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Antimicrobial peptide cecropin B functions in pathogen resistance of *Mythimna separata*

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Research Paper

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Abstract

Mythimna separata (Lepidoptera: Noctuidae) is an omnivorous pest that poses a great threat to food security. Insect antimicrobial peptides (AMPs) are small peptides that are important effector molecules of innate immunity. Here, we investigated the role of the AMP cecropin B in the growth, development, and immunity of *M. separata*. The gene encoding *M. separata* cecropin B (*MscecropinB*) was cloned. The expression of *MscecropinB* was determined in different developmental stages and tissues of *M. separata*. It was highest in the prepupal stage, followed by the pupal stage. Among larval stages, the highest expression was observed in the fourth instar. Tissue expression analysis of fourth instar larvae showed that *MscecropinB* was highly expressed in the fat body and haemolymph. An increase in population density led to upregulation of *MscecropinB* expression. *MscecropinB* expression was also upregulated by the infection of third and fourth instar *M. separata* with *Beauveria bassiana* or *Bacillus thuringiensis* (*Bt*). RNA interference (RNAi) targeting *MscecropinB* inhibited the emergence rate and fecundity of *M. separata*, and resulted in an increased sensitivity to *B. bassiana* and *Bt*. The mortality of *M. separata* larvae was significantly higher in pathogen plus RNAi-treated *M. separata* than in controls treated with pathogens only. Our findings indicate that *MscecropinB* functions in the eclosion and fecundity of *M. separata* and plays an important role in resistance to infection by *B. bassiana* and *Bt*.

Introduction

Mythimna separata (Lepidoptera: Noctuidae; commonly called the northern armyworm, oriental armyworm, or rice ear-cutting caterpillar) is a migratory, omnivorous, and intermittent explosive pest that eats gramineous crops and can damage maize, rice, and wheat (Chen *et al*., 2020; Xu 2021; Yang *et al*., 2021). The first and second instar larvae of *M. separata* feed on plant leaves, causing holes, and the third instar and above (referred to as armyworms) gnaw on leaf edges, causing irregular nicks (Ma *et al*., 2019b). During the period of most intensive feeding (fifth and sixth instars), the larvae consume vast amounts of food; they nibble crop leaves to the stalk, resulting in crop yield reduction over large areas, or even a complete failure of the harvest. The food intake in this period accounts for >90% of that throughout the larval stage (Zhang *et al*., 2018; Li *et al*., 2020). The average number of eggs laid by this high-fecundity pest is >1000, and it can mate and lay eggs multiple times (Wei, 2012). In addition, adult *M. separata* have strong flight ability (Liu *et al*., 2022). Migratory pests have the characteristics of attacking suddenly and explosively, which makes them difficult to monitor, forecast, prevent, and control. Effective pest management and control of *M. separata* is crucial. Chemical control is still the most commonly used method, but chemical use over the years has caused the '3R' problem: pesticide residue, resistance, and resurgence (Wu, 2017; Li *et al*., 2021). To help solve these problems, the coordinated application of other control methods, including biological control, which has a lasting and stable control effect on pests with no toxic effects on the surrounding environment and organisms, should be improved (Ma *et al*., 2021; Yang *et al*., 2021). At present, the most commonly used biological control methods for *M. separata* are application of the fungus *Beauveria bassiana* and the bacterium *Bacillus thuringiensis* (*Bt*).

B. bassiana is a typical entomogenous fungus (i.e. a fungus that grows on insects), which has the characteristics of a wide host range, strong adaptability, and high pathogenicity; it has become one of the most widely used entomogenous fungi in biological pest control, and does not pollute the environment (Duan *et al*., 2023). *B. bassiana* can invade and infect insects through the body wall, digestive tract, trachea, and wounds. After *B. bassiana* enters the insect body, the hyphae begin to reproduce and enter the haemolymph of the insect body and produce mycelia. Secondary metabolites produced by the fungal pathogen begin to gradually destroy physiological processes of the insect body and attack the host defence system (Zhuang *et al*., 2019; Duan *et al*., 2023). Following this, the mycelia begin to grow vigorously in the insect body. Finally, after the death of the infected insect, the hyphae penetrate the epidermis

to produce conidia, which spread to transmit the fungus (Li and Zhang, 2005). Conidia of entomopathogenic fungi can survive for months to years in the natural environment, and they can grow on dead insects and continue to act as a source of infection, and thus their control effect on pests lasts for a long time (Hajek and Leger, 1994; Scholte *et al.*, 2004).

Bt is a facultative anaerobic Gram-positive bacterium that is widely distributed in soil, water, air, and vegetation. *Bt* and its products have proved effective in controlling Lepidoptera, Diptera, Orthoptera, Coleoptera, and other pests (Roh *et al.*, 2007). Moreover, *Bt* and its products are non-toxic and harmless to non-target organisms, and thus *Bt* has become the most widely used biological insecticide in agriculture (Cui *et al.*, 2023). *Bt* produces many insecticidal substances in the process of growth and metabolism, including chitinase, phosphoesterase C (α-exotoxin), thuringiin (β-exotoxin), insecticidal crystal protein (δ-endotoxin), haemolysin, enterotoxin, and nutritive phase insecticidal protein (Cao and Ni, 2014). The insecticidal crystal protein is the main toxin component of *Bt* as an insecticide, and is produced by the parasporal crystal of *Bt* (Schnepf *et al.*, 1998). δ-Endotoxins are divided into two groups: Cry proteins and Cyt proteins. Cry protein is considered to be the most effective component of *Bt* in insect control; it specifically binds to insect midgut receptors and is inserted into the cell membrane of midgut cells, and then ion pores are formed in the membrane of the midgut epithelial cells. At this stage, insects stop feeding and the intestinal mucosa is soon destroyed and cell permeability is affected, which results in insect cell autolysis and ultimately insect death. Cyt protein is specifically toxic to insects, causes haemolysis, and seriously affects the activity and physiological function of insect cells (Aronson and Shai, 2001; Song *et al.*, 2005; Li *et al.*, 2012). However, control of *M. separata* by *B. bassiana* and *Bt* is slow, and some *M. separata* survive after being infected with these pathogens. We speculate that there is a resistance gene in *M. separata*, which helps them resist or even kill these pathogens.

Antimicrobial peptides (AMPs), small polypeptides of 12–60 amino acids, exist in all organisms, protect the host from infection, and serve as a major component of the innate immune system, playing a variety of functions (Wang, 2020). Insect AMPs are synthesised by the fat body and released into the haemolymph. AMPs have a broad spectrum of antibacterial activity and they are not prone to bacterial resistance (Xue *et al.*, 2023). They have rapid killing activity against bacteria, viruses, parasites, fungi, and even cancer cells (Zasloff, 2002). AMPs can also promote the proliferation of immune cells and strengthen the function of phagocytes in mammals (Wang, 2023). They have high thermal stability and water solubility, and acid and alkali resistance. The primary structure is conserved; the N-terminus of the peptide chain is rich in hydrophilic basic amino acids and carries a positive charge, while the C-terminal end of the peptide chain is rich in hydrophobic amino acids, which are often amidated and neutral. AMPs have an amphiphilic molecular structure (affinity for both water and lipids), which may be a prerequisite for efficient and rapid sterilisation by AMPs (Zhang and Hua, 2023).

On the basis of differences in structure and function, AMPs can be divided into cecropins, defensins, proline-rich peptides, and glycine-rich peptides. The main mechanisms of action of AMPs are action on the cell wall, action on the membrane, and action on a target in the cell. When acting on bacteria, AMPs first come in contact with the cell wall and can exert an antibacterial effect by destroying the cell wall structure. AMPs can bind to lipopolysaccharides and teichoic acids in the cell wall, thereby damaging the cell wall (Kang *et al.*, 2021). AMPs fuse with the lipid membrane of cells to form pores. Because of the different physicochemical properties of the AMPs, the modes of pore formation are different, but they are interrelated. Hypotheses about pore-forming methods include the bucket plate model, carpet model, ring hole model, and polymerisation model (Li *et al.*, 2019). After entering the cell, AMPs act on nucleic acids, affect the synthesis and replication of DNA and RNA, and affect the synthesis of proteins. They can also act on protein molecules, cause changes in the structure of proteins, and make many enzymes inactive. Some AMPs can enter mitochondria, affect the activity of enzymes in the mitochondria, and cause disorder of metabolism (Feng *et al.*, 2019).

Cecropin B belongs to the cecropin class of AMPs, with a positive charge and two α-helices. It was first isolated from the haemolymph of *Hyalophora cecropia* (cecropia moth). Studies have shown that insect cecropins can inhibit Gram-positive bacteria, Gram-negative bacteria, and some fungi, and that cecropin B is the most active cecropin (De Lucca *et al.*, 1998; Hu *et al.*, 2013). Cecropin B has the ability to form specific amphipathic α-helices that target non-polar lipid in cell membranes. Upon membrane targeting, ion-permeable channels form resulting in cell depolarisation, irreversible cytolysis, and finally death (Boman, 2003).

The present study was undertaken to investigate the effect of *M. separata* cecropin B on *B. bassiana* and *Bt* infection in *M. separata*. We show that cecropin B helps *M. separata* survive infection by these entomopathogenic microorganisms. Importantly, this research provides a molecular target for development of 'green' pesticides that inhibit the synthesis of AMPs in insects, so as to enable control of these pests through biological means.

Materials and methods

Experimental insects and pathogens

M. separata were established from a culture at the experimental base of Northeast Agricultural University, Harbin, China, in 2020, and maintained in the laboratory. The larvae were fed on fresh maize leaves every day in a climate-controlled chamber at 26 ± 2°C and relative humidity of 70 ± 5% with a light/dark period of 14 h/10 h. Adults were maintained under the same conditions, and fed on 5% honey water. Three days after eclosion, egg strips were hung in the cage, and female laid eggs on the egg strips. *B. bassiana* (*Bb170428*) (Yu *et al.*, 2023) and *B. thuringiensis* strain 46 (*Bt46*) used in this study were provided by Northeast Agricultural University, Harbin, China.

Cloning of the gene encoding M. separata *cecropin B*

M. separata larvae were collected and flash-frozen in liquid nitrogen for subsequent transcriptome sequencing, which was performed by Annoroad Gene Technology Company (Beijing, China). The sequences of AMP-encoding genes were identified based on public protein databases from NCBI. The total RNAs of larvae were extracted by using TRIzol reagent (1 ml 100 mg^{-1}, Invitrogen, Carlsbad, CA, USA), and first-strand cDNA was synthesised using PrimeScript™ RT Master Mix (TaKaRa, Beijing, China). Reaction conditions were as follows: 37°C for 15 min and 85°C for 5 s. The reaction system was as follows:

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500 ng RNA, 2 µl mix, and double-distilled water (ddH_2O) were added to yield a total volume of 10 µl. All primers in this research were designed using Primer Premier 5.0 software and synthesised by Jiansu Gene (Heilongjiang, China) (table 1). The gene encoding *M. separata* cecropin B was amplified by polymerase chain reaction (PCR) using Premix *Taq* (TaKaRa). PCR conditions were as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 44°C for 30 s, and 72°C for 1 min; 72°C for 10 min. The reaction system was as follows: 1 µl cDNA, 2 µl sense and anti-sense primers (10 µmol), 25 µl mix, and ddH_2O were added to yield a total volume of 50 µl. PCR products were checked by agarose gel electrophoresis and purified by using a MiniBEST DNA Fragment Purification Kit (TaKaRa). The purity and concentration of the target genes were detected using a microspectrophotometer. The target gene was linked to vector pMD™18-T at 16°C overnight (reaction system: the amount of target genes was ≥3× that of the plasmid backbone, 5 µl solution I was added, and ddH_2O was added to yield a total volume of 10 µl) (TaKaRa). The resulting plasmid was transformed into *Escherichia coli* DH5α. Positive clones were selected by conventional blue-white screening and confirmed by colony PCR. The reaction system was as follows: 0.5 µl bacterial culture, 5 µl mix, 1 µl sense and anti-sense primers (10 µmol) (sense primer: 18-T vector universal primer; anti-sense primer: anti-sense primer of the target gene), and ddH_2O was added to yield a total volume of 10 µl (Premix *Taq*, TaKaRa). Reaction conditions were as follows: 95°C for 15 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; 72°C for 10 min. Plasmid isolated from bacteria positive for the target band was confirmed by sequencing.

Bioinformatic analysis

The obtained sequence of *MscecropinB* (the gene encoding *M. separata* cecropin B) was registered and deposited in the NCBI database (https://www.ncbi.nlm.nih.gov/). The open reading frame was predicted and translated using ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Insect cecropin B amino acid sequences were downloaded from the NCBI database for construction of a phylogenetic tree by the neighbour-joining method with best-fitting model in MEGA 7.0 software with 1000 bootstrap replicates. The isoelectric point, molecular weight, and protein hydrophobicity were predicted using the ExPASy Compute pI/MW online tool (https://www.expasy.org/). For signal peptide prediction, SignalP 5.0 online software was used (http://www.cbs.dtu.dk/services/SignaIP/). Protein transmembrane structure was predicted by TMHMM server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Protein secondary structure was predicted by SOPMA (http://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl). The tertiary structure of protein was modelled using SWISS-MODEL (http://swissmodel.expasy.org/interactive).

Table 1. Primer sequences used in this study

Primer name	Primer sequence (5′–3′)	Use
MscecropinB-F	GCAGTCTTCGTGAATTC	Gene cloning
MscecropinB-R	AAATGCTATGGCGATA	Gene cloning
MscecropinB-q-F	GGTGTTCGTGTTCGCTTGT	qRT-PCR
MscecropinB-q-R	TGATGTTGCGTCCCATTT	qRT-PCR
Msβ-actin-q-F	CCAACGGCATCCACGAGACCA	qRT-PCR
Msβ-actin-q-R	TCGGCGATACCAGGGTACAT	qRT-PCR
MsGAPDH-q-F	GCTACAGTCGTTGCCATCAA	qRT-PCR
MsGAPDH-q-R	GAGGACGGAGATTTTGTTGC	qRT-PCR

Spatiotemporal expression pattern of MscecropinB

Total RNA was extracted from insects at different developmental stages (egg, first-day first to sixth instar larvae, prepupae, pupae, and adults) and from various tissues (foregut, midgut, hindgut, salivary gland, Malpighian tubules, integument, fat body, and haemolymph), and then reverse-transcribed into cDNA for quantitative real-time PCR (qRT-PCR). The reaction system for reverse transcription was as follows: 1000 ng RNA, 4 µl mix, and ddH_2O were added to yield a final volume of 20 µl. The expression levels of *MscecropinB* in different *M. separata* developmental stages and tissues were analysed with *M. separata β-actin* (GenBank: GQ856238) and *GAPDH* (GenBank: HM055756) as reference genes. qRT-PCR system was as follows: 2 µl cDNA, 6.8 µl ddH_2O, 0.6 µl sense and anti-sense primers, and 10 µl THUNDERBIRD SYBR qPCR Mix (Toyobo, Shanghai, China). The reaction conditions were as follows: 95°C for 3 min; 35 cycles of 94°C for 10 s, 56°C for 30 s, and 72°C for 30 s. The data were recorded using Bio-Rad CFX Manager 3.1 software. Melting curves were checked to assess the specificity of the qRT-PCR. The qRT-PCR was performed in three technical replicates each with three biological replicates.

Determination of LC_{50} values of two pathogens towards M. separata

To prepare conidia of *B. bassiana* cultured on potato dextrose agar, spore suspension was dissolved in sterile water containing 0.1% Tween 80, and suspensions of 1×10^9, 1×10^8, 1×10^7, and 1×10^6 spores ml^{-1} were prepared for later use. We used a haemocytometer to count the filtered spore suspensions. Healthy *M. separata* third and fourth instar larvae of the same size were treated by the spray method (Wang *et al.*, 2012), and spore suspensions of different concentrations were uniformly sprayed on the surface of the larvae using a 30 ml hand-held plastic aerosol sprayer (JS-0119). The blank control was sterile water containing 0.1% Tween 80. To prepare *Bt46* cultured in Luria–Bertani medium, the bacterial suspension was dissolved in sterile water, and the cells were evenly dispersed. Then, bacterial suspensions of 1×10^{10}, 1×10^9, 1×10^8, and 1×10^7 cells ml^{-1} were prepared for later use. Fresh maize leaves were treated with the bacterial suspension by the leaf soaking method (Yu *et al.*, 2023) for 1–2 min. They were then dried and fed to healthy *M. separata* third and fourth instar larvae of the same size. The blank control was fresh maize leaves soaked in sterile water.

Each group included 20 larvae, and the experiments were repeated three times. After infection, larvae were maintained at constant temperature (26 ± 2°C) and humidity (70 ± 5%), and fed fresh, untreated maize leaves. Over 10 days of continuous observation, the number of living and dead insects was counted. Regression statistical analysis was performed by using DPS7.5 software to calculate LC_{50} values.

Effect of insect population density on MscecropinB expression

Immediately after egg hatching, *M. separata* were transferred to feeding boxes (0.5 litres) at a density of 2 larvae per box, 4 larvae

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per box, 8 larvae per box, 16 larvae per box, and 32 larvae per box. Each experiment was repeated three times. The larvae were fed until the first day of the fourth instar whereupon total RNA was extracted, and then reverse-transcribed into cDNA for qRT-PCR analysis.

Effect of infection on MscecropinB *expression*

Healthy third and fourth instar larvae were treated with the LC_{50} of *B. bassiana* spore suspension or *Bt46* bacterial suspension for the corresponding instar; the treatment methods were the same as described in the section 'Determination of LC_{50} values of two pathogens towards *M. separata*'. Each group included 25 larvae and the experiment was repeated three times. The *B. bassiana* treatment group was observed once daily for 5 days; for *Bt46* treatment, samples were collected at 12, 24, 36, 48, and 70 h. At each time point, larvae were quickly frozen in liquid nitrogen. Total RNAs were extracted, reverse-transcribed into cDNA, and then qRT-PCR was performed to detect *MscecropinB* expression.

Fourth instar larvae were treated with the LC_{50} of *B. bassiana* after 3 days. *Bt46*-treated larvae were fed after 48 h with maize leaves soaked in bacterial suspension at the LC_{50}. Control larvae were treated with 0.1% Tween 80 in sterile water and sterile water only, respectively. The larvae were dissected to obtain the fat bodies and haemolymph. Each group contained 25 larvae, and each experiment was repeated three times. The total RNA extracted from tissues was reverse-transcribed into cDNA and then qRT-PCR was performed.

siRNA synthesis and interference efficiency measurement

A siRNA sequence for *MscecropinB* and a negative control siRNA were synthesised by Harbin Ruibo Xingke Biotechnology (table 2). The siRNAs were diluted into diethylpyrocarbonate-treated water to a final concentration of 20 µM. First-day fourth instar larvae were injected with 2 µl siRNA for *MscecropinB* or the negative control siRNA into the internode membrane of the last two to three segments of the abdomen using a 10 µl microinjector (needle tip outer diameter: 0.5 mm). The larvae were fed normally after the injection. Each group included 20 larvae, and the experiments were repeated three times. Larvae were collected 3, 6, 12, 24, 48, 72, and 96 h after injection. Total RNAs were extracted from the larvae and then reverse transcribed to cDNA for analysis of *MscecropinB* expression by qRT-PCR.

Effects of RNA interference (RNAi) on growth and development of M. separata

The death rate of *M. separata* larvae 12, 24, 48, and 96 h after interference was recorded, and the subsequent pupation rate, pupal weight, emergence rate, and egg-laying capacity of surviving larvae were observed and calculated. The pupal weight on the second day was determined using an Electronics Weighing Scales (One Over Ten-thousand Analytical Balance). Each treatment group contained 50 larvae, and the experiments were repeated three times.

Table 2. Sequences of siRNAs used in this study

Target gene	Sequence couple (5′–3′)
SiMscecropinB	GCUUGUUUGGUCGCCAUGUTT
	ACAUGGCGACCAAACAAGCTT
SiNegative control	UUCUCCGAACGUGUCACGUTT
	ACGUGACACGUUCGGAGAATT

Synergistic effects of pathogens and RNAi on MscecropinB *expression*

Healthy third instar larvae were treated with *B. bassiana*, and fourth instar larvae were treated with *Bt46*, at the LC_{50} values, respectively. Thirty-six hours after infection, all larvae were treated by siRNA. Controls were treated with *B. bassiana* or *Bt46* and injected with the negative control siRNA. Each treatment group included 25 larvae, and the experiments were repeated three times. Larvae were collected 3, 6, 12, 24, 48, 72, and 96 h after the injection of siRNA for analysis of *MscecropinB* expression. For growth and development research, each treatment group included 50 larvae. The death rate of the treated *M. separata* larvae was recorded 12, 24, 48, and 96 h after interference, and the subsequent pupation rate, pupal weight, emergence rate, and egg-laying capacity of surviving larvae were observed and calculated. The pupal weight on the second day was determined by using a balance (One Over Ten-thousand Analytical Balance).

Statistical analysis

The relative expression of genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Differences between multiple treatment groups were analysed by one-way analysis of variance, and differences between treatment and control groups were analysed using the *t*-test in software GraphPad Prism 9.3.0 and IBM SPSS Statistics 23. Duncan's multiple comparison method was used to analyse the significance of differences ($P < 0.05$). Graphs were drawn in GraphPad Prism 9.3.0 software. Simple numerical operations were performed using Microsoft Excel 2010.

Results

Cloning of MscecropinB

The gene encoding *M. separata* cecropin B (referred to here as *MscecropinB*) was amplified using fourth instar larvae as the template, as shown by polyacrylamide gel electrophoresis (fig. S1). A recombinant plasmid of *MscecropinB* was successfully constructed and transformed into *E. coli* DH5α (fig. S2). After blue-white screening, white colonies were randomly selected and checked by colony PCR detection. Colonies that presented a single band of the expected amplicon size (approximately 200 bp) were sent for verification of the *MscecropinB* expression plasmid by DNA sequencing (fig. S3). The amplified *MscecropinB* sequence was consistent with the sequence from the transcriptome.

Bioinformatic analysis of MscecropinB

The *MscecropinB*-encoding cDNA (GenBank: OQ914365) is 765 bp long, with an open reading frame of 192 bp. The encoded protein (63 amino acids) (fig. 1) has a predicted isoelectric point of 10.15 and molecular weight of 6.79 kDa. Amino acids 1–22 at the N-terminus form a predicted signal peptide. Residues 7–24 form a predicted transmembrane domain. In the predicted

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1    CGTGCGGCTGTTATTATCTAGCCCACTAAGTTAGCTTCACACTAACTTTAACACCGCCAG
61   AGCAAAAATATGATCATGCACTTTTGTAAAGATTATTAATAAATAATAATTATTAAAAAA
121  CAACTAAAATAATAACAAAGAGATTGACACGGGGCATAACATTTATCATACCAGAAAAGG
181  GGCTTTCCTGGAGTTCATATAAATAAGAAGAGTTATCAGTACGCAGACATACGTTACGTC
241  AGCCACAATTAATAGTAACAGAACAGGAGGTCATACTATTACGGTCACAAGTTTGTTTAC
301  AAATAAGAGTACTATATAAAACGAGATTTATTCTAGTAGAGCATCATTCAACTCGAAGCA
361  GTCTTCGTGAATTCAAATAAATAATAATTTGAATTAAGAACAATAATAGTAAAAATGAAT
1                                                        M   N
421  TTCTCTCGCGTGTTGGTGTTCGTGTTCGCTTGTTTGGTCGCCATGTGCGCTGTGTCGGCG
3    F . . S . . R . . V . . L . . V . . F . . V . . F . . A . . C . . L . . V . . A . . M . . C . . A . . V . . S . . A .
481  GCGCCCGAGCCACGGTGGAAGGTCTTCAAGAAAATTGAGAAAATGGGACGCAACATCAGA
23   . A . . P . . E . . P . . R . . W . . K . . V . . F . . K . . K . . I . . E . . K . . M . . G . . R . . N . . I . . R .
541  GATGGCATCATCAAGGCTGGCCCAGCTGTTGCTGTTCTCGGCGACGCCAAAGCTTTAGGA
43   . D . . G . . I . . I . . K . . A . . G . . P . . A . . V . . A . . V . . L . . G . . D . . A . . K . . A . . L . . G .
601  AAATAGTTCTTATTTATATCGCCATAGCATTTAAATTTGTACAGTGTGTCTTTTTGTCTT
63     K   *
661  AAAAATAATGCCAAAGTATTTATTTTAATTAGTTAAGAACCACCTATTTTATGTAAAGCT
721  TAAAGTGTCGAAATATAAAATATTTTTAAGTTAAAAAAAAAAAAA
```

Figure 1. cDNA and peptide sequences of *M. separata* cecropin B (*MscecropinB*). The signal peptide is shaded in grey, the transmembrane domain is underlined, and the two predicted helices are boxed.

secondary structure of *MscecropinB*, α-helix accounts for 63.49%, random coil for 20.63%, extended chain for 12.70%, and β-angle for 3.17%. The tertiary structure of *MscecropinB* was predicted based on the secondary structure of *Bombyx mori* cecropin E (PDB: Q308S4.1.A) as a template; the confidence was 74.9%, and the sequence coverage was 98%. As shown in fig. S4, *MscecropinB* is predicted to have two α-helices, which is consistent with cecropin structure studies. Multiple-sequence alignment with cecropin B sequences from other insects (fig. S5) showed that they are highly conserved. In a phylogenetic tree constructed for *MscecropinB* and some insect cecropin B sequences, *MscecropinB* was clustered with the cecropin B of *Trichoplusia ni* (ABV68872.1) and *Spodoptera exigua* (AKJ54503.1) (fig. 2), indicative of a relatively close relationship. The next closest relationships were with the cecropins B from *Danaus plexippus plexippus* (XP_032523475.1), *H. cecropia* (AAA29184.1), and *Antheraea pernyi* (QDH09591.1), which are again closely related species to *M. separata*. *M. separata* cecropin B was distant in the tree from cecropins B from mosquitoes and fruit flies.

Spatiotemporal expression pattern of MscecropinB

qRT-PCR was used to analyse the expression of *MscecropinB* in *M. separata* at different developmental stages. The results (fig. 3A) ($F(9, 80) = 9943.4358$; $P < 0.05$) showed that *MscecropinB* was expressed in all developmental stages, with the highest expression in the prepupal stage, followed by the pupal stage. The lowest *MscecropinB* expression level was in the egg stage. The *MscecropinB* expression level in the prepupal stage was 123.34

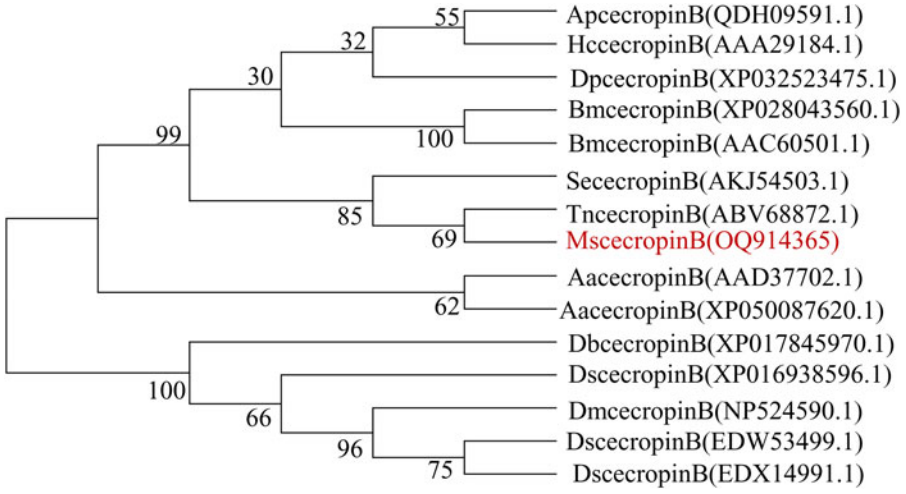

Figure 2. Phylogenetic analysis of cecropin B sequences from Lepidoptera and Diptera. *Ap*: *A. pernyi*; *Hc*: *H. cecropia*; *Dp*: *D. plexippus*; *Bm* (XP028043560.1): *Bombyx mandarina*; *Bm* (AAC60501.1): *B. mori*; *Se*: *S. exigua*; *Tn*: *T. ni*; *Aa* (AAD37702.1): *Aedes albopictus*; *Aa* (XP050087620.1): *Anopheles aquasalis*; *Db*: *Drosophila busckii*; *Ds* (XP016938596.1): *Drosophila suzukii*; *Dm*: *Drosophila melanogaster*; *Ds* (EDW53499.1): *Drosophila sechellia*; *Ds* (EDX14991.1): *Drosophila simulans*.

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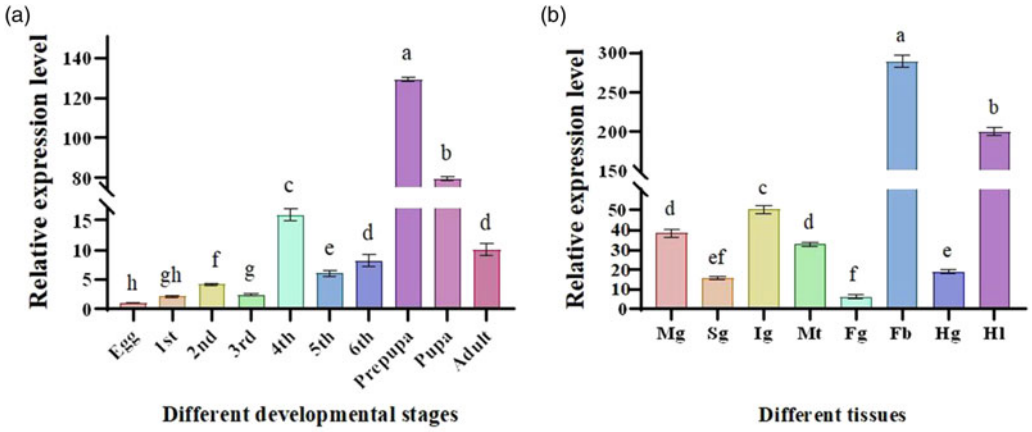

Figure 3. Spatiotemporal *MscecropinB* expression pattern in different developmental stages (a) and in diverse tissues (b) of *M. separata*. Mg, midgut; Sg, salivary gland; Ig, integument; Mt, Malpighian tubules; Fg, foregut; Fb, fat body; Hg, hindgut; Hl, haemolymph. Different lowercase letters above the columns indicate significant differences ($P < 0.05$).

times that in the egg stage, and 1.64 times that in the pupal stage. In the larval stage, the highest expression level was in the fourth instar, 15.63 times that in the egg stage.

To explore the expression patterns of *MscecropinB* in different tissues, we dissected fourth instar larvae and obtained eight tissues: foregut, midgut, hindgut, salivary gland, Malpighian tubules, integument, fat body, and haemolymph. *MscecropinB* was most highly expressed in the fat body, followed by the haemolymph; the lowest expression was in the foregut (fig. 3B) ($F(7, 64) = 2641.6824$; $P < 0.05$). *MscecropinB* expression in the fat body was 46.63 times that in the foregut, and 1.43 times that in the haemolymph.

Determination of LC_{50} of two pathogens towards M. separata

Larvae were treated with four different concentrations of *B. bassiana* or *Bt46* suspension for 10 days, and the mortality rate was observed for each treatment (fig. 4). The LC_{50} value of *B. bassiana* towards third instar *M. separata* was 1.07×10^7 spores ml^{-1}, the virulence equation was $y = 2.3678 + 1.3363x$ ($r = 0.9422$, y: mortality, x: concentration). The LC_{50} of *B. bassiana* towards fourth instar *M. separata* was 1.08×10^8 spores ml^{-1} ($y = 1.7174 + 1.0937x$, $r = 0.9528$). The LC_{50} of *Bt46* towards third instar *M. separata* was 1.24×10^8 cells ml^{-1} ($y = 1.7583 + 1.0574x$, $r = 0.9411$). The LC_{50} of *Bt46* towards fourth instar *M. separata* was 1.13×10^9 cells ml^{-1} ($y = 0.4551 + 1.1298x$, $r = 0.9448$).

The effect of population density on expression of MscecropinB

The expression level of *MscecropinB* was significantly impacted by the population density. As shown in fig. 5 ($F(4, 41) = 1074.757$; $P < 0.05$), when *M. separata* were exposed to high-density conditions, the expression of *MscecropinB* was significantly increased. The expression of *MscecropinB* at 32 larvae per box was 11.63 times that for 2 larvae per box. However, there was no significant difference in larval *MscecropinB* expression in several low-density conditions, indicating that there is a threshold insect population density for the regulatory effect of population density on *MscecropinB* expression.

Pathogens upregulated the expression of MscecropinB

There were significant differences in the expression of *MscecropinB* at different times after infection of third and fourth instar *M. separata* with the LC_{50} of *B. bassiana* (fig. 6A, B) ($P < 0.05$). During the second day after *B. bassiana* infection, the *MscecropinB* expression in third instar larvae was 9.49 times that in control. The expression levels on the third, fourth, and fifth days of infection were 3.52, 3.26, and 2.58 times those in the control, respectively. In fourth instar larvae, *MscecropinB* showed significant differential expression compared with controls from the first day of infection. The expression reached 9.91 and 7.36 times that in the controls on days 3 and 4, respectively.

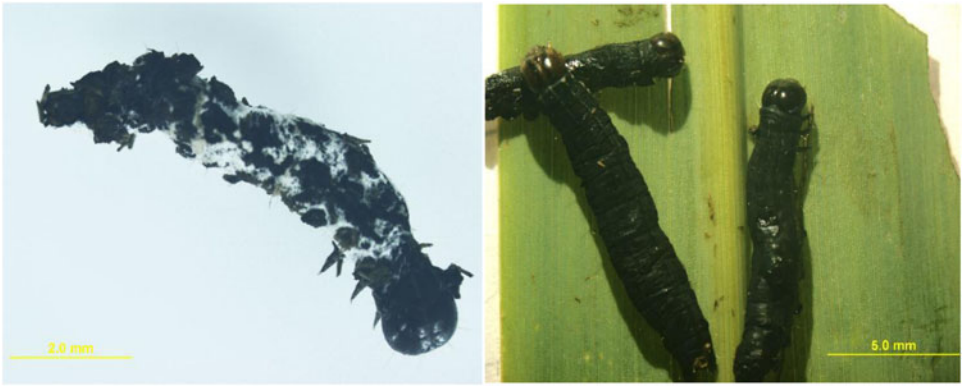

Figure 4. Third instar *M. separata* larvae infected by *B. bassiana* and fourth instar larvae infected by *Bt46*.

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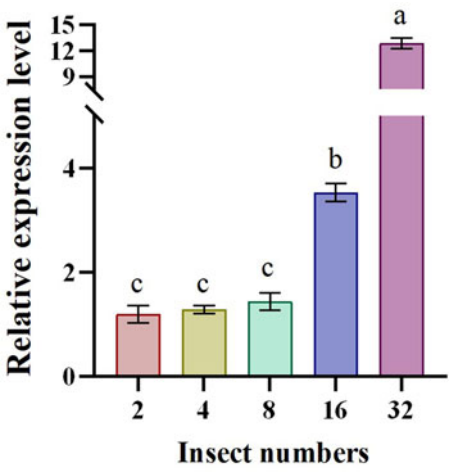

Figure 5. Expression of *MscecropinB* under different conditions of larval density. Different lowercase letters above the columns indicate significant differences ($P < 0.05$).

There were significant differences in the expression of *MscecropinB* at different times after infection of third and fourth instar *M. separata* with the LC_{50} of *Bt46* (fig. 7A, B) ($P < 0.05$). At 36 and 48 h after *Bt46* infection, the *MscecropinB* expression in third instar larvae was 7.63 and 8.88 times that in the controls, respectively. In fourth instar larvae, the *MscecropinB* expression was significantly different compared with controls 12 h after infection. The expression reached 5.40 and 9.66-times that in controls at 36 and 48 h, respectively.

Because different pathogens take effect at different times, fourth instar larvae treated with the LC_{50} of *B. bassiana* (3 days) and *Bt46* (48 h) were dissected to obtain the fat bodies and haemolymph. Compared with controls, qRT-PCR showed that *MscecropinB* expression in the fat body and haemolymph increased 5.62- and 2.20-fold after *B. bassiana* infection, and 2.63- and 2.19-fold after *Bt46* infection (fig. 8) ($P < 0.05$).

RNAi efficiency

To confirm the efficiency of RNAi, fourth instar larvae were injected with equal amounts of siRNA for *MscecropinB* or negative control siRNA. The *MscecropinB* expression level was analysed 3, 6, 12, 24, 48, 72, and 96 h after injection. At 6, 12, and 24 h, the expression of *MscecropinB* decreased by 80.01, 96.52, and 81.09%, respectively, in the treatment group compared with the negative control group (fig. 9) ($P < 0.05$). There was no significant interference effect at 3, 48, or 96 h. Therefore, the time of maximal interference efficiency was about 12 h after injection.

Effects of RNAi on growth and development of M. separata

The silencing of *MscecropinB* had no effect on the mortality of the fourth instar *M. separata* for up to 96 h (table 3). The RNAi-treated *M. separata* were further observed for pupation

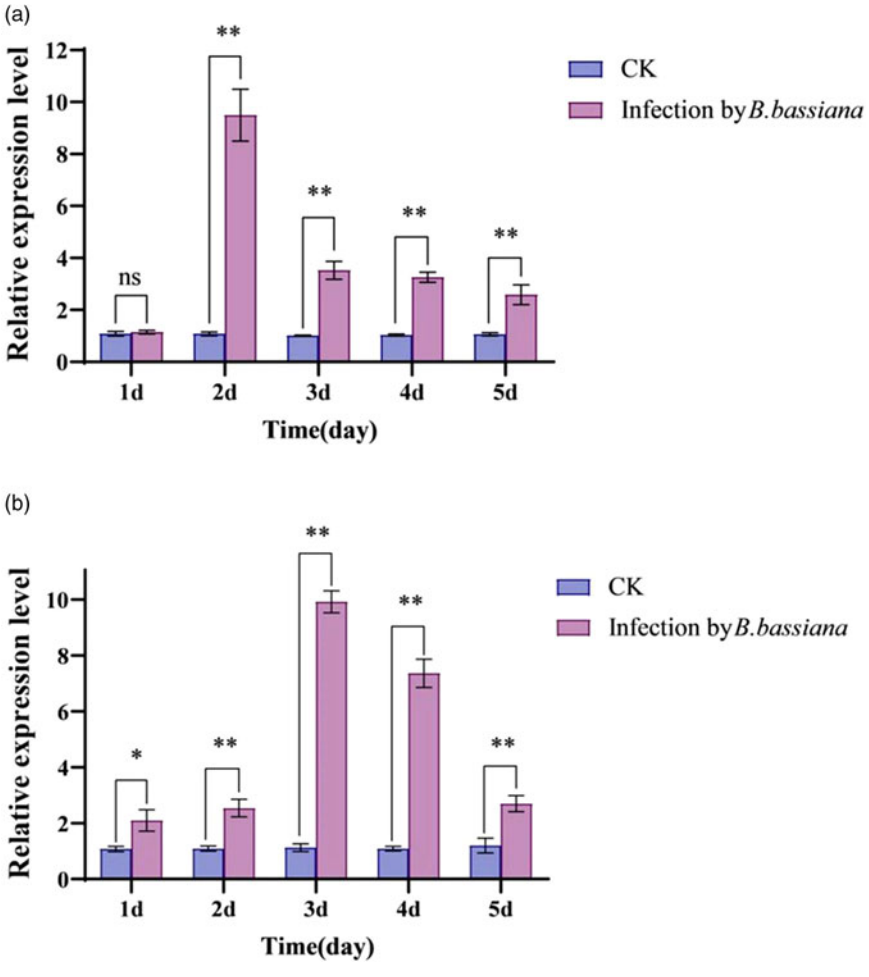

Figure 6. Expression of *MscecropinB* in third (a) and fourth (b) instar *M. separata* infected with *B. bassiana*. Healthy third and fourth instar larvae were treated with *B. bassiana* at the LC_{50} values. Controls were treated with sterile water containing 0.1% Tween 80 (*$P < 0.05$; **$P < 0.01$; ns, $P > 0.05$).

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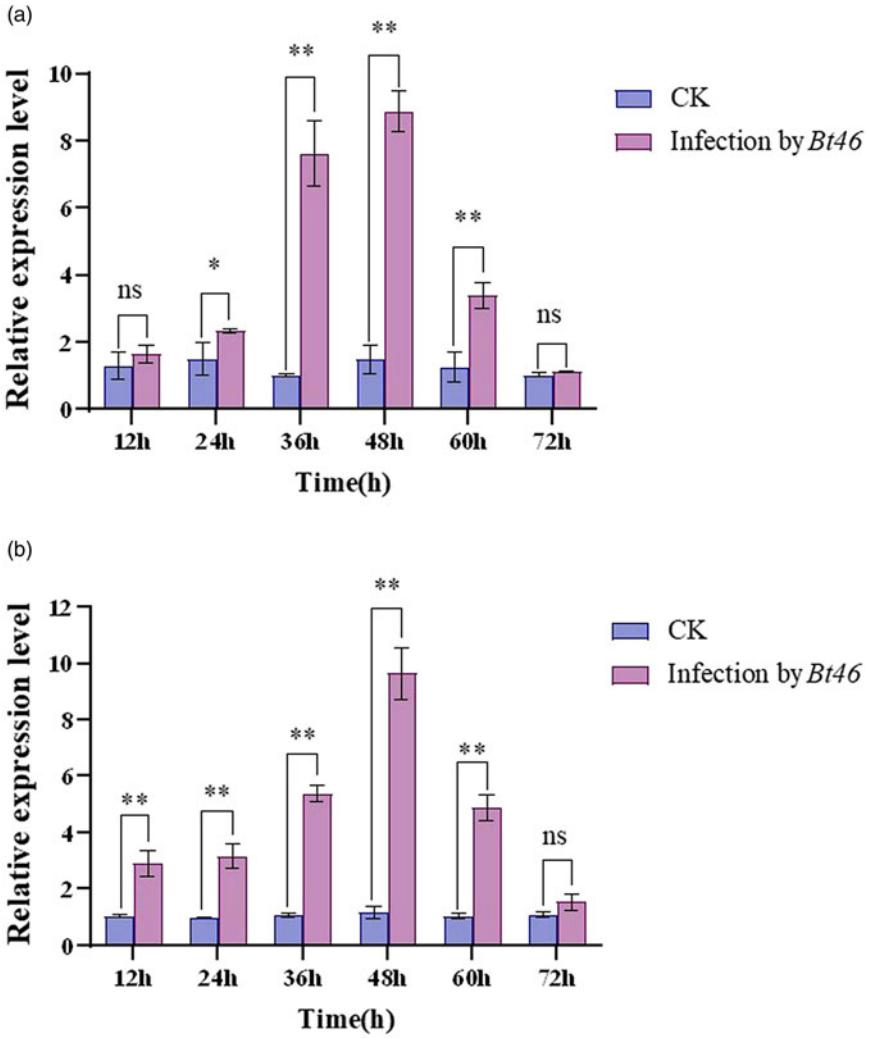

Figure 7. Expression of *MscecropinB* in third (a) and fourth (b) instar *M. separata* infected with *Bt46*. Healthy third and fourth instar were treated with *Bt46* at the LC_{50} values. Controls were treated with sterile water (*$P < 0.05$; **$P < 0.01$; ns, $P > 0.05$).

rate, pupal weight, emergence rate, and single female egg production. The interference with *MscecropinB* had no effect on the pupation rate or pupal weight, but the emergence rate has decreased by 22.22%, and the single female egg production has decreased by about 272 eggs.

Synergistic effect of pathogens and RNAi on MscecropinB

qRT-PCR results showed that RNAi treatment significantly suppressed *MscecropinB* expression 3–24 h after interference in *B. bassiana*-infected third instar larvae, and 3–48 h after interference in *Bt46*-infected fourth instar larvae (fig. 10) ($P < 0.05$). Comparing the *B. bassiana*-infected group with the control group (infected, but not subjected to siRNA), the *MscecropinB* expression levels at 3, 6, 12, and 24 h were decreased by 69.92, 82.22, 80.66, and 90.16%, respectively. In the *Bt46* plus RNAi treatment group, the *MscecropinB* expression levels at 3, 6, 12, 24, and 48 h were decreased by 47, 74.61, 85.76, 90.35, and 50.69%, respectively, compared with controls (infected, but not

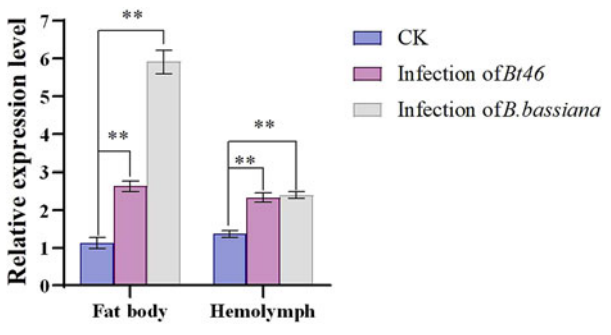

Figure 8. Expression of *MscecropinB* in fat body and haemolymph after infection by pathogens (*$P < 0.05$; **$P < 0.01$; ns, $P > 0.05$).

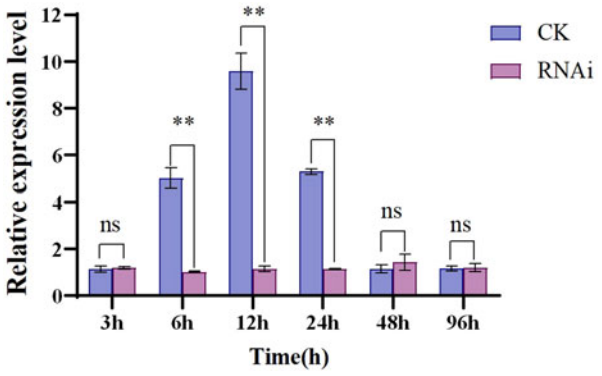

Figure 9. Efficiency of RNAi with *MscecropinB* (*$P < 0.05$; **$P < 0.01$; ns, $P > 0.05$).

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Table 3. Mortality, growth, and development of RNAi-treated *M. separata*

Treatment	Mortality (%)				Pupation rate (%)	Pupal weight (g)	Emergence rate (%)	Number of eggs
	12 h	24 h	48 h	96 h				
SiNC	0.67 ± 0.67	2.67 ± 0.67	8.00 ± 0.58	12.00 ± 1.15	90.83 ± 0.83	0.35 ± 0.0025	88.89 ± 2.22	1747.97 ± 64.49
siMscecropinB	0.67 ± 0.67	3.33 ± 0.67	10.00 ± 1.15	13.33 ± 0.67	87.50 ± 1.44	0.34 ± 0.0049	66.67 ± 1.93**	1475.30 ± 35.47*

Note: Data are presented as the mean ± SE. SiNC, negative control RNAi.
*$P < 0.05$, **$P < 0.01$ (*t*-test).

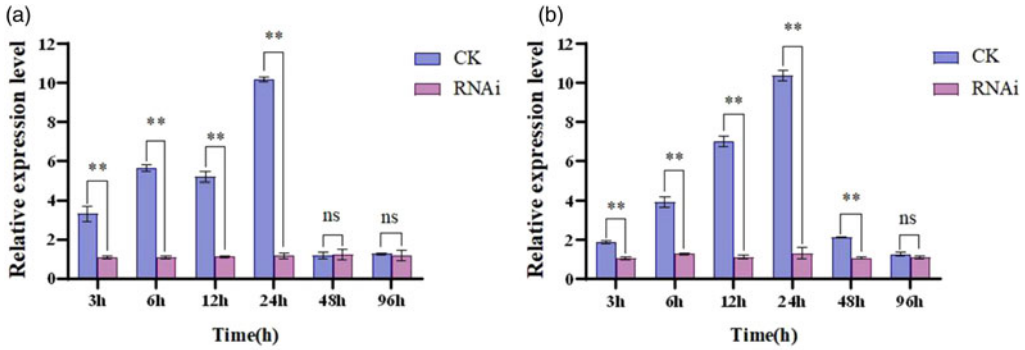

Figure 10. Analysis of *MscecropinB* expression after pathogen infection and RNAi treatment. Infection with (a) *B. bassiana* and (b) *Bt46* (*$P < 0.05$; **$P < 0.01$; ns, $P > 0.05$). Healthy third instar larvae were treated with *B. bassiana*, and fourth instar were treated with *Bt46* at the LC_{50} values, respectively. Thirty-six hours after infection, all larvae were treated by siRNA. Controls were treated with *B. bassiana* or *Bt46* and injected with negative control siRNA.

subjected to siRNA). It can be seen that in infected larvae, the RNAi was most effective at about 24 h after injection.

Effect of RNAi of MscecropinB *on susceptibility of* M. separata *to pathogens*

Changes in physiological parameters were observed in pathogen plus RNAi-treated third and fourth instar *M. separata*. The mortality rates in both treatment groups (*B. bassiana* + siRNA or *Bt46* + siRNA) were significantly higher than that of the control group (infected but not treated by siRNA) after interference for 24 h (table 4). After 96 h of interference, the death rate in the *B. bassiana* group was 19.34% higher than that in the control group; the death rate in the *Bt46* group was 22% higher than that in the control. The pupation rate, emergence rate, and single female egg production were also significantly influenced by RNAi treatment of *M. separata* infected with either *B. bassiana* or *Bt46*, while the pupal weight was unaffected. The pupation rate in the *B. bassiana* plus RNAi-treated group was 59.17% lower, the emergence rate was 31.11% lower, and the single female egg production was about 397 eggs per female lower than those in the control group (all $P < 0.05$). In the *Bt46* plus RNAi-treatment group, the pupation rate was 40.83% lower, the emergence rate was 18.89% lower, and the single female egg production was about 347 eggs per female lower than those in the control. The results showed that interference with *MscecropinB* expression increased the sensitivity of *M. separata* to *B. bassiana* and *Bt46*, and the synergism between RNAi and pathogen infection markedly affected the growth and development of *M. separata*.

Discussion

AMPs are important effector molecules of insect innate immunity and are involved in many defence processes, such as neutralising

Table 4. Mortality of *M. separata* treated with pathogens and RNAi

Treatment	Mortality (%)				Pupation rate (%)	Pupal weight (g)	Emergence rate (%)	Number of eggs
	12 h	24 h	48 h	96 h				
B. bassiana + SiNC	2.67 ± 1.33	12.67 ± 0.67	20.00 ± 1.15	31.33 ± 0.67	87.50 ± 1.44	0.35 ± 0.0043	97.78 ± 1.11	1473.77 ± 34.81
B. bassiana + siMscecropinB	4.00 ± 2.31	22.67 ± 1.76**	30.00 ± 1.15**	50.67 ± 1.33**	38.33 ± 0.83**	0.34 ± 0.0053	66.67 ± 1.93**	1077.17 ± 51.94**
Bt46 + SiNC	2.67 ± 1.33	14.00 ± 1.15	20.00 ± 1.15	31.33 ± 0.67	90.83 ± 0.83	0.33 ± 0.0026	96.67 ± 1.93	1398.30 ± 32.03
Bt46 + siMscecropinB	3.33 ± 0.67	36.67 ± 0.67**	42.00 ± 1.15**	53.33 ± 1.33**	50.00 ± 1.44**	0.32 ± 0.0020	77.78 ± 2.94**	1051.50 ± 47.02**

Note: Data are presented as the mean ± SE. SiNC, negative control RNAi.
*$P < 0.05$, **$P < 0.01$ (*t*-test).

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endotoxins, regulating immune responses, and killing bacteria, viruses, and fungi (Singh *et al.*, 2014). AMPs show broad-spectrum antibacterial activities. In this experiment, a new *M. separata* AMP, cecropin B, was discovered and the encoding gene was successfully cloned. Bioinformatic analysis showed that *MscecropinB* is a positively charged small peptide with amphiphilicity and two amphiphilic α-helices, which corresponds with previous structure research (Brogden *et al.*, 1996; Schittek *et al.*, 2001). The positive charge and amphiphilic nature of AMPs endow them with capacity to efficiently and quickly participate in immune reactions. They act on the cell membrane or cell wall of pathogenic microorganisms, form pores to destroy the ion balance, and kill the microorganism (Singh *et al.*, 2014; Holdbrook *et al.*, 2018; Li *et al.*, 2019; Zhang and Hua, 2023). *MscecropinB* has a signal peptide sequence and transmembrane domain, indicating that it is a secreted protein. Tissue-specific expression analysis revealed that the highest expression of *MscecropinB* was in the fat body, followed by the haemolymph. Studies have shown that several classes of AMPs are synthesised in fat bodies, enter the haemolymph of insects, and react to pathogens throughout the body (Uvell and Engstrom, 2007; Hwang *et al.*, 2009). Our results show that *MscecropinB* demonstrates these characteristics.

Previous studies have found that the AMP gene *CPAlo-13768* is highly expressed in adult and fourth instar larvae of *Conogethes punctiferalis* (yellow peach moth), and highly expressed in the fat body (Dong, 2021) which is consistent with *MscecropinB* expression patterns. Numerous field studies have shown that fourth instar *M. separata* larvae show significant resistance to insecticides and pathogens (Dong *et al.*, 2014; Zhao *et al.*, 2018). Analysis of the expression pattern of *MscecropinB* in the larval stage in the present study suggests that the high tolerance of fourth instar *M. separata* to insecticides and pathogens is most likely associated with xenobiotic metabolism. In this research, *MscecropinB* was highly expressed during the prepupal stage followed by the pupal stage, suggesting *MscecropinB* has a potential function in increasing innate immune responses and adaptability to external adverse effects (Ma *et al.*, 2019a).

The expression of *MscecropinB* was upregulated by increased *M. separata* population density. Yuan (2022) studied cAMP response element-binding protein (CREB) in *M. separata* and found that with the gradual increase of larval density, the expression level of *MsCREB-B* increased significantly. After RNAi with *MsCREB-B*, the expression levels of *AMPs* (*cecropin*, *lebocin*, and *gloverin*) were significantly downregulated, as was the survival rate of the treatment group after *Bt* infection. Jing (2021) found that the expression levels of *cecropin* and *defensin* were significantly higher in larvae raised at high density. After RNAi with *AMPs*, the larval survival rate after *Bt* infection decreased significantly. In summary, *M. separata* larvae exhibit typical density-dependent disease resistance. High density induces upstream genes that affect the expression of AMPs, enhancing the disease resistance of *M. separata*.

Previous research has found that the expression of *Mdatta2* in *Musca domestica* (housefly) larvae was significantly upregulated 3 and 6 h after stimulation by *E. coli* and *Staphylococcus aureus*, which proved that *Mdatta2* plays a role in the immune response to these bacteria (Liu *et al.*, 2011). In the present study, *B. bassiana* and *Bt46* were used to infect third and fourth instar *M. separata* larvae, respectively. The expression of *MscecropinB* was significantly upregulated after infection by *B. bassiana* or *Bt46*. Comparing the regulation of *MscecropinB* by *B. bassiana* in third and fourth instar larvae, *MscecropinB* expression was significantly upregulated on the first day of infection in fourth instar larvae, but a day later in third instar larvae. The expression analysis indicated that the immune response in fourth instar larvae was stronger and faster than that in third instar larvae. The difference in the regulatory effect between the two instars decreased over an observation period of 5 days. The response trends of *MscecropinB* were similar between *Bt46* and *B. bassiana* treatment, in terms of timing. It takes time for pathogens to enter the insect body from the insect wall (Ferreira and de Freitas Soares, 2023). *MscecropinB* expression responded more rapidly in fourth instar larvae than in the third instar, and thus the fourth instar *M. separata* had stronger resistance to the pathogens than the third instar larvae. These results indicate that *MscecropinB* plays an important role in the immune process of *M. separata* against *B. bassiana* and *Bt46*.

Chu (2022) found that the kinases *Spätzle* and *Relish* are key in Toll and immune deficiency signalling pathways of AMP secretion. After 6 h of interference with *Spätzle* and *Relish*, the mortality rate of *Monochamus alternatus* (Japanese pine sawyer) after *B. bassiana* infection was significantly increased, and the relative expression levels of *cecropin* and *defensin* were significantly decreased. The effect of RNAi on gene expression is time-sensitive; in the present study, the maximal period of interference efficiency with *MscecropinB* was about 12 h after injection. After an extended period, the expression levels of the genes that have been interfered with return to normal levels. We found that interference with *MscecropinB* directly impacted the emergence rate and egg production of *M. separata*. It might be that interference with *MscecropinB* weakened the resistance of *M. separata* to the external environment. AMPs are important molecules in insect innate immunity and participate in defence processes. Insects lack adaptive immunity and their survival depends on the production of AMPs, which enables them to establish strong defence mechanisms to combat infections (Zhao *et al.*, 2019). Interference with *MscecropinB* significantly increased the mortality rate of *M. separata* after *B. bassiana* or *Bt46* infection. The pupation rate, emergence rate, and single female egg production were also significantly reduced in the treatment group. This indicates that *MscecropinB* plays an important role in the resistance of *M. separata* to infection by *B. bassiana* or *Bt46*. Controlling this pest by inhibiting the synthesis of *MscecropinB* combined with pathogen treatment will have excellent results.

Conclusions

MscecropinB is a small alkaline peptide. The encoding gene was expressed in all developmental stages of *M. separata*; the highest expression level was in the prepupal stage. In fourth instar larvae, the highest expression level was in the fat body among the tested tissues. Infection of *M. separata* with *B. bassiana* or *Bt46* induced the expression of *MscecropinB*. After *MscecropinB* silencing using RNAi, *M. separata* showed increased sensitivity to *B. bassiana* and *Bt46*, which improved the insecticidal efficiency of these pathogens. The rates of pupation, emergence, and single female egg laying were also significantly reduced by RNAi with *MscecropinB* following *B. bassiana* or *Bt46* infection. The results showed that *MscecropinB* plays an important role in the immune processes of *M. separata* defending against infection by *B. bassiana* and *Bt46*. Our findings indicate that the biocontrol effect of entomopathogenic microorganisms can be improved by changing the internal environment of the target pest. This article promotes

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the development of green pesticides for pest control that inhibit the synthesis of AMPs in insects.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485324000130.

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Competing interests. None.

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