Bulletin of Insectology ***65*** *(2): 233-238, 2012*
ISSN 1721-8861

Cloning and characterisation of the gallerimycin gene from immunised *Spodoptera litura*

Zhongkang WANG, Wendi ZHANG, Huan CHEN, Youping YIN
Genetic Engineering Research Center, College of Bioengineering, Chongqing University, PR China

Abstract

We report the cloning of *Sl-gallerimycin* from the armyworm *Spodoptera litura* (F.) (Lepidoptera Noctuidae). Full length *Sl-gallerimycin* cDNA encodes a 75-amino acid protein sharing 80-95% amino acid sequence identity with the defensin-like antifungal peptide gallerimycin, and phylogenetic analysis reveals that *Sl-gallerimycin* is closely related to other known lepidopteran gallerimycin genes. Using quantitative real-time PCR (RT-qPCR) analysis, we show that *Sl-gallerimycin* expression is very low in naive larvae but can be strongly induced in larval fat bodies by the entomopathogenic fungus *Nomuraea rileyi* (Farlow) Samson. Knocking-down *Sl-gallerimycin* transcripts by dsRNA interference accelerates death in insects infected with *N. rileyi*. This is the first report showing that *Sl-gallerimycin* plays a significant role in resisting *N. rileyi* infection.

Key words: antifungal peptide, *Sl-gallerimycin*, RNAi, *Spodoptera litura*, RT-qPCR.

Introduction

Unlike mammals, insects do not have antibodies, T- and B-lymphocytes, or a complement system. Immune defence in insects is achieved through haemocyte and humoral responses. Haemocyte responses include phagocytosis and encapsulation and humoral responses include the synthesis of antimicrobial peptides (Brey, 1998, Lavine and Strand, 2002). Antimicrobial peptides are synthesised either in the fat body or by haemocytes. They are secreted into the haemolymph after being infected by microbes, which they rapidly kill. More than 500 different antimicrobial peptides have been reported in insects (Theis and Stahl, 2004; Schuhmann *et al*., 2003), including dozens of antifungal peptides (Wu *et al.*, 2009). Antimicrobial peptides are classified into three major groups: (i) peptides with α helical conformation (insect cecropins, magainins, etc.), (ii) cyclic and open-ended cyclic peptides with pairs of cysteine residues (defensins, protegrins, etc.), and (iii) peptides with an over representation of some amino acids (proline rich, histidine rich, etc.) (Bulet *et al.*, 2004).

Gallerimycin is a defensin-like antifungal peptide gene which was first cloned in the great wax moth *Galleria mellonella* (L.) from haemocytes taken from lipopolysaccharide (LPS) pretreated larvae. Kazuhiko Hashimoto (2008) isolated a cDNA encoding gallerimycin from the immunised larval fat body of wild silk worm moth, *Samia cynthia ricini* (Boisduval). Their studies showed that gallerimycin expression can be highly induced by bacteria, the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin, peptidoglycan and zymosan (Hashimoto *et al.*, 2008). Gallerimycin is an inducible peptide with a conserved domain containing a unique spacing of six cysteine residues. It has no obvious effects on Gram-positive and Gram-negative bacteria or yeast, but does have clear effects on filamentous fungi (Schuhmann *et al.*, 2003). Other studies have shown that gallerimycin expression can be induced in *G. mellonella* larvae by several kinds of microbial infection and plays an important role in the immune response of *G. mellonella* larvae (Bergin *et al.*, 2006, Wojda and Jakubowicz., 2007, Mowlds *et al.*, 2008, Wojda *et al.*, 2009). Further documentation of the antifungal role of gallerimycin comes from its transfection into tobacco plants, where it confers resistance to fungal pathogens (Langen, 2006).

Spodoptera litura (F.) causes serious damage to a wide variety of agricultural crops throughout the globe every year. In the field, the fungus *Nomuraea rileyi* (Farlow) Samson is the most important suppressor of *S. litura* populations. However, there is to date no documentation of the gallerimycin gene in *S. litura*. In this study, we report the cloning of *Sl-gallerimycin* from the immunised larval fat body of *S. litura*, and describe its relationship with other insect gallerimycin genes. In addition, we investigate its gene function using RNAi and real time quantitative PCR (RT-qPCR).

Materials and methods

Rearing of *S. litura*

The *S. litura* larvae used for immunisation were reared in our laboratory and fed with an artificial diet (Qi-Jin *et al*., 2000) until the 5th instar larval stage. During the entire rearing period, temperature (27 °C) and humidity (75%) were maintained 16L: 8D cycles.

N. rileyi culture and preparation of spores

The *N. rileyi* strain CQNr01 isolated by Z. K. Wang from natural fungi infected dead larvae of *S. litura*, was reactivated and incubated in medium containing 50 g/L lactose, 5 g/L yeast extract, 5 g/L peptone, and 40 mg/L vitamin B1 for about 2 weeks at 27 °C. The spores were collected and filtered to remove medium, washed twice with sterile apyrogenic water and once with 0.05% Tween-80 after which they were resuspended in 0.05% Tween-80 and diluted to a concentration of about 10^7 spores/ml.

Immunisation of *S. litura* and RNA extraction

S. litura were immunised by first anaesthetising the 5th instar larvae on ice for 30 min. After sterilising the surface with 70% ethanol, the larva was injected with 5 µl 1×10^7 ml^{-1} fungal spore suspension using a sterile syringe. The same volume of 0.05% Tween-80 was injected into another larva as a control. The larvae were subsequently reared normally as described above.

The fat body was collected at 0 h, 8 h, 16 h, 24 h and 48 h after fungal spore injection, and five larvae were used for each time point. The fat body for each treatment was ground to a fine powder in liquid nitrogen and total RNA was isolated using TRIzol (Invitrogen). RNA samples were further purified by the addition of RNase free DNaseI (Promega) (1 U/ml) at 37 °C for 1 h.

Cloning and sequencing of *Sl-gallerimycin*

Due to the absence of *S. litura* gallerimycin expressed sequence tags (ESTs) in GenBank, a pair of degenerate primers for *Sl-gallerimycin* (galF: 5'-TGY GTD TTT TAC GARTGC-3'; galR: 5'-ATC GMA GAC ATT GRC ATC-3'; (R = A / G, Y = C / T, M = A / C, D = A / G / T) was designed using oligonucleotides from highly conserved sequence motifs obtained from the conserved sequence (CVFYECIA*CRQ*****GGYCTINGCQCLR) of gallerimycin of *S. cynthia ricini* (gi|169264911), *Trichoplusia ni* (Hubner) (gi|157704333), *Spodoptera frugiperda* (Smith) (gi|33439716), *G. mellonella* (gi|21311450). Primers for 3' RACE (gal5: 5'-AAC GAG GCT ACA AGT CAG GTG GCT-3') and 5' RACE (outside primer-galw3: 5'-TGA CTA TCG CAG ACA TTG GCA TCC-3', inside primer-galn3: 5'-CCG ACT TGT AGC CTC GTT GT-3') were designed using conserved sequences. 3' RACE and 5' RACE were carried out using SMARTTM RACE cDNA Amplification Kit (Clontech). After obtaining both ends, the full length cDNA was obtained by sequencing across the splice joint.

Reverse transcription and RT-qPCR

One microgram of total RNA was used to synthesise cDNA according to the manufacturer's protocol (M-MLV, Promega). RT-qPCR was performed with gene-specific primers (galqr: 5'-CAA CGA GGC TAC AAG TCA GG-3'; galqf: 5'-CGA ACA TGG TAA GAC GAG AGC-3'). Actin was used as a reference gene (QActinS: 5'-TGA GAC CTT CAA CTC CCC CG-3'; QActinR 5'-GCG ACC AGC CAA GTC CAG AC-3'). RT-qPCR was carried out using the SYBR® Green I mix (TaRaKa) in a volume of 25 µl. The amplifying conditions were: 94 °C, 5 min; 42 cycles (95 °C, 5 sec; 59 °C, 30 sec), 95 °C, 30 sec; 55 °C, 30 sec; 55-95 °C, 0.5 °C was reduced per cycle. The standard curve was established with several serial dilutions of cDNA template from immunised larvae and a slope was obtained. The amplification efficiency of genes was estimated using the equation $E = 10^{-1/slope}$. The homogeneity of the PCR product was confirmed by melting curve analysis. The expression of *Sl-gallerimycin* was calculated using the $2^{-\Delta\Delta Ct}$ method.

RNAi and survival assay

The interference dsDNA sequence (196 bp) for the *Sl-gallerimycin* gene was cloned from total cDNA from the fat body using the following *Sl-gallerimycin* specific sequences followed by the T7 promoter sequence which was underlined (galrF: 5'-TAA TAC GAC TCA CTA TAG GGG CGT GGT TCT AGC TCT GT-3'; galrR: 5'-TAA TAC GAC TCA CTA TAG GGC TGA CTT GTA GCC TCG TT-3'). RNA was synthesised *in vitro* using the MEGAscript® High Yield Transcription Kit (Ambion). Sense and antisense RNA were annealed in DEPC treated water. The dsRNA was diluted to 300 ng/µl and stored at –80 °C until use. The GFP (Green Flour Protein) gene (GenBank accession no. U87973. 1) was obtained by PCR from the plasmid C:BarGFP using primers GFPF: 5'-TAA TAC GAC TCA CTA TAG GAG AGG GTG AAG GTG ATG C-3' and GFPR: 5'-TAA TAC GAC TCA CTA TAG GCT TGA AGT TGG CTT TGA T-3. A negative dsRNA was used as a control. The quality and quantity of dsRNA was determined by electrophoresis and ultraviolet spectrophotometry.

RNAi of *Sl-gallerimycin* was carried out by dsRNA injection followed by pathogen infection. 5 µl *Sl-gallerimycin* or GFP dsRNA was injected 6 h prior to injection of *N. rileyi* spores (referred to as time 0). After RNAi injection, treated insects were incubated at 27 °C for 18 h after which the fat body was isolated as described above and fat body RNA was extracted (Eleftherianos and Millichap, 2006). Five *N. rileyi* spore injected insects were used for each time point. Finally, RT-qPCR was performed to measure the efficiency of RNAi.

For each time point in the survival assay, we assayed thirty *S. litura* larvae injected with a 10 µl *N. rileyi* CQNr01 spore (1×10^7 spores/ml) suspension (secondary injection) 6 h after the dsRNA injection (primary injection). The GFP gene was used as a control. The two injection controls were (1) the injection of DMPC treated water without dsRNA in the primary injections and (2) the injection of 0.05% Tween-80 without *N. rileyi* CQNr01 in the secondary injections. The larvae mortality rate was recorded 72 h after treatment and blank control mortality did not exceed 10%. All time points were assayed three times. The LT_{50} value was estimated by probit analysis using DPS software.

Results

cDNA of *Sl-gallerimycin*

The full-length cDNA sequence of gallerimycin was obtained from cDNA from the fat body of *S. litura* larvae, and called *Sl-gallerimycin* (GenBank accession number is HM747937). The gene sequence and deduced amino acid sequence are shown in figure 1. The cDNA length is 380 bp, with a single open reading frame encoding 75 amino acid residues. A polyadenylation site can be found behind the stop codon. The calculated molecular weight of the deduced protein is 8.1 kDa and the pI (isoelectric point) is 8.79 (http://expasy.org/tools/protparam.html). The mature protein contains a signal peptide encoded by the first 20 amino acids (http://www.cbs.dtu.dk/services/SignalP/).

```
1       GACACTTTGAAAGGTTTCAGTCATGAAGGCTTGCGTGGTTCTAGCTCTGTTCCTCGTCGC
1                            M  K  A  C  V  V  L  A  L  F  L  V  A
61      TTTTGCGGTCGCGTCCTCCGCCGCTGATTTAGGACATACAGAAGCAGCTCACATAGTAAA
21       F  A  V  A  S  S  A  A  D  L  G  H  T  E  A  A  H  I  V  K
121     AAGAGAAACGATCCGAGGACCAGAGTTCCCGAACCGATGCGTGTTTTACGAATGCATTGC
41       R  E  T  I  R  G  P  E  F  P  N  R  C  V  F  Y  E  C  I  A
181     TAGCTGCAGACAACGAGGCTACAAGTCAGGTGGCTACTGCACCCTTGGTGGATGCCAATG
61       S  C  R  Q  R  G  Y  K  S  G  G  Y  C  T  L  G  G  C  Q  C
241     TGTGCGATAGTCATGTGTAATTTTAAATCTTGTATTGAATTTGAAGCTCTCGTCTTACCA
81       V  R
301     TGTTCGTATTATTAGTTGTAAGCCAAACATAAAATAAACTGTTTTACATTACAAAAAAAA
361     AAAAAAAAAAAAAAAAAAAA
```

Figure 1. Nucleotide sequence of *Sl-gallerimycin* cDNA. The deduced amino acid sequence is shown below the cDNA sequence. The polyadenylation sequence AATAAA is underlined. The open reading frame (ORF) codes for a putative protein of 75 amino acids. The signal peptide is underlined.

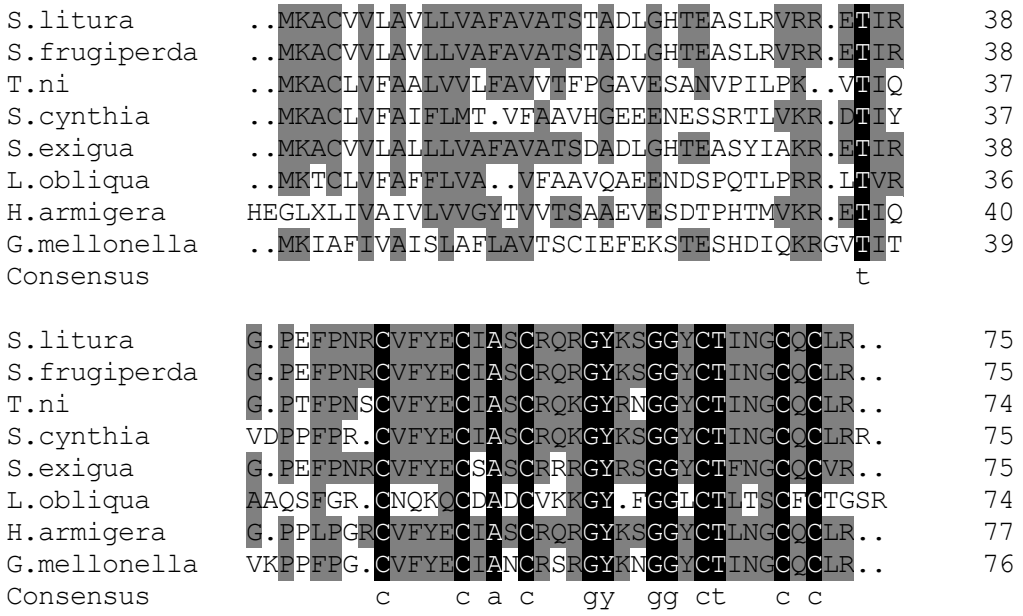

Figure 2. The amino acid sequence alignment of *Sl-gallerimycin* with other known gallerimycins. The high-consensus amino acid residues are highlighted in black and all six cysteine residues are within this category.

We compared the amino acid sequence of the deduced protein with the seven gallerimycin sequences available in GenBank (figure 2). The results showed that all gallerimycins have a high shared homology. *Sl-gallerimycin* has the strongest identity (93%) with gallerimycin from *S. frugiperda*. Like other gallerimycins, *Sl-gallerimycin* contains six cysteine residues in its C-terminus which, as in other defensins, are possibly involved in intra molecular disulphide bonds. The amino acid residues in this region are highly conserved among gallerimycins. A bootstrapped neighbour-joining tree was generated from these homologous genes using MEGA 4 (figure 3). Although gallerimycin is related to heliomycin and drosomycin, and was named according to drosomycin, the genetic relationship between heliomycin and drosomycin is closer.

Sl-gallerimycin expression in the larval fat body after induction by *N. rileyi*

After induction by *N. rileyi*, the expression of the *Sl-gallerimycin* gene in the larval fat body of immunised fifth instar *S. litura* larvae was analysed by RT-qPCR. The expression level of *Sl-gallerimycin* mRNA is 20 times higher than in naive control larvae, and increases in a time-dependent manner for the first 24 h (figure 4). The induction of *Sl-gallerimycin* can be observed at 8 h

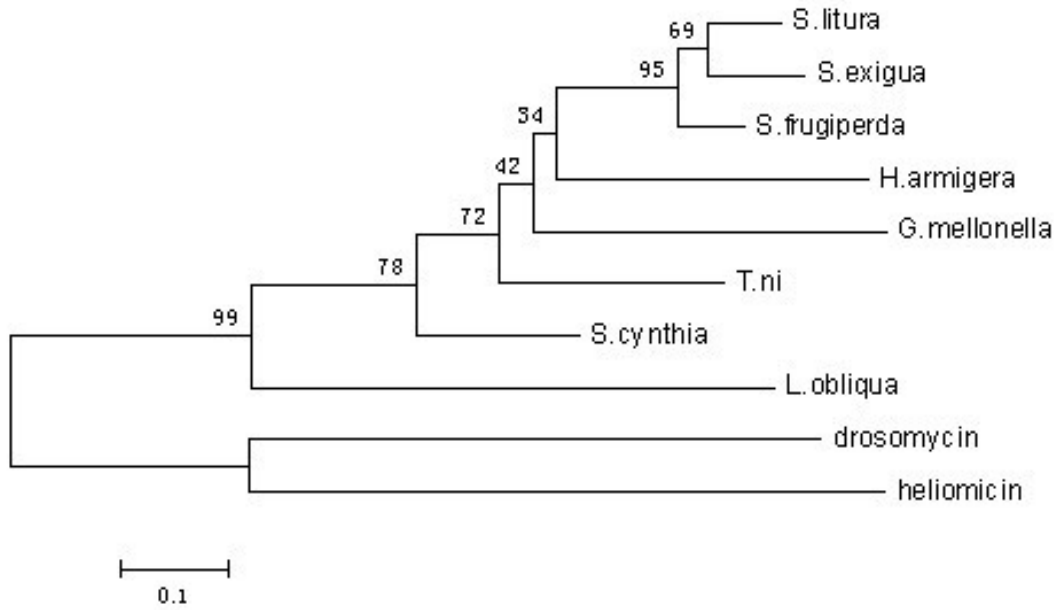

Figure 3. Bootstrapped neighbour-joining tree generated using gallerimycins, drosomycin and heliomycin.

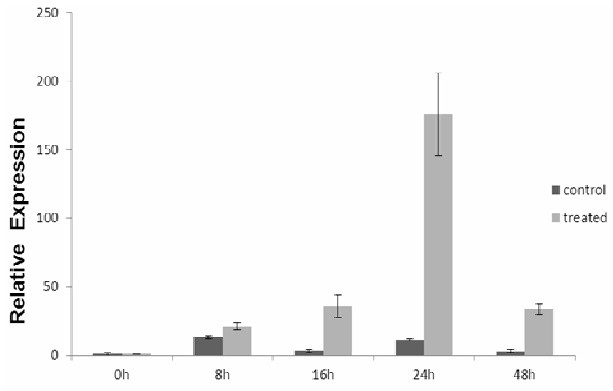

Figure 4. Relative quantification of *Sl-gallerimycin* expression in the 5th instar larva fat body at 0 h, 8 h, 16 h, 24 h, and 48 h after infection by *N. rileyi*. The expression levels were normalised to those of actin mRNA.

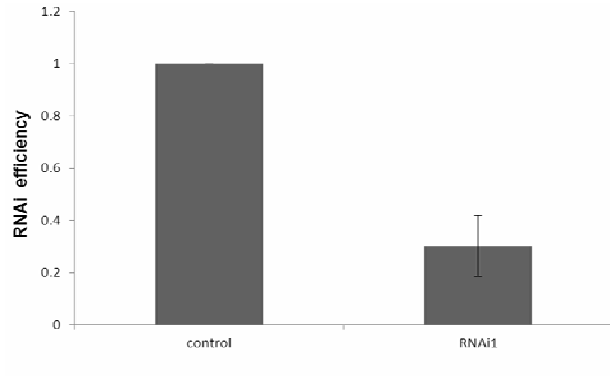

Figure 5. The efficiency of RNAi of *Sl-gallerimycin*. The control group was injected with GFP dsRNA. The experimental group (RNAi) was injected with *Sl-gallerimycin* dsRNA.

post-infection and increases from 8 h to 16 h at a slower rate. A remarkable increase of *Sl-gallerimycin* expression level was observed at 24 h after infection, and also represents the maximum level of expression at this larval stage. At 48 h, there is a large decrease of *Sl-gallerimycin* expression.

Susceptibility to *N. rileyi* after *Sl-gallerimycin* knockdown

To test if RNAi-mediated knockdown of *Sl-gallerimycin* mRNA could lead to increased susceptibility to *N. rileyi* CQNr01 infection, we analysed the expression of *Sl-gallerimycin* in *S. litura* larval fat bodies by RT-qPCR at 18 h after *N. rileyi* CQNr01 infection. Compared to GFP dsRNA, *Sl-gallerimycin* dsRNA injection led to significantly lower *Sl-gallerimycin* mRNA expression levels, with RNAi efficiency measured that 69.8% of *Sl-gallerimycin* mRNA was knocked down compared to control as shown in figure 5 (difference significant at $p \leq 0.05$) (Livak and Schmittgen, 2001).

We also performed a survival assay in which we quantified the survival of *N. rileyi* CQNr01 immunised larvae pre-treated with *Sl-gallerimycin* dsRNA. Knockdown of gallerimycin increased the mortality rate of infected larvae (figure 6). The larvae of the treatment group showed a higher susceptibility to *N. rileyi* CQNr01 than the control group injected with GFP dsRNA. After treatment with interference RNA followed by a *N. rileyi* CQNr01 injection, the larvae turned a pale yellow in 24 h and their activity weakened after 48 h. The first dead larva was found 96 h post infection and a significant difference in mortality was observed for the treated vs. control group after 120 h and 132 h of infection. All the larvae (both experimental and control) were found dead at 156 h and 168 h, respectively. The LT_{50} (time required to reach 50% mortality) of the experimental group (eg) was 118.97 h, and 127.21 h for the control group (difference significant at $p \leq 0.05$).

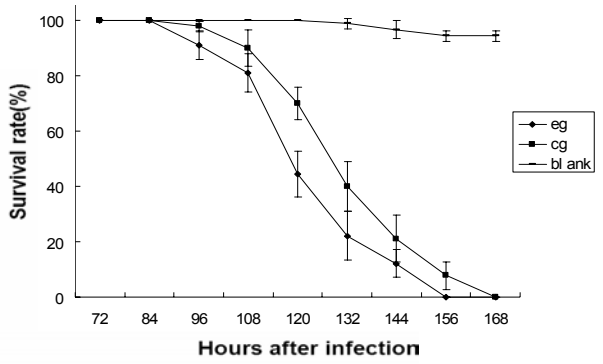

Figure 6. *Sl-gallerimycin* RNAi inhibits the antifungal immune response of *S. litura*. Survival rate of *S. litura* after infection by *N. rileyi*; the larvae were injected with $1x10^7$ ml^{-1} fungal spore suspension after a 5 µl dsRNA injection. Every 12 hours, the live larvae from the control group (cg, injected with GFP dsRNA), the experimental group (eg, injected with gallerimycin dsRNA) and the blank group were counted.

Discussion

In this study we report the cloning of the *Sl-gallerimycin* gene obtained from cDNA from the larval fat body of *S. litura* immunised by the entomopathogenic fungus *N. rileyi*. The amino acid sequence encoded by *Sl-gallerimycin* is highly conserved with that of other gallerimycins. *Sl-gallerimycin* can be strongly induced by *N. rileyi* and RT-qPCR showed that up-regulation of transcripts is associated with the infection, revealing a maximum increase during the first 24 h after septic injury, which is consistent with previous studies (Volkoff *et al.*, 2003, Mowlds and Kavanagh, 2008). However, the results of the present study differ from other studies, which found that the maximum up-regulation of gallerimycin occurred 48 h after injection (Wojda *et al.*, 2009). These studies also showed that maximum transcription varied with different induction conditions, occurring at 8 h after infection by *Candida albicans* (Robin) Berkhout or *Saccharomyces cerevisiae* Meyen ex Hansen, but 24 h after injection with laminarin (Bergin *et al.*, 2006). Together these results suggest that the transcription level of gallerimycins depends on a number of factors, such as insect species, the injected material or fungal strain, the injected dose, and even the conditions for growth and development. In naive larvae, we detected trace *Sl-gallerimycin* expression, which was unexpected because previous research suggests that there should be no expression without pathogenic induction by infection (Hashimoto *et al.*, 2008). A reasonable explanation for our findings is the existence of a low level of induction from occasional microorganisms. Thus, our observation demonstrates that *Sl-gallerimycin* is not only up-regulated after entomopathogenic fungal spore injection but is also induced by microorganisms, an event which is likely to play a significant role in resistance against fungi in the field.

We used RNAi technology (Fire *et al.*, 1998) to study *SI-gallerimycin* gene function. Knockdown of *Sl-gallerimycin* expression using a long dsRNA of *Sl-gallerimycin* increased larvae's susceptibility to *N. rileyi* infection and shortened LT_{50} values. In this context the *Sl-gallerimycin* gene is likely to play a vital role in immunisation against fungal infection. However, further study is required to understand the immunological mechanisms of *Sl-gallerimycin* function.

Acknowledgements

This work was supported by National Special Fund for Agro-scientific Research in the Public Welfare (Grant No. 201103002) and the Key Technologies R & D Program of Chongqing (Grant No. CSTC 2008AB1009).

References

BERGIN D., MURPHY L., KEENAN J., CLYNES M., KAVANAGH K., 2006.- Pre-exposure to yeast protects larvae of *Galleria mellonella* from a subsequent lethal infection by *Candida albicans* and is mediated by the increased expression of antimicrobial peptides.- *Microbes and Infection*, 8: 2105-2112.

BREY P. T., 1998.- *Molecular mechanisms of immune responses in insects*.- CRC Press, Boca Raton, USA.

BULET,P., STOCKLIN R., MENIN L., 2004.- Antimicrobial peptides: from invertebrates to vertebrates.- *Immunological Reviews*, 198: 169-184.

ELEFTHERIANOS I., MILLICHAP P. J., 2006.- RNAi suppression of recognition protein mediated immune responses in the tobacco hornworm *Manduca sexta* causes increased susceptibility to the insect pathogen *Photorhabdus*.- *Developmental & Comparative Immunology*, 30: 1099-1107.

FIRE A., XU S., MONTGOMERY M. K., KOSTAS S. A., DRIVER S. E., MELLO C. C., 1998.- Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*.- *Nature*, 391: 806-811.

HASHIMOTO K., YAMANO Y., MORISHIMA I., 2008.- Cloning and expression of a gene encoding gallerimycin, a cysteine-rich antifungal peptide, from eri-silkworm, *Samia cynthia ricini*.- *Comparative Biochemistry and Physiology, Part B*, 150: 229-232.

LANGEN G., IMANI J., ALTINCICEK B., KIESERITZKY G., KOGEL K. H., VILCINSKAS A., 2006.- Transgenic expression of gallerimycin, a novel antifungal insect defensin from the greater wax moth *Galleria mellonella*, confers resistance to pathogenic fungi in tobacco.- *Biological chemistry*, 387: 549-557.

LAVINE M., STRAND M., 2002.- Insect hemocytes and their role in immunity.- *Insect Biochemistry and Molecular Biology*, 32: 1295-1309.

LIVAK K. J., SCHMITTGEN T. D., 2001.- Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔ CT method.- *Methods*, 25: 402-408.

MOWLDS P., KAVANAGH K., 2008.- Effect of pre-incubation temperature on susceptibility of *Galleria mellonella* larvae to infection by *Candida albicans*.- *Mycopathologia*, 165: 5-12.

MOWLDS P., BARRON A., KAVANAGH K., 2008.- Physical stress primes the immune response of *Galleria mellonella* larvae to infection by *Candida albicans*.- *Microbes and Infection*, 10: 628-634.

QI-JIN C., GUANG-HONG L., YI P., 2000.- A simple artificial diet for mass rearing of some noctuid species.- *Entomological Knowledge*, 37: 325-327.

SCHUHMANN B., SEITZ V., VILCINSKAS A., PODSIADLOWSKI L., 2003.- Cloning and expression of gallerimycin, an antifungal peptide expressed in immune response of greater wax moth larvae, *Galleria mellonella*.- *Archives of Insect Biochemistry and Physiology*, 53: 125-133.

THEIS T., STAHL U., 2004.- Antifungal proteins: targets, mechanisms and prospective applications.- *Cellular and Molecular Life Sciences*, 61: 437-455.

VOLKOFF A.-N., ROCHER J., D'ALENÇON E., BOUTON M., LANDAIS I., QUESADA-MORAGA E., VEY A., FOURNIER P., MITA K., DEVAUCHELLE G., 2003.- Characterization and transcriptional profiles of three *Spodoptera frugiperda* genes encoding cysteine-rich peptides. A new class of defensin-like genes from lepidopteran insects?- *Gene*, 319: 43-53.

WOJDA I., JAKUBOWICZ T., 2007.- Humoral immune response upon mild heat-shock conditions in *Galleria mellonella* larvae.- *Journal of Insect Physiology*, 53: 1134-1144.

WOJDA I., KOWALSKI P., JAKUBOWICZ T., 2009.- Humoral immune response of *Galleria mellonella* larvae after infection by *Beauveria bassiana* under optimal and heat-shock conditions.- *Journal of Insect Physiology*, 55: 525-531.

WU J-J., WANG H., YE G-Y., 2009.- Insect antifungal peptides.- *Chinese Bulletin of Entomology*, 46 (2): 317-323.

Authors' addresses: Youping YIN (corresponding author, ypy128@vip.sina.com), Zhongkang WANG (w-zk@163.com), Wendi ZHANG (wendyzhang0111@sina.com), Huan CHEN (chenhuan_ch@hotmail.com), Genetic Engineering Research Center, College of Bioengineering, Chongqing University, 174 Shazhengjie, Shapingba Dist., Chongqing 400030, PR China.

Received October 22, 2011. Accepted July 30, 2012.