Available online at www.sciencedirect.com
ScienceDirect
Journal of Invertebrate Pathology 98 (2008) 218–227
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Detection of genes encoding antimicrobial peptides in Mexican strains of *Trichoplusia ni* (Hübner) exposed to *Bacillus thuringiensis*

P. Tamez-Guerra [a,*], J.A. Valadez-Lira [a], J.M. Alcocer-González [a], B. Oppert [b], R. Gomez-Flores [a], R. Tamez-Guerra [a], C. Rodríguez-Padilla [a]

[a] *Departmento de Microbiología e Inmunología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León (FCB-UANL), AP 46-F, 66450 San Nicolás de los Garza, N.L., Mexico*
[b] *USDA-ARS Grain Marketing and Production Research Center, 1515 College Avenue, Manhattan, KS 66502, USA*

Received 15 October 2007; accepted 13 February 2008
Available online 20 February 2008

Abstract

The systemic immune response of *Trichoplusia ni* after *Bacillus thuringiensis* (Bt) exposure was evaluated by comparing the expression of genes encoding antimicrobial peptides (AMPs) in Bt-susceptible and -resistant *T. ni* strains that were either exposed or not to XenTari® (Bt-XT). AMP genes were detected by RT-PCR using primers for attacin, gloverin, lebocin, lysozyme, and peptidoglycan recognition peptide (PGRP). In general, AMP genes were detected more frequently in Mexican field strains previously exposed to Bt (SALX and GTOX) than in a Mexican laboratory strain (NL), but expression was similar to the AMP expression in USA laboratory strains (US and USX). Among the AMPs, transcripts for lebocin were the least detected (11.7%) and those for lysozyme were the most detected (84.8%) in all samples. Lebocin was detected only in 2nd instar and pupa. All untreated controls expressed attacin. Attacin and gloverin were not detected in any midgut sample, and their highest detection was in pupa. Lysozyme was rarely detected in 2nd instar larvae from any strain or treatment but was detected in almost all midgut and hemolymph samples. Overall, AMPs were found more in *T. ni* strains previously exposed to Bt-XT, especially lebocin and globerin (1.8-fold increase) and PGRP (3.8-fold increase). The data suggest that the expression of AMPs in *T. ni* correlates to previous Bt exposure.

Keywords: *Trichoplusia ni* immune response; *Bacillus thuringiensis*; Antimicrobial peptides; Bt-susceptibility

1. Introduction

Insects represent three-fourths of all animal species and have confronted many potentially pathogenic microorganisms, including those used in pest control. However, insects have developed protective mechanisms to evade the pathogenic effects of microbes (Vilmos and Kurucz, 1998; Gillespie et al., 1997). Insect defense mechanisms are diverse and involve cellular and systemic type reactions (Jarosz, 1996; Hultmark et al., 1983; Marchini et al., 1993). Cellular-mediated reactions mainly involve phagocyte cells and the formation of a capsule produced by hemolymph cells (Jarosz, 1996). The systemic responses usually involve a rapid synthesis of small cationic peptides, such as defensins, cecropins, and attacins (Marchini et al., 1993; Natori, 1995). Following a bacterial infection, antimicrobial peptides are produced in the insect fat body (analogous to the liver in mammals) and in hemolymph cells, and accumulate in the hemolymph of the infected insect (Gillespie et al., 1997; Hoffmann, 1997).

Bacillus thuringiensis (Bt) has been the most commercially used bioinsecticide among entomopathogenic microorganisms. The role of insect immune protective mechanisms to evade Bt infection is unknown, but the production of inhibitory factors from Bt strains can interfere with the insect immune response (Edlund et al., 1976). Insect immunity was reported to play an important role in the overall pathogenicity of another bacterium, *Serratia marcescens* (Flyg et al., 1980). However, *S. marcensces*

* Corresponding author. Fax: +5281 8352 4212.
E-mail address: patamez@hotmail.com (P. Tamez-Guerra).

0022-2011/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2008.02.008

spreads through the hemolymph, whereas Bt enters through the digestive system.

Insects with different Bt-susceptibility have been demonstrated to have variations in (1) toxin receptors in the midgut (DeMaagd et al., 2001), or (2) the production of digestive proteases resulting in differences in enzymatic activity levels (Oppert et al., 1997). However, other protective mechanisms may be responsible for varying susceptibility to bacteria harboring Bt toxins in insects (Tabashnik et al., 1997). Therefore, we compared the expression of genes encoding antibacterial peptides (AMPs) in strains of the cabbage looper *Trichoplusia ni* (Hübner) with varying susceptibility to Bt protoxins and toxins.

2. Materials and methods

2.1. Insects

Trichoplusia ni strains were selected based on differences in susceptibility to Bt toxins and protoxins (Tamez-Guerra et al., 2006) (Table 1). Strains included NL (a Mexican strain collected in Nuevo Leon and reared in our laboratory for 3 years), US (kindly provided by Dr. Behle from the National Centre for Agriculture Utilization Research, United States Department of Agriculture, Peoria, IL), and two wild strains from east central México, Guanajuato State (SAL and GTO). Selected strains were exposed in the laboratory to XenTari® (Bt-XT) (Valent Biosciences Corporation, Libertyville, Illinois, E.U.A.; imported and distributed in Mexico by DuPont México, S.A. de C.V.) over several generations (Tamez-Guerra et al., 2006) and were coded with an "X" (USX, SALX, and GTOX). All insect populations were reared on artificial diet (McGuire et al., 1997) at 25 °C ± 2 °C, 55 ± 10% relative humidity and 16 h light/8 h dark photoperiod.

Table 1
Trichoplusia ni strains used in this study, and comparison of relative susceptibilities to Cry 1Aa, Cry1Ab, and Cry1Ac toxins and protoxins[a]

Strain code[b]	Source	Resistance ratio[c]
NL	Laboratory UANL-FCB, Mexico	91, pAa vs. US
US	USDA-ARS, Peoria, IL, USA	None
USX		4.5, pAa vs. US 21, pAc vs. NL
SALX	Field, Salvatierra, Gto. Mexico	6.2, Ab vs. US 16, Ab vs. US
GTOX	Field, San Luis de la Paz, Gto., Mexico	16, pAa vs. US 40, Ab vs. US 50, pAb vs. NL 87, pAc vs. NL 22, pAc vs. US

[a] Data from Tamez-Guerra et al., 2006.
[b] Strains that were selected in the laboratory with XenTari were denoted by the designation of the strain followed by an "X".
[c] Resistance ratio was based on confidence limits of the LC_{50} value. Aa, Ab, or Ac, and pAa, pAb, and pAc = resistance to Cry1Aa, Cry1Ab, or Cry1Ac toxins or protoxins, respectively, compared with laboratory strains (NL or US), as indicated.

2.2. Bioassays

We determined the presence of genes encoding AMPs in each *T. ni* strain under different Bt toxin exposures, using two bioassays: an overlayer bioassay and a stained droplet-feeding bioassay (Tamez-Guerra et al., 2006; Behle et al., 2000). Bt-XT treatments consisted of five doses of XenTari®, based on international units (IU), as follows: 0, 500, 500* (exposed to Bt-XT until larvae reached a specific instar and analyzed 20 h after exposure), 2500, or 3500 IU/ml. Other treatments consisted of a fresh culture or a mixture of spore–crystals from Bt var *thuringiensis* BtUANL01001 (Btt) and *Escherichia coli* strain DH5-α (EC), either alone or with 500 IU/ml Bt-XT. The only *T. ni* strain that was susceptible to both Cry protoxin and toxin was US (Tamez-Guerra et al., 2006), and therefore the response of US to *E. coli* exposure was consistently tested for microbial peptide production. We tested fresh culture and spore–crystals of Btt to compare the infective stage (fresh culture) versus the insecticidal toxins present in crystals. *Escherichia coli* was tested because it is a Gram-negative bacterium commonly used as a positive control for AMP detection by PCR (Sugiyama et al., 1995; Axen et al., 1997; Lundstrom et al., 2002; Hara and Yamakawa, 1995; Spies et al., 1986). Btt and *E. coli* were kindly provided by the Laboratory of Immunology, Biological Sciences College, Autonomous University of Nuevo Leon, MX. Btt was originally isolated from soil in Guanajuato, MX, whereas *E. coli* was originally obtained from ATCC (Manassas, VA). *Escherichia coli* and Btt cultures (100 µl) were at an optical density$_{600\ nm}$ of 0.6 U when harvested (Gene Quant Pro, Amersham Bioscience, Brazil). To obtain a spore–crystal mixture from a slant sample, Btt was inoculated in a Petri dish on nutrient agar (Spectrum) and incubated at 28 °C. After 5 days of culture, if spores and crystals were observed, culture was collected into 30 ml of sterile saline solution (0.1 M sodium chloride and 0.01% Triton-X 100 in distilled water) and washed three times, mixing it in distilled water and centrifuging at 20,000*g* for 30 min (Beckman, Avanti, J-25I). Spore–crystals were lyophilized (Labconco 77500-20, Kansas City, MO). The dried spore–crystal mixture was tested at a dose of 100 mg/ml.

Treatments of bacterial challenge consisted of Btt, Btt-EC, Bt-XT, and EC in the overlayer bioassay in selected strains and developmental stages. Treatments were mixed in PBS, and 50 µl of each treatment were applied to 2 cm^2 artificial diet mixed with 0.1% bovine serum albumin (BSA) in 12-well trays (Costar). PBS only was applied as a control (Tamez-Guerra et al., 2006). Doses were air-dried for 2 h and infested with one *T. ni* larva per well. For the Bt-XT treatments, larvae were fed artificial diet until they reached a specific instar, when they were transferred to treated diet and were collected and analyzed after 20 h

exposure, based on previous reports of AMP gene expression time analysis (Chowdhury et al., 1995; Liu et al., 2000). For pupae analysis, larvae were fed on treated diet throughout larval development and were collected as pupa. Each bioassay was performed on different days with triplicate determinations.

Neonates were exposed to Bt-XT in a droplet-feeding bioassay. Neonates were fed on a colored solution with distilled water or a solution containing specific doses of each treatment. Positively treated larvae were selected by color from each treatment and were incubated in 14:10 light:dark photoperiod at 28 °C to the specific instar or pupae stage.

2.3. Antimicrobial peptide (AMP) gene detection

RT-PCR was used to detect genes encoding AMP in *T. ni* exposed to different bacterial treatments. Samples included the whole body of 2nd, 3rd, and 4th instar larvae and pupae (Kang et al., 1996a), and hemolymph or midgut of 4th instar larvae, exposed or not to the treatments described above (Wang et al., 2004; Hara and Yamakawa, 1995). The fat body was not analyzed separately, but was considered as the contributing factor if hemolymph and midgut were negative. In general, whole body, hemolymph or midgut samples were obtained after fasting larvae for 2–4 h, as recommended for midgut enzyme detection (Lam et al., 2000). For whole larvae or pupae, three individuals from each selected instar were homogenized in 1.5 ml of pre-chilled, 50 mM Tris–HCl buffer pH 8.0, containing 0.01 M $CaCl_2$ (PRO-250, PRO Scientific, Monroe, CT). From these samples, 50–100 mg were homogenized with 1 ml of TRI reagent (Molecular Research Center, Inc., Connecticut, OH). Midgut mRNA was obtained by a previous procedure (Chomczynski and Sacchi, 1987). In brief, three midguts with food bolus were dissected from 4th instar larvae and homogenized and added to 1 ml of TRI reagent. Hemolymph was obtained by a syringe from samples after the midgut was removed and added to pre-chilled TRI reagent (1:5 v:v). Samples were incubated 5 min at room temperature. In each sample, 0.2 ml of chloroform was added and vigorously mixed for 15 s and incubated at room temperature for 2–3 min. Samples were then centrifuged at 12,000*g* for 8 min at 2–8 °C. The upper layer (transparent phase) was isolated and transferred into a new tube, 500 μl of isopropanol was added, and the sample was mixed in a vortex, incubated at room temperature for 5–10 min, and centrifuged at 12,000*g* for 8 min. The supernatant was discarded, and the remaining pellet with RNA was washed with 1 ml of ethanol 75% in DEPC water (milli Q water mixed vigorously with 0.1% diethylpyrocarbonate for 2 h and autoclaved). The sample was centrifuged for 5 min at 7500*g*. The supernatant was discarded and the pellet was air-dried for 5–10 min. The pellet was dissolved by pipetting in 50–200 μl of DEPC water and was incubated at 55–60 °C for 10 min. AMP gene detection was performed in triplicate from randomly selected larvae from the same generation.

RT-PCR was used to synthesize complementary DNA (cDNA) from RNA. In each tube, 10 μl of 5× reaction buffer (250 mM Tris–HCL, pH 8.3, 375 mM KCl, and 1.5 mM $MgCl_2$), 1 μl of 50 mM dithiothreitol, 1 μl of 1 U of RNAase inhibitor, 2 μl of 800 μM of dNTPs, 2 μl of 2.5 μM of primer dT12-18, and 1 μl of 200 U of Moloney murine leukaemia virus (MMLV) reverse transcriptase (PROMEGA) were added to 5 μg of RNA samples. This mixture was adjusted to 50 μl with DEPC water and was incubated at 37 °C for 2 h. The enzyme was inactivated by increasing the temperature to 60 °C for 10 min.

To identify transcripts of the constitutive ribosomal protein S3a (RPS3a, as positive internal expression gene) and AMPs (attacin, gloverin, lebocin, lysozyme, and peptidoglycan recognition peptide, PGRP), specific internal gene primers were used in a polymerase chain reaction (PCR). In a final volume of 50 μl, 1× buffer (200 mM Tris–HCl at pH 8.4, 500 mM KCl), 5 μl of template (cDNA), 3 μl 1.5 mM $MgCl_2$, 1 μl of 800 μM of dNTP's, and 10 pmol of each primer were mixed with 1 U of DNA taq polymerase (Bioline). RPS3a was amplified using the primers RPS3a-1-AGGCACC GTCTAGTTCACC-, RPS3a-2-GCCAGCGAGACTTCA AAAAC-) (Borovsky and Wuyts, 2001). Attacin was amplified using the primers ATA1-CAAATTGATTTTGGGA TTGG and ATA2-CACTTATTACCAAAAGACCGGC, for an expected product of 750 bp (GenBank: U46130; Sugiyama et al., 1995; Kang et al., 1996b). Gloverin was amplified using the primers GLO1-GAATCGTTCACCAT GCAGTC, and GLO2-TCCTCATTTTAACCATACACG AAA, for an expected product of 808 bp (GenBank: AF233590; Lundstrom et al., 2002). Lebocin was amplified using the primers LEB1-TCTGGTGTTGTGTGTGC TCTC, and LEB2-GGACAAAAACAGAAAAGTGCAA, for an expected product of 857 bp (GenBank: AF233589; Yamakawa and Tanaka, 1999). Lysozyme was isolated from *T. ni* (Kang et al., 1996a) using the primers LISHv-ATTC GCTAACCAGTGGTCGT, and LISHv2-GGTACAGT GCCTTTTTAATTTGC, and these primers were used to detect an expected product of 925 bp (GenBank: U50551; Spies et al., 1986). PGRP was amplified using the primers PGRP1-GACTGTGAGTGGAGATTGCG, and PGRP2-TTTTGGTCTATTTCACCATTTACG, to obtain a 605 bp product (GenBank: AF076481).

Preliminary tests were performed to select the amplification cycle number for each sample. AMP transcripts were amplified for 25 cycles for whole body, 35 cycles for midgut, and 40 cycles for hemolymph in a thermocycler (Touchgene Techne, Cambridge, England). Each cycle had a denaturing step of 94 °C/1 min, an annealing step of 55 °C/1 min, and an extension step of 72 °C/2 min and a final extension of 72 °C/7 min. Ten microliters of the amplified sample was analyzed in a 1.5% agarose gel and ethidium bromide stain under UV light using a UVP trans-illuminator (VWR, USA). Optical densities of the DNA bands were detected using a UVP spectrometer (BioSpectrum Imaging Systems, UVP, Inc. Upland, CA). For each transcript, the RPS3 detection was used as internal

control. If inconclusive results were obtained in any replicate, samples were retested using more amplification cycles (up to 40).

3. Results

To detect genes that encode antimicrobial peptides (AMPs), mRNA was isolated from larval and pupal whole body and 4th instar larval hemolymph and midgut, and was used as a template for cDNA amplification by RT-PCR. The products of typical amplification reactions with US and GTOX are shown in Fig. 1. Constitutive RPS3 gene was used as internal positive control and was detected in all samples.

When all of the samples were combined from all developmental stages, the most frequently detected AMP genes were lysozyme and PGRP, found in 80.5% and 70.2% of the samples, respectively (Fig. 2A). Gloverin was found in 33.0% of samples, whereas attacin and lebocin were in less than 20.0% of all samples.

The expression of AMP genes (attacin, gloverin, lebocin, lysozyme, or PGRP) differed within the various developmental stages of the *T. ni* strains (Fig. 2B). However, lysozyme and PGRP were the most frequently detected AMP genes in all but 2nd instar larvae. Attacin was not detected in the midgut tissue of any strain, and was found in only 4.17% of 2nd instar and hemolymph samples, 16.0 and 8.70% of 3rd and 4th instar larvae, respectively, but

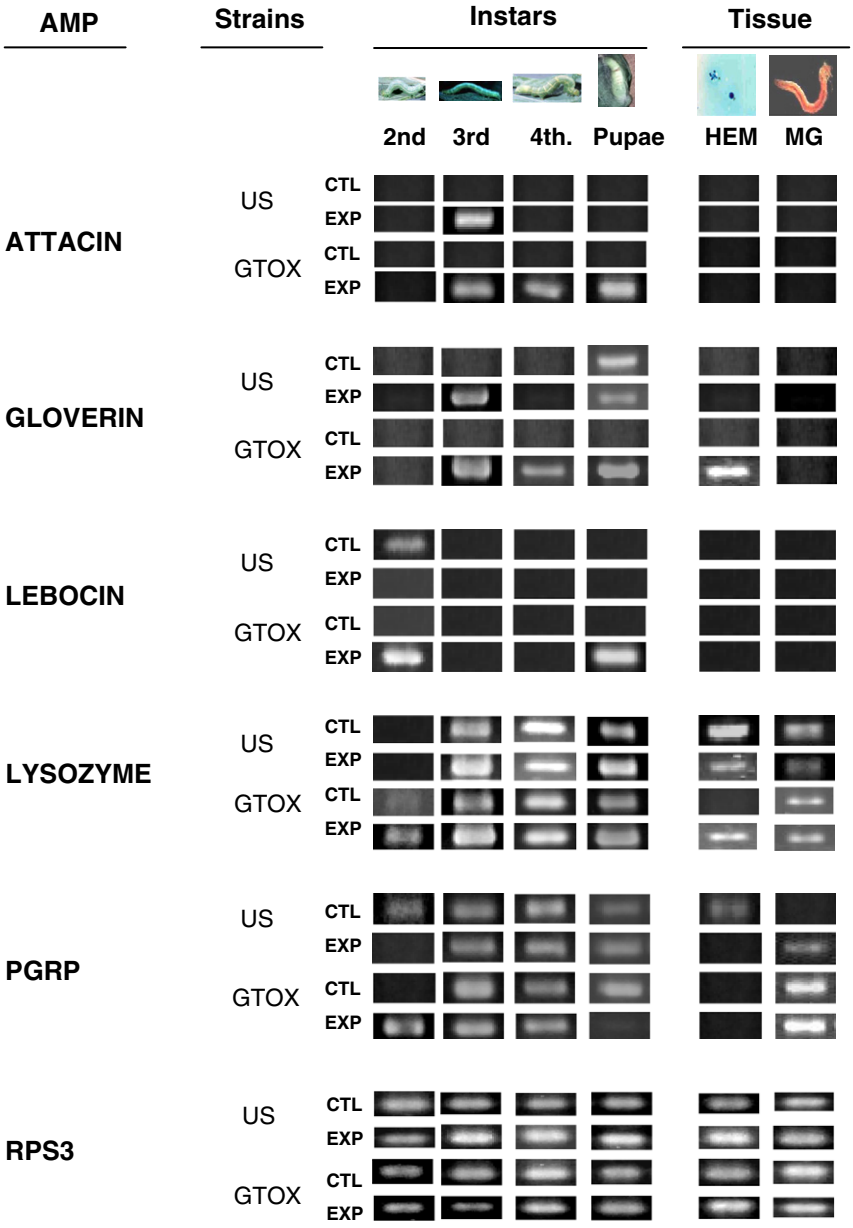

Fig. 1. Comparision of relative expression of antimicrobial peptide genes (attacin, gloverin, lebocin, lysozyme, and PGRP) in two *T. ni* strains (GTOX and US) in different larval instars and tissue samples by RT-PCR. CTL, unexposed control; EXP, exposed to Bt-XT (500 IU/mL) until larvae reached the indicated instar and analyzed 20 h after exposure; HL, hemolymph; MG, midgut.

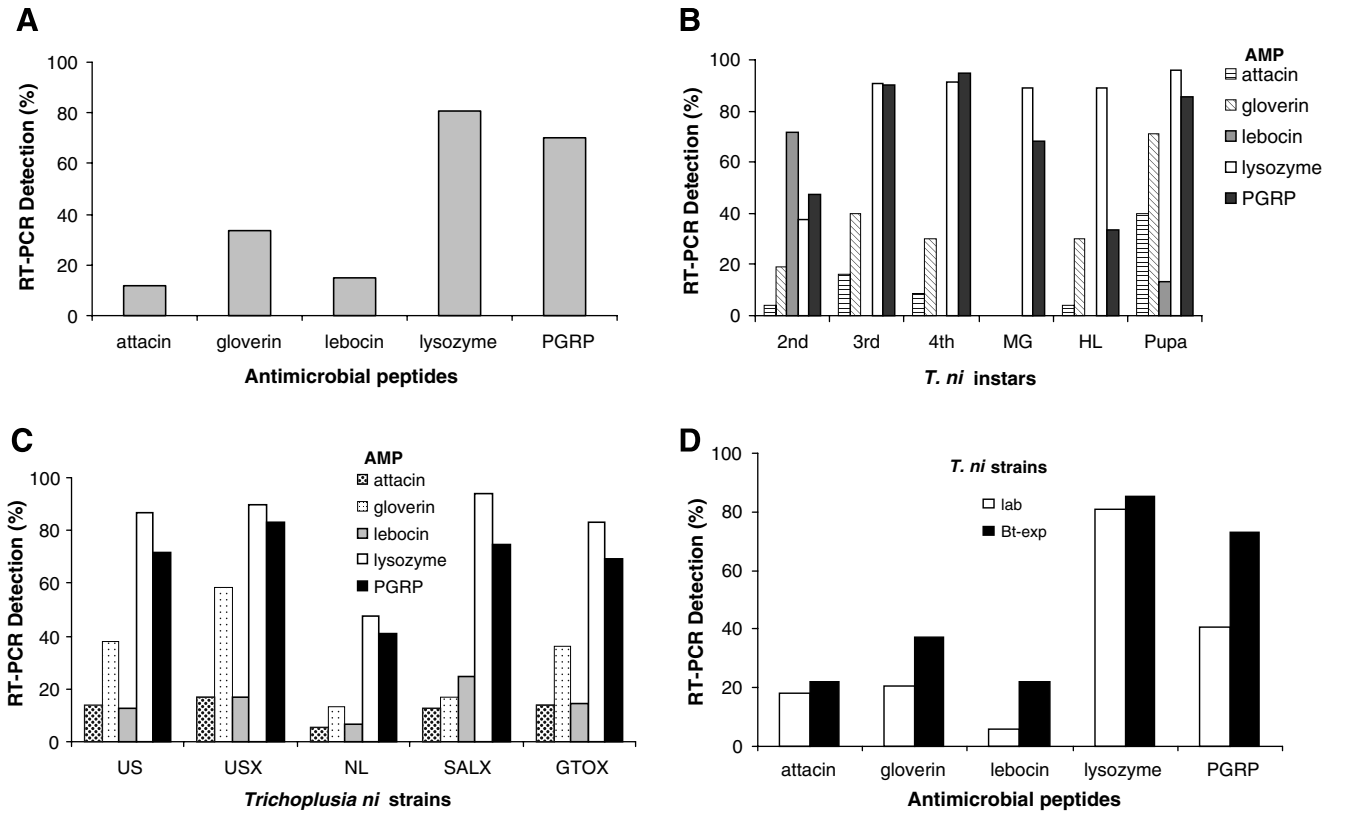

Fig. 2. Expression of antimicrobial peptide (AMP) genes in *T. ni* strains, as detected by RT-PCR and including all samples from all instars tested. (A) Percentage of total positive samples expressing AMP; (B) percentage of AMP detection in different *T. ni* instars; (C) comparison of AMP detection among *T. ni* strains; (D) percentage of AMP detection among control and treated samples. Lab = NL + US strains; Bt-exp, previously exposed to *Bacillus thuringiensis* (XenTari®, Bt-XT) for more than three generations in the laboratory (a combination of responses from USX + SALX + GTOX).

45.8% of the pupae samples. Similar to attacin, gloverin was not detected in any midgut sample, but was found in 19.4%, 40.0%, 30.0%, and 38.7% of 2nd, 3rd, and 4th instar and hemolymph samples, respectively, and in 71.0% of pupae samples. Lebocin was detected in 71.4% of 2nd instar larvae, 13.0% of pupae, but not in any of the other developmental stages. Lysozyme was detected in more than 90.0% of the samples, except for 2nd instar larvae, of which 37.5% expressed the gene. PGRP also was highly expressed in most samples, detected in 33.3% of the hemolymph samples, 47.6%, 90.0%, and 95.0% of 2nd, 3rd, and 4th instar, respectively, 85.0% of pupa, and 65.4% of the midgut samples.

Regardless of the strain, lysozyme and PGRP were the more prevalent AMPs found in *T. ni* (Fig. 2C). In general, AMPs were detected more in Mexican strains that were previously exposed to Bt in the field and in the laboratory (SALX and GTOX) compared with a laboratory strain (NL), but expression was similar to that of the USA strains (US and USX). Surprisingly, the US laboratory strain had a similar AMP expression pattern compared to that of the Mexican field strain selected with Bt-XT, GTOX. The relative expression patterns of lysozyme and PGRP were the same in all of the strains, with lysozyme followed closely by PGRP as the most frequently encountered AMP genes. However, gloverin also was prevalent, expressed in 36.1–58.3% of US, USX, and GTOX strains.

In comparisons of the relative expression of AMP genes in laboratory *T. ni* strains (NL + US, lab) and those exposed to Bt-XT (USX, SALX, and GTOX) in all treatments and samples tested, the expression of all AMPs was increased in the Bt-XT exposed strains, especially for gloverin, lebocin, and PGRP (Fig. 2D). The smallest increase was observed with lysozyme, with only a 1.05-fold increase in the Bt-exposed *T. ni* strains.

3.1. Attacin expression

The attacin transcript was not detected in any developmental stage or tissue of untreated controls of *T. ni* strains (Tables 2 and 3, Fig. 1). Attacin gene expression generally increased in response to Bt-XT or Btt exposure, and most positive samples were from US, USX, and GTOX strains. The highest percentage of attacin expression was 66.7% in the GTOX strain exposed to 500 IU of Bt-XT and analyzed after 20 h. Expression of the attacin gene transcript was found in two treatments: first, exposed to Bt-XT in one given instar and tested after 20 h or as neonate using higher doses of Bt-XT (US, USX, and GTOX); or second, exposed to a lower dose of Bt-XT dose as a neonate (NL and SALX). Attacin gene expression was found in less than

Table 2
Relative expression of antimicrobial peptides (AMPs) in American *Trichoplusia ni* strains fed control or different diet treatments

T. ni strains	AMP	Treatments[a]							
		None	Doses of Xen-Tari® (Bt-XT, IU/ml)				EC	Btt	Btt-SC
			500	500*	2500	500 + EC			
US	Attacin	0.0	0.0	16.7	33.3	0.0	0.0	16.7	16.7
	Gloverin	16.7	33.3	33.3	33.3	50.0	6.7	33.3	33.3
	Lebocin	16.7	0.0	0.0	16.7	16.7	16.7	6.7	16.7
	Lysozyme	83.3	75.0	**83.3**	83.3	100	83.3	100	83.3
	PGRP	100.0	25.0	66.7	33.3	83.3	100	66.7	83.3
USX	Attacin	0.0		**33.3**	33.3	**16.7**		16.7	16.7
	Gloverin	16.7		**66.7**	**50.0**	**83.3**	66.7	**50.0**	**66.7**
	Lebocin	16.7		**16.7**	16.7				
	Lysozyme	83.3		66.7	**100**	100			**100.0**
	PGRP	100.0	**50.0**	**83.3**	**83.3**				

Data represent the percentage of all insects tested within each strain (including all developmental stages and tissues).
[a] Treatments are detailed in Section 2. Numbers in bold show detection values at higher percentages in the same treatment/AMP comparing US versus USX. EC, *Escherichia coli*. Btt, fresh culture of *Bacillus thuringiensis* serovar *thuringiensis*. Btt-SC, spores and crystals of *B. thuringiensis* serovar *thuringiensis*. Blanks indicate that results were either inconclusive or were not tested.

Table 3
Relative expression of antimicrobial peptides in Mexican *Trichoplusia ni* strains fed control or different diet treatments

T. ni strains	AMP	Treatments[a]					
		None	Doses of Xen-Tari® (Bt-XT, IU/ml)			Btt	Btt-SC
			500	500*	2500		
NL	Attacin	0.0	16.7		0.0		
	Gloverin	0.0	**50.0**	0.0	16.7		
	Lebocin	0.0	16.7				
	Lysozyme	33.3	66.7	**100**	**100**	0.0	
	PGRP	50.0	50.0	0.0	0.0		
SALX	Attacin	0.0	**25.0**				
	Gloverin	**16.7**	5.0				
	Lebocin	**16.7**		33.3			
	Lysozyme	**100.0**	**100**	75.0	**100**		
	PGRP	**80.0**	0.0	**100**	0.0		
GTOX	Attacin	0.0		66.7	16.7		
	Gloverin	0.0	16.7	**66.7**	**50.0**	50.0	
	Lebocin	0.0	0.0	33.3	33.3		
	Lysozyme	66.7	66.7	**100**	83.3	**83.3**	100
	PGRP	66.7	**66.7**	83.3	**66.7**	83.3	50.0

Data represent the percentage of all insects tested within each strain (including all developmental stages and tissues).
[a] Treatments are detailed in Section 2. Numbers in bold show detection values at higher percentages in the same treatment/AMP comparing NL versus SALX and GTOX strains. EC, *Escherichia coli*. Btt, fresh culture of *Bacillus thuringiensis* serovar. *thuringiensis*. Btt-SC, spores and crystals of *B. thuringiensis* serovar. *thuringiensis*.

half of pupae samples but not the hemolymph, except for NL, where it only was found in the hemolymph but not the pupae (Fig. 3A). After bacterial challenge trials, US and USX strains exposed to either fresh Btt culture or Btt-SC expressed attacin only in pupae stage, whereas the GTOX strain did not (data not shown).

3.2. Gloverin expression

Gloverin was detected in pupae samples of the untreated controls of US and USX (16.7%) and SALX (50.0%) (Tables 2 and 3, Fig. 1). SALX, USX, and GTOX strains had higher levels of gloverin when they were exposed to 500 IU of Bt-XT and tested after 20 h exposure, or when given a higher dose (2500 IU). Similar to attacin, gloverin gene expression was generally at higher percentage in samples exposed to bacterial challenge. Bacterial challenge by Bt-XT and EC induced the highest gloverin expression in the USX strain (Table 2). Gloverin was expressed in all instars, hemolymph, and pupae of US, USX, and GTOX, but expression was variable (Fig. 3B). SALX was the only strain that did not express gloverin in the hemolymph.

3.3. Lebocin expression

Increased lebocin gene expression was noted only in field strains SALX and GTOX exposed to 500 IU of Bt-XT and tested after 20 h exposure, or when given a higher dose

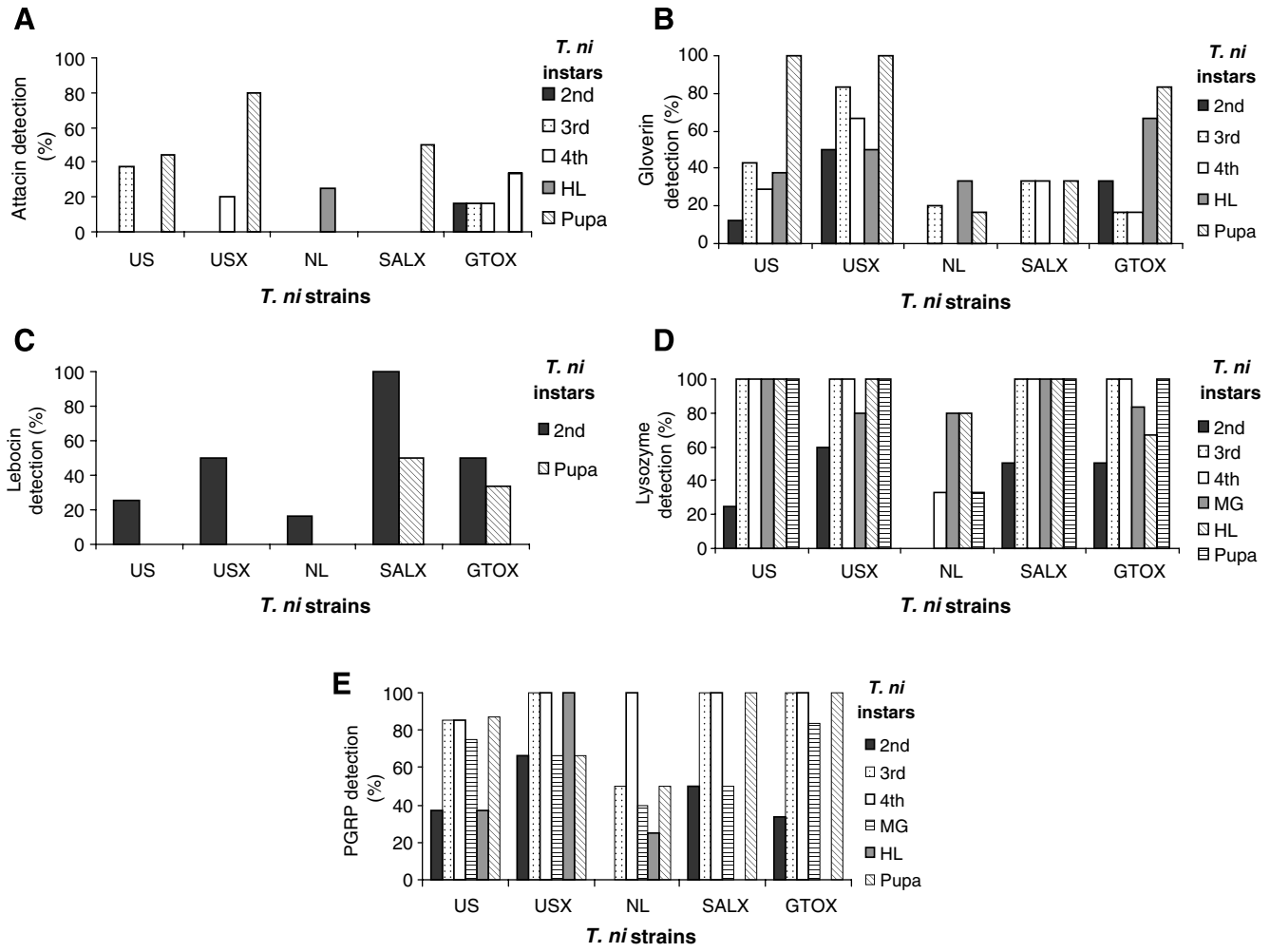

Fig. 3. Detection of antimicrobial peptides (AMPs) by RT-PCR among instars of *T. ni* strains, as a percentage of the total samples tested. (A) Attacin; (B) gloverin; (C) lebocin; (D) lysozyme; (E) peptidoglycan recognition protein (PGRP).

(2500 IU/ml) (Table 3). No differences in lebocin expression were observed in US and USX after bacterial challenge (Table 2). Lebocin gene expression was found in the 2nd instar of all strains, except by NL and GTO untreated controls. Lebocin was found only in pupae samples of the field strains SALX and GTOX (Fig. 3C). Of the 2nd instar samples, untreated controls of the US strain were positive but treatments with 500 or 500* IU/ml for were negative for lebocin, whereas the opposite was observed with GTOX (Fig. 1). None of the other developmental stages or tissues samples expressed lebocin.

3.4. Lysozyme expression

Lysozyme expression was found in all strains (Tables 2 and 3). Increased lysozyme expression was observed in NL and GTOX, in particular when exposed to 500 IU of Bt-XT and assayed 20 h later. Increasing the Bt-XT dose to 2500 IU generally caused an increased lysozyme gene expression in all strains except US. In general, after bacterial challenge the USX, NL, and GTOX strains expressed lysozyme in higher percentage compared with US and SALX strains. Whereas attacin, gloverin, and lebocin transcripts were not detected in midgut from 4th instar larvae of any *T. ni* strain, lysozyme was detected in many of the midgut samples (Fig. 3D). In contrast, only the midgut and hemolymph samples of NL had higher lysozyme expression, and lysozyme was not detected in NL 2nd instar larvae.

3.5. PGRP expression

PGRP was found in all US and USX untreated controls, but bacterial challenge resulted in a decrease in PGRP expression (Table 2 and Fig. 1). The only samples that demonstrated increased PGRP gene expression were from SALX and GTOX larvae exposed to Bt-XT at 500 IU/ml and analyzed after 20 h. PGRP was not detected in hemolymph samples from SALX and GTOX nor in 2nd instar larvae of NL (Fig. 3E). Almost all midgut GTOX samples expressed PGRP, with the exception of those exposed to the Btt-SC (data not shown). Pupae from US and USX strains did not have PGRP after Btt challenge (data not shown).

4. Discussion

Innate immune systems in insects recognize microorganisms through a series of pattern recognition receptors that are highly conserved in evolution. For example, in *Drosophila*, the activation of the innate immune response involves recognition of the infectious agent and subsequent activation of cellular and humoral responses (Kurata, 2004). Humoral reactions depend on a primary response, which is mediated by the activation of cascades of constitutive proteins present in the hemolymph, and a secondary response, which requires transcriptional activation of AMPs (Kurata, 2004). These peptides accumulate in the infected insect's hemolymph and have the fundamental role of protecting the insect from the pathogenic microorganism (Hoffmann, 1997).

Because AMPs are transcriptionally induced peptides, the secondary humoral response of insects may be defined by a set of peptides that are absent in the naïve state, which has been especially noted with attacin. Bt is more than an infectious agent, because it produces insecticidal toxins that interact with specific receptors in the insect midgut that elicit secondary effects. Based on previous immunity studies (Gillespie et al., 1997), we hypothesized that the production of AMP in Bt exposed insects was the result of a secondary humoral response that result in differences in susceptibility to an infection. Similar results were reported by Crowley and Houck (2002), who found an increased immune response and, more specifically, the expression of cecropin B in pupae of *Calliphora vicina* after *E. coli* exposure. In this study, several comparisons were made based on the results from untreated diets. For example, NL and GTOX strains on untreated diets did not induce gloverin in the pupae stage, and lebocin in the 2nd instar, whereas other strains did.

It was previously reported that attacin expression by *Bombyx mori* larvae was rapidly induced by the injection of *E. coli* bacilli into larvae, and continued at least for 48 h, mainly in fat bodies and hemocytes (Sugiyama et al., 1995). Similarly, 5th instar *B. mori* larvae injected with lipopolysaccharide from *E. coli* showed a significant induction of attacin-like polypeptides, but the level decreased 48 h after injection (Wang et al., 2004). In addition, attacin was not detected in the midgut of lipopolysaccharide injected *B. mori* (Wang et al., 2004) or in immunized *Samia cynthia ricini* larvae (Kishimoto et al., 2002). Whereas attacin has been reported in midgut of *Drosophila* (Tzou et al., 2000), we did not detect attacin in the midgut samples of any *T. ni* strain. Expression of attacin in bacterial challenges of *T. ni* larvae was previously reported from hemolymph samples (Kang et al., 1996a). In the present study, attacin was expressed infrequently in *T. ni* strains exposed to bacteria, but it was induced in GTOX if the sample was analyzed following 20 h of exposure to Bt-XT.

Gloverin is an inducible antibacterial insect protein isolated from pupae of *Hyalophora cecropia* and suppresses *E. coli* growth at very low concentrations (Axen et al., 1997). In our study, we observed that the expression of gloverin was, in many aspects, similar to that of attacin, as previously reported by Axen et al. (1997). We found that gloverin expression was related to insect stage, where the lowest expression was found in 2nd instar, increasing in 3rd and 4th instar larvae, and the highest at the pupae stage. In many cases, gloverin was induced in US or USX larvae under bacterial challenge. Other tests with *T. ni* have demonstrated that gloverin is expressed in hemolymph and hemocytes of bacteria-immunized larvae (Lundstrom et al., 2002). Based on these results, the gloverin expression in *T. ni* appears to be very specific.

Early studies of lebocin detection in *B. mori* indicated a gene expression pattern typical of an insect antibacterial peptide (Chowdhury et al., 1995). In this study, lebocin was the least detected AMP transcript, only detected in 2nd instar larvae and in three pupae samples. GTOX was the only strain that demonstrated an increase in lebocin in response to bacterial challenge. In a previous study with *T. ni*, a Northern blot analysis demonstrated that lebocin expression was inducible upon bacterial challenge, mainly in fat body and hemocytes, and that its expression reached its highest level after 20 h challenge and continued at least until 60 h after bacterial injection (Liu et al., 2000). Similarly, *B. mori* studies showed that lebocin gene expression occurred tissue-specifically in the fat bodies and continued at least 48 h post bacterial injection (Chowdhury et al., 1995). This may explain our detection of lebocin transcript primarily in 2nd instar larvae. These results also suggest a longer induction time for attacin and gloverin gene expression (and longer expression period) compared with that of lebocin.

One of the most recognized AMPs is lysozyme. Lysozyme has been detected in *H. cecropia* (Hultmark et al., 1983) and *Manduca sexta* larvae (Spies et al., 1986) after *E. coli* exposure. In the present study, lysozyme was the most detected AMP and was constitutively expressed. Lysozyme was detected at high levels in most instars, pupae, and tissues, and its expression levels fluctuated in response to bacterial challenge.

PGRPs are pattern recognition molecules that recognize bacteria and peptidoglycan molecules (Dziarski, 2004). PGRP expression has been reported after the exposure of Gram-positive bacteria or bacterial cell wall fragments in a differential display screen for up-regulated immune genes in *T. ni* (Kang et al., 1998). PGRPs are differentially expressed in various cells and tissues, their expression is often up-regulated by bacteria, and they mediate host responses to bacterial infections (Dziarski, 2004). In *Drosophila*, PGRP are the initial sensors of infecting bacteria that then trigger a cascade leading to the expression of antimicrobial peptides (Girardin and Philpott, 2004). Recent data suggested that in diverse PGRPs are involved in distinguishing between invading bacteria and activation of appropriate immune reactions (Kurata, 2004). Similar to observations with lysozyme, PGRP gene expression was constitutively expressed and fluctuated in response to bac-

terial challenge in the *T. ni* strains. PGRP was not detected in any hemolymph sample of SALX or GTOX, but was detected in all Bt-exposed midgut treatments, suggesting that it is expressed as a response to bacteria challenge or an immune signaling molecule.

Overall, the expression of attacin, gloverin, and lebocin gene in different stages of selected *T. ni* strains was related to bacterial challenge and instar/tissue, whereas lysozyme and PGRP were not. Different patterns of AMP gene expression were found in strains from the US and Mexico. However, all *T. ni* strains previously exposed to Bt demonstrated an increased AMP gene expression. More studies with individual AMP and Bt resistant strains are needed to determine a conclusive relationship between Bt-susceptibility and insect immunity.

Acknowledgments

We want to thank Ludek Zurek at Kansas State University and Pablo Zapata at University of Nuevo Leon for their thoughtful comments to improve the manuscript. This study was supported by the research and scientific exchange division, United States Department of Agriculture-Agricultural Research Services, Grant 210-22310-003-03S, PAICYT-UANL CN1355-06, and CONACyT 36815-B. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US. Department of Agriculture.

References

Axen, A., Carlsson, A., Engstrom, A., Bennich, H., 1997. Gloverin, an antibacterial protein from the immune hemolymph of *Hyalophora pupae*. Eur. J. Biochem. 247, 614–619.

Behle, R.W., McGuire, M.R., Tamez-Guerra, P., 2000. Effect of light energy on alkali released virions from *Anagrapha falcifera* nucleopolyhedrovirus. J. Invertebr. Pathol. 76, 120–126.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Chowdhury, S., Taniai, K., Hara, S., Kadono-Okuda, K., Kato, Y., Yamamoto, M., Xu, J., Choi, S.K., Debnath, N.C., Choi, H.K., et al., 1995. cDNA cloning and gene expression of lebocin, a novel member of antibacterial peptides from the silkworm, *Bombyx mori*. Biochem. Biophys Res Commun. 214, 271–278.

Crowley, L.D., Houck, M.A., 2002. The immune response of larvae and pupae of *Calliphora vicina* (Diptera: Calliphoridae), upon administered insult with *Escherichia coli*. J. Med. Entomol. 39, 931–934.

DeMaagd, R.A., Bravo, A., Crickmore, N., 2001. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Rev. Trends Genet. 17, 193–199.

Dziarski, R., 2004. Peptidoglycan recognition proteins (PGRPs). Mol. Immunol. 40, 877–886.

Edlund, T., Siden, I., Boman, H.G., 1976. Evidence for two immune inhibitors from *Bacillus thuringiensis* interfering with the humoral defense system of saturniid pupae. Infect. Immun. 14, 934–941.

Flyg, C., Kenne, K., Boman, H.G., 1980. Insect pathogenic properties of *Serratia marcescens*: phage-resistant mutants with a decreased resistance to *Cecropia* immunity and a decreased virulence to *Drosophila*. J. Gen. Microbiol. 120, 173–181.

Gillespie, J.P., Kanost, M.R., Trenczek, T., 1997. Biological mediators of insect immunity. Annu. Rev. Entomol. 42, 611–643.

Girardin, S.E., Philpott, D.J., 2004. Mini-review: the role of peptidoglycan recognition in innate immunity. Eur. J. Immunol. 34, 1777–1778.

Hara, S., Yamakawa, M., 1995. A novel antibacterial peptide family isolated from the silkworm, *Bombyx mori*. Biochem. J. 310, 651–656.

Hoffmann, J.A., 1997. Immune responsiveness in vector insects. Proc. Natl. Acad. Sci. USA 94, 11152–11153.

Hultmark, D., Engstrom, A., Anderson, K., Steiner, H., Bennich, H., Boman, H.G., 1983. Insect immunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia*. EMBO J. 2, 571–576.

Jarosz, J., 1996. Anti-infective defense strategies and methods of escape from entomologic pathogens under immunologic control of insects. Wiad. Parazytol. 42, 3–27.

Kang, D., Liu, G., Gunne, H., Steiner, H., 1996a. PCR differential display of immune gene expression in *Trichoplusia ni*. Insect Biochem. Mol. Biol. 26, 177–184.

Kang, D., Liu, G., Lundstrom, A., Gelius, E., Steiner, H., 1998. A peptidoglycan recognition protein in innate immunity conserved from insects to humans. Proc. Natl. Acad. Sci. USA 95, 10078–10082.

Kang, D., Lundstrom, A., Steiner, H., 1996b. *Trichoplusia ni* attacin A, a differentially displayed insect gene coding for an antibacterial protein. Gene 174, 245–249.

Kishimoto, K., Fujimoto, S., Matsumoto, K., Yamano, Y., Morishima, I., 2002. Protein purification, cDNA cloning and gene expression of attacin, an antibacterial protein, from eri-silkworm, *Samia cynthia ricini*. Insect Biochem. Mol. Biol. 32, 881–887.

Kurata, S., 2004. Recognition of infectious non-self and activation of immune responses by peptidoglycan recognition protein (PGRP)-family members in *Drosophila*. Dev. Comp. Immunol. 28, 89–95.

Lam, W., Cosat, G.M., Rayne, R.C., 2000. Characterization of multiple trypsin from the midgut of *Locusta migratoria*. Insect Biochem. Mol. Biol. 30, 85–94.

Liu, G., Kang, D., Steiner, H., 2000. *Trichoplusia ni* lebocin, an inducible immune gene with a downstream insertion element. Biochem. Biophys. Res. Commun. 269, 803–807.

Lundstrom, A., Liu, G., Kang, D., Berzins, K., Steiner, H., 2002. *Trichoplusia ni* gloverin, an inducible immune gene encoding an antibacterial insect protein. Insect Biochem. Mol. Biol. 32, 795–801.

Marchini, D., Giordano, P.C., Amons, R., Bernini, L.F., Dallai, R., 1993. Purification and primary structure of ceratotoxin A and B, two antibacterial peptides from the female reproductive accessory glands of the med fly *Ceratitis capitata* (Insecta: Diptera). Insect Biochem. Mol. Biol. 23, 591–598.

McGuire, M.R., Galán-Wong, L.J., Tamez-Guerra, P., 1997. Bacteria: bioassay of *Bacillus thuringiensis* against lepidopteran larvae. In: Lacey, L. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, New York, pp. 91–99.

Natori, S., 1995. Antimicrobial proteins of insect and their clinical application. Nippon Rinsho 52, 1297–1304.

Oppert, B., Kramer, K.L., Beeman, R.W., Johnson, D., McGaughey, W.H., 1997. Proteinase-mediated resistance to *Bacillus thuringiensis* insecticidal toxins. J. Biol. Chem. 272, 23473–23476.

Spies, A.G., Karlinsey, J.E., Spence, K.D., 1986. Antibacterial hemolymph proteins of *Manduca sexta*. Comp. Biochem. Physiol. B 83, 125–133.

Sugiyama, M., Kuniyoshi, H., Kotani, E., Taniai, K., Kadono-Okuda, K., Kato, Y., Yamamoto, M., Shimabukuro, M., Chowdhury, S., Xu, J., et al., 1995. Characterization of a *Bombyx mori* cDNA encoding a novel member of the attacin family of insect antibacterial proteins. Insect Biochem. Mol. Biol. 25, 385–392.

Tabashnik, B.E., Liu, Y.-B., Malvar, T., Heckel, D.G., Masson, L., Ballester, V., Granero, F., Ménsua, J.L., Ferré, J., 1997. Global variation in the genetic and biochemical basis of diamondback moth resistance to *Bacillus thuringiensis*. Proc. Natl. Acad. Sci. USA 94, 12780–12785.

Tamez-Guerra, P., Damas, G., Iracheta, M.M., Gomez-Flores, R., Oppert, B., Rodríguez-Padilla, C., 2006. Differences in susceptibility and physiological fitness of *Trichoplusia ni* (Hübner) strains to *Bacillus thuringiensis* exposure. J. Econ. Entomol. 99, 937–945.

Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., Reichhart, J.M., Lemaitre, B., Hoffmann, J.A., Imler, J.L., 2000. Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. Immunity 13, 737–748.

Vilmos, P., Kurucz, E., 1998. Mini review. Insect immunity: evolutionary roots of the mammalian innate immune system. Immunol. Lett. 62, 59–66.

Wang, Y., Zhang, P., Fujii, H., Banno, Y., Yamamoto, K., Aso, Y., 2004. Proteomic studies of lipopolysaccharide-induced polypeptides in the silkworm, *Bombyx mori*. Biosci. Biotechnol. Biochem. 68, 1821–1823.

Yamakawa, M., Tanaka, H., 1999. Immune proteins and their gene expression in the silkworm, *Bombyx mori*. Dev. Comp. Immunol. 23, 281–289.