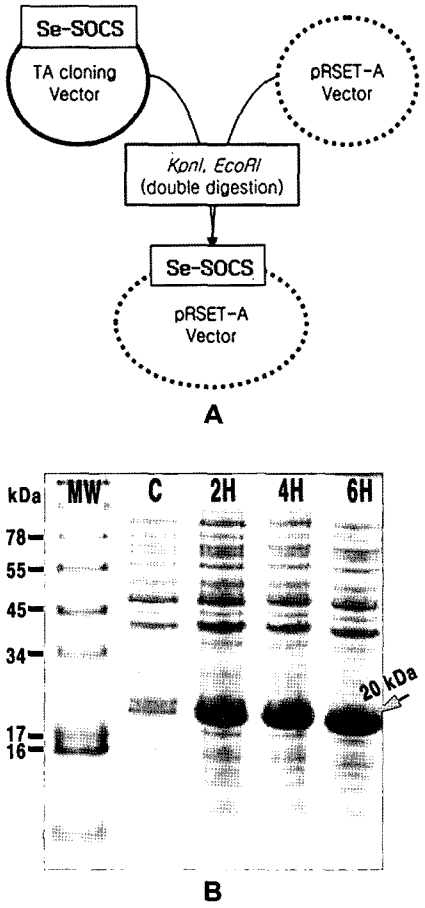


**Fig. 4.** Outline of *SeSOCS* box subcloning into pRSET expression vector. IPTG induction patterns of recombinant *SeSOCS* protein in Tricine SDS gel. Arrow indicates the apparent molecular weight of the 20 kDa protein of recombinant *SeSOCS*.

in the beet armyworm, *S. exigua,* in response to SeNPV. It showed that the level of *SeSOCS* mRNA in NPV-uninfected control larvae was very low. And then a sharp increase at 4 hrs, a steep decrease at 8 hrs and the highest level at 12 hrs was observed during the process of virus infection. Thus, it will be very intriguing to further elucidate the role of second wave of *SeSOCS* at 12 hrs. On the other hand, inhibitor of apoptosis 2 (SeNPV-IAP2) used as a positive marker of SeNPV infection in the SeNPV-infected larvae, did not show the biphasic induction expression profile, indicating that the biphasic induction is dependent on SeNPV infection (Fig. 2). Interestingly, the highest level of SeIAP2 was detected at 8 hrs when the level of *SeSOCS* mRNA was almost undetectable. Generally, these results showed that SeIAP2 works as a good molecular marker in studying the systematic time course of SeNPV infection.

However, little is known regarding the effect of JAK/STAT activation and overexpression of SOCS in the entomopathogenic SeNPV infected larvae. Therefore, the role of induction of the *SeSOCS* within the NPV-infected cells in *S. exigua* is not clear. Recently, there is a very interesting report that infection of herpes simplex virus type 1 (HSV-1) rapidly induced the suppressor of cytokine signaling-3 (SOCS3), a host negative regulator of the JAK/STAT pathway, and that the induced SOCS is correlated with efficient viral replication (Yokota *et al.*, 2005). Considering the mammalian model reported previously, it is possible that SeNPV-dependent inducible SOCS may be associated with SeNPV replication and infection in *S. exigua*. However, further studies with a sophisticated experimental design using RNA interferences are necessary to verify whether the biphasic induction of *SeSOCS* is directly linked to SeNPV infection.

Another set of time course experiments was also performed to see if laminarin known as an immune elicitor originated from *Laminaria digitata*, up-regulates the transcripts of *SeSOCS* (Fig. 3). It shows that similar biphasic expression patterns were also induced by laminarin. Taken together, our results clearly indicate that both SeNPV and laminarin induce *SeSOCS* in *S. exigua*. Yet, we do not know whether this type of biphasic fluctuation in response to known immune elicitors such as LPS, LTA, and laminarin is a universal phenomenon. It remains to be further studied.

**Overexpression of recombinant *SeSOCS* box protein**

As seen in Fig. 4A, the strategies of the subcloning of the *SeSOCS* box are pictorially described. Following transformation, the expression of the recombinant *SeSOCS* was monitored on 16.5% tricine SDS-PAGE. Uninduced cells did not contain this new protein whereas induced cells carrying pRSET vector with *SeSOCS* insert produced apparent molecular weight of the 20 kDa protein (Arrow in Fig. 4B). We are in the middle of generating a polyclonal antibody against the purified *SeSOCS* antigen. And the polyclonal antibody will be useful to functionally characterize the role of *SeSOCS* in the progression of SeNPV pathogenesis.

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## References

Alexander, W. S. and D. J. Hilton (2004) The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Ann. Rev. Immunol.* **22**, 503-529.

Barillas-Mury, C., Y. S. Han, D. Seeley and F. C. Kafatos (1999) *Anopheles gambiae* Ag-STAT, a new insect member of the STAT family, is activated in response to bacterial infection. *Embo J.* **18**, 959-967.

Barillas-Mury, C., B. Wizel and Y. S. Han (2000) Mosquito immune responses and malaria transmission: lessons from insect model systems and implications for vertebrate innate immunity and vaccine development. *Insect Biochem. Mol. Biol.* **30**, 429-442.

Dostert, C., E. Jouanguy, P. Irving, L. Troxler, D. Galiana-Arnous, C. Hetru, J. A. Hoffmann and J. L. Imler (2005) The JAK/STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. *Nature Immunol.* **6**, 946-953.

Han, Y. S., J. Thompson, F. C. Kafatos and C. Barillas-Mury (2000) Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes. *EMBO J.* **19**, 6030-6040.

Han, Y. S. and C. Barillas-Mury (2002) Implications of time bomb model of ookinete invasion of midgut cells. *Insect Biochem. Mol. Biol.* **32**, 1311-1316.

Kanost, M. R., H. Jiang and Y. Xq (2004) Innate immune responses of a lepidopteran insect, *Manduca sexta. Immunol. Rev.* **198**, 97-105.

Lin, C. C., C. M. Chou, Y. L. Hsu, J. C. Lien, Y. M. Wang, S. T. Chen, S. C. Tsai, P. W. Hsiao and C. J. Huang (2004) Characterization of two mosquito STATs, AaSTAT and CtSTAT. Differential regulation of tyrosine phosphorylation and DNA binding activity by lipopolysaccharide treatment and by Japanese encephalitis virus infection. *J. Biol. Chem.* **279**, 3308-3317.

Sliva, D. and L. A. Haldosen (1996) STAT-like DNA-binding activity in *Spodoptera frugiperda* cells. *Biochem. Biophys. Res. Commun.* **225**, 562-569.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin and D. G. Higgins (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876-4882.

Vlachou, D. and F. C. Kafatos (2005) The complex interplay between mosquito positive and negative regulators of Plasmodium development. *Current Opinion Microbiol.* **8**, 415-421.

Wang, X. H., Aliyati, R., Li, W. X., Li, H. W., Kim, K., Cartherw, R., Atkinson, P., and S. W. Ding (2006) RNA interference directs innate immunity against viruses in adult *Drosophila. Science* **312**, 452-454.

Yokota, S.-I., N. Yokosawa, T. Okabayashi, T. Suzutani and N. Fujii (2005) Induction of suppressor of cytokine signaling-3 by herpes simplex virus type 1 confers efficient viral replication. *Virology* **338**, 173-181.