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INDUCTION OF ANTIMICROBIAL PEPTIDES IN INFECTED TISSUES OF LARVAL *HELIOTHIS VIRESCENS* (LEPIDOPTERA: NOCTUIDAE)

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ABSTRACT

Effective biological control of destructive heliothine larvae via application of microbial entomopathogens or parasitoids is limited by a robust immune response against infection and parasitization. To better understand the immune response of budworms against microbial entomopathogens, several orthologs of antibacterial response proteins were identified and extracted from a tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), expressed sequence tag database for study of their upregulation by bacterial infection. Transcript sequences corresponding to precursors of 3 antimicrobial peptides from *H. virescens* hemolymph (virescein, heliomicin and heliocin) have been identified and their induction by bacterial elicitation analyzed. Further, a gloverin-like antibacterial and an *H. virescens* ortholog of the Lepidopteran hemocyte-specific antibacterial Spod-X-Tox have been identified. Induction of these selected antibacterial protein transcripts by bacterial infection were quantitated using quantitative RT-PCR in hemocytes and fat bodies. Transcript levels of all were elevated by bacterial elicitation in both tissues as early as 3 hours post-treatment.

Key Words: antimicrobial peptides, antibacterial peptides, bacteria, infection, budworm, hemocyte, fat body, gloverin-like, lebocin-like, heliomicin, virescin, heliocin

RESUMEN

El control biológico eficaz de larvas destructivas de la subfamilia Heliothinae por medio de la aplicación de microbios entomopatógenos o parasitoides está limitada por una robusta respuesta inmune contra la infección y parasitismo. Para entender mejor la respuesta inmune del gusano bellotero contra microbios entomopatógenos, varios ortólogos de proteínas de respuesta antibacterianos fueron identificados y extraidos del gusano bellotero del tabaco, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) que expresa secuencias etiquetadas de base de datos para el estudio de su regulación positiva por la infección bacteriana. Las secuencias de transcripción correspondientes a los precursores de 3 péptidos antimicrobianos de la hemolinfa, virescein, heliomicin y heliocin de *H. virescens* fueron identificados y su inducción por elicitación bacteriana fue analizada. Además, un antibacteriano parecido de gloverin y un ortólogo de *H. virescens* antibacteriano Spod-X-Tox específico de los hemocitos de lepidóptero fueron identificados. Se cuantificaron la inducción de estas transcripciones de proteínas antibacterianas seleccionadas por la infección bacteriana por medio de RT-PCR cuantitativa en los hemocitos y cuerpos grasos. Todos los niveles de transcripción fueron elevados por elicitación bacteriana en ambos tejidos tan rápido como 3 horas después del tratamiento.

Palabras Clave: péptidos antimicrobianos, péptidos antibacterianos, bacterias, infección, gusano bellotero, hemocitos, grasa corporal, gloverin, lebocin, heliomicin, virescin, heliocin

The innate immune system of insects is generally described as having well-coordinated interacting humoral and cellular components, each of which express constitutive and inducible antimicrobial proteins (Lemaitre & Hoffmann 2007; Broderick et al. 2009). Insect antimicrobial peptides were among the first immune effectors to be characterized historically, and a large and growing number of peptides active against a taxonomically broad range of microorganisms have been isolated from a wide variety of insect species (Bulet et al. 2004; Lamberty et al. 1999). By consensus, known lepidopteran antimicrobial peptides are arranged into the families of defensins, cecropins and moricins, attacins, gloverins, and lebocins (Rayaprolu et al. 2010). Transcription of antimicrobial peptides within the fat bodies and other tissues is rapidly upregulated upon microbial detection by host PRRs, leading to activation of the signal transduction pathways, *toll* (fungi and gram-positive bacteria), or *imd* (gram-negative bacteria; Broderick et al. 2009; Ragan et al. 2009).

To better understand and disrupt the heliothine immune response against microbial entomo-

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pathogens, we have initiated a project to study the functional immunogenomics of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) by identification of antimicrobial response orthologs (Terenius et al. 2009; Shelby & Popham 2009; Shelby & Popham 2008). To this end, an *H. virescens* transcriptome database has been created by pooling the efforts of several laboratories (Terenius et al. 2009; Shelby & Popham 2009; Shelby & Popham 2008; Vogel et al. 2010; Govind et al. 2010; Clem et al. 2010; Breitenbach et al. 2011; Shelby & Popham 2012). Study of these assembled immune-stimulated *H. virescens* larval transcripts revealed many immune system effectors orthologous to other insects. A number of antibacterial and antifungal proteins of *H. virescens* orthologous to other insects also were mined from this database. Induction of transcripts encoding the antimicrobial peptides virescein, heliomicin, heliocin, gloverin, and Hv-3-Tox, in the major immune-responsive tissues of the fat bodies and hemocytes, by bacterial infection is reported here.

Materials and Methods

Insects

Heliothis virescens eggs were received from the North Carolina State University Dept. of Entomology (Raleigh, North Carolina) and reared individually on an artificial wheat germ-based diet under standard conditions of 14:10 h (L:D), 55% RH, and 28 °C (Popham et al. 2005). To document the pattern of antibacterial proteins stemming from immune-elicitation, qPCR measurements of the transcript levels of 6 transcripts was performed. To activate the antibacterial immune response, early 5th instar *H. virescens* larvae were immunized by septic puncture with a tungsten needle dipped into a suspension of heat killed *Micrococcus lysodeikticus/Escherichia coli* in phosphate-buffered saline (Shelby & Popham 2008). In order to eliminate the possibility that wounding per se affects transcript levels, mock controls received a sterile puncture. At each time point 0-24 h post infection (hpi) hemolymph was collected from 10 larvae bled through a punctured anterior proleg into ice cold phosphate buffered saline as described (Shelby & Popham 2008). Following hemocyte pelleting by centrifugation at 5000 × g for 4 min., plasma supernatant was removed to a separate tube. Fat bodies were dissected from the same larvae under extensive washes with phosphate buffered saline to dislodge adherent hemocytes (Terenius et al. 2009). All were stored at -85 °C for later use. Three or more complete, separate biological replicates were collected for each treatment time series.

Transcript Assembly and Analysis

Heliothis virescens transcripts were pooled from a variety cDNA libraries (Shelby & Popham 2009; Vogel et al. 2010; Govind et al. 2010; Shelby & Popham 2012; Breitenbach et al. 2011) and assembled. Clusters with genome ontologies indicative of immune response were examined and putative orthologs of insect antibacterial and antifungal proteins or peptides were selected for closer examination. Orthologs with full or near-full length sequences were retained for further analysis. Five full length transcripts encoding antimicrobial peptides were selected for further sequence and expression analysis: virescein, heliomicin, heliocin, gloverin, and Hv-3-Tox. Contigs were reassembled from single reads and subjected to further comparisons with their respective orthologs from several insect species (http://www.ebi.ac.uk/). All sequences used in this study have been deposited with NCBI GenBank (FJ546342.1, FJ546343.1, FJ546346.1, FJ546344.1, FJ161976.1, and FJ577645.1).

RNA Isolation and qPCR.

Total RNA was extracted from fat bodies and hemocytes using the RNeasy™ kit and reverse transcribed using the Omniscript® *Reverse Transcription kit* (www.qiagen.com) exactly as previously described (Shelby & Popham 2009). Quality and concentration of extracted RNA samples were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Delaware) and a Fragment Analyzer Automated CE System (Advanced Analytical Technologies, Ames, Iowa).

Reactions were carried out in a total volume of 20 µL using the cDNA synthase buffer, dNTP mix, oligo-dT, RT enhancer, Verso Enzyme mix and 5 µL of template RNA. The reaction was incubated at 37 °C for 60 min and inactivated at 95 °C for 5 min. The samples were either used immediately for qPCR or frozen at -20 °C until use. Forward and reverse primer pairs were designed from the transcripts discussed above to allow rapid and specific quantification of transcript levels following treatments (Table 1). PCR reactions were performed at 95 °C for 15 min followed by 50 cycles of 95 °C for 15 sec, 55 °C for 30 sec, and 72 °C for 30 sec using the Qiagen QuantiFast™ SYBR Green® qRT-PCR Kit (www.qiagen.com). All reactions were performed in duplicate and the ribosomal protein L4 (*Hv*RPL4) internal control normalized expression ratio was calculated using the $2(-\Delta\Delta C_T)$ method (Livak & Schmittgen 2001) exactly as previously described (Shelby & Popham 2009). A Holm-Sidak multiple comparison was used with a p value of less than 0.05 considered significant. All results are a composite of at least 3 independent biological replicates, where quantitative data represent the mean ± SEM.

Results

Some of the data on sequence analyses are presented in supplementary material for this article in Florida Entomologist 97(3) (2014) online at

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TABLE 1. LIST OF PRIMERS USED FOR AMPLIFICATION OF TRANSCRIPT LEVELS OF ANTIMICROBIAL PEPTIDES IN THIS STUDY.

Target	NCBI #	Forward	Reverse	Reference
Heliomicin	FJ546343	TGTCAACTACACCTCCGACTGCAA	TCGCACCAACAGTTGCACTTAGCG	This report
Virescein	FJ546342	TTGTTGTGATTGCTCTGCTGGTGC	CCGGAGTCCTTTGCCAATTGCTTT	Bulet *et al.* (unpub.)
Heliocin	FJ546346	TGATCATCCGATCCTGCCTTCAGT	TCCAGTATCCCGGCTTGAAGAAGA	Bulet *et al.* (unpub)
Gloverin	FJ546344	AACCGATGGAATGGT TCCAGGCT	AGAATCGTCAGTGCTTCCCAAGGT	This report
Hv-3-Tox	FJ161976	TTTCCGAAATGCAGAAGGAGTGCC	ACCAGTGAAAGTGAGTCTGCGACA	This report
Serpin	FJ577645	GCATCATCGGACCTGGCAATTGAT	ATCATAATCAGCTGGAGCTGGGCT	This report
HvRPL4	EU523116	AGATGCTGAACGTGGACAAGCTGA	TTCAGCGGGTTGAGTTCCTGGTA	(Shelby & Popham 2008)

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Assembly and Analysis of Antimicrobial Protein Sequence Expression

From the *H. virescens* sequence database, a 664 base pair contig encoding a 199 amino acid polypeptide with 3 CS- αβ motifs highly homologous to the X-Tox proteins isolated previously from other Lepidoptera. In accordance with proposed nomenclature of this novel class of lepidopteran antimicrobial peptides was named Hv-3-Tox (NCBI accession# FJ161976.1). Multiple sequence alignment with other lepidopteran orthologs demonstrated that the position of the cysteine residues within each toxin cassette was exactly conserved (Suppl. Fig. S1A). However, much lower amino acid similarity was observed within each of the toxin cassettes (Suppl. Fig. S1B). Excluding the much longer *Spodoptera frugiperda* Spod-11-Tox from the alignment substantially improved the alignment of Hv-3-Tox with the remaining orthologs (Suppl. Fig. S1C).

Virescein was among the 5 antimicrobial peptide orthologs identified within the *H. virescens* transcriptome database. Virescein is a moricin-like 41 amino acid peptide sequence isolated and sequenced by Bulet et al. (2004) (NCBI accession #P83146) from immune-stimulated *H. virescens* hemolymph. A 682 base pair contig encoding the 66 amino acid virescein precursor was identified within the *H. virescens* transcriptome (Suppl. Fig. S1A; NCBI# FJ546342.1). When other lepidopteran moricin-like sequences were recovered from NCBI, and with aligned the *H. virescens* precursor polypeptide, the mature peptides were found to be closely homologous throughout their length (Suppl. Fig. S2B).

Heliomicin is an antifungal defensin peptide previously isolated from hemolymph of *H. virescens* larvae (NCBI#P81544). A 552 base pair contig encoding the full length transcript of the 60 amino acid heliomicin precursor was identified (Fig. S3A; NCBI# FJ546343.1). The 44 amino acid heliomicin peptide sequence within this precursor coincided exactly with the P81544 peptide sequence, containing the 6 cysteine residues within the CS-αβ motif characteristic of this protein family. Multiple sequence alignment revealed extremely close homology with the *G. mellonella* galiomicin precursor and trimmed peptide with exact alignment of the cysteine residues (Suppl. Fig. S3B).

A lebocin-like peptide was purified from *H. virescens* plasma by Bulet et al. (2004) (NCBI#P83427) which they named "heliocin". Their nomenclature will be accepted for the purposes of this report. A 618 base pair contig encoding the 122 amino acid heliocin precursor was identified within the EST sequence database (Suppl. Fig. S4A; NCBI# FJ546346.1). The polypeptide precursor encoded the 22 amino acid mature heliocin peptide with the predicted PTXXP glycosylation site (Suppl. Fig. S4A). Multiple sequence alignment of the *H. vire-*

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scens heliocin transcript with other lepidopteran orthologs revealed extremely close sequence homology to lebocins which have been isolated from other species (Suppl. Fig. S4B).

A 718 base pair contig encoding a gloverin-like sequence was identified (Suppl. Fig. S5A; NCBI# FJ546344.1). The 173 amino acid polypeptide encoded 2 tryptic peptides isolated from a two-dimensional polyacrylamide electrophoretic gel of plasma from bacterially induced *H. virescens* larvae (UniProtKB accession #P86358; Suppl. Fig. S5A) confirming translation from this or related transcripts in immune-stimulated larvae. Multiple sequence alignment with other lepidopteran gloverin-like antimicrobial peptides demonstrated that the *H. virescens* peptide was most similar in sequence to those isolated from *Plutella xylostella* and *Trichoplusia ni* (Suppl. Fig. S5B).

A serpin-like transcript was selected to serve as an immune-responsive control (NCBI#FJ577645.1). The predicted amino sequence (Suppl. Fig. S6A) when aligned with other lepidopteran serpins demonstrated that the *H. virescens* peptide was most similar in sequence serpins previously isolated from *Plutella xylostella* and *Trichoplusia ni* (Suppl. Fig. S6B).

Induction of Antimicrobial Protein Transcripts

Transcript levels of all 5 antimicrobial peptides were quantitated in fat bodies and hemocytes of 5th instar *H. virescens* larvae before, and following septic puncture using quantitative RT-PCR (qPCR; Fig. 1). Transcripts of all 5 measured antimicrobial peptides were undetectable in fat bodies and hemocytes of control larvae that received a sterile puncture wound. Primers used for these amplifications are shown in Table 1.

Hv-3-Tox transcript levels were significantly induced by bacterial infection in the hemocytes and fat bodies at roughly equal levels (Fig. 1). Peak transcript levels were observed in hemocytes at 6 hpi. Virescein transcript levels in bacterially elicited *H. virescens* larvae were the highest measured relative to control uninduced larvae (Fig. 1). Expression levels were roughly equal between hemocytes and fat bodies, but peaked at 6 hrs post-infection (hpi) in hemocytes ($F_{5, 17} = 2.851$; $P < 0.05$). Expression levels declined in both tissues by 18 hpi. Heliomicin transcripts quantitated by qPCR showed increased levels within 3 hpi, peaking at 6 hpi in hemocytes and at 9 hpi in fat bodies ($F_{5, 17} = 4.041$; $P < 0.015$; Fig. 1). Heliomicin transcript levels were most evident in hemocytes compared to fat bodies, with peak expression at 6 hpi, persisting at elevated levels out to 18 hpi (Fig. 1). Heliocin was significantly induced by bacterial infection in fat bodies ($F_{5, 17} = 8.403$; $P < 0.01$) and to a lesser degree in hemocytes ($F_{5, 17} = 3.829$; $P < 0.02$) peaking at 6 hpi in the former and 9 in the latter tissue. Fat body expression persisted at high levels even at 18 hpi where hemocyte expression had already declined almost to control uninduced levels (Fig. 1). Gloverin-like antimicrobial peptide transcripts exhibited a different pattern of expression, being nearly equal in both tissues at all time points post-infection (Fig. 1). However induction was already present at high levels as early as 3 hpi in fat bodies and hemocytes. Expression of serpin transcripts was observed primarily in hemocytes and was rapidly induced by immune stimulation, remaining at high levels for all time points sampled (Fig. 1). A slower induction of serpin transcription was observed up to 12 hours post stimulation in hemocytes (Fig. 1).

Discussion

Heliothine moths are major polyphagous pests of commodity crops such as maize, cotton, soybeans and vegetables throughout the world (Gordon et al. 2009; Oppenheim & Hopper 2009). Control of larvae of the North American pest moth *Heliothis virescens*, also known as the tobacco budworm, and other closely related heliothines would be fundamentally advanced by understanding and disrupting the basic mechanisms underlying resistance to entomopathogens and parasitoids used for the biological control of these pests. To identify candidate genes involved in resistance to microbial entomopathogens as possible immunosuppressive targets a survey of the transcripts expressed in hemocytes and other tissues of *H. virescens* was undertaken (Shelby & Popham 2009; Shelby & Popham 2012; Breitenbach et al. 2011). For these experiments hemocytes were selected because in addition to being first responders to infection, they can be collected quickly and easily with very low contamination by other tissues. Selected full length open reading frames were identified within the transcriptome database and were subjected to expression analysis using qPCR. However, fat bodies being the other major immune-responsive tissue, these also were dissected from larvae and monitored by qPCR for expression of the same antimicrobial genes. Larvae were challenged with bacterial and baculoviral microbial infections. Per os infection with the baculovirus HzSNPV showed little effect upon transcript levels of prophenoloxidases-1 and -2 (Shelby & Popham 2008), hemolin (Terenius et al. 2009), or of scolexin, growth blocking peptide binding protein, or C-type lectin (Shelby & Popham 2009). In contrast, transcript levels of the antimicrobial immune responders scolexin-B, growth-blocking peptide binding protein, C-type lectin (Shelby & Popham 2009), and the lepidopteran-specific hemolin (Terenius et al. 2009), were rapidly induced to high levels by bacterial elicitation, or showed a slightly elevated response to infection, *e.g.* prophenoloxidase-1 (Shelby & Popham 2008). Lysozyme transcription and translation in *H. virescens* larvae also were upregulated by bacterial infection (Shelby et al. 1998), but in addition were elevated by injection of *Cam-*

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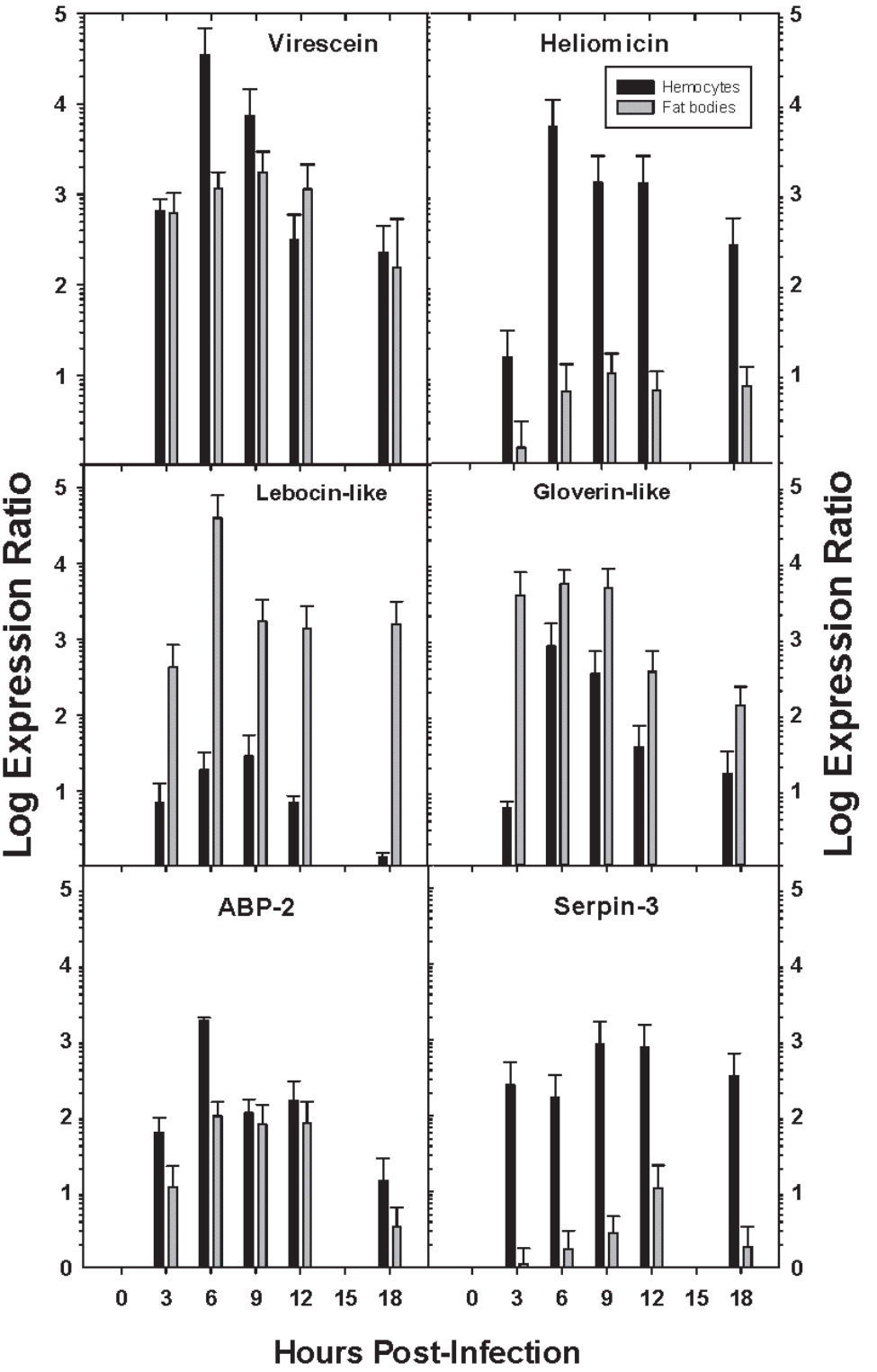


Fig. 1. Effect of bacterial infection on 5th instar *Heliothis virescens* hemocyte and fat body antibacterial peptide transcript levels. Quantitative PCR Expression data were normalized to the expression of ribosomal protein L4 (*Hv*RPL4). Mean of at least 3 biological replicates (± SEM) at each time point. Hemocyte transcript levels (black bars); fat body transcript levels (gray bars).

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poletis sonorensis ichnovirus (Nusawardani et al. unpublished). Bacterial induction of lysozyme and cecropin transcripts also was inhibited by *Cotesia plutella* bracovirus infection of factitious host larvae of *Spodoptera exigua*, but not in larvae of the normal host *Plutella xylostella* (Shrestha et al. 2009).

The X-Tox peptides are defensin-related antimicrobial peptides found only in Lepidoptera which encode variable repeats of a cysteine stabilized alpha-beta sheet (CS-αβ) scorpion toxin related motif (Destoumieux-Garzon et al. 2009). An earlier EST effort identified Hv-3-Tox in transcripts from immune-stimulated larval *H. virescens* hemocytes (Shelby & Popham 2009). This transcript encoded only 3 of the CS-αβ toxin motifs along its length. The *S. frugiperda* ortholog of this protein, encoding 11 CS-αβ-motifs (Girard et al. 2008, Destoumieux-Garzon et al. 2009) was expressed solely in the granulocytes and plasmatocytes (Destoumieux-Garzon et al. 2009). However, equivalent expression levels of Hv-3-Tox transcripts in *H. virescens* fat bodies and hemocytes were observed. Despite great care taken during dissections of fat bodies it is likely that substantial numbers of hemocytes remained adherent and thus all fat body mRNA samples are likely to contain hemocyte transcripts as well. Further experiments must resolve the tissue-specificity of Hv-3-Tox expression in *H. virescens*.

Several additional antimicrobial immune-response transcripts have recently been isolated and identified in the tissues of immune-stimulated *H. virescens* larvae (Shelby et al. 1998; Shelby & Popham 2008; Terenius et al. 2009; Shelby & Popham 2009). Immune-stimulated antimicrobial or antifungal proteins and peptides also have been isolated from larval *H. virescens* hemolymph such as cecropins (P83413, P83414, P83415), heliocin (P83427; Gennaro et al. 2002), attacin (P86359), virescein (P83416), and heliomicin (P81544; Lamberty et al. 1999). Herein is reported the identification and expression analysis of the transcript sequences encoding virescein, heliomicin and heliocin. A separate peptide isolated from immune-stimulated larval *H. virescens* plasma, virescin (P86354; Chung & Ourth 2000) matches a transcript annotated as "chemosensory protein" but was not included in the present survey of antimicrobial peptide expression. Microarray experiments demonstrated induction of larval *H. virescens* antimicrobial peptides and other immune system components by lipopolysacharides and by laminarin (Chung & Ourth 2000). Their confirmatory qPCR experiments performed with lipopolysacharides-injected larvae showed that fat body and hemocyte transcripts of lysozyme, gloverin-like, cecropin, attacin and lebocin-like (here named "heliocin") peptides were induced with temporal expression profiles slightly delayed from those in this report. However, RNA-seq experiments conducted with bacterial and fungally induced *H. virescens* tissues demonstrated upregulation of cecropins, Hv-3-Tox, gloverin-like, heliocin, heliomicin, virescin, and virescein transcripts (Shelby & Popham 2012). The confirmatory qPCR studies in this report demonstrate that these antibacterial peptide transcripts were upregulated by the same treatments of *H. virescens* larval fat bodies and hemocytes.

The defensin-related peptide heliomicin was first isolated from *H. virescens* hemolymph by Lamberty et al. (1999). Chromatographically purified heliomicin exhibited only antifungal activity even though, as in this report, its synthesis was induced by septic injury with a suspension of gram positive and gram negative bacteria (Lamberty et al. 1999). Two other antimicrobial peptides were isolated from immune-stimulated *H. virescens* hemolymph by the same group: virescein and heliocin. Virescein is a moricin-like antibacterial peptide, while heliocin is a lebocin-like glycosylated antibacterial peptide (Gennaro et al. 2002). The identification of transcripts for all 3 of these previously isolated *H. virescens* peptides allowed expression analysis to be performed using qPCR. Further, identification of a gloverin-like antibacterial peptide transcript, as well as cecropins and attacins (Vogel et al. 2010) has demonstrated all major classes of antibacterial/antifungal peptide families are present in *H. virescens*. Induction of all 4 antimicrobial peptides was observed in the primary immune-responsive fat bodies and hemocytes of bacterially elicited *H. virescens* 5th instars.

These findings extend the generalization of inducible humoral innate immune reactions to bacterial infections of the polyphagous larvae of *H. virescens*, a major pest of many North American crops. Possession of the full transcript sequences encoding primary immune responders such as the sentinel effectors and antimicrobial peptides of *H. virescens* (Terenius et al. 2009; Shelby & Popham 2009; Vogel et al. 2010) and of *Helicoverpa armigera* (Pauchet et al. 2009; Wang et al. 2010), will enable studies of the role of immunity leading to improved microbial entomopathogen control of the heliothines.

Conflict of Interest

The author declares no conflict of interest.

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