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Characterization and transcriptional profiles of three *Spodoptera frugiperda* genes encoding cysteine-rich peptides. A new class of defensin-like genes from lepidopteran insects?

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Abstract

The present work describes sequence and transcription of three *Spodoptera frugiperda* genes encoding 6-cysteine-rich peptides. Sequence alignments indicate that the predicted peptides belong to the insect defensin family, although phylogenetic analyses suggest they form a cluster distinct from that of other neopteran insect defensins. The three genes were identified in a non-immune-challenged Sf9 cells cDNA (DNA complementary to RNA) library (Landais et al., Bioinformatics, in press) and were named *spodoptericin*, *Sf-gallerimycin* and *Sf-cobatoxin*. *Spodoptericin* is a novel defensin-like gene that appears to be weakly up-regulated following injection of bacteria and fungi. Interestingly, no sequence motif clearly homologous to *cis* regulatory element involved in the regulation of antimicrobial genes was found. An homologue of the *spodoptericin* gene was identified in the SilkBase *Bombyx mori* cDNA library. *Sf-gallerimycin* is related to the *Galleria mellonella* gallerimycin gene and is induced after immune challenge by injection of bacteria in the larval fat body as well as in hemocytes. In silico analysis of the sequence upstream from the cDNA reveals the presence of at least one motif homologous to a nuclear factor kappaB (NF-κB) binding site. Finally, *Sf-cobatoxin* is related to the *G. mellonella* cobatoxin-like gene. Despite high levels of constitutive expression compared to the two previous genes, transcription of *Sf-cobatoxin* is increased after immune, in particular, bacterial challenge. We therefore confirm that these three genes encode potential candidate molecules involved in *S. frugiperda* innate humoral response.

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Abbreviations: aa, amino acid(s); BAC, bacterial artificial chromosome; cDNA, DNA complementary to RNA; CSab, cysteine-stabilized alpha beta motif; EST, expressed sequence tag; nt, nucleotide(s); Imd, immune deficiency (pathway); kb, kilobase(s); kDa, kilodalton(s); ORF, open reading frame; PFAM, protein families database of alignments; PCR, polymerase chain reaction; Prosite, database of protein families and domains; SCOP, database of structural classification of proteins; Brn-2, POU factor Brain-2-; CdxA, caudal homeobox gene A; Dfd, Deformed; GATA-1, GATA-binding factor 1; IL-6 RE-BP, interleukin-6 response element binding protein; c-Myb, cellular homolog of avian myeloblastosis virus oncogene; NF-κB, nuclear factor kappaB; Oct-1, octamer-binding factor 1; UTR, untranslated region.

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1. Introduction

In insects, cysteine-rich peptides constitute a diverse and widely distributed family of defense molecules against bacterial and fungal infections (Dimarcq et al., 1998). The insect defensins, which belong to this family, are peptides with a cysteine-stabilized αβ (CSαβ) motif involving six conserved cysteine residues that confers to the molecules high stability and resistance to the proteases. Insect defensins present sequence similarities with mammalian defensins but they are structurally unrelated since the latter consist of β-sheets and lack the α-helix characteristic of insect defensins (Dimarcq et al., 1998). In all multicellular

organisms, including humans and plants, defensins appear to have a wide range of actions directed against various microbes (Hoffmann et al., 1999). Mammalian defensins disrupt microbial membranes and chemically attract T cells whereas plant defensins are primarily directed against fungal pathogens. In *Drosophila*, defensins have been shown to form voltage-dependent ion channels in Gram-positive cells (Cociancich et al., 1993).

Insect defensins are generally cationic peptides composed of 34–46 residues, with molecular masses ranging from 2 to 6 kilodaltons (kDa). Initially discovered in diptera (Matsuyama and Natori, 1988), defensins have since been described in many neoptera insect orders. Most act against a broad spectrum of Gram-positive bacteria but have little effect on Gram-negative bacteria and fungi (Dimarcq et al., 1998). Defensin-like peptides have recently been identified in lepidoptera. The best known of these is heliomicin, a defensin-like peptide identified in the moth *Heliothis virescens*. Interestingly, heliomicin has mainly antifungal properties and is more related to the *Drosophila* antifungal drosomycin than to defensins (Lamberty et al., 1999).

Studies on the immune system of *Drosophila* suggest that innate immunity is an ancient defense mechanism (Hoffmann et al., 1999). In insects, all promoters of immune-inducible peptide genes contain responsive elements homologous to those involved in the regulation of genes-encoding acute-phase proteins in mammals. The most prominent and often mandatory are binding sites for transcriptional activators of the Rel family. In *Drosophila*, the functional importance of the κ B-related sequences has been demonstrated for several antibacterial and antifungal genes (Meister et al., 1994). Genetic and functional studies have shown that production of subsets of peptides against different pathogens is based on the activation of two distinct but maybe cross-talking signaling cascades—the Toll dorso-ventral-signaling pathway and the immune deficiency (Imd) pathway (reviewed in Khush et al., 2001; Hoffmann and Reichhart, 2002). For example, the *Drosophila* defensin gene requires both Toll and Imd pathways for full inducibility following immune challenge (Lemaitre et al., 1997). In lepidopterans, κ B-like sequences occur in the promoter regions of the *B. mori* antibacterial cecropins, attacin and lebecins genes (Ponnuvel and Yamakawa, 2002), suggesting that antimicrobial lepidopteran genes are regulated through similar pathways. To date, however, there is no published information on lepidopteran genes encoding cysteine-rich peptides such as defensins.

In this study, we used a Sf9 cDNA (DNA complementary to RNA) library constructed from non-immune-challenged cells recently made available to us (Landais et al., 2003) to detect defensin-like peptide genes in lepidopterans and to investigate their transcriptional regulation. Genes encoding three cysteine-rich defensin-like peptides expressed in the lepidopteran *Spodoptera frugiperda* were

identified from the Sf9 cells of this cDNA library. The first encodes a novel peptide, named spodoptericin, distinct from previously described lepidopteran defensin-like peptides such as heliomicin and the *Mamestra brassicae* defensin (GenBank AAL69980). The other two are related to recently identified cysteine-rich peptides of the greater wax moth *Galleria mellonella* and resemble scorpion potassium-channel blocking peptides named cobatoxins (Seitz et al., 2003). To assess the role of these three predicted peptides in the immune response in *S. frugiperda*, we conducted a transcription analysis of the genes following immune-challenge with germs such as bacteria and/or the entomopathogenic fungus *Beauveria bassiana*. We also analyzed the regions upstream from the transcription start for the genes encoding two of these peptides in order to identify sequence motifs homologous to *cis* regulatory elements involved in the regulation of genes encoding antimicrobial peptides.

2. Material and methods

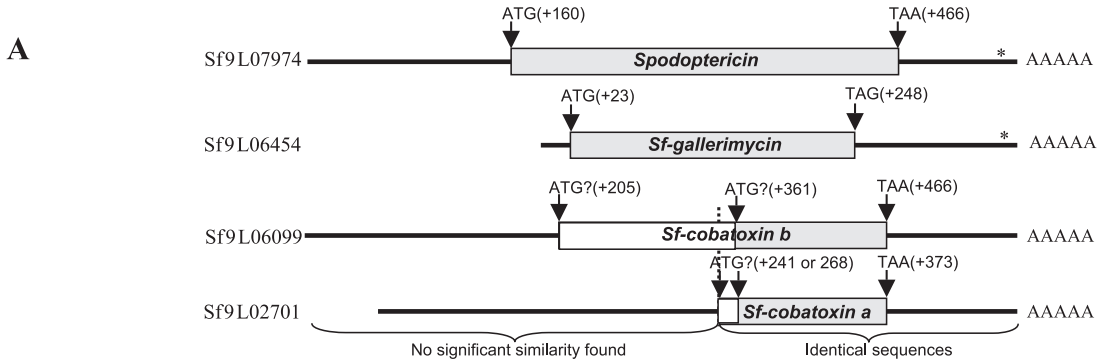
2.1. Sf9 cells cDNA expression library

The clones used are indicated in Fig. 1A and belong to Sf9 cells (ATCC CRL1711) cDNA expression library (Landais et al., 2003). The clones #Sf9L00498 and Sf9L07974 correspond to a *S. frugiperda* defensin-like gene we named *spodoptericin* as no previously described homologues were found. Based on similarity with previously described genes, the clones #Sf9L06137 and Sf9L06454 correspond to a gene we named *Sf-gallerimycin*. Four clones, #Sf9L00509, Sf9L02701, Sf9L03867 and Sf9L06099 correspond to genes we named *Sf-cobatoxins*. Sf9L00509 and Sf9L02701, as well as Sf9L03867 and Sf9L06099, are overlapping clones and all four clones have a common 3'-end sequence. For clarification purposes, the nomenclature of the clones will be simplified in all subsequent text. For example, SF2701 will be used instead of Sf9L02701.

2.2. Isolation of the complete coding sequence

To isolate the genomic sequences, polymerase chain reactions (PCRs) were conducted using Sf9 genomic DNA as a template and internal primers within the cDNA sequences. The primer pairs were SF7974_For 5'-GGT GTC GGC TTG TCT GAT ACA-3' (17 nt after the ATG codon), and SF7974_Rev 5'-GCA GCT ACA TGT GTG ACT GAC-3' (4 nt before the TAA codon) for the *spodoptericin* gene; SF6454_For 5'-AAG GTT TCA GTC ATG AAG GCT TGC-3' (12 nt before the ATG codon), and SF6454_Rev 5'-TAC AAA CAT GGC AAG ATG GAG AGC-3' (37 nt after the TAA codon) for the *Sf-gallerimycin* gene; and SF6099_For 5'-AGC GTT GTC GAC TTT TTC ACC TCA-3' (70 nt before the ATG codon), and SF6099_Rev 5'-GGC TGA AAC GCA GTT TCC ATA TTT-3' (22 nt before

EST clones	BlastX best match	E-score	gene name
Sf9L00498 (AY238438,322bp); Sf9L07974 (AY238439,561bp)	<i>Palomena prasina</i> defensin, GenBank#P80407	2,4	<i>spodoptericin</i>
Sf9L06137 (AY238440,375bp); Sf9L06454 (AY238440,375bp)	<i>Galeria mellonella</i> gallerimycin, GenBank#AF453824	e-12	<i>Sf-gallerimycin</i>
Sf9L00509 (AY238441,450bp); Sf9L02701 (AY238442,504bp)	<i>G. mellonella</i> cobatoxin-like, GenBank#AF394590	9.e-7	<i>Sf-cobatoxin a</i>
Sf9L03867 (AY238443,319bp); Sf9L06099 (AY238444,568bp)	idem		<i>Sf-cobatoxin b</i>

**B****Spodoptericin (SF7974)**

MGVKVINVFLLI AVSACLIHAVAG↓KPNPRDSSVVEEQSLGPIHNEDELVKVKPETTTTPEPRIPGR↓VSCDFEEANEDAVCQEHCLPKGYTYGICVSHTCSCI
 precursor: 102aa; 11kDa; pI=5.04 propeptide: 78aa; 8.6kDa; pI=4.7 peptide?: 36aa; 3.95 kDa; pI=4.35

Sf-gallerimycin (SF6454)

MKACVVLAVLLVAFAVATSTA↓DLGHTEASLRVRRRETIRGPEFP↓NRCVfyECIASCRQRGYKSGGYCTINGCQCLR
 Pre-: 75aa; 8.2 kDa; pI=9 pro-: 54aa; 6.2 kDa; pI=8.9 peptide?: 32aa; 3.6 kDa; pI=8.9

Sf-cobatoxin (SF6099)

MKLIILFVVCAMVMAAYA↓VEVISVDDLLRAYDVSHNKIDGESVKMATEALYV**PMSL**↓**RACTSSACNFVCKFLGKYGNCSAETCRCYS**
 Pre-: 87aa; 9.6 kDa; pI=6.5 propeptide: 70aa; 7.7 kDa; pI=5.6 !! peptide?: 32aa; 3.5 kDa; pI=8.65
 !SF2701 peptide: 44aa; 4.8 kDa; pI=8.3

Fig. 1. Sf9 cells cDNAs encoding cysteine-rich peptides. (A) The table indicates the EST clone references, their best BlastX match and score and the deduced name of the corresponding genes. A schematic representation of the longest clone is shown below the table. Boxes represent the open reading frames. For *Sf-cobatoxins*, shaded boxes correspond to the ORF predicted if sequence is compared to the *G. mellonella* cobatoxin. The start and stop codons are indicated by vertical arrows, position +1 corresponds to the start of the EST clone. Asterisk indicates a polyA signal. (B) Predicted amino acid sequences. The position of possible cleavage sites is indicated by a vertical arrow. For each peptide [precursor (pre-), pro-peptide (pro-) and putative mature peptide (peptide)], the length, the calculated molecular weight and the theoretical pI are indicated. For *Sf-cobatoxin*, sequence of the peptide predicted to be encoded by SF2701 (SF2701 peptide) is underlined, and the most probable sequence for this peptide (predicted peptide, by analogy with *G. mellonella*) is in bold characters. The conserved six cysteine residues are highlighted.

the TAA codon) for the *Sf-cobatoxin* gene. PCR was conducted with Taq DNA polymerase according to standard procedure. The obtained amplicons were cloned in pGEM-TEasy vector (Clontech) and sequenced (GenomeExpress, France).

2.3. Analysis of the gene copy number by Southern blot analysis

The gene copy number was analyzed by Southern blot analysis of (1) *S. frugiperda* genomic DNA digested with restriction enzymes absent from the known sequence (*Pst*I or *Hind*III) and (2) a *S. frugiperda* bacterial artificial chromosome (BAC) library (potential 10 × coverage, d'Alençon et al., in preparation). Genomic DNA was extracted from *S. frugiperda* 4th instar larvae according to standard procedures. Hybridizations were conducted as described previously (Volkoff et al., 1999; d'Alençon et al., in preparation), using the cDNA inserts as probes.

2.4. Isolation of the *spodoptericin* and *Sf-gallerimycin* genes upstream sequence

Cloning of the region upstream from the *spodoptericin* cDNA was accomplished by inverse PCR. Digestion of the genomic Sf9 DNA with the restriction enzyme *Sma*I resulted in a 2-kbp hybridization band with the *spodoptericin* probe in preliminary Southern blot analysis. *Sma*I digested genomic DNA was then recircularized and the ligation product used as template for a PCR using the primers SF7974_invF 5'-ATA CCT GGC GTT GTG GTG GTT TCT G-3' and SF7974_invR 5'-GAA GTA GCG GCT GCG ATA AAC CTC TTA-3'. The resulting PCR product was cloned in pGEM-TEasy vector and approximately 790 bp were sequenced upstream from the cDNA sequence.

To obtain *Sf-gallerimycin* upstream sequence, two positive BAC clones (BAC61_G18 and BAC68_H5) were sequenced using the gene specific primer BAC6454 5'-GAA CCA CGC AAG CCT TCA TGA CTG-3' (5 nt upstream the

ATG codon, underlined). Approximately 600 bp were sequenced upstream from the *Sf-gallerimycin* cDNA sequence.

2.5. Transcription analysis by Northern blot

To collect total RNA samples, dissected fat body cells were placed directly into 0.5 ml of Trizol reagent (Gibco). Hemocyte samples were collected by bleeding larvae into 0.75 ml of Trizol reagent. To analyze transcription during *S. frugiperda* development, total RNA was extracted from whole larvae of 1st, 2nd and 3rd instars, and from fat body of 4th, 5th, pupa and adult instars.

Hybridizations were conducted as described previously (Volkoff et al., 1999), using the cDNA inserts as probes. The same membrane was used for all three probes by stripping the membrane after each hybridization. A *S. frugiperda actin* probe was generated by PCR from Sf9 DNA, using the forward primer 5'-CAA CTG GGA CGA CAT GGA GAA GAT-3' and the reverse primer 5'-CCA CCG ATC CAT ACG GAG TAT TTC-3' and was used as an internal standard.

2.6. Microorganisms used for immunization assays

The bacterial strains used were the Gram-positive *Bacillus subtilis* (strain 168, gift from E. Le Chatelier, INRA Jouy-en-Josas), and the Gram-negative *Escherichia coli* (strain JM83). The fungus strain used was the entomopathogenic *B. bassiana* (strain 80.2 from our laboratory's collection). The polydnavirus used was HdIV, which is associated with the ichneumonid wasp *Hyposoter didymator* (Volkoff et al., 1999).

2.7. Immunization of insects

In a first assay, 1-day old *S. frugiperda* 5th instar larvae were immune-challenged by injection of either 8×10^2 *B. bassiana* conidia or 8×10^5 live bacteria (mix of *B. subtilis* and *E. coli*) per insect. For bacterial challenge, we chose to use a mix of both Gram-negative and Gram-positive bacteria, as each bacterial type activates different regulation pathways: the first activates the Imd pathway whereas the second activates preferentially the Toll pathway. Larvae were infected with HdIV as described in Volkoff et al. (2002) and immune-challenged 4 h after infection. HdIV infection of *S. frugiperda* larvae was controlled using a probe composed of viral cDNAs. Hemocytes and fat bodies were recovered 24 and 48 h after immune challenge ($n = 5$ larvae per assay). For fat bodies, two repeats were done for the *Sf-gallerimycin* and Sf-cobatoxin probes. For the *spodoptericin* probe, two additional assays were done with individual larvae.

In a second assay, 1-day old *S. frugiperda* 5th instar larvae were injected with two suspensions of *B. bassiana* conidia in PBS, corresponding to either 8×10^2 or 8×10^3 conidia per insect. Fat bodies were recovered 24 and 48 h after injection ($n = 5$ larvae per assay).

2.8. Immunization of cells in culture

The Sf9 cells were treated with a cell medium suspension of previously heat-inactivated *E. coli*, *B. subtilis* and *B. bassiana* conidia or with a suspension of LPS and zymozan. Samples were collected 24 and 48 h after treatment.

2.9. Sequence analysis

Comparisons of sequences in nonredundant databases were carried out using BLASTX Sequence Similarity Searches (<http://www.ncbi.nlm.nih.gov>). Molecular weight of the deduced proteins was calculated with the ProtParam tool (<http://www.expasy.ch/cgi-bin>). Comparison with protein family databases was performed with the Family Pairwise Search (<http://fps.sdsc.edu>). Transcription factor binding consensus sites in the putative promoter regions were identified using TFSEARCH (<http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html>) software, with the threshold score varying from 70 to 85 (default). Sequence were aligned and bootstrapped neighbor-joining trees were calculated using ClustalX and TreeView software, respectively.

3. Results and discussion

3.1. Isolation of *S. frugiperda* cDNAs encoding predicted cysteine-rich peptides and in silico analysis of the predicted amino acid sequences

Analysis of the non-immune challenged Sf9 cells cDNA library revealed the existence of several clones related to three distinct genes encoding cysteine-rich peptides (Fig. 1A). One is a novel gene first described in the present work that we named *spodoptericin*. Based on their similarity with other genes, the other two were named *Sf-gallerimycin* and *Sf-cobatoxin*. Only the longest and most probable full-length cDNA will be discussed here: SF7974 (*spodoptericin*), SF6454 (*Sf-gallerimycin*) and SF6099 (*Sf-cobatoxin*). The clones SF6099 and SF2701 share the terminal 280 nt in the 3' end of the sequence (Fig. 1A, diagram) but differ in their 5' regions. Indeed, comparison of the divergent 5' regions show no significant similarity between the two sequences. As opposed to the two other cDNAs, no polyadenylation signal was found in the 3' untranslated region (UTR) region of the *Sf-cobatoxin* cDNAs. However, since only the open reading frame (ORF) sequence is available for the *G. mellonella* cobatoxin-like (GenBank AF453824), we do not know if the absence of a polyadenylation signal is a feature of this gene family. In all *S. frugiperda* expressed sequence tag (EST) clones, a putative ORF is present (diagram, Fig. 1A; sequences in Fig. 1B). However, the ORFs for the two *Sf-cobatoxin* cDNAs may be shorter than that predicted. Indeed, if the sequences are compared with their *G. mellonella* homologue, the actual ORF may correspond only to the last 105 nt of the

sequences and be the same for the two clones (shaded boxes in diagram, Fig. 1A).

The three *S. frugiperda* predicted peptides each contains six cysteine residues that are probably involved in intramolecular disulfide bonds. They all present similarities with the arthropod defensins signature (Prosite signature: C-x(2,3)-[HN]-C-x(3,4)-[GR]-x(2)-G-G-x-C-x(4,7)-C-x-C) when compared with domain databases [database of protein families and domains (Prosite), protein families database of alignments (PFAM) and database of structural classification of proteins (SCOP)]. In contrast to other known insect defensins, they also present to some extent the scorpion short toxins signature (Prosite signature: C-x(3)-C-x(6,9)-[GAS]-K-C-[IMQT]-x(3)-C-x-C). Although unusual, the similarity between scorpion toxins and insect defensins is not surprising: both use a similar CSαβ motif folding pattern, suggesting that these molecules may have a common ancestor with a similar structural organization. Among the *S. frugiperda* predicted peptides, only spodoptericin presents a stretch of 10 amino acids (aa) between the first two cysteine residues,

comparable to what is found in other insect defensins (alignment in Fig. 2A). Nevertheless, based on their amino acid sequences, the three *S. frugiperda* predicted peptides might be considered as defensin-like peptides.

Sequence and features of the predicted peptides are shown in Fig. 1B. As mentioned previously, Sf-cobatoxin is probably shorter than predicted if compared to the sequence available for the *G. mellonella* homologue (57% identity). Data in Fig. 1B are given for all predicted sequences, although only the most probable 35 aa predicted Sf-cobatoxin peptide will be discussed here (Fig. 1B, bolded sequence). Spodoptericin and Sf-gallerimycin contain a putative hydrophobic signal sequence and are most likely cleaved. For all three proteins, other potential cleavage sites located two amino acids before the first cysteine residue were determined by aligning the sequences with known insect defensins (Fig. 1B, peptides). Cleavage would result in the formation of predicted mature peptides of 36, 32 and 32 amino acids for spodoptericin, Sf-gallerimycin and Sf-cobatoxin, respectively, which is consistent with the length of other known insect

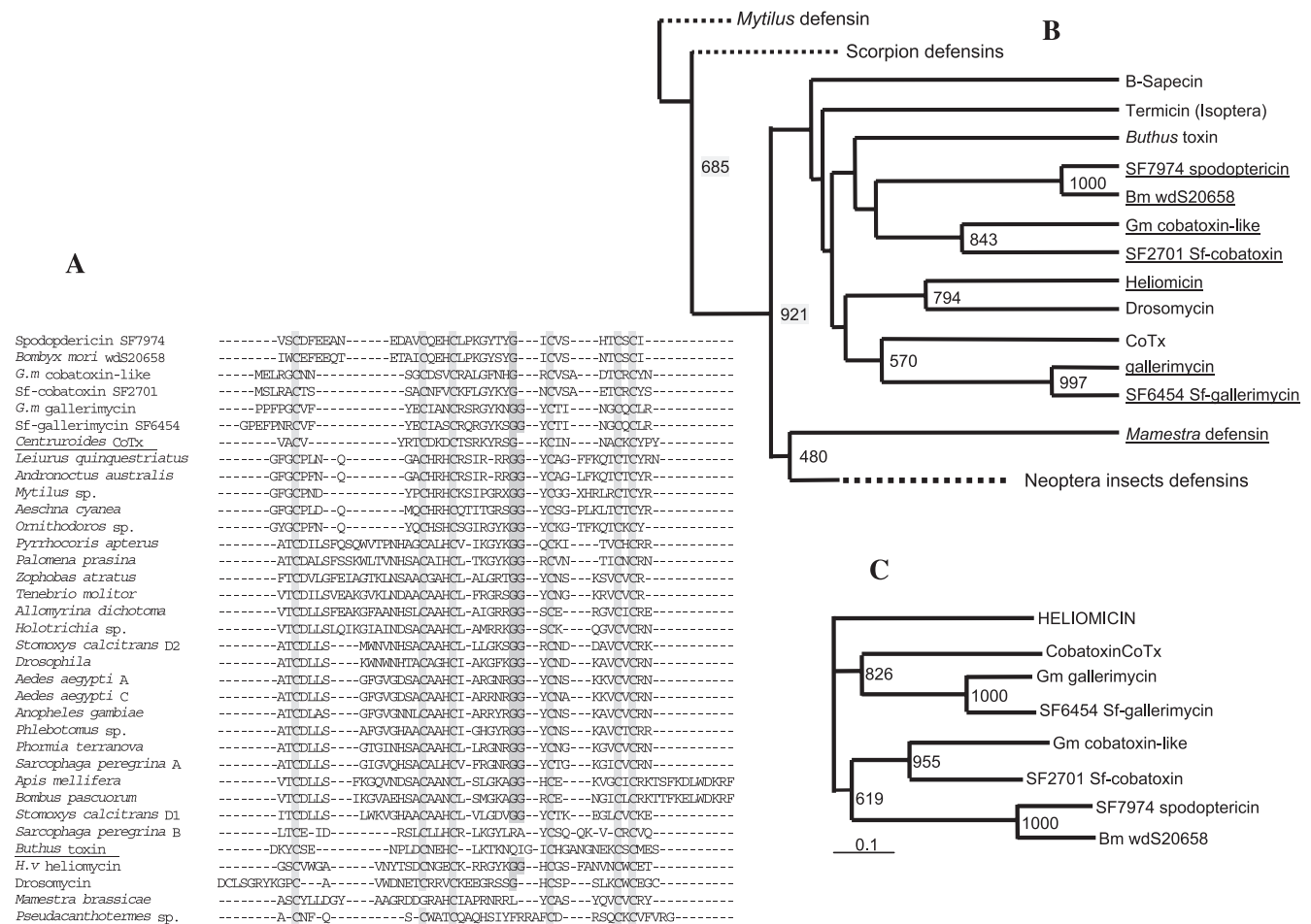


Fig. 2. Phylogenetic analysis of cysteine-rich defensin-like peptides. (A) ClustalX alignment of several arthropod defensins, two scorpion toxins (underlined) and the lepidoptera peptides using the cysteines as landmarks (highlighted). (B) Simplified phylogenetic tree generated from the sequences aligned in (A) using the neighbor-joining method with the mussel *Mytilus* sp. defensin defined as outgroup. (C) Neighbor-joining tree generated from the lepidopteran cysteine-rich peptides and cobatoxin (CoTx) sequences with heliomicin defined as an outgroup. Bootstrap values (of 1000 replicates) are indicated at the nodes of branches when >480. The scale at the bottom of (C) corresponds to 10% divergence between sequences.

defensins. Based on calculated pIs, Sf-gallerimycin and Sf-cobatoxin are predicted to be cationic, with or without cleavage, which is in agreement with insect defensins. Interestingly, spodoptericin is predicted to be anionic. Cationic peptides are more often antibacterial whereas neutral peptides are more often antifungal (Hetru, personal communication), therefore, spodoptericin is a novel anionic cysteine-rich peptide for which the function is still unknown.

3.2. Phylogenetic analysis of the lepidopteran defensin-like peptides

To analyze the lepidopteran defensin-like family, a large array of arthropod defensins and some scorpion toxins were aligned using ClustalX then manually using the cysteines as landmarks (Fig. 2A). Analysis of the *Bombyx mori* SilkBase EST library (<http://www.ab.a.u-tokyo.ac.jp/silkbase/>; Mita et al., 2002) allowed us to identify a silkworm homologue of the spodoptericin gene (clone wdS20658), which was added in the alignment (Fig. 2A). A bootstrapped neighbor-joining tree was then generated from the aligned sequences using the same software (Fig. 2B).

When the tree was rooted on the mussel sequence by defining this sequence as an outgroup, the sequences were split into two groups, the scorpion defensins and the other sequences (Fig. 2B). The latter was further split into two subsets, the branching being supported by a high bootstrap value (921 of 1000 replicates). One subset grouped the previously described defensins from neopteran insects (Hemiptera, Coleoptera, Diptera and Hymenoptera) and included the *M. brassicae* defensin. The second subset grouped the cysteine-rich lepidopteran peptides from *S. frugiperda*, *B. mori* and *G. mellonella* including *H. virescens* heliomicin and scorpion toxins (*Buthus* and *Centruroides* spp.). Interestingly, spodoptericin and its silkworm homologue appear more related to cobatoxin-like peptides than to gallerimycin peptides, the latter being more related to scorpion cobatoxin than cobatoxin-like peptides themselves. This result was confirmed by the high bootstrap values obtained when only lepidopteran cysteine-rich peptides were analyzed, with heliomicin defined as an outgroup (Fig. 2C).

It thus appears that *S. frugiperda* peptides belong to families of defensin-like molecules that are widespread within the Lepidoptera order. They form a cluster distinct from the previously described insect defensins and are characterized by some degree of similarity, at a sequence level, to short scorpion toxins. As suggested by our tree, *H. virescens* heliomicin is included in this defensin-like group whereas *M. brassicae* defensin, in spite of five instead of six cysteine residues, is more related to the other neopteran insect defensins. It is noteworthy that two forms of defensins are also present in the diptera *Sarcophaga peregrina*, with one of the two forms, sapecin-B, also showing structural similarity to potassium-channel-blocking scorpion toxins (Yamada and Natori, 1993). Note that sapecin B groups with the lepidopteran defensin-like peptides in our analysis.

3.3. Transcription analysis of the *S. frugiperda* defensin-like genes

Control experiments showed that all three genes were constitutively expressed in Sf9 cells although at a low level (data not shown). In *S. frugiperda* larvae, in absence of immune challenge, *Sf-gallerimycin* transcripts are detected during the last larval instar, *spodoptericin* transcripts during all larval development at low level, and the *Sf-cobatoxin* gene is essentially transcribed during the two last larval instars (data not shown). The constitutive expression of the *Sf-cobatoxin* gene is higher than that of the other two genes, particularly in the fat body. In diptera, spontaneous expression of defensin has often been reported (Dimarcq et al., 1994; Lowenberger et al., 1999). As induction of transcription often occurs at the onset of pupal development, it has been suggested that these genes may have a dual role in defense and development (Matsuyama and Natori, 1988).

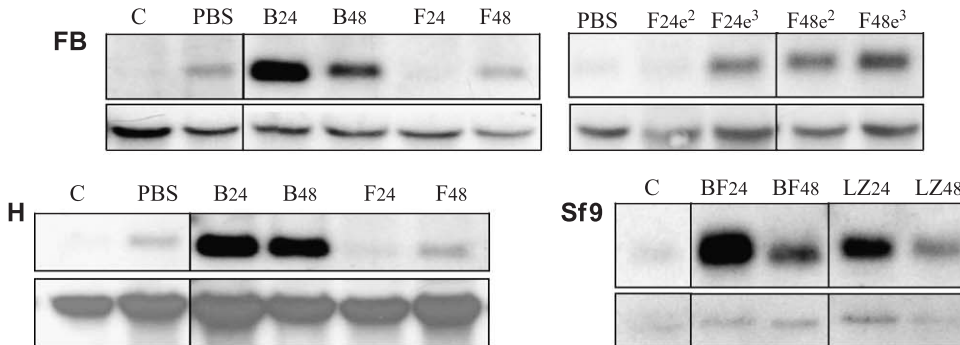
Since the three *S. frugiperda* genes are putatively antimicrobial genes, we analyzed their response following immune challenge in insects as well as in cells in culture. In insects, transcription was analyzed in two immune tissues of 5th instar larvae, i.e., hemocytes and fat bodies. Immune challenge consisted in injection of live bacteria or fungi. For bacterial challenge, Gram-negative and Gram-positive bacteria were injected together to ensure activation of one of the two regulation pathways, Toll and Imd, leading to expression of antibacterial genes. The effect of polydnavirus infection was also analyzed since these viruses are immune-suppressive parasitoid wasps symbionts (Webb, 1998). They have been previously shown to inhibit translation, but not transcription, of antibacterial lysozyme genes (Shelby et al., 1998). In all three genes examined, injections of HdIV alone did not induce an increase in transcription (data shown for the spodoptericin, Fig. 3C, panel H, lane V).

After immune challenge, the *Sf-gallerimycin* gene exhibited a significant up-regulation. Increases in the expression of the two other *S. frugiperda* cysteine-rich genes after a bacterial or fungal challenge are less pronounced, suggesting that they are up-regulated to a lesser extent than the *Sf-gallerimycin* gene.

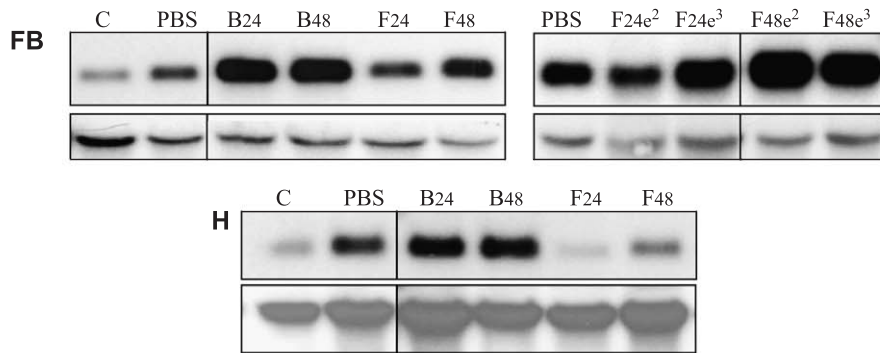
3.3.1. The *Sf-gallerimycin* gene is significantly up-regulated after bacterial challenge

In the caterpillar, injection of *E. coli* and *B. subtilis* induced a strong up-regulation of the *Sf-gallerimycin* gene 24 h after immune challenge (Fig. 3A, panels FB and H, lanes B24 and B48). Transcription of this gene occurred essentially in the fat body cells. Conversely, injection of *B. bassiana* did not significantly increase the level of transcription compared to saline-injected larvae (Fig. 3A, panels FB and H, lanes F24 and F48). A slight increase was observed in the fat body 48 h post-injection (pi). We thus investigated if this response was related to the dose of conidia injected. We found that, in fat body cells, 24 h pi, a response was observed for the higher dose only (Fig. 3A, panel FB, lane F24e³) whereas 48 h pi,

A- Sf-Gallerimycin



B- Sf-Cobatoxin



C- Spodoptericin

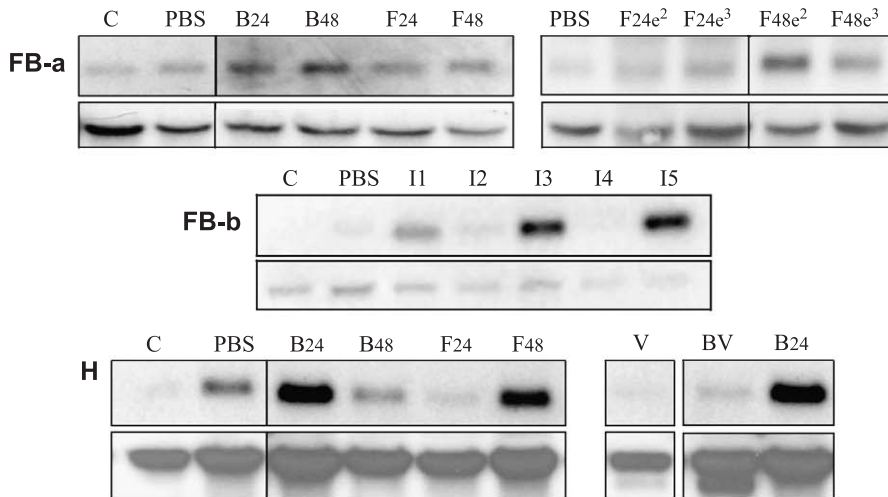


Fig. 3. Effects of immune challenge on transcription of (A) Sf-gallerimycin, (B) Sf-cobatoxin and (C) Spodoptericin. Assays were conducted with *Spodoptera frugiperda* 5th instar larvae fat body (panels FB) and hemocytes (panels H). One-day-old larvae were immune-challenged by injection of either a mix of Gram+ and Gram- bacteria (lanes B) or *B. bassiana* conidia (lanes F). To examine the dose-dependent effects of *B. bassiana* injection on transcription in the fat body, 8×10^2 (lanes Fe²) or 8×10^3 conidia per insect (lanes Fe³) were injected. Lanes C and PBS indicate nonchallenged and PBS-injected controls. All samples were collected and processed 24 (lanes B24 and F24) and 48 (lanes B48 and F48) h postinjection and subsequently hybridized successively with cDNA probes. An actin probe was used to quantify initial amounts of RNA loaded per lane (lower panels). In (A) Sf-gallerimycin, panel Sf9 shows up-regulation of *Sf-gallerimycin* transcripts in Sf9 cells after an immune challenge. Cells were treated either with a suspension of heat-inactivated Gram+, Gram- bacteria and *B. bassiana* conidia (lanes BF) or with a suspension of LPS and zymozan (lanes LZ). Samples were collected 24 and 48 h after treatment. Lane C indicates the nonchallenged control. In (C) spodoptericin, panel FB-b shows results obtained from individual 5th-instar larvae 48 h after injection of a mix of bacteria and *B. bassiana* (lanes I1–5). In panel H, results are shown from HdIV-infected larvae without (lane V) or 24 h after challenge with bacteria (lane BV).

both doses resulted in higher levels of transcripts (Fig. 3A, panel FB, lanes F48e² and F48e³). It is noteworthy that in natural *B. bassiana* infections, the blastospores and hyphae, and not the conidia, are in direct contact with the hemocoel (Vey and Fargues, 1977). This suggests that the gene up-regulation seen here could be related to the presence of the main development stage of *B. bassiana*, which invades the host cavity, e.g., the blastospores.

In immune-challenged Sf9 cells, *Sf-gallerimycin* transcripts were detected in large amounts 24 h after presence of a mixture of heat-inactivated bacteria and fungi, or after addition of LPS and zymozan (Fig. 3A, panel SF9). Induction of antimicrobial peptides in cell lines has previously been reported in insects. For example, upon immune stimulation with heat-killed bacteria, hemocyte-derived mosquito cell lines secrete different classes of immunity factors, including defensins (Fallon and Sun, 2001; Gao et al., 1999).

3.3.2. The *Sf-cobatoxin* gene is constitutively expressed and up-regulated following bacterial challenge in fat body cells

In the *S. frugiperda* larvae, the *Sf-cobatoxin* gene is constitutively well expressed, and is up-regulated by saline injection only (Fig. 3B, lane PBS). Note that septic injury by itself is known to induce transcription, albeit at low levels, of antibacterial peptides in *Drosophila* (Hoffmann and Reichhart, 2002). Following bacterial injection, the *Sf-cobatoxin* gene was up-regulated essentially in the fat body (Fig. 3B, lanes B24 and B48). Hemocyte response to bacterial challenge, if compared to saline injection, was not significantly increased (Fig. 3B, panel H, compare lanes PBS and B24). As for the *Sf-gallerimycin* gene, injection of *B. bassiana* induces an increase of *Sf-cobatoxin* transcription, after 48 h, in the fat body (Fig. 3B, lanes F24 and F48), and response levels appear to be correlated to the dose of injected conidia.

3.3.3. The *spodoptericin* gene exhibits low levels of induced transcription after immune challenge

In the caterpillar fat body, after bacterial challenge, little difference between saline- and germ-injected samples could be seen for *spodoptericin* transcription (Fig. 3C, panel FB-a, compare lanes PBS and B24, increase of approximately 2-fold). These results are consistent with previous reports that defensin genes often exhibit lower levels of infection-induced transcription compared to other antibacterial peptide genes (Dimarcq et al., 1994). As for the previous two genes, injection of *B. bassiana* induced a slight increase in gene transcription mainly after 48 h. We completed several tests using isolated caterpillars injected with a mix of bacteria and fungi and found a great inter-individual variability in the response (Fig. 3C, panel FB-b). This could explain a lower overall response when pooled larvae are analyzed.

In hemocytes, injection of bacteria led to an increase in the number of *spodoptericin* transcripts, although this increase is not clearly significant and may be related to a

larger amount of loaded RNA (Fig. 3C, compare lanes PBS and B24, *spodoptericin* and actin probes). Conversely to the other two genes, we found a strong hybridization signal in the hemocytes 48 h after injection of fungi (Fig. 3C, panel H, lane F48). Also in contrast to the other two genes (data not shown), HdIV infection of the hemocytes appeared to inhibit full inducibility of the *spodoptericin* gene (Fig. 3C, panel H, compare lanes BV and B24). Thus, our data suggest that, in addition to their known effects on cellular immune response (Webb, 1998), polydnviruses may also affect, to some extent, host gene transcription.

To conclude on the transcription of the three *S. frugiperda* defensin-like genes, the *Sf-gallerimycin* and *Sf-cobatoxin* are up-regulated, to variable degrees, mainly in the fat body cells, and essentially in response to a bacterial infection. The *spodoptericin* gene also undergoes up-regulation although it is not as prominent. *In vitro*, the induction of the *spodoptericin* and the *Sf-cobatoxin* genes was not detected (data not shown) but we found induction of the *Sf-gallerimycin* gene. This observation suggests that the necessary circulating receptors or pathogen recognition proteins known to be required for induction of antibacterial peptide genes (Choe et al., 2002; Michel et al., 2001) are present *in vitro* and that Sf9 cells contain sufficient amounts of specific necessary transcription factors. Indeed, differential regulation of Rel proteins, for example, can lead to preferential expression of specific target genes (Han and Ip, 1999).

3.4. Structure of the *S. frugiperda* genes encoding defensin-like peptides

To date, genomic data on defensin genes are still scarce and most of our knowledge pertains to dipteran genes. As opposed to genomes of flies and mosquitoes, arthropod genomes are still poorly characterized. Furthermore, most known peptides in arthropods have been identified from immune-challenged hemolymph by mass spectrometry so that no information on nucleic acid sequences is available. This led us to further characterize the *S. frugiperda* defensin-like genes since it should provide valuable information on this gene family.

3.4.1. Gene copy number in the *S. frugiperda* genome

Our hybridization results strongly suggest that the *spodoptericin* and *Sf-gallerimycin* genes are encoded by a single copy sequence (data not shown). Southern blot analysis of *Pst*I digested *S. frugiperda* DNA resulted in unique hybridization bands of ~ 10 and ~ 8 kbp for the *spodoptericin* and *Sf-gallerimycin*, respectively. This result was confirmed by probing our 10 × coverage BAC library with the *spodoptericin* coding sequence, resulting in 11 positive spots. Similar experiment on half of the BAC library resulted in seven positive spots for the *Sf-gallerimycin* probe. A single gene copy is also present in the *Drosophila* (Dimarcq et al., 1994) and the *Anopheles gambiae* (Eggleston et al., 2000) genomes, whereas *Aedes aegypti* displays two copies of this gene

(Lowenberger et al., 1999). Because of unexplained additional hybridization bands, no conclusions could be drawn for the *Sf-cobatoxin* gene.

3.4.2. Splicing

The *Drosophila* defensin gene displays no introns (Dimarcq et al., 1994) whereas one short intron is present in *A. gambiae* (Eggleston et al., 2000) and *A. aegypti* (Cho et al., 1997) genes (Fig. 4, upper diagram). In *S. frugiperda*, alignment of cDNA and PCR-amplified genomic sequences showed that the *spodoptericin*, *Sf-gallerimycin* and *Sf-cobatoxin* genes all contain one intron (597, 465 and 822 nt long, respectively) located within the ORF (GenBank # AY128091, AY236867 and AY236868), 8, 18 and 19 aa before the cysteine motif (Fig. 4, upper diagram). Note that *A. gambiae* and *A. aegypti* introns are located 21 aa upstream the cysteine motif. The positions within the coding sequence and length of introns are thus quite variable among the three *S. frugiperda* genes and differ from the mosquitoes defensin genes. Interestingly, in the *Sf-cobatoxin* gene, the 3' sequence shared by the SF2701 and SF6099 clones starts with the beginning of the second exon. Since the SF6099 sequence was amplified by PCR, it could be hypothesized that the RNA corresponding to SF2701 results from alternative splicing, the corresponding first exon being upstream from the SF6099 sequence. However, attempts to amplify a genomic sequence by PCR using a forward primer in the SF2701 sequence (46 nt upstream the SF2701 putative ATG codon) failed. Thus, because two *Sf-cobatoxin* cDNAs exist and share the second exon sequence, only one RNA is detected in Northern blot, and hybridization with genomic DNA leads to inconclusive results, the genomic structure of *Sf-cobatoxin* and the hypothesis of an alternative splicing will be further investigated in the near future.

3.4.3. cDNA upstream region analysis

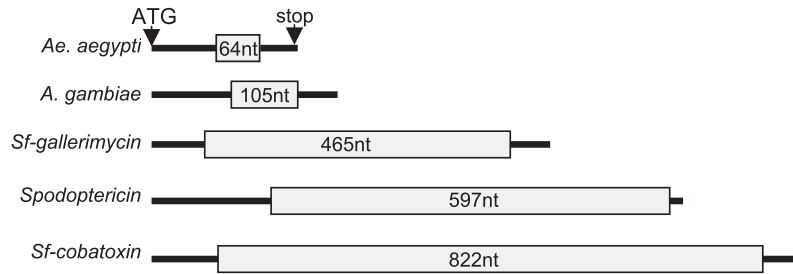
Partial promoter sequences were analyzed for the well-induced *Sf-gallerimycin* gene (GenBank # AY236867) and the poorly induced *spodoptericin* gene (GenBank # AY128091). In the *Sf-gallerimycin* sequence, a putative TATA box is located 26 nt upstream from the start of the cDNA sequence. Binding sequences for transcription factors known to be involved in the immune response were identified. A clear consensus NF- κ B binding site was found 250 bp upstream from the cDNA first nucleotide (nt) (Fig. 4A). Several GATA-binding factor 1 (GATA-1) motifs were also identified, as well as two binding sites for interleukin-6 response element binding protein (IL-6 RE-BP) (Fig. 4A). For the *spodoptericin* gene, the only found TATA box sequence was located in the SF9 cDNA 5'UTR. In contrast to the *Sf-gallerimycin* gene, no clear consensus binding site was found for NF- κ B transcription factor (Fig. 4B). A few low conserved GATA-1 and two dorsal putative binding sites were found approximately 500 bp upstream from the cDNA start (Fig. 4B). Interestingly, the upstream region contains a 39-nt imperfectly repeated sequence, which

includes a 10-nt conserved motif (Fig. 4B). TFSEARCH analysis of the complete gene, including introns, did not reveal other relevant transcription factor binding sites that could suggest how this gene is regulated. All three genes as well as the *Drosophila* and *Anopheles* defensin genes present several sites of factors possibly involved in development [for example, octamer-binding factor 1 (Oct-1), Brn-2, c-Myb, Deformed (Dfd), CdxA], but we are, at this point, unable to clearly interpret these data.

Thus, the two *S. frugiperda* defensin-like genes differ by their promoter sequences as well as by their response to immune challenge. Because of the presence of known *cis* regulatory elements, *Sf-gallerimycin* transcription is likely to be regulated by the classical pathways, Toll and/or Imd, known to control Rel/NF- κ B-like mediated immune antimicrobial response in insects. On the other hand, the absence of such consensus binding sites in the 700-nt upstream sequence of *spodoptericin* might indicate that the regulation of the transcription of this gene, which occurs at low level, depends on these pathways, and in this case, binding sites are more distant from the coding sequence, or depends on other pathways and/or other unknown transcription factors. Indeed, a number of unresolved questions in insect innate immunity remain and the possible existence of a third pathway, distinct from the known Imd and Toll pathways, has been proposed even in the well-known *Drosophila* immunity model (Hoffmann and Reichhart, 2002).

3.5. Conclusion

1. To our knowledge, this work is the first report on genomic and transcriptional characteristics of lepidopteran cysteine-rich defensin-like genes in fat bodies and hemocytes.
2. Three defensin-like *S. frugiperda* genes are described. Spodoptericin is a novel putative anionic peptide that exhibits low levels of induced transcription after immune challenge. In contrast, Sf-gallerimycin is a putative cationic peptide that is up-regulated in both immune tissues and Sf9 cells following immune challenge. Sf-cobatoxin is a putative cationic peptide, which exhibits high levels of constitutive transcription and is up-regulated in immune tissues following immune challenge.
3. These defensin-like genes are widespread in lepidopterans, with homologues found in at least two other species. They differ from previously described neoptera insect defensins and are characterized by their similarity with short scorpion toxins. This suggests the existence of two types of defensins in lepidoptera—as previously described in *S. peregrina*—one type similar to neoptera insect defensin (such as the *M. brassicae* defensin), the other more similar to short scorpion toxins.
4. On the basis of information provided by the partial promoter sequence analyses, the *Sf-gallerimycin* transcription could be regulated by the classical pathways known to control Rel/NF- κ B-like mediated immune



A. Upstream sequence of the *Sf-gallerimycin* gene

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c caatccccactcataaaaaacacatatcagtatatccttaatacactgtaatcctaatttacttagataag 72
a aattacatactgtatattgaataattataaaatgcaaaaattaaaggaaaatgaatgacaaacattgtaaa 144
c tgaatatttacctctaaccacagataacaagaacatacaatgataaactgttcacaataccaacaacacat 216
c accctgtttaagattcatacaaaacaaggataatcaactttaatctcgttctgatatagtggtaaattga 288
a tgacgaatacgggaatgtatcacttgcaattctatgccagaacagggactcagggattcccctccacataca 360
a tattaatgtggtagcgtatagtgcaagcgactgattgcacgggactgtttctcgggtatccatttccgctc 432
t gaacacgactgtaaaacggttacgagaatagtttagcagcaatgtatgtttaagtgaaatgtatttagatt 504
t ggtcgtgtgtttatcgatctggtggtatagtttgggtgaagtatacgtgggtctgagttagcataaaata 576
a aggtggagctggaaagtcgcacacattgaaaggtttcagtc ATG AAG GCT TGC GTG GTT CTC 639
                                     M K A C V V L 7

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B. Upstream sequence of the *Spodoptericin* gene:

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c ccgggaattgaaatattaatgcaattctttgccattgtgcccgatcgtgtggaggcgatgcagtccttg 72
c catagagttagataattcaaaaactgacaaaggattatgctaccatcacggtagaaaatctaagtaa 144
a gtaaagaaaactaagtaatggtaaaatggtgaatagacttcatagggtgaatacacacaggaatggggtta 216
a tagaattcaaaagggtgaatagatacaagaatgtgggaatagatgtaggtattttccaaaattttattc 288
a acccctgaattctctttacgacgttcaaattttttcacattataaaaaaatattcaaattcttcacc 360
g tttctttaattaatgctttgtaattaatgtgtgtgtcaacatgcattgttctagttaagtaaggtttg 432
t atgtacctttagacctaattaatacaagctaataaggtacgttttatatttgcacgtaataatattcacg 504
a tggtaggttttagtcggaatccaaattaataatcaagcaatacttatttaggtaaatatgctcgttgctt 576
t cctgttgatctaccataagcatgtaaaattgttattgttcgaacgagacagtaataaatagtttctaaat 648
a agtagaaacagacttgtaacctatgattaatttccgcgaacaatgtgacgacagtagtagtaaatcaatg 720
c attagtcattgcagtgccgagtggtcgtagtggtgtacctacgttcatattgactgtccactgacagc 792
t tgaatgcacaaaatataaggcgcgttctgttgaccttcatcattgtgtctgtctatttttgtgcccga 864
a tacgtcggctattcgttaattagctgtttaacaataaccacgttcatcttctgtatcaataattggcgca 936
c gaagaaata ATG GGT GTA AAG GTA ATA AAT GTG TTT CTG CTG ATT GCG GTG TCG 991
                                     M G V K V I N V F L L I A V S 15

```

Fig. 4. Genomic sequence analysis. The upper diagram shows intron position and length within the three *S. frugiperda* coding sequences compared to *A. aegypti* and *A. gambiae* defensin genes. (A) *Sf-gallerimycin* upstream sequence (AY236867). (B) *Spodoptericin* upstream sequence (AY128091). Start of cDNA sequences is indicated above sequence. Putative TATA boxes are boxed. NF- κ B consensus binding sites are highlighted; italics indicate a weak consensus. Predicted GATA-1 binding sites are in bold characters, and predicted IL6-RE are underlined. In the *spodoptericin* gene, horizontal arrows limit the two tandem repeats, the conserved 10 nt are in grey.

antimicrobial response in insects whereas regulation of the *spodoptericin* gene may rely on other unknown transcription factors.

5. Although the assessment of the antimicrobial activity of the three *S. frugiperda* peptides will require their purification and assays of inhibition of bacteria and

fungi growth, transcriptional analyses suggest that these genes do encode for antimicrobial peptides.

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