

Prior infection of *Manduca sexta* with non-pathogenic *Escherichia coli* elicits immunity to pathogenic *Photobacterium luminescens*: Roles of immune-related proteins shown by RNA interference

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Abstract

Prior infection of *Manduca sexta* caterpillars with the non-pathogenic bacterium *Escherichia coli* elicits effective immunity against subsequent infection by the usually lethal and highly virulent insect pathogen *Photobacterium luminescens* TT01. Induction of this protective effect is associated with up-regulation of both microbial pattern recognition protein genes (*hemolin*, *immulectin-2* and *peptidoglycan recognition protein*) and anti-bacterial effector genes (*attacin*, *cecropin*, *lebocin*, *lysozyme* and *moricin*). We used RNA interference to knock down over-transcription of members of both these sets of genes one at a time. Interfering with expression of individual recognition proteins had a drastic adverse effect on the *E. coli* elicited immunity. RNAi knock-down of *immulectin-2* caused the greatest reduction in immunity, followed by *hemolin* and *peptidoglycan recognition protein* (PGRP) in that order, to the extent that knock-down of any one of these three proteins left the insects more susceptible to *P. luminescens* infection than insects that had not experienced prior infection with *E. coli*. Interfering with the expression of individual antibacterial effector proteins and peptides had a much less marked effect on immunity. Knock-down of *attacin*, *cecropin* or *moricin* caused treated insects to be more susceptible to *P. luminescens* infection than controls that had been pre-infected with *E. coli* but which had not received the specific RNAi reagents, but they were still less susceptible than insects that had not been pre-infected with *E. coli*. RNAi knock-down with expression of *lebocin* or *lysozyme* had no effect on *E. coli*-induced immunity to *P. luminescens*, indicating that these effectors are not involved in the response. By bleeding pre-infected caterpillars and growing the pathogen directly within cell-free insect haemolymph, we showed that at least part of the protection elicited by previous exposure to *E. coli* is due to the presence of factors within the blood plasma that inhibit the growth of *P. luminescens*. The production of these factors is inhibited by RNAi treatment with ds-RNA reagents that knock down *hemolin*, *immulectin-2*, and PGRP. These results demonstrate that the insect immune system can be effectively primed by prior infection with non-pathogenic bacteria against subsequent infection by a highly virulent pathogen. Given the continuous normal exposure of insects to environmental and symbiotic bacteria, we suggest that prior infection is likely to play a significant and underestimated role in determining the level of insect immunity found in nature.

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1. Introduction

Unlike mammals, insects lack antibody-mediated defences. The insect immune system is therefore often compared with the innate immune system of mammals

(Kanost et al., 2004) and it is sometimes asserted that in insects there is no ‘memory’ of prior infection. But it has been recognised for many years that ‘immunisation’ with killed bacteria (e.g. with *Pseudomonas aeruginosa*—Stephens, 1959, 1962) or prior infection with living bacteria (e.g. with *Enterobacter cloacae*—Boman et al., 1972, 1974) can induce in insects a temporary state of markedly enhanced resistance to

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subsequent infection, either to the same bacterium or a different one.

Recognition of microbial infection is an essential first step in immunity in insects, just as in mammals (Leulier et al., 2003; Royet, 2004). As a consequence of the activation of signalling pathways dependent on such recognition (Royet et al., 2005), the invading pathogen is either restricted or eliminated by antimicrobial effectors. Although in insects antimicrobial actions also include cell-mediated responses, the best known effectors of the insect immune system are antimicrobial peptides (AMPs), which are secreted into the haemolymph. The first of these to be identified were the cecropins (Steiner et al., 1981) and many others have since been discovered (Boman and Hultmark, 1987; Otvos, 2000). Although the original observation of induced immunity has led to a large quantity of research on the nature of the recognition proteins, the induced signalling pathways, and the identities of the humoral microbe-killing agents of insects, there has been remarkably little work on the actual phenomenon of induced immunity to infection by pathogens.

In this paper we investigate the active immune response of the model lepidopteran insect *Manduca sexta* to prior infection with live cells of a harmless laboratory strain of the Gram-negative bacterium *Escherichia coli*. We show that this confers on the treated insects a remarkable resistance to subsequent challenge with the virulent insect pathogen *Photorhabdus luminescens*, another Gram-negative organism. We have utilised this model system, together with the powerful technique of RNA interference (RNAi) (Dillon et al., 2005), to investigate the relative contributions to induced immunity of various recognition and effector proteins.

Many *Manduca* genes are upregulated following bacterial challenge (Zhu et al., 2003), and the sequences of these immune-related genes are a powerful resource that assist the investigation of immune responses in this insect. In this work we have studied three different representative pattern recognition proteins: hemolin, peptidoglycan recognition protein (PGRP), and immunelectin-2 (Yu et al., 2002), and five different AMPs, attacin, cecropin, lebecin, lysozyme and moricin (Kanost et al., 2004).

As expected from previous work on these genes, we found that prior infection of *Manduca* caterpillars with *E. coli* resulted in the up-regulation of all of these recognition and effector genes. We also found that overtranscription of these genes can be individually prevented by injecting the corresponding double-stranded (ds) RNAs, and that this RNAi-mediated 'knock-down' of immune gene over-transcription was associated with dramatically increased mortality when the treated insects were exposed to the pathogen.

2. Materials and methods

2.1. Insects and bacteria

Larvae of the tobacco hornworm, *M. sexta* (L.) (Lepidoptera: Sphingidae), were maintained individually

on a wheat germ-based artificial diet at 25 °C and a photoperiod of 17 h light:7 h dark (Reynolds et al., 1985). Newly moulted (day 0) fifth stage larvae were used for all injection experiments. At this stage, the insects weighed about 1.2 g.

Bacterial strains used were *E. coli* strain DH5 α and *P. luminescens* subsp. *laumondii* strain TT01. All bacteria were cultured on Petri dishes containing 2.5% Luria–Bertani (LB) and 1.5% agar (Difco Laboratories, Detroit, MI). For pre-infection with *E. coli*, 50 μ l of a phosphate-buffered saline (PBS) suspension containing approximately 3×10^7 washed DH5 α cells were injected directly into the haemocoel of *M. sexta* larvae using a 100 μ l disposable syringe with a 30-gauge needle. The numbers of injected bacteria were estimated by optical density at 600 nm and subsequently confirmed by plating a known volume of injected suspension on 2.5% LB agar plates. PBS injected insects served as controls. Larvae were stored at 28 °C. At 18 h after injection they were either surface sterilised with 70% ethanol and then dissected to collect fat body tissues, or further challenged with pathogenic bacteria.

For pathogen injection primary-form *P. luminescens* TT01 colonies were inoculated into sterile tubes containing 5 ml of 2.5% LB broth and the cultures were incubated for 24 h at 30 °C on a rotary shaker at 340 rpm. Approximately 1×10^8 washed TT01 cells were injected into the test insects. Ten *M. sexta* individuals were used for each treatment, and the experiments were repeated three times. After injection, larvae were held individually with diet at 28 °C between and after treatments. Mortality, defined as failure to react to a needle poke, was scored at intervals up to 96 h after the final injection.

2.2. Pathogen growth in vitro

To determine the ability of the *P. luminescens* TT01 pathogen to grow within the plasma of infected *M. sexta*, insects were injected with 50 μ l of ds-RNA (2 μ g/ml) specific for hemolin, immunelectin-2 or PGRP or 50 μ l PBS (control), and 6 h later with 50 μ l of *E. coli* (approx. 3×10^7 cells) or PBS. Five insects were used for each treatment. They were maintained at 28 °C for 24 h and then chilled on ice for 15 min, swabbed with 70% ethanol, cut at the midpoint of the dorsal horn and individually bled into prechilled sterile polypropylene tubes to collect their total haemolymph fluids (approximately 500 μ l per larva). Tubes were centrifuged at 200g for 5 min at 4 °C to pellet the haemocytes and the supernatant haemolymph plasma samples were transferred to fresh tubes following the addition of 20 mM phenylthiocarbamide (PTC), a known inhibitor of phenoloxidase. All samples were inoculated with 3 μ l of TT01 (approximately 1×10^8 cells) and then incubated at 28 °C with constant shaking for 24 h. TT01 growth in *M. sexta* plasma was measured by optical density at 600 nm.

2.3. RNA extraction, RT-PCR and cDNA cloning

One hundred mg of fat body tissue was homogenised in 1 ml of TRI reagent (Sigma). RNA was then isolated following the manufacturer's protocol. RNA samples were further purified by the addition of RNase free DNaseI (Invitrogen) (1 U/ μ l) at 37 °C for 1 h and the enzyme was subsequently inactivated by incubation for 10 min at 75 °C. Lack of DNA contamination was confirmed in every RNA sample via 35 cycles of PCR using RNA as the template. Single step reverse transcription (RT)-PCR was performed with the 'OneStep' RT-PCR kit (Qiagen). Each reaction was carried out in a 50 μ l volume containing 0.6 μ M of forward and reverse gene primers (Table 1) and 2 μ g of RNA template. All primers were specific with the exception of PGRP. In this case the primers used did not distinguish between two almost identical PGRP genes (PGRP-1 and PGRP-2) present in the *M. sexta* genome (Zhu et al., 2003). However, since the mature proteins encoded by these two genes are identical the results accurately reflect the presence of mRNAs encoding PGRP. Amplifications were performed on a PTC-100 thermal controller (MJ Research) under the following cycling conditions: RT at 50 °C for 30 min, 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 45 °C for 30 s and 72 °C for 1 min with a final extension of 72 °C for 10 min. RT-PCR control reactions for ribosomal protein S3 (rpS3) (Jiang et al., 1996) were performed as outlined above. PCR products were visualised by agarose gel electrophoresis. RT-PCR samples were then purified using the QIAquick gel extraction kit (Qiagen) by following the manufacturer's protocol.

cDNA cloning and transformation were performed using the 'TOPO TA' cloning kit (Invitrogen). Purified cDNAs were cloned into pCR4-TOPO vector (Invitrogen) and transformed into 'One Shot' TOP10 electrocompetent

E. coli cells (Invitrogen). Transformed cells were plated onto LB agar plates containing 2 μ g/ml ampicillin and incubated overnight at 37 °C to select for recombinants. Positive transformants were identified by PCR. Each reaction was carried out in a 50 μ l volume containing 20 μ M of T3 sense and T7 antisense primers and individual colonies as DNA templates. Amplifications were performed on a PTC-100 thermal controller (MJ Research) under the following cycling conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min. Clones with the correct sized insert for each gene were picked and cultured in 5 ml LB/ampicillin broth by horizontal shaking and incubation at 37 °C overnight. Plasmids were then purified from the cells using the 'Miniprep' kit (Qiagen) by following the product's manual. DNA inserts were finally amplified from the purified plasmids using PCR with T7 (TAATAC-GACTCACTATAGGG) and T3 (ATTAACCCTCAC-TAAAGGGA) sequencing primers. PCR reaction conditions were as given above.

2.4. RNAi and Western blotting

Products from the amplification of inserts using T3 and T7 primers were used as a template to generate ds RNAs for each gene. Sense and antisense strands were synthesised using the T3 and T7 'Megascript' kits (Ambion), respectively, according to manufacturer's instructions. DNA templates were removed with RNase free DNase I as previously outlined, and reaction products recovered and purified using lithium chloride precipitation following the kit's protocol. Single-stranded (ss) RNAs were dissolved in dimethyl pyrocarbonate (DMPC) treated water and complementary strands were annealed by combining equal molar amount of each strand, heating to 70 °C for 15 min

Table 1
Nucleotide sequences of primers used in RT-PCR of *Manduca* RNA

Gene	Primer	Sequence	Product size (bp)
<i>Hemolin</i>	Forward	ACAGCAACAACACAGGTGAA	1273
	Reverse	TTAAGCAACAATCACGAGCG	
<i>Immulectin-2</i>	Forward	GACTCTTGCGAGTCGTGTGA	953
	Reverse	GACTGTTGGGTCCTTTTCG	
<i>PGRP</i>	Forward	ACGGTATCACTTCCGTCCAC	516
	Reverse	CATTCTGGCCATCTCCTGAT	
<i>Attacin B</i>	Forward	GGTCACGGCGCTACTCTTAC	341
	Reverse	TTGGGCATCTCGAACTTCTT	
<i>Cecropin D</i>	Forward	TTCTTCGTCTTCGCTTGCTT	161
	Reverse	CCTTTGAAAATGGCGGTTG	
<i>Lebocin</i>	Forward	ACGTGCGTAGTGTGAACGAG	170
	Reverse	CGCAGATTATGAGTTACGACGA	
<i>Lysozyme</i>	Forward	TACAAACGCCACAAGTTCCA	309
	Reverse	AGGCTTGTAACCAAGGACGA	
<i>Moricin 2</i>	Forward	TCGCGTTGTGCTATTATTTT	327
	Reverse	CAGAAGATTCCGAAGGGAGA	
<i>rpS3</i>	Forward	CTGGCTGAGGATGGCTACTC	186
	Reverse	TTTCTCAGCGTACAGCTCCA	

All sequences read 5' to 3' left to right.

and cooling overnight at room temperature. The annealing of ss-RNAs was found to be complete when examined by agarose gel electrophoresis. ds-RNAs were diluted to 2 µg/ml in DMPC treated water, and stored at -20 °C until required. As a negative ds-RNA control we used a gene from a plant, *Manihot esculenta* catalase *CAT1* (GenBank accession no. AF170272). The template for synthesis of ds-RNA control (dsCON) was a pBluescript IKS+ plasmid containing the cloned gene, which was amplified by PCR using T3 and T7 sequencing primers, and dsCON was then synthesised as described above. For RNAi, ds-RNAs (50 µl) were injected into fifth instar *M. sexta* 6 h before challenge with *E. coli*. Controls involving DMPC water without ds-RNA in the primary injections and PBS without *E. coli* in the secondary injections were also used. After RNAi injection, treated insects were held at 28 °C for 18 h and then dissected in order to isolate fat body as before. Two insects were used for each treatment. RT-PCR reactions using extracted RNA as a template and suitable pair of primers were performed to determine mRNA transcription patterns of each gene.

For Western analysis, *M. sexta* haemolymph plasma samples were diluted in sample buffer (2 × sample buffer: 0.1 Tris, 4% SDS, 5% 2-mercaptoethanol, 0.01% Coomassie blue G250, pH 6.8) and separated by SDS-PAGE using a 12% acrylamide separating gel and a 5% acrylamide stacking gel. After electrophoresis, plasma protein samples were stained with Coomassie blue or transferred electrophoretically (Mini Trans-Blot Transfer Cell, Bio-Rad, UK) to PVDF membranes (Bio-Rad, UK). The blots were then blocked in 5% skimmed milk powder (Marvel) in transblotting solution (TBS, 20 mM Tris, 0.9% NaCl, pH 7.2) for 12 h in at 4 °C. The blots were washed (three times for 10 min) in TBS followed by incubation in 3% Tween transblotting milk solution (TTBS, 20 mM Tris, 0.9% NaCl, 0.1% Tween-20, pH 7.2) containing 1/10,000 dilution of the appropriate primary antibody (anti-serum raised in rabbit reactive against *M. sexta* hemolin, PGRP or immunectin-2; all generous gifts from Michael Kanost) for 1 h at room temperature. After subsequent washing (three times for 10 min in TTBS) the blots were incubated in 3% TTBS milk solution containing 1/10,000 dilution of horseradish peroxidase-labelled goat anti-rabbit IgG secondary antibody (Upstate) for 1 h at room temperature. After further washing the bound antibodies were detected using a chemiluminescence Western blotting kit (Visualizer, Upstate) and the membranes were exposed to X-ray film (Biomax, Kodak) for 1 s to detect the signals.

3. Results

3.1. Prior-infection with *E. coli* protects *Manduca sexta* against pathogen infection

Following injection of 1×10^8 cells of the *P. luminescens* TT01 pathogen into day 0 fifth stage larvae of *M. sexta*, all of the injected insects died within 48 h (Fig. 1A). However,

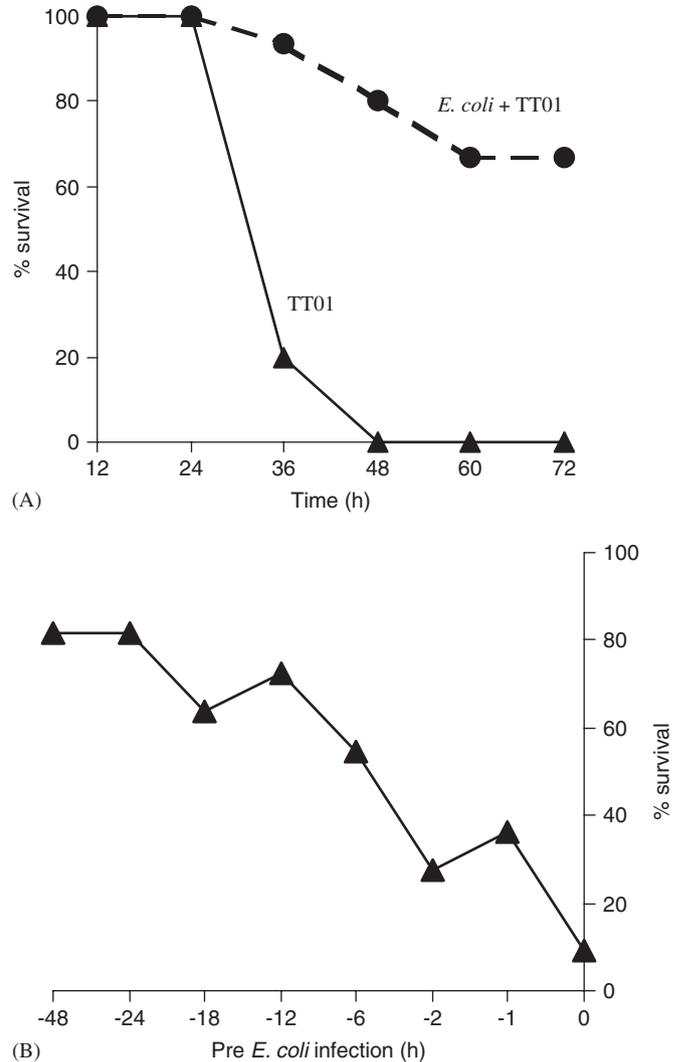


Fig. 1. Prior-injection of non-pathogenic *Escherichia coli* DH5 α protects *M. sexta* larvae against the lethal insect pathogen *Photobacterium luminescens* TT01. (A) Normally injection of *P. luminescens* TT01 (1×10^8 cells) kills all *Manduca* within 48 h (TT01). However, prior infection with *E. coli* (3×10^7 cells) 18 h earlier protects against the subsequent lethality of a TT01 injection (*E. coli* + TT01). (B) The protective effect of prior infection of *E. coli* increases with increasing time between exposure of insects to *E. coli* and the time of subsequent challenge with *P. luminescens* TT01. Note, that 18 h (shaded box) was chosen as a standard time window for subsequent pathogen exposure as the protective effect is nearing its maximum by this time.

when they had been exposed to a prior infection with *E. coli* 18 h earlier, 80% of the insects exposed to *Photobacterium* survived to 48 h and 70% survived to 72 h (Fig. 1A). This represents the elicitation of a protective immune response by one Gram-negative bacterium (*E. coli*) against another (*P. luminescens*).

To investigate the extent and timing of this protective effect we pre-injected insects with *E. coli* at differing time intervals prior to pathogen injection. We found that the pre-infected insects were protected against *P. luminescens* infection to at least some extent as early as 1–2 h after injection of *E. coli*. The extent of protection was

progressively greater as more time elapsed between the time of the *E. coli* pre-infection and the time of the *P. luminescens* challenge. Maximal protection (70–80% survival at 48 h after pathogen challenge) was achieved when animals were pre-infected with *E. coli* 18 h before *P. luminescens* or earlier (Fig. 1B). In all subsequent experiments 18 h was used as the standard time for pre-injection of *E. coli*.

3.2. Up-regulation and knock-down of *Manduca recognition and effector genes*

To investigate the molecular basis of the protective effect associated with *E. coli* immunisation, we used RT-PCR to detect up-regulation of both immune recognition (*hemolin*, *immulectin-2* and *peptidoglycan recognition protein*) and effector (*attacin*, *cecropin*, *lebobcin*, *lysozyme* and *moracin*) genes in fat body cells of *M. sexta*. For all eight genes, prior injection of *E. coli* led to significant over-transcription of both recognition (Fig. 2A) and effector genes (Fig. 2B) compared to control insects injected with PBS, or uninjected controls, in which RT-PCR did not detect any of these mRNAs at all.

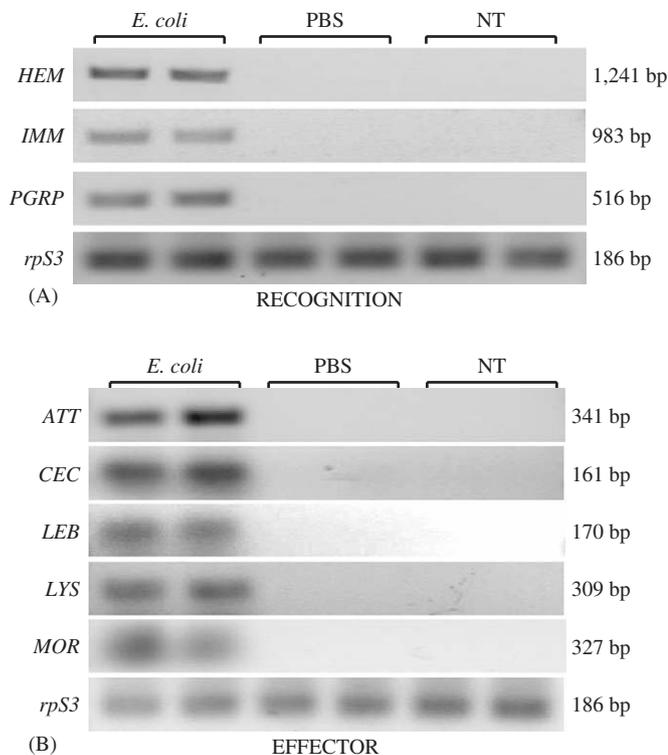


Fig. 2. RT-PCR shows that mRNA levels of both microbial pattern recognition and antimicrobial effector genes of *M. sexta* are up-regulated by prior *E. coli* infection. (A) The recognition genes *hemolin* (HEM), *immulectin-2* (IMM) and *peptidoglycan recognition protein* (PGRP) are all over-transcribed 18 h after *E. coli* infection. Note that no signal is seen in negative controls including *Manduca* injected with PBS alone (PBS) or in untreated animals (NT). (B) The effector genes *attacin* (ATT), *cecropin* (CEC), *lebobcin* (LEB), *lysozyme* (LYS) and *moracin* (MOR) are all over-transcribed 18 h after prior *E. coli* infection. Two replicate experiments (different insects) are shown for each treatment. RT-PCR amplification of the constitutively expressed gene *rpS3* is used as a loading control.

To demonstrate that RNAi could ‘knock-down’ this *E. coli*-induced gene expression, we pre-treated animals with individual ds-RNA reagents specific to each gene of interest. Following RNAi, gene over-transcription was

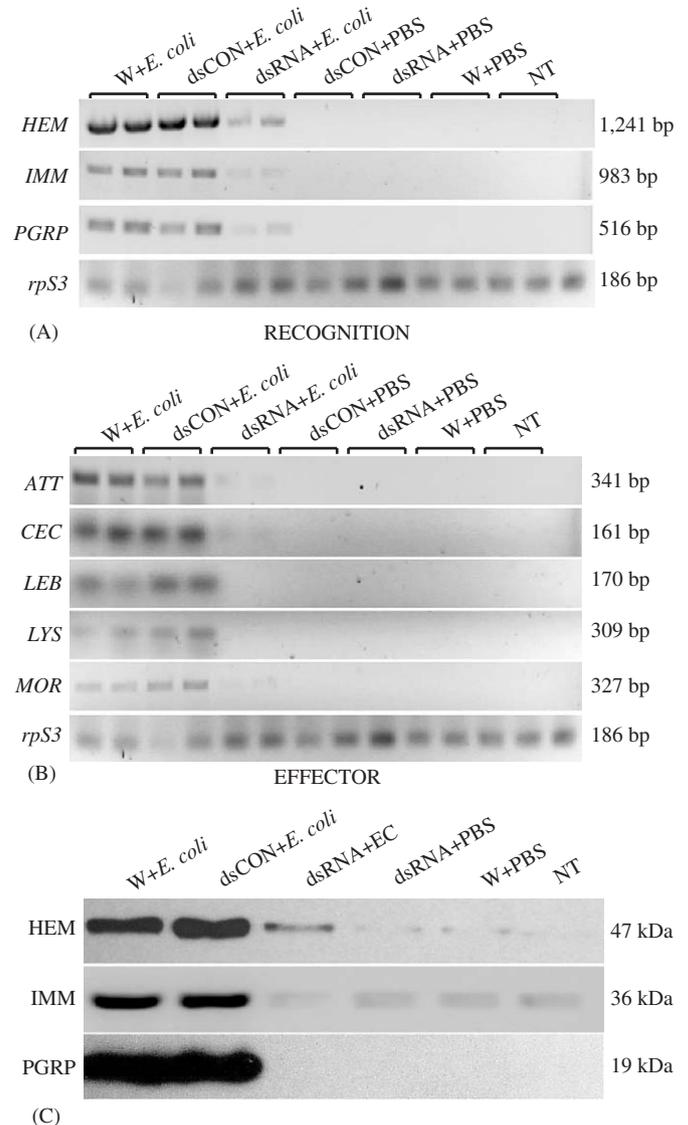


Fig. 3. Injection of double-stranded RNA knocks down *E. coli*-induced over-transcription of *Manduca* immune genes. (A) Injection of *E. coli* again leads to over-expression of the three recognition genes (*E. coli*). However, prior injection of ds-RNA specific for each of the three genes either abolishes, or greatly reduces, gene transcription (ds-RNA + *E. coli*). (B) Similarly *E. coli* injection again induces over-transcription of all the effector genes, which in turn can be knocked down by prior injection with each of the respective ds-RNAs. In contrast, the control ds-RNA reagent dsCON has no effect on immune gene transcription. Note that in both panels all negative controls (either injection of ds-RNA alone with no *E. coli* infection) (ds-RNA + PBS), injection of PBS alone (PBS) or no treatment (NT) produce no immune response (see Fig. 2 for gene names). (C) Western blot using anti-recognition protein antibodies to confirm that RNAi of these genes also results in reduced protein expression. However, protein expression of the three recognition genes is not reduced in insects pre-injected with dsCON. Specificity of staining was checked by omitting the primary antibody, which led to complete absence of bands (results not shown). Two replicate experiments (different insects) are shown for (A) and (B).

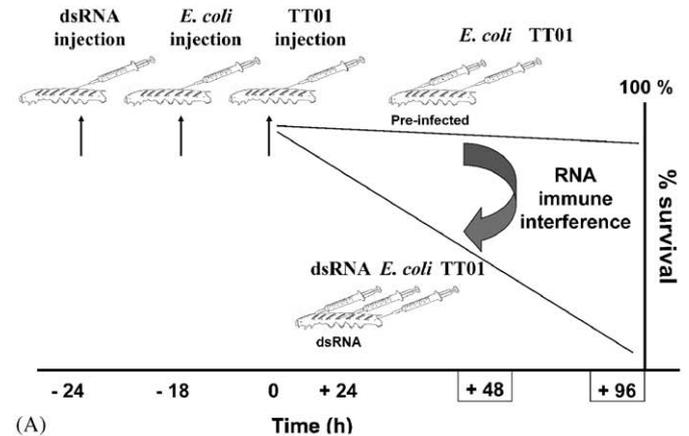
either strongly reduced or not detectable at all by RT-PCR in fat body cells (Fig. 3A and B). That the knock-down response was specific to the ds-RNA for each gene was shown by the observation that a ds-RNA control reagent prepared from an 'irrelevant' gene (from a plant) had no effect on the expression of any of the genes that we investigated. Negative controls, including injection of ds-RNA alone with no subsequent *E. coli* infection, showed no detectable effect on the transcription of any of the immune-response genes. Thus, *E. coli* induces over-transcription of immune response genes in *M. sexta*, and this can be specifically knocked down by RNAi.

To confirm that RNAi-induced reductions in the amount of mRNA led to a corresponding reduction in protein levels we also performed Western analysis using anti-recognition protein antibodies (Fig. 3C). The results confirmed that RNAi knock-down of fat body mRNA resulted in similar changes in haemolymph protein levels. Following ds-RNA treatment, PGRP was undetectable in haemolymph, while levels of haemolin and immuectin-2 were strongly reduced to levels comparable with those seen in controls which had not been pre-infected.

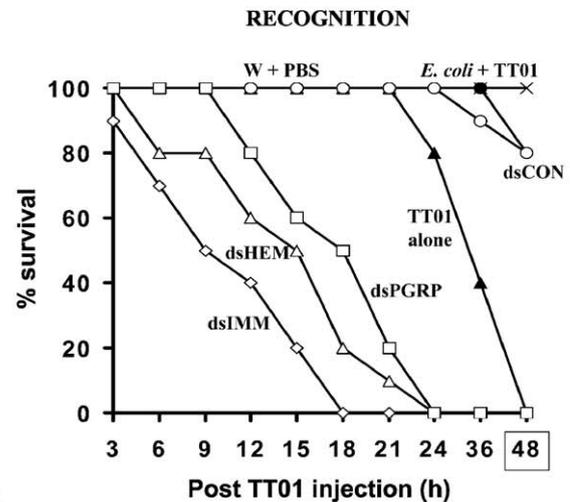
3.3. Knock-down of immune-related genes increases susceptibility to *Photorhabdus* infection

To test if RNAi-mediated knock-down of either recognition or effector proteins could lead to a reduction in the protective effect conferred by prior *E. coli* infection, we tested the survival of immunised larvae pre-treated with ds-RNA (Fig. 4A). In these experiments, knock-down of any of the three recognition proteins, immuectin-2, hemolin and PGRP, led to faster insect death (Fig. 4B). The increase in mortality at 24 h was statistically highly significant (χ^2 test, $P < 0.001$). Similarly, knock-down of any one of three effectors (cecropin, attacin and moricin) also increased the rate at which RNAi treated insects died. These insects were significantly more susceptible than pre-infected controls given a control ds-RNA (72 h mortality, χ^2 test, $P < 0.001$)

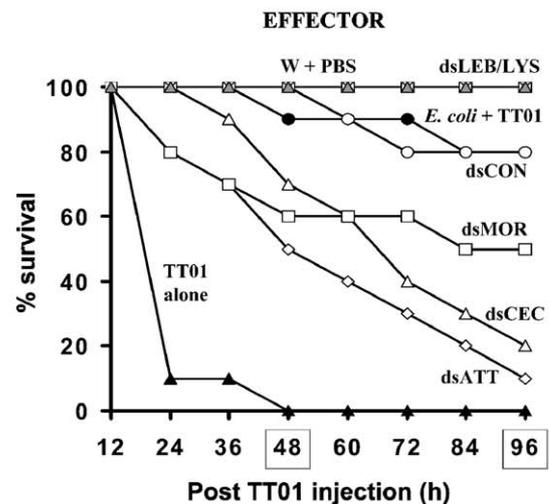
but they were still less susceptible than those insects that had not been pre-infected with *E. coli*. This indicates that cecropin, attacin and moricin are all involved in induced protection, but that no single effector can account for the protective effect. By contrast, RNAi suppression of lysozyme and lebecin expression had no detectable effect on the susceptibility of the treated insects (Fig. 4C), indicating that these effectors probably do not play any part in *E. coli*-induced immunity to *P. luminescens*.



(A)



(B)



(C)

Fig. 4. RNAi-mediated knock-down of immune recognition or effector genes is also associated with decreased survival following pathogen exposure. (A) Diagram illustrating experimental design for RNAi survival experiments. *Manduca* larvae are given sequential injections of ds-RNA, non-pathogenic *E. coli* and then the insect pathogenic *P. luminescens* TT01. Their survival is then monitored over 96 h. We postulated that animals with immune systems compromised by the injection of specific ds-RNAs would also show reduced survival after exposure to the TT01 pathogen (arrow). (B) RNAi induced knock-down of each of the three immune recognition genes speeds the rate at which *Manduca* die after TT01 infection. Note that TT01 alone kills all insects by 48 h and that knock-down of any one of the recognition genes kills all the insects within 18–24 h following the same dose of pathogen. RNAi of the recognition genes therefore effectively makes them 'super-susceptible' to the pathogen. (C) Similarly, RNAi knock-down of most of the effector genes increases mortality associated with the normally non-lethal *E. coli*. Note, however, that two of the RNAi treatments (dsLYS and dsLEB) have no effect (see text for discussion and Fig. 2 for gene names). In both cases injection of the unrelated ds-RNA control dsCON prior to pathogen infection has no effect on insect survival.

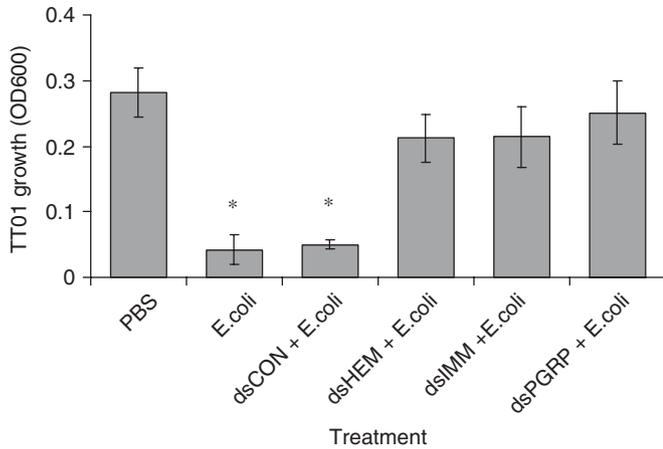


Fig. 5. The RNAi-inhibitable antibacterial immune response is associated with cell-free haemolymph plasma. Histogram showing the final density (optical density at 600A, OD₆₀₀) of the *P. luminescens* TT01 pathogen after 18 h growth in cell-free plasma collected from *M. sexta* larvae after different individual treatments. Insects were injected with PBS alone (PBS) or *E. coli* (*E. coli*) with or without prior injection of ds-RNA for hemolin (dsHEM + *E. coli*), immunectin-2 (dsIMM + *E. coli*) or peptidoglycan recognition protein (dsPGRP + *E. coli*). Note that *Photobacterium* grows poorly in plasma from insects that had experienced prior infection with *E. coli*, but that this growth inhibiting effect can be overcome by pre-injection of ds-RNA from any one of the recognition genes. Again, pre-injection of the control RNA dsCON prior to *E. coli* infection has no effect on *Photobacterium* growth. Bars show means + SE ($n = 5$). The asterisks show values that were significantly different ($P < 0.01$) from the PBS controls.

3.4. The protective effect is associated with cell-free haemolymph

Given that the induced immunity to infection of pre-infected insects has long been known to be accompanied by the presence of microbicidal activity in haemolymph (Stephens, 1962) and that insects are now known to secrete both recognition proteins and antimicrobial effectors into the haemolymph (Kanost et al., 2004), we examined the rate of growth of the *Photobacterium* pathogen in the cell-free plasma of haemolymph bled from treated insects. The results (Fig. 5) show that plasma of animals pre-infected with *E. coli* supports the growth of *P. luminescens* TT01 significantly less well than does plasma from animals pre-treated with buffer (PBS) alone. Moreover this protective effect, now isolated to the blood plasma, can be abolished by prior RNAi knock-down of the induced expression of any one of the three recognition genes (*hemolin*, *immunectin-2* or *PGRP*), but not by treatment with control ds-RNA. This shows that all three immune recognition proteins are involved in the induction of synthesis and secretion of effectors into the haemolymph of *E. coli* infected insects.

4. Discussion

In this study we have shown that pre-infection of fifth stage larvae of *M. sexta* with a harmless Gram-negative

bacterium (a laboratory cloning strain of *E. coli*) protects previously unchallenged tobacco hornworms against subsequent infection with a virulent pathogen (*P. luminescens* TT01). The roles of a number of immune-related genes, including recognition and effector proteins have been probed using RNA interference. It is evident that the induced expression of three known recognition proteins, hemolin, PGRP and immunectin-2 is strongly implicated in the protective effect of prior infection with *E. coli*, and that the protective response involves the recognition protein-dependent secretion into the haemolymph of one or more effectors that prevent the growth of pathogenic bacteria. Attacin, cecropin and moricin appear to be among these effectors; although leibocin and lysozyme are upregulated following the *E. coli* prior infection, they are not involved in protecting the insect against infection by the pathogenic *P. luminescens*.

The fact that RNAi knock-down of any one of hemolin, PGRP and immunectin-2 causes the treated insects to be even more susceptible to pathogen infection than those insects that had not previously experienced prior infection with *E. coli*, implies that these three proteins must play a part in a normally occurring but ultimately ineffective defence against *P. luminescens*. This is in accord with our previous finding (Eleftherianos et al., 2006) that these three genes are strongly expressed following exposure to this pathogen and that RNAi knock-down results in faster death when previously uninfected insects are challenged with *Photobacterium*.

Several studies have used RNAi to modulate genes involved in the lepidopteran immune system. The first report to demonstrate gene silencing in Lepidoptera was a study of the effects of *hemolin* RNAi in *Hyalophora cecropia*. This study showed that hemolin, a member of the immunoglobulin superfamily, is required for normal embryo development (Bettencourt et al., 2002). More recently, RNAi has been used to investigate the role of a haemocyte-specific *Manduca* integrin in the encapsulation of polymer beads (Levin et al., 2005).

The specificity of the RNAi technique for the targeted gene is indicated by the fact that although all RNAi treatments were effective in knocking down the targeted gene, their effects on immunity differed markedly. In previous work (Eleftherianos et al., 2006) we found that treatment with a ds-RNA reagent directed against any one of the same recognition proteins studied here did not affect the expression of the other two. In the present experiments we included numerous control treatments (e.g. a control ds-RNA, injection-specific ds-RNA without subsequent infection, or no treatment) all of which were consistent with the supposition that the effects of ds-RNA treatment are specific to the targeted gene. Importantly, we did not observe any case in which administration of a ds-RNA reagent alone elicited expression of the targeted gene. This contrasts with the study of Hirai et al. (2004), in which up-regulation of hemolin expression in *H. cecropia* pupae was reported in response to the specific ds-RNA reagent alone.

The reasons for the effect observed by Hirai et al. (2004) remain unknown.

The timing of the protective effect of prior-infection with *E. coli* against subsequent pathogen infection is interesting (Fig. 1). Reduced susceptibility to *Photorhabdus* appeared rapidly, within a few hours, and persisted for at least 48 h. This is consistent with previous observations (Stephens, 1959, 1962) in which immunity was induced within hours of exposure to microbial pattern molecules. There was no sign in our experiments of any decline in immunity during the 48 h period of the experiment. We considered it inappropriate to extend the duration of the experiment beyond this time, because at later times the control insects would be preparing to pupate, when new immune defences begin to appear according to a developmental programme (Dunn et al., 1994). In previous work with *Galleria mellonella* larvae (Stephens, 1959, 1962) immunity induced by injection of killed *P. aeruginosa* cells was found to decline after about 24 h. The possibility that the progressive loss of immunity with time seen in Stephens' experiment was due to the onset of pupation cannot be excluded. Further work is required to define more precisely the duration of the protection afforded by pre-infection. Although the induced immunity to infection may be temporary, it should be realised that the insect's larval life is in any case relatively short, so that even temporary protection against infection is significant.

The protective effect of *E. coli* pre-infection has been shown here to involve all of the recognition genes and several of the effector genes studied. This is consistent with previous observations in *Drosophila* demonstrating that numerous recognition and effector proteins are deployed against a single pathogen (Tzou et al., 2002). Interestingly, however, knock-down of any one individual *Manduca* recognition gene by RNAi caused 'super-susceptibility' to subsequent pathogen infection (Fig. 4B). This observation is surprising as one might expect recognition proteins to show some level of functional redundancy, i.e. that a full immune response could still be mounted in the absence of any single recognition factor. These data therefore reinforce the suggested complexity of the insect immune system, and demonstrate that a normal immune response (even in the absence of pre-infection) requires the presence of a full complement of recognition genes. Similarly, RNAi knock-down of individual effector genes, with the exception of lysozyme and lebecin, also reduced the protective effect of prior *E. coli* infection (Fig. 4C). This suggests that attacin, cecropin and moricin are all involved in defence against *P. luminescens*.

Interestingly, the effect of knocking down any one of the three recognition proteins was much greater than for any of the effectors that we studied. The reason for this is not entirely clear. Recognition proteins have been suggested to play individual but overlapping roles in activating the insect immune system (Yu et al., 2002) but the strong effects observed here of interfering with any one individual pattern recognition protein suggest instead that these

proteins co-operate during immune response activation. Thus, removing any one of them might have unexpectedly severe consequences for immunity. This is an area deserving further research. It is known, for example, that *Manduca* recognition proteins including immuelectin-2 form protein complexes in the haemolymph that are involved in prophenoloxidase activation (Yu et al., 2003).

The failure of lebecin and lysozyme knock-down to influence *Photorhabdus* virulence suggests that these two antimicrobial effector proteins are ineffective in the destruction of these Gram-negative pathogens in *Manduca*, despite their induction by pre-infection with another Gram-negative bacterium, *E. coli* (both *E. coli* and *P. luminescens* are Gram negative members of the Enterobacteriaceae—Liu et al., 1997). This shows that the immune response elicited by pre-infection is not limited to the expression of agents specific to a particular kind of bacteria. The inefficacy of lebecin against *Photorhabdus* is perhaps not surprising since this AMP has only limited antibacterial activity except under low-salt conditions, and may not normally act alone as an antimicrobial agent (Hara and Yamakawa, 1995). The lack of effect of RNAi knock-down of lysozyme was also to be expected since this enzyme is principally active against Gram-positive bacteria (Hughey and Johnson, 1987). We also point out that the lack of effect of these two RNAi reagents on induced immunity is further evidence that the marked effects of the other RNAi reagents tested were not non-specific responses that would have occurred following the introduction of any ds-RNA.

In their broadest context, these results also suggest that previous experiments conducted on naïve and antibiotic-treated animals may not accurately reflect the normal range of immune responses shown by insects in their natural environments. Given the continuous and potentially chronic exposure of most insects to a range of viruses, fungi, yeasts and bacteria in the environment, as well as the continuous presence of their microbial symbionts (both intra- and extra-cellular) we might expect that most insects effectively have their immune systems 'primed' all the time. Our results therefore beg a detailed examination of the range of immune responses presented by insects in their native environments. Only by understanding this range of response can we begin to understand the true ability of insect populations to withstand, or persist with, their microbial pathogens and symbionts.

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References

- Bettencourt, R., Terenius, O., Faye, I., 2002. Hemolin gene silencing by ds-RNA injected into cecropia pupae is lethal to next generation embryos. *Insect Mol. Biol.* 11, 267–271.
- Boman, H.G., Hultmark, D., 1987. Cell-free immunity in insects. *Ann. Rev. Microbiol.* 41, 103–126.
- Boman, H.G., Nilsson, I., Rasmusson, B., 1972. Inducible antibacterial defence system in *Drosophila*. *Nature* 237, 232–235.
- Boman, H.G., Nilsson-Faye, I., Paul, K., Rasmusson Jr., T., 1974. Insect immunity I. Characteristics of an inducible cell-free antibacterial reaction in hemolymph of *Samia cynthia* pupae. *Infection and Immunity* 10, 136–145.
- Dillon, C.P., Sandy, P., Nencioni, A., Kissler, S., Rubinson, D.A., van Parijs, L., 2005. RNAi as an experimental and therapeutic tool to study and regulate physiological and disease processes. *Ann. Rev. Physiol.* 67, 147–173.
- Dunn, P.E., Bohnert, T.J., Russell, V., 1994. Regulation of antibacterial protein-synthesis following infection and during metamorphosis of *Manduca sexta*. *Ann. New York Acad. Sci.* 712, 117–130.
- Eleftherianos, I., Millichap, P.J., French-Constant, R.H., Reynolds, S.E., 2006. RNAi suppression of recognition protein mediated immune responses in the tobacco hornworm *Manduca sexta* causes increased susceptibility to the insect pathogen *Photorhabdus*. *Dev. Comp. Immunol.*, in press.
- Hara, S., Yamakawa, M., 1995. A novel antibacterial peptide family isolated from the silkworm, *Bombyx mori*. *Biochem. J.* 310, 651–656.
- Hirai, M., Terenius, O., Li, W., Faye, I., 2004. Baculovirus and ds-RNA induce Hemolin, but no antibacterial activity, in *Antheraea pernyi*. *Insect Mol. Biol.* 13, 399–405.
- Hughey, V.L., Johnson, E.A., 1987. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. *Appl. Environ. Microbiol.* 53, 2165–2170.
- Jiang, H., Wang, Y., Kanost, M.R., 1996. Primary structure of ribosomal proteins S3 and S7 from *Manduca sexta*. *Insect Mol. Biol.* 5, 31–38.
- Kanost, M.R., Jiang, H., Yu, X.Q., 2004. Innate immune responses of a lepidopteran insect, *Manduca sexta*. *Immunol. Rev.* 198, 97–105.
- Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J.H., Caroff, M., Lee, W.J., Mengin-Lecreux, D., Lemaitre, B., 2003. The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition. *Nature Immunol.* 4, 478–484.
- Levin, D.M., Breuer, L.N., Zhuang, S., Anderson, S.A., Nardi, J.B., Kanost, M.R., 2005. A hemocyte-specific integrin required for hemocytic encapsulation in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* 35, 369–380.
- Liu, J., Berry, R., Poinar, G., Moldenke, A., 1997. Phylogeny of *Photorhabdus* and *Xenohabdus* species and strains as determined by comparison of partial 16S rRNA gene sequences. *Int. J. System Bact.* 47, 948–951.
- Otvos, L., 2000. Antibacterial peptides isolated from insects. *J. Peptide Sci.* 6, 497–511.
- Reynolds, S.E., Nottingham, S.F., Stephens, A.E., 1985. Food and water economy and its relation to growth in 5th-instar larvae of the tobacco hornworm, *Manduca sexta*. *J. Insect Physiol.* 31, 119–127.
- Royet, J., 2004. Infectious non-self recognition in invertebrates: lessons from *Drosophila* and other insect models. *Mol. Immunol.* 41, 1063–1075.
- Royet, J., Reichhart, J.M., Hoffmann, J.A., 2005. Sensing and signaling during infection in *Drosophila*. *Curr. Opin. Immunol.* 17, 11–17.
- Steiner, H., Hultmark, D., Engström, Å., Bennich, H., Boman, H.G., 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* 292, 246–248.
- Stephens, J.M., 1959. Immune responses of some insects to some bacterial antigens. *Can. J. Microbiol.* 5, 203–228.
- Stephens, J.M., 1962. Bactericidal activity of the blood of actively immunized wax moth larvae. *Can. J. Microbiol.* 8, 491–499.
- Tzou, P., De Gregorio, E., Lemaitre, B., 2002. How *Drosophila* combats microbial infection: a model to study innate immunity and host–pathogen interactions. *Curr. Opin. Microbiol.* 5, 102–110.
- Yu, X.Q., Zhu, Y.F., Ma, C., Fabrick, J.A., Kanost, M.R., 2002. Pattern recognition proteins in *Manduca sexta* plasma. *Insect Biochem. Mol. Biol.* 32, 1287–1293.
- Yu, X.Q., Jiang, H.B., Wang, Y., Kanost, M.R., 2003. Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* 33, 197–208.
- Zhu, Y., Johnson, T.J., Myers, A.A., Kanost, M.R., 2003. Identification by subtractive suppression hybridization of bacteria-induced genes expressed in *Manduca sexta* fat body. *Insect Biochem. Mol. Biol.* 33, 541–559.