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# Prominent Down-Regulation of Storage Protein Genes After Bacterial Challenge in Eri-Silkworm, *Samia cynthia ricini*

**Yan Meng, Naoko Omuro, Shunsuke Funaguma, Takaaki Daimon, Shinpei Kawaoka, Susumu Katsuma, and Toru Shimada***

We constructed two independent cDNA libraries from the fat body of *Escherichia coli*- or *Candida albicans*-challenged eri-silkworm *Samia cynthia ricini* larvae. We performed comparative expressed sequence tag (EST) analysis of the two cDNA libraries and found that two putative storage protein genes, *ScSP1* and *ScSP2*, were markedly repressed by *E. coli* injection as compared with *C. albicans* injection. By quantitative real-time RT-PCR analysis, we showed that *ScSP1* mRNA significantly reduced to 1/32–1/3 in the fat body of the female larvae, and *ScSP2* mRNA reduced to 1/7–1/3 and 1/22–1/5 in the females and males, respectively, 12–36 h after *E. coli* injection as compared with PBS injection. In addition, SDS-PAGE analysis revealed that the accumulation of both the ScSP proteins in the larval hemolymph apparently decreased up to 36 h after *E. coli* injection. However, the amounts of the two ScSP proteins returned to the same level as those in the larvae injected with PBS by 48 h after injection, showing that the reduction in ScSPs caused by the bacterial challenge was transient. Moreover, potential binding sites for the *Drosophila* Rel/NF-κB protein Dorsal were found in the 5′ upstream regulatory regions of *ScSP1* and *ScSP2*, suggesting the participation of the Rel/NF-κB proteins in controlling the bacterial suppression of the *ScSP* genes. These results suggested the hypothesis that *S. c. ricini* has a genetic program to shut down temporarily dispensable gene expression in order to induce an acute and efficient expression of immune-related genes. These findings may provide new insight into the innate immune system in lepidopteran insects. Arch. Insect Biochem. Physiol. 67:9–19, 2008. 

KEYWORDS: storage proteins; microbial injection; insect immunity; comparative EST analysis

## INTRODUCTION

Insects are capable of initiating an efficient innate immune response against microbial pathogens although they lack an adaptive immune system like that of mammals (Khush and Lemaitre, 2000). When insects encounter microbial invaders such as bacteria or fungi, two major signaling pathways, Toll and Imd, are activated, and this induces the transcription of genes encoding antimicrobial peptides (AMPs), lysozymes, peptidoglycan recognition proteins (PGRPs), lectins, and other immune-related molecules (Koizumi et al., 1999; Yamakawa and Tanaka, 1999; De Gregorio et al., 2002; Girardin and Philpott, 2004).

Using microarray technology, genome-wide analyses of insect immune response have been presented in the fruit fly *Drosophila melanogaster* (De Gregorio et al., 2001), the malaria mosquito *Anopheles gambiae* (Christophides et al., 2002), and the

Laboratory of Insect Genetics and Bioscience, Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Science, The University of Tokyo, Tokyo, Japan

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*Correspondence to: Toru Shimada, Laboratory of Insect Genetics and Bioscience, Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Science, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan. E-mail: shimada@ss.ab.a.u-tokyo.ac.jp

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honey bee *Apis mellifera* (Evans et al., 2006), providing a large number of novel genes associated with insect immunity. The activation mechanisms of immune response genes have been sought intensively in *Drosophila* (De Gregorio et al., 2002; Kim and Kim, 2005). Findings on the activation mechanism of immune-related genes in other insects are limited, although numerous AMP and PGRP genes have been characterized from non-*Drosophila* insects (Hultmark et al., 1983; Yamakawa and Tanaka, 1999; Kishimoto et al., 2002; Tanaka et al., 2005). Furthermore, there have been very few genome-wide analyses of genes that are affected by the microorganism challenge in lepidopteran insects, which are important economic and agricultural pests. Hence, little is known about the differences and diversity of immune systems among insect species.

Storage proteins are the major larval hemolymph proteins in most holometabolous insects. These proteins are well known to play important roles in the larval–pupal transformation, metamorphosis, and reproduction in adults during nonfeeding periods (Kanost et al., 1990; Haunerland, 1996). They are homo- or hetero-hexameric proteins (the so-called hexamerins), consisting of six subunits (~80 kDa each) with native molecular weights of approximately 500 kDa (Telfer and Kunkel, 1991). The storage protein genes belong to a large arthropod protein gene family including hemocyanins and prophenoloxidases (Burmester et al., 1998). These proteins differ in several properties, such as the amino acid composition, stage- and/or sex-specific synthesis, and antigenic reactivity. Insects actively produce storage proteins in the fat body during the larval feeding period, especially during the last larval instar. These proteins are released into the hemolymph, where they accumulate to reach high concentrations. The proteins are then sequestered by the fat body cells during the transition from larva to pupa and are stored as protein granules for use in the formation of numerous new proteins and structures (Pan and Telfer, 1996). We previously isolated and characterized the arylphorin-type storage protein from the eri-silkworm *Samia cynthia ricini* (Lepidoptera: Saturniidae) (Shimada et al., 1987). To date, there have been no reports on the relationship between storage proteins and immunity in lepidopterans.

In this study, in order to find genes that are activated or repressed by the microbial challenge in lepidopteran insects and to compare immune responses among insect species, we constructed two cDNA libraries from the fat body mRNA of *S. c. ricini* larvae injected with *Escherichia coli* or *Candida albicans*. Comparative analysis of the expressed sequence tags (ESTs) showed for the first time that the expression of two putative storage protein genes, *ScSP1* and *ScSP2*, which are orthologs of *SP1* and *SP2*, respectively, in *Bombyx mori* (Sakurai et al., 1988; Fujii et al., 1989), was strikingly suppressed in the fat body of the *E. coli*-challenged larvae. We verified the repression of *ScSP1* and *ScSP2* at the mRNA level by quantitative real-time RT-PCR (qRT-PCR) and the reduced accumulation of the ScSP1 and ScSP2 proteins in the larval hemolymph by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), when *E. coli* was injected into the body cavity of the *S. c. ricini* larvae. Moreover, potential *cis*-regulatory elements in the 5′-upstream regions of the two genes are also discussed. Our results provide new knowledge to help understand the immune system in lepidopteran insects.

## MATERIALS AND METHODS

### Insects

Larvae of the Vietnamese strain of the eri-silkworm *S. c. ricini* were reared on the leaves of the ailanthus *Ailanthus altissima* at 25°C in a breeding chamber. The fifth-instar larvae were used in the experiments. Normal larvae and the larvae injected with phosphate-buffered saline (PBS) or microbes were maintained under the same conditions.

### Microbial Injections

*E. coli* (JCM5491; ATCC 25299) and *C. albicans* (JCM1621; ATCC 10264) grown overnight at 37°C in Luria–Bertani medium and potato-dextran me-

dium, respectively, were injected into the body cavity of the *S. c. ricini* larvae on day 3 of the fifth instar. The larvae were anesthetized in ice water for 5 min and then sterilized with 70% ethanol-permeated soft paper around the injection position. One hundred microliters of overnight-cultured microbes diluted to 1:100 at $OD_{600}$ in sterile PBS were injected into the hemocoel from the third–fourth intersegmental membrane at the lateral thorax using a 27-gauge needle. The control larvae were injected with an equal volume of PBS.

### Total RNA Preparation

The fat bodies from the fifth-instar *S. c. ricini* larvae of normal and *E. coli*- or PBS-injected individuals were dissected separately for the two sexes in ice-cold sterile PBS at each desired time point. The tissues were rinsed in PBS, quickly frozen with liquid nitrogen, and stored at –80°C until use. Total RNA was extracted using TRIzol reagent (Invitrogen), treated with RNase-free DNase I (TaKaRa), and further purified with the RNeasy Mini Kit (Qiagen) as recommended by the manufacturers.

### cDNA Lbraries and Comparative EST Analysis

From the fat bodies collected 12–24 h after injection of *E. coli* or *C. albicans*, two cDNA libraries of *S. c. ricini* were constructed into the vector pBluescript II SK(+) (Stratagene). For generating the EST database, cDNA clones were randomly chosen from the two libraries and sequenced from the 5′ end with primer RV-M (TaKaRa). The EST sequences were clustered with the Phrap computer program, and the counts of ESTs in each cluster were comparatively analyzed between the two cDNA libraries. The DNA sequences of the cDNA clones containing *ScSP1* and *ScSP2* were determined completely using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems) and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The nucleotide sequences have been submitted to the DDBJ/EMBL/GenBank data bank under the accession numbers AB288051 (*ScSP1*) and AB288052 (*ScSP2*).

### qRT-PCR

The first-strand cDNA was synthesized from 1 μg of the total RNA using the TaKaRa RNA PCR Kit (TaKaRa). The expression profiles of *ScSP1* and *ScSP2* in both the females and males were determined quantitatively by qPCR, with forward and reverse primers designed based on their cDNA sequences. qPCR was conducted using SYBR Green PCR Master Mix and the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The thermal cycling program was performed for one cycle of 50°C for 2 min and 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 1 min, followed by a dissociation stage cycle of 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s. The relative amounts of the *ScSP1* and *ScSP2* mRNAs were normalized using *S. c. ricini* actin mRNA as the internal control. The expression difference values of triplicates between *E. coli* and PBS injection were calculated using the $2^{-Ct}$ method (Livak and Schmittgen, 2001). ANOVA was performed using MS Excel 2003. Nucleotide sequences of the primers used here and in the following experiments are available when requested.

### SDS-PAGE

Hemolymph specimens at different time points were simultaneously collected into microcentrifuge tubes on ice containing a few phenylthiourea crystals and diluted with 10 volumes of PBS (10 mM, pH 7.0). After removal of the hemocytes by centrifugation, the supernatants were boiled for 2 min in equal volumes of loading buffer (62.5 mM Tris–HCl, pH 6.6, containing 1% SDS, 2% β-mercaptoethanol, and a little glycerol and bromophenol blue), and subjected to 10% SDS-PAGE according to the method of Laemmli (1970). Gels were stained with Quick CBB (Wako), and LAS 1000 (Fujifilm) was used to photograph.

### Inverse PCR

Genomic DNA of *S. c. ricini* was isolated from the silk gland by the phenol-chloroform method

as described previously (Meng et al., 2006). Inverse PCR was conducted to amplify the 5′-flanking promoter regions of *ScSP1* and *ScSP2*. One microgram of the genomic DNA was used for the restriction enzyme digestion, T4 DNA ligase reaction (TaKaRa), and subsequent PCR reaction. The DNA fragments were gel-purified (Qiagen) and subjected to TA cloning with pGEM-T Easy Vector (Promega).

### Southern Blot Analysis

Genomic DNA of *S. c. ricini* was isolated as described above. Ten micrograms of the genomic DNA was fully digested with restriction enzymes at 37°C overnight and subjected to Southern blot analysis as described previously (Meng et al., 2006). A probe for *ScSP1* or *ScSP2* was amplified using PCR DIG Probe Synthesis Kit (Roche).

### DNA Sequencing and Analysis

DNA sequences were determined as described above. Transcriptional initiation sites were predicted with Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html). Potential transcription factor binding sites in the 5′-flanking regions were searched using the Patch program (available at http://www.gene-regulation.com/pub/programs.html) with a maximum of 2 mismatches and a lower score boundary of 87.5.

## RESULTS

### Characterization of Microbe-Challenged EST Database From Fat Body of *S. c. ricini*

The microbe-challenged EST database of *S. c. ricini* comprises 13,456 random sequences from the two cDNA libraries that were constructed from the fat body mRNA of the fifth-instar larvae injected with *E. coli*- or *C. albicans*. We comparatively analyzed the EST frequencies of the assembled genes and found many genes that were differentially expressed between the two libraries. As listed in Table 1, of the ESTs principally derived from the *E. coli*-challenged library, several have already been characterized as immune-inducible genes in *S. c. ricini*. They include *Attacin* (*SattA*, AB059394; *SattB*, AB059395; Kishimoto et al., 2002), *lysozyme* (AB048258, Fujimoto et al., 2001), and *PGRP*s (*PGRP-A*, AB232695; *PGRP-B*, AB232693; *PGRP-C*, AB232694). In addition, two putative *S. c. ricini* Cecropin genes, *ScCecropin B* and *ScCecropin D*, respectively homologous to *Cecropin B* (M10309, van Hofsten et al., 1985; D11114, Taniai et al., 1992) and *Cecropin D* (X06673, Lidholm et al., 1987; AB010825, Yang et al., 1999) of the cecropia moth *Hyalophora cecropia* and the silkworm *B. mori*, were mainly observed in the *E. coli*-injected library (Table 1), as well as the *Lebocin-like* gene (Bao et al., 2005).

Most of the genes abundantly expressed in the *C. albicans*-challenged library have not been isolated from *S. c. ricini*. However, their homologs in other insect species are well identified (Table 1). The most remarkable expression in the *C. albicans*-challenged library was two putative hemolymph storage protein genes, *ScSP1* and *ScSP2*. Possible prophenoloxidase-activating proteinase (proPAP) of *S. c. ricini* (AB264091, Bao et al., 2007) showed to be transcripted more abundantly in the *C. albicans*-challenged library than in the *E. coli*-challenged one. The following also showed dominant expression in the *C. albicans*-injected library (Table 1): riboflavin-binding hexamerin (RfBP) homolog of *H. cecropia* RbH (AF032397; Burmester et al., 1998), *M. sexta* serine proteinase-like protein 2 (AF518768; Yu et al., 2003) homolog, and serine proteinase inhibitor (Serpin) homologous to *B. mori* Serpin-2 (AF242200) and Serpin-4A (AY566164). The expression of *30K1* and putative imaginal disc growth factor, *IDGF*, was not changed by injection of either *E. coli* or *C. albicans*.

We observed that 95.2% of the ESTs of *ScAttacin* and 95.3% of *ScPGRPs* were derived from the *E. coli*-challenged cDNA library, whereas 97.4% ESTs of *ScSP1* and 93.4% of *ScSP2* originated from the *C. albicans*-challenged library (Table 2). However, the EST frequency of another major plasma protein gene, *Sc30K1*, showed no difference between *E. coli* and *C. albicans* injection (Table 2). The expression of *ScSP1* and *ScSP2* seemed markedly re-

TABLE 1. Classification of Representative Genes in Microbe-Challenged *S. c. ricini* EST Database*

| Expression biased to *E. coli* challenge (>80% EST) | Expression biased to *C. albicans* challenge (>80% EST) | Equally expressed genes |
|---|---|---|
| Attacin | Methionine-rich hexamerin precursor (SP1) | 30K1 protein |
| Lysozyme | Arylphorin precursor (SP2) | Imaginal disc growth factor |
| Cecropin B | Prophenoloxidase-activating proteinase | Hemolin |
| Cecropin D | Riboflavin-binding hexamerin | Ribosomal protein |
| Peptidoglycan recognition protein | Serine proteinase-like protein | Glutathione-S-transferase |
| Lebocin-like protein | Serpin | Secreted protein acidic rich in cysteine |
| Ommochrome-binding protein | Immulectin | Cytoplasmic actin A3 |
| Cathepsin L-like proteinase | Apolipophorin | |
| Transferrin Moricin | Chymotrypsin inhibitor | |

*Appearance of more than 80% of ESTs in either the *E. coli*- or the *C. albicans*-challenged cDNA library was regarded as biased gene expression.

pressed by the *E. coli* challenge, which prompted us to quantitatively investigate the actual mRNA transcripts of the *ScSP* genes after bacterial injection.

## Expression Patterns of ScSP Genes in Fifth Instar Larvae

We cloned and sequenced full-length cDNAs of *ScSP1* and *ScSP2*. *ScSP1* potentially encodes a 753–amino acid peptide containing 9.03% methionines while the translated product of *ScSP2* is an arylphorin-type storage protein containing 18.61% aromatic amino acids among 704 deduced residues. The coding regions of both the *ScSP* genes are dispersed in five exons by four introns, which are organized in a manner similar to those of *B. mori SP1* and *SP2* (Fujii et al., 1989). Southern blot analysis revealed that they are one-copy genes in the haploid genome (data not shown).

The expression patterns of *ScSP1* and *ScSP2* in the normal females and males of the fifth larval instar were quantitatively investigated by qRT-PCR. *ScSP1* was female-specifically expressed in the late fifth-instar larvae (Fig. 1A), whereas *ScSP2* was expressed in both the males and females (Fig. 1B).

TABLE 2. Comparison of EST Frequencies Distributed in Two cDNA Libraries

| Genes | ESTs from *E. coli* (%) | ESTs from *C. albicans* (%) | Total |
|---|---|---|---|
| *ScSP1* | 4 (2.6) | 149 (97.4) | 153 |
| *ScSP2* | 43 (6.6) | 611 (93.4) | 654 |
| *ScAttacin*[a] | 922 (95.2) | 46 (4.8) | 968 |
| *ScPGRP*[b] | 82 (95.3) | 4 (4.7) | 86 |
| *Sc30K1* | 11 (28.9) | 27 (71.1) | 38 |

[a]EST number of *ScAttacin* comprises *Satt A* and *Satt B* (accession numbers AB059394 and AB059395, respectively).
[b]EST number of *ScPGRP* comprises *PGRP-A*, *PGRP-B*, and *PGRP-C* (accession numbers AB232693, AB232694, and AB232695, respectively).

## Transcriptional Repression of *ScSP*s After *E. coli* Injection

For monitoring the effects of bacterial injection on the *ScSP* transcription, we performed qRT-PCR experiments. As shown in Figure 2A, the *ScSP1* mRNA in the females markedly reduced to 1/32–1/3 from 12 to 36 h, and then slightly recovered to half of the control level by 48 h after *E. coli* injection (Fig. 2A). In addition, it was observed that the transcripts of *ScSP2* significantly decreased to 1/7–1/3 and 1/22–1/5 in the females (Fig. 2B) and males (Fig. 2C), respectively, 12–36 h after *E. coli* injection relative to values after PBS injection. These results were well consistent with our comparative EST analysis described above.

## Reduced Accumulation of ScSP Proteins in Larval Hemolymph

We next analyzed the accumulation of the ScSP proteins in the hemolymph of the *E. coli*- or PBS-injected larvae. SDS-PAGE analysis showed that the accumulation of both the ScSP1 and ScSP2 proteins in the hemolymph after *E. coli* injection was reduced as compared with PBS injection between 12 and 36 h after injection (Fig. 3). However, 48 h after injection the two ScSP proteins in the hemolymph of the *E. coli*-injected larvae were present at levels similar to those in the PBS-injected larvae (Fig. 3). The reduction profiles of the ScSP pro-

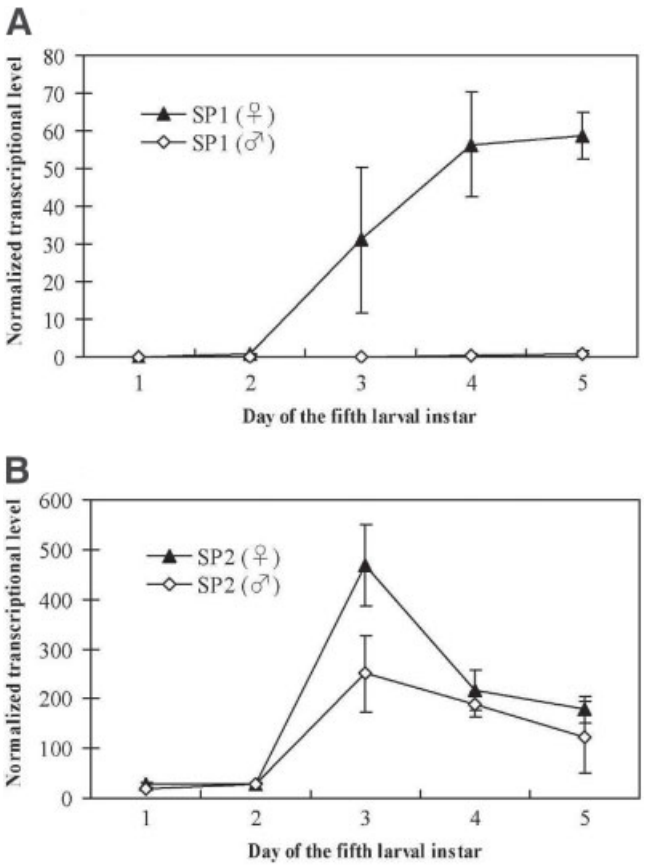


Fig. 1. Transcriptional profiles of (**A**) *ScSP1* and (**B**) *ScSP2* in the fat body of the fifth-instar larvae. Total RNA was extracted from the fat bodies collected from three female and three male larvae at each time point. First-strand cDNAs were synthesized from 1 μg of the total RNA. qRT-PCR was performed as described in Materials and Methods. Relative transcription levels of *ScSP1* and *ScSP2* were internally normalized by those of *S. c. ricini* actin, *ScActin*. The data are the mean ± SD of triplicates.

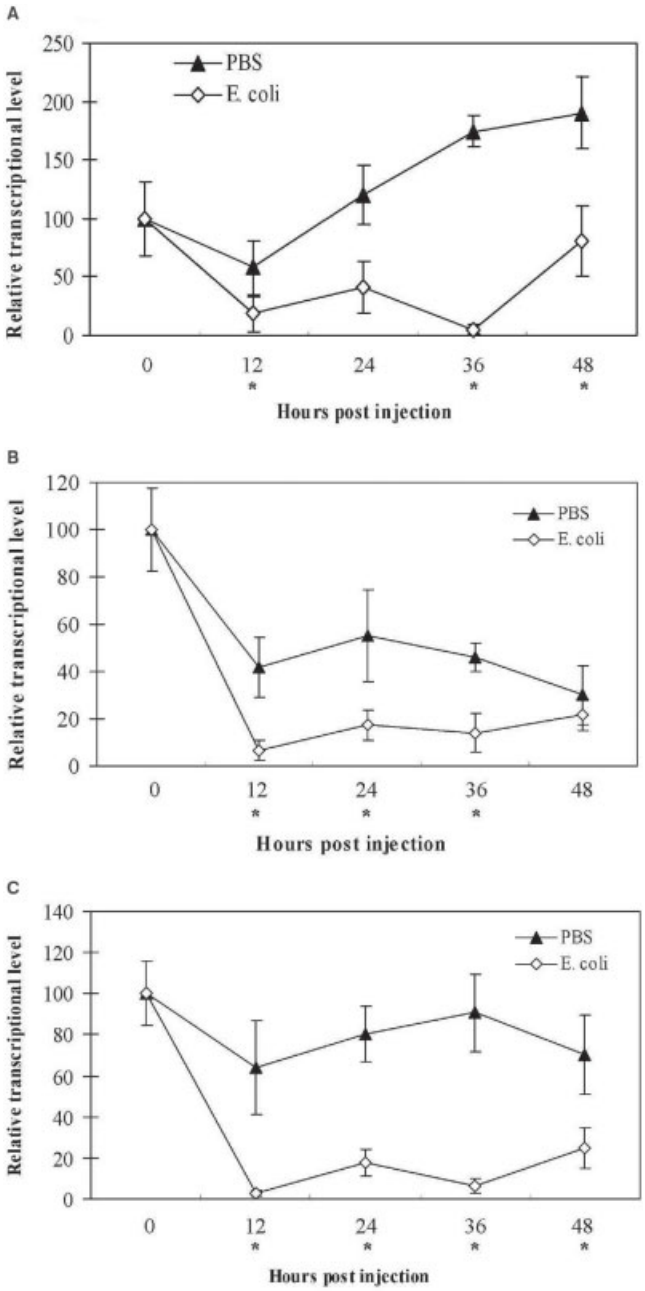


Fig. 2. Repression of (**A**) *ScSP1* and (**B**) *ScSP2* in females and of (**C**) *ScSP2* in males after *E. coli* injection. Fat bodies were collected from at least three female and/or male larvae at each time point. mRNA amounts at 0 h (day 3 of the fifth larval instar, just before injection) were designated as 100%. The data are means ± SD of triplicates, and significance ($P < 0.05$) is shown by asterisks below corresponding time points.

teins in the hemolymph were consistent with those of the *SP1* and *SP2* mRNA amounts in the fat body (Figs. 2 and 3). These results suggest that the *E. coli*-induced reduction of the ScSP proteins in the hemolymph was temporary and recovered by about 48 h after bacterial injection (Fig. 3).

## Characterization of 5′-Flanking Sequences of *ScSP1* and *ScSP2*

To find immune-related factor binding sequences, such as NF-κB motifs for Rel proteins, the 5′-flanking regulatory regions of 738 bp for *ScSP1*

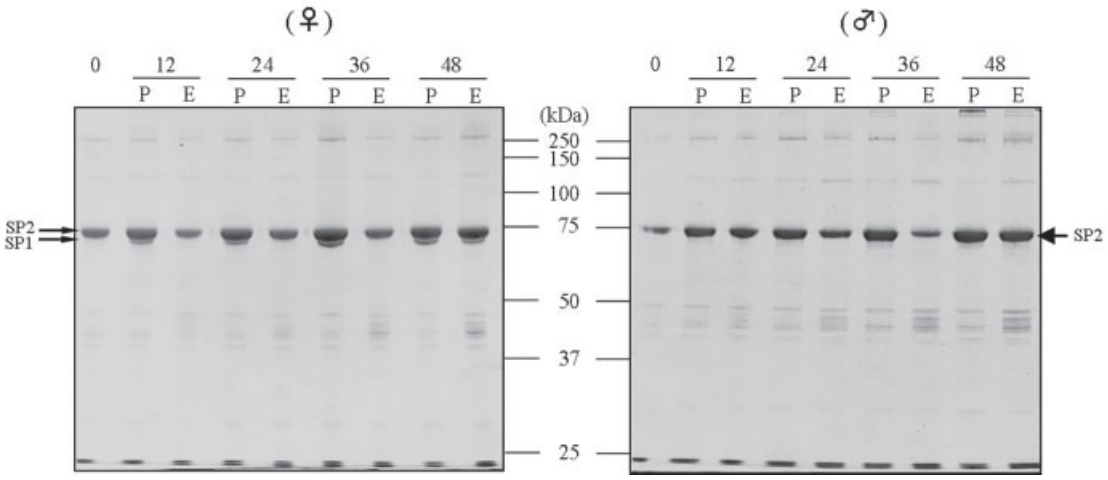


Fig. 3. SDS-PAGE analysis of reduction of ScSP1 and ScSP2 in larval hemolymph after *E. coli* injection(left, female; right, male). Hemolymph of three larvae at 0 h (V3 stage, just before injection) and 12–48 h after PBS or *E. coli* injection was subjected to electrophoresis. ScSP1 and ScSP2 are indicated by arrows. The molecular weights of protein standards are also indicated. P, PBS injection; E, *E. coli* injection.

and 604 bp for *ScSP2* upstream from the transcription initiation site were obtained. In the 5′ upstream region of *ScSP1*, there were two potential Rel/NF-κB binding sites with one nucleotide mismatch to those for Dl, a Dorsal protein isolated from the fruit fly *Drosophila melanogaster* (CGGAAAACAA at position –589 to –580 nucleotides, and GGGTTTTGAT at position –406 to –415 nucleotides) (Fig. 4A). Similarly, in the 5′ upstream region of *ScSP2*, at the position of –246 to –237 nucleotides, one nucleotide mismatched site, CAGATAAATC, was also predicted to be a Rel/NF-κB regulatory element for Dl (Fig. 4B). These results suggest that the binding of Dl-related proteins to the upstream regions might be responsible for the transcriptional repression of the *ScSP* genes. Additionally, putative binding sites for a doublesex protein, DSXF/M, were found in the 5′-flanking regions of both the genes (Fig. 4A,B), which suggests a contribution of the *cis*-regulator, DSXF or DSXM, to the transcription of *ScSP2* in both females and males, and the female-specific expression of *ScSP1* (Jinwal et al., 2006).

## DISCUSSION

To find immune-related genes of *S. c. ricini* that are induced or repressed by microbial injection, we constructed two microbe-challenged cDNA libraries from the fat body mRNA of *S. c. ricini* larvae injected with *E. coli* or *C. albicans*, and performed comparative analysis of the 13,456 ESTs obtained from the two libraries.

The EST frequencies of many genes seemed notably different between the *E. coli*- and *C. albicans*-challenged libraries. As expected, we observed that the *E. coli* challenge stimulated the transcriptional activation of the genes encoding attacin, cecropin, lysozyme, and PGRP (Table 1). These are well known as immune-inducible antibacterial peptides, bacterial cell-destroying enzymes and important pattern recognition molecules of bacterial cell components in *S. c. ricini*, *B. mori*, and other insects (Hultmark et al., 1983; Yamakawa and Tanaka, 1999; Ochiai and Ashida, 1999; Fujimoto et al., 2001; Kishimoto et al., 2002; Girardin and Philpott, 2004). More than 95% of the ESTs of *ScAttacin* in our EST database came from the *E. coli*-injected cDNA library (Table 2). qRT-PCR analysis showed that the *ScAttacin* mRNA was almost absent in the normal fifth larvae, but was acutely upregulated by *E. coli* injection by more than 1,000-fold 12 h after injection, and the induction was still greater than 100-fold 48 h after injection (data not shown).

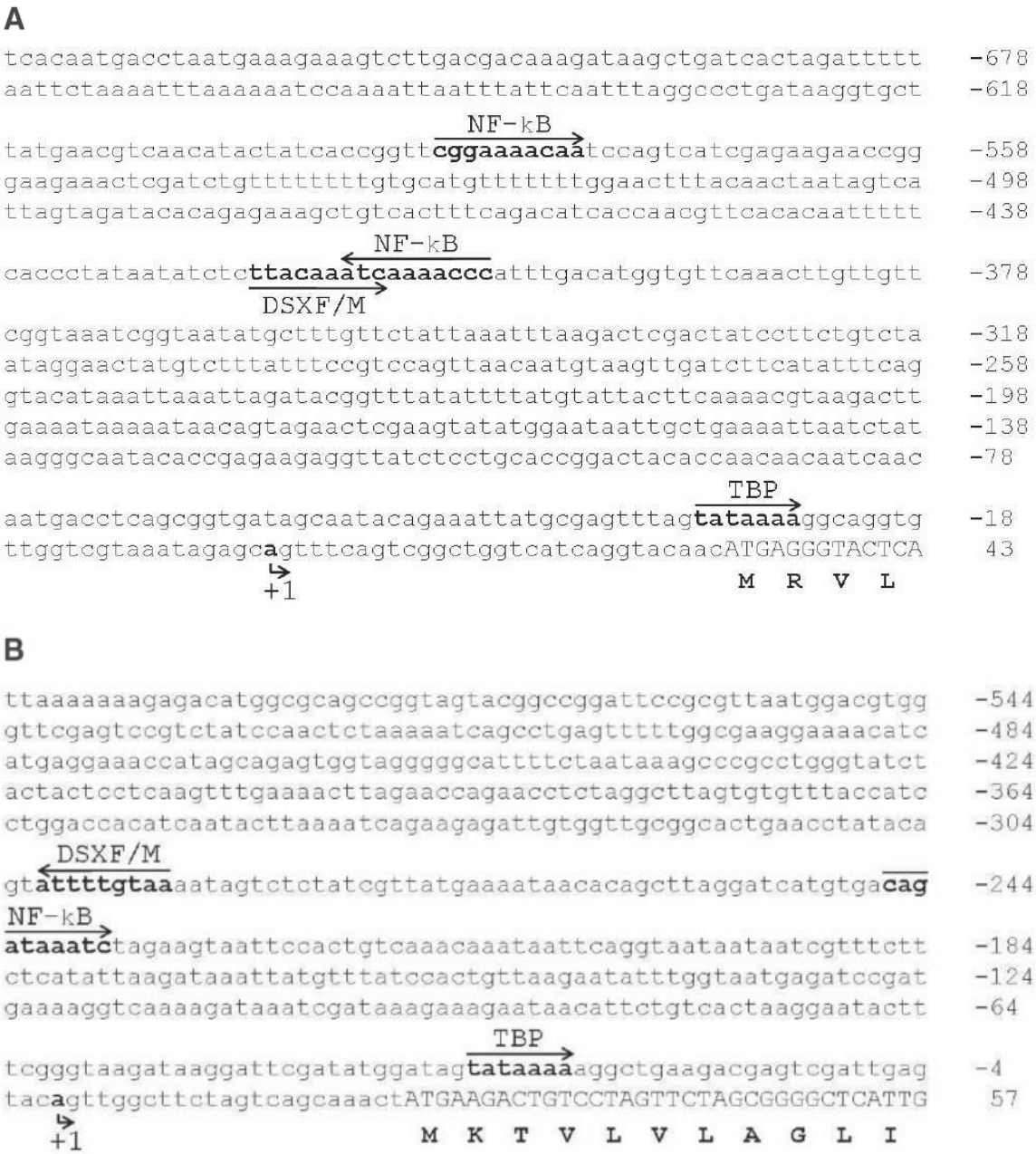


Fig. 4. Nucleotide sequence in the 5′-upstream regions of (**A**) *ScSP1* and (**B**) *ScSP2*. The putative transcription initiation site is numbered +1 with a right-directed arrow. Amino acid residues adjacent to the translation start site are shown in boldface under the uppercased nucleotides. Typical TATA boxes and predicted *cis*-acting elements for the binding of Rel/NF-κB and DSXF/M are indicated in bold letters.

Interestingly, the EST frequencies of two major hemolymph storage protein genes, *ScSP1* and *ScSP2*, manifested the biggest difference between the two microbe-challenged libraries. Fewer than 10% of the transcripts were derived from the *E. coli*-challenged library, inferring the transcriptional repression of *ScSP*s by bacterial attack (Table 2). *ScSP1* and *ScSP2* are orthologous to the genes *SP1* and *SP2*, respectively, of the silkworm *B. mori* (Sakurai et al., 1988; Fujii et al., 1989). By qRT-PCR, we confirmed that *E. coli* injection resulted in the marked repression of the *ScSP* transcription (Fig.

2). In addition, we observed that the accumulation of both the storage proteins in the larval hemolymph apparently decreased until 36 h after *E. coli* injection (Fig. 3). In contrast, many immune-related genes, such as those encoding the AMPs, were transcriptionally induced at a continuously high level during the early period after bacterial injection (data not shown). In view of these results, we speculate that there may be a relationship between the transcriptional repression of the *ScSP* genes and the acute induction of the AMP genes in the fat body of bacterial-challenged *S. c. ricini* larvae.

For holometabolous insects, storage proteins, as well as larval serum proteins (LSPs), are of great importance because they are utilized as amino acid reserves for the formation of numerous new proteins and structures during non-feeding periods. ScSP1 belongs to the methionine-rich type hexamerins, which are always expressed in a female-specific manner, so that they are generally considered as playing special roles in insect egg formation (Pan and Telfer, 2001). ScSP2 is a member of the aromatic amino acid-rich arylphorins, which contain large amounts of phenylalanine and tyrosine to support cuticle deposition (Webb and Riddiford, 1988). The properties of ScSP1 and ScSP2 are quite similar to those of their orthologs in *B. mori* (Tojo et al., 1980; Mine et al., 1983; Shimada et al., 1987). If repressed expression of *ScSP*s by an *E. coli* challenge results in a decrease in storage protein endocytosis by fat body cells before pupation, there will be some physiological and/or biochemical effects on the subsequent development of pupae and adults. We observed that 48 h after injection, the differences in the amounts of the *ScSP* mRNA between *E. coli* and PBS injection diminished to some extent and that the ScSP proteins that had accumulated in the *E. coli*-challenged larval hemolymph recovered almost to the same level as when PBS was injected (Figs 2 and 3). Hence, we conclude that the repressed expression of the ScSP proteins was notable but temporary because it was limited to the early period after *E. coli* injection (up to 36 h). It is well documented that the innate immune system of insects is activated during a short period in response to microorganism invasion, and that many immune-related genes, such as those encoding the AMPs, are quickly promoted to express in the fat body (Koizumi et al., 1999; De Gregorio et al., 2002; Girardin and Philpott, 2004). These acute immune responses utilize large amounts of energy, amino acids, and other biosynthesis-related substances as compared with the normal condition. Taking these results together with our present results, we speculate that there is a genetic program to shut down some temporarily dispensable protein syntheses in the fat body of lepidopteran insects when bacteria invade the body cavity, so that immune-related genes can be induced rapidly and efficiently. De Gregorio et al. (2001) reported that, among a large number of genes displaying changes in the mRNA expression in *Drosophila*, 400 immune-regulated genes were selected. These comprised 230 induced and 170 repressed genes; of these, two LSP genes, four cuticle protein genes, and several enzymes involved in general metabolism were repressed after microbial infection. These results suggest that there may be some common defense mechanisms in the innate immune response among insects.

Three mammalian Rel/NF-κB protein homologs in *Drosophila* have been shown to upregulate AMP genes through binding to the κB sites (Ghosh et al., 1998; Silverman and Maniatis, 2001). Dl and Dif proteins are activated by the Toll pathway to control antifungal and anti-gram-positive bacterial peptide genes, and Relish is activated by the Imd pathway to control anti-gram-negative bacterial peptides (Dushay et al., 1996; Lemaitre et al., 1997). *LSP1* and *LSP2* in *Drosophila* are known to be repressed by both the Relish/Imd and the Spaetzle/Toll pathways (De Gregorio et al., 2002).

To examine whether the Rel/NF-κB proteins are involved in the repression of *ScSP* genes, we searched the possible *cis* elements in the 5′-upstream regulatory regions of *ScSP1* and *ScSP2*. Two putative 10-nucleotide NF-κB sites for Dl in the 5′-upstream regulatory region of *ScSP1* and one in that of *ScSP2* were found (Fig. 4). However, binding motifs for GATA factors, usually adjacent coactivators of Rel/NF-κB in regulating immune genes (Kadalayil et al., 1997; Cheng et al., 2006), were

not found in either of the upstream regions. Dl is known to function as either an activator or a repressor of transcription in determining dorsoventral fate in the *D. melanogaster* embryo (Govind and Steward, 1991). Further studies are required to confirm that Rel/NF-κB proteins act not only as activators of AMP genes but also as repressors of *ScSP* genes. Together, our findings provide new insight into the immune response system in lepidopteran insects.

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