Bulletin of Entomological Research

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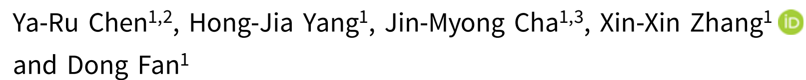

Expression patterns and antifungal function study of KaSPI in *Mythimna separata*

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Research Paper

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Abstract

Kazal-type serine protease inhibitors (KaSPI) play important roles in insect growth, development, digestion, metabolism and immune defence. In this study, based on the transcriptome of *Mythimna separata*, the cDNA sequence of *MsKaSPI* with Kazal domain was uploaded to GenBank (MN931651). Spatial and temporal expression analysis showed that *MsKaSPI* was expressed at different developmental stages and different tissues, and it was induced by 20-hydroxyecdysone in third-instar larvae of *M. separata*. After 24 h infection by *Beauveria bassiana*, the expression level of *MsKaSPI* and the corresponding MsKaSPI content were significantly up-regulated, being 6.42-fold and 1.91-fold to the control group, respectively, while the activities of serine protease, trypsin and chymotrypsin were inhibited. After RNA interference interfered with *MsKaSPI* for 6 h, the expression decreased by 73.44%, the corresponding content of MsKaSPI protein decreased by 55.66% after 12 h, and the activities of serine protease and trypsin were significantly enhanced. Meanwhile, both the larval and pupal stages of *M. separata* were prolonged, the weights were reduced and the number of eggs per female decreased by 181. *Beauveria bassiana* infection also increased the mortality of *MsKaSPI*-silenced *M. separata* by 18.96%. These prove *MsKaSPI* can not only result in slow growth and low fecundity of *M. separata* by regulating the activity of related protease, but also participate in the resistance to pathogenic fungi by regulating the serine protease inhibitor content and the activities of related serine protease.

Introduction

Protease inhibitors exist widely in all organisms and play an important role in physiological processes such as growth and development (Gubb *et al.*, 2010). Protease inhibitors can be divided into four categories: serine protease inhibitors (SPIs), cysteine protease inhibitors, metalloproteinase inhibitors and aspartic protease inhibitors (Getti and Peter, 2002), among which SPIs is a class of structurally conserved enzyme activity regulators, which can inhibit target enzymes to participate in important metabolic processes of life, such as anticoagulation, reproduction, inhibition of excessive phagocytosis, tissue remodelling, antibacterial and immune responses (Zhao *et al.*, 2016). Insects produce innate immune response when pathogens infect, which requires a variety of serine proteases (SPs) to ensure the transmission and expansion of immune signals. SPIs regulate the activity of SPs to make the immune response rapid and intense, and limit it to a certain extent and range to protect from parasitic organisms (Irving *et al.*, 2000; Ferrandon *et al.*, 2007).

Kazal-type serine protease inhibitor (KaSPI) is the most conservative class of SPIs, and its extensive biological functions make it a research hotspot (Laskowski and Kato, 1970; Rimphanitchayakit and Tassanakajon, 2010; Hoef *et al.*, 2013). KaSPI has been identified from many insects, including *Drosophila melanogaster* (Niimi *et al.*, 1999), *Apis cerana* (Kim *et al.*, 2013), *Aedes aegypti* (Torquato *et al.*, 2017), *Pachycrepoideus vindemiae* (Yang *et al.*, 2020), etc. It was expressed in the foregut and midguts of *Bombyx mori*, and was positively regulated by 20-hydroxyecdysone (20E) (Zheng *et al.*, 2007; Gan *et al.*, 2016). In addition, the messenger RNA (mRNA) levels of *KaSPI* in *B. mori* (Zhao *et al.*, 2018) and *Antheraea pernyi* (Wang *et al.*, 2014) were up-regulated under the attack of bacteria and virus, but the degree and time of up-regulation were different. Interestingly, the KaSPI gene may be involved in the reproductive process of insects. The inhibitor gene *Greglin* was isolated from the yolk membrane of *Locusta migratoria*. The study showed that the inhibitor was induced by the moulting hormone, and was expressed at a very high level during vitellogenesis. Knockout of *Greglin* in adults could accelerate the degradation of SP substrate and significantly reduce the level of *Greglin* protein in haemolymph and ovary. This hinders the maturation of oocytes and regulates the reproductive process of insects (Guo *et al.*, 2019).

Mythimna separata seriously endangers food security of its migratory, clustering, polyphagous and fulminant characteristics. In this study, the cDNA sequence of the *MsKaSPI* was

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cloned and the effects of *MsKaSPI* silencing on the growth and development of *M. separata* and the sensitivity to *B. bassiana* were studied. By exploring the role of KaSPI in insect growth, development and pathogen infection, this research lays the foundation for the follow-up study of the physiological functions of KaSPI in insects and the use of pathogenic microorganisms for biological control of agricultural pests.

Materials and methods

Experimental insect and strain

Adults of *M. separata* were collected from a light trap at the experimental field of Xiangfang Experimental Base of the Northeast Agricultural University, Harbin, China. The adults were raised in net cages and routinely fed with 5% honey water. After copulation, the females laid eggs in folded plastic ropes. Newly hatched larvae were transferred to plastic boxes and fed with fresh corn leaves in an artificial climate box at 25 ± 2°C and 70% relative humidity, with a photoperiod of 14:10 (light: dark). *Beauveria bassiana* was provided by the Agricultural Insect and Pest Control Group, Northeast Agricultural University. It was activated on potato dextrose medium (PDA) medium for 1–2 generations, inoculated on PDA medium and cultured in 25°C incubator for 15 days. After the strains are fully sporulated, they are stored in a refrigerator at 4°C for later use.

Acquisition and sequence analysis of MsKaSPI

The first to sixth-instar larvae, pupae and adults of *M. separata* were collected in a 1.5 ml Eppendorf tube, and transcriptional sequencing was carried out by Annuoyoda Genome Technology Company (Beijing, China). *MskaSPI* of *M. separata* was screened from transcriptome database. After homology comparison on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), full-length primers (table S1) were designed using Primer Premier 5.0. Polymerase chain reaction (PCR) amplification procedure was as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 4 min, and a final extension at 72°C for 10 min. PCR products were purified and sequenced for confirmation by comparing with the transcriptome sequences. The MsKaSPI and other insect KaSPIs from NCBI database were used to construct a phylogenetic tree by using MEGA 5.1. The neighbour-joining method with arithmetic averages was used, and a bootstrap analysis of 1000 replications was performed to evaluate the branch strengths of the phylogenetic tree.

Analysis of MsKaSPI expression patterns

Total RNAs were extracted from eggs, each instar larvae, pupae, female and male adults of *M. separata* as well as seven different tissues of third-day third-instar larvae using Trizol® reagent (Invitrogen, Carlsbad, CA, USA). The extracts were treated with 1% diethylpyrocarbonate to prevent ribonuclease contamination. An Implen NanoPhotometer® P300 was used to determine the RNA concentration, and placed in an −80°C refrigerator for later use. Complementary DNA (cDNA) was synthesised using ReverTra Ace qPCR RT Kit (TOYOBO, Shanghai, China) according to the manufacturer's instructions.

The expression patterns of *MsKaSPI* at different developmental stages and in different tissues were analysed by reverse-transcription quantitative real-time PCR (RT-qPCR). β-actin and GAPDH were used as reference genes. The primers were listed in table S1. The qRT-PCR procedure was as follows: initial denaturation of the cDNA at 94°C for 1 min, followed by 40 cycles of 95°C for 30 s, 59°C for 30 s and 72°C for 10 min. A melting curve was constructed to confirm the amplification specificity of qRT-PCR.

Induction of MsKaSPI by 20-hydroxyecdysone

20E solutions were obtained at concentrations of 5, 10, and 20 μg μl^{-1} by dissolving 5, 10, and 20 mg 20E (A506554; Sangon Biotech, Shanghai, China) in 100 μl 1% dimethylsulfoxide (DMSO) then adding double-distilled water to 1 ml, respectively. The concentration was set according to Beckstead *et al.*'s (2007) and Li *et al.*'s (2016) study. For each concentration, three replicates with each 45 larvae were performed. The larvae in the experimental groups were injected with 2 μl of 20E at different concentrations using 10 μl Micro Sample Syringe (F519160; Sangon Biotech), while an equal volume of 0.1% DMSO solution was injected in the control group. After 6, 12, 24, 48 and 72 h, larvae were sampled and total RNAs were extracted. The expression levels of *MsKaSPI* injected with different 20E concentrations were quantified using qRT-PCR.

Infection of B. bassiana *and determination of MsKaSPI expression response*

We collected the spores of *B. bassiana* cultured on the PDA medium, washed the conidia with 0.1% Tween-80 sterile water, stirred evenly with a magnetic agitator and filtered the hyphae with four layers of gauze. Then, the spore suspensions with concentrations of 4×10^9, 4×10^8, 4×10^7, 4×10^6 and 4×10^5 spore ml^{-1} were prepared for use, respectively. The third-day third-instar larvae of *M. separata* were treated by spray method. Five millilitres of each spore suspensions were evenly sprayed on the surface of third-instar larvae, and reared in culture box containing absorbent paper. The fresh feed and absorbent paper in the box were replaced every 24 h, and 0.1% Tween 80 sterile water was used as the control group. We observed continuously for 15 days and calculated the LC_{50} and the spore suspension with the LC_{50} value was 4.75 × 108 spores ml^{-1} according to the pre-experiment. Eighteen insects were retained in each treatment; after 3, 6, 12, 24, 48 and 72 h, half of the larvae were collected to detect the expression of *MsKaSPI* treated with *B. bassiana* at different time points by qRT-PCR, and the other half were used for the determination of the *MsKaSPI* protein content in subsequent infection.

Functional analysis of MsKaSPI by RNA interference

For RNA interference, specific primers of siMsKaSPI (accession No: MN931651) and the negative control (siNC) were designed and synthesised by Shanghai Jima Company (shown in table S1). When designing siMsKaSPI and siNC, do not target the 5′ and 3′ non-coding regions, and the GC content of the sequence should be around 30−60%. Starting from the AUG start codon of the transcript (mRNA), search for the 'AA' binary sequence and record its 19 base sequences at the three ends as potential siRNA targets. Both the sense and antisense chains are designed using these 19 bases (excluding AA repeats). Compare the selected sequences in the public database to ensure that the target sequence has no homology with other genes using BLAST

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(www.ncbi. nlm. nih. gov/BLAST/). Selecting suitable target sequences for siRNA synthesis can directly provide 21 base sequences starting with AA. The negative control (siNC) is a gene sequence that has the same composition as the selected siRNA sequence, but has no significant homology with the target gene and has no biological effect in *M. separata*.

The third-day third-instar larvae of *M. separata* were selected, and 2 μl of 20 $\mu mol\,l^{-1}$ siMsKaSPI was injected into the 2–3 segments on the abdomen side of larvae *M. separata*, while the control group was injected with the same amount of siNC. After injection, the larvae were reared as above and collected at 3, 6, 12, 24 and 48 h. These RNA interference (RNAi)-treated larvae were used for interference efficiency detection, determination of MsKaSPI content and recording the spore suspension with the LC_{50} value of 4.75×108 spores ml^{-1} treated with *B. bassiana* spores at 12, 24, 48 and 96 h. Corrected mortality (%) = [(pest mortality of the treatment group–pest mortality of the control group)/(1–pest mortality of the control group)] × 100. Meanwhile, the RNAi-treated larvae were also used to observe the developmental duration of larvae and pupae, as well as the preoviposition duration and longevity of male and female moth. The weight of larvae (first day of fourth instar) and pupae (second day of pupation), pupation rate, emergence rate and number of eggs per female were also calculated.

Determination of MsKaSPI protein content

The above-mentioned third-day third-instar larvae of *M. separata* treated with *B. bassiana* and RNAi at different time points were grounded in a glass homogeniser for 5 min, then centrifuged at 4°C for 15 min at 4 500 × ***g***. The supernatant was and reserved. We used the insect KaSPI enzyme-linked immunosorbent assay (ELISA) kit (Meibiao, Jiangsu) to determine the change of MsKaSPI content in *M. separata* treated with *B. bassiana* and RNAi by double-antibody sandwich method. There were three biological replicates for each treatment, and three technical replicates for each sample.

Determination of serine protease activity

The insect SP, trypsin, and chymotrypsin ELISA kits (Meibiao) were used to determine the corresponding enzymatic activity by double-antibody sandwich method. There were three biological replicates for each treatment, and three technical replicates for each sample. The total protein content of the samples from the whole body of third-instar larvae were determined with reference to the Coomassie Brilliant Blue G-250 staining method described by Bradford (1976). Sample converted enzyme activity = the actual enzyme activity of the sample/the total protein content of

```
1    ATTCGACGCTGACATCGAGCTCAGTTATCCCGAACATTTTCGCTTTTTTAAATAAAAGCT
61   TCATTTAATTTTGTTAATATTCAGAGCAGGTTTGGAAGTAACTCAGAAAATATGGATTTT
1                                                          M  D  F
121  AAATTCGGAGTACTCCTGGTCTTCAGCGCCTACATCTGCAGCTCTCTCGCGCTTCCTCCG
4    K  F  G  V  L  L  V  F  S  A  Y  I  C  S  S  L  A  L  P  P
181  TGCACGTGCACCAGGAACTACCTGCCCATCTGCGGTTCCGACGGCGAGACCTACAGCAAC
24   C  T  C  T  R  N  Y  L  P  I  C  G  S  D  G  E  T  Y  S  N
241  GCCTGCTGGACTGCGCCAACTACAACACGCACAAGGACATCGTCGCCGTCAAGGAA
44   Q  C  L  L  D  C  A  N  Y  N  T  H  K  D  I  V  A  V  K  E
301  GGTCCCTGCGACGGCAACCTGCCCGTCGTCGAATTAGAATGTATATGTCCCTTCAACTAC
64   G  P  C  D  G  N  L  P  V  V  E  L  E  C  I  C  P  F  N  Y
361  CTGCCGGTGTGCGGTACTGATGGCGTTACCTACTCCAACCAGTGCAGCCTCAACTGCCAG
84   L  P  V  C  G  T  D  G  V  T  Y  S  N  Q  C  S  L  N  C  Q
421  AAGCAGCGCAGCGCAGGTCTCGAGGTGAAGCACATGGGCCAGTGCGTGGAGGCGTCAACT
104  K  Q  R  S  A  G  L  E  V  K  H  M  G  Q  C  V  E  A  S  T
481  GAGGCCTTCCAGTGCATGTGCGGGAGGGACAAGAAGCCCGTGTGTGGCAGCGACGGCAAC
124  E  A  F  Q  C  M  C  G  R  D  K  K  P  V  C  G  S  D  G  N
541  ACCTACAGCAACGCCTGCCTGCTCAACTGCGCTACCGCTAAAGACAGCTCGCTCTTCATC
144  T  Y  S  N  A  C  L  L  N  C  A  T  A  K  D  S  S  L  F  I
601  GCTAAGTACGGACCCTGTGACGACAAAGTGAAGGTCGCCGAGCTCTCCTAAGTCATTTGA
164  A  K  Y  G  P  C  D  D  K  V  K  V  A  E  L  S  *
661  ATACTACGTTAAGTTTTTAGTACTGCCTTATGCATTTGAGTAGTCACATATGTAGATGCA
721  TTCAAAATATCACTCATATTATTTAAGTTTATTTTTATGAACTGTTACGATCTGATGCAT
781  TTTATTTAAATTTTAAAATGTTTAATGTTTATTGTCGAGTTTAATGAATTTGTTTATTGC
841  TATTTATTAAATCGACCTTCTAATCAAAA
```

Figure 1. Nucleotide and deduced amino acid sequences of *MsKaSPI* cDNA. The start codon (ATG) and stop codon (TAA) are boxed. The signal peptides are underlined with double underline. Conserved cysteines are underlined with a single line. Kazal structure domain residues are indicated by grey shade.

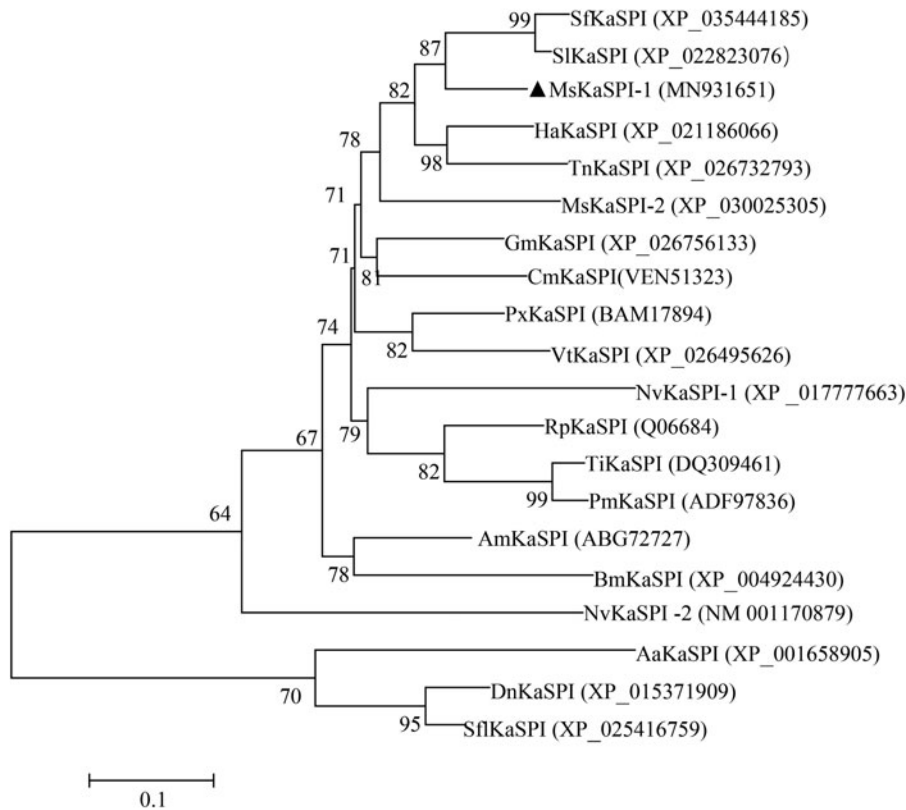

Figure 2. Phylogenetic tree of KaSPI proteins from *Mythimna separata* and other insects by neighbour-joining method based on amino acid sequence (1 000 replicates). Origin species of KaSPI proteins: SfKaSPI: *Spodoptera frugiperda*; SlKaSPI: *Spodoptera litura*; AcKaSPI: MsKaSPI-1, MsKaSPI-2: *M. separata*; HaKaSPI: *Helicoverpa armigera*; TnKaSPI: *Trichoplusia ni*; MsKaSPI: *Manduca sexta*; GmKaSPI: *Galleria mellonella*; CmKaSPI: *Callosobruchus maculatus*; PxKaSPI: *Papilio xuthus*; VtKaSPI: *Vanessa tameamea*; NvKaSPI: *Nicrophorus vespilloides*; RpKaSPI: *Rhodnius prolixus*; TiKaSPI: *Triatoma infestans*; PmKaSPI: *Panstrongylus megistus*; AmKaSPI: *Antheraea mylitta*; BmKaSPI: *Bombyx mori*; NvKaSPI-1, NvKaSPI-2: *Nasonia vitripennis*; AaKaSPI: *Aedes aegypti*; DnKaSPI: *Diuraphis noxia*; SflKaSPI: *Sipha flava*.

the sample, and the converted enzyme activity is used as the sample enzyme activity in results.

Statistical analysis

The relative expression levels were measured using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and mean expression ratio (±SE) of three biological replicates were calculated. Statistical analyses were performed using SAS 9.0 software (IBM Corp., Armonk, New York, USA) single-factor variance Duncan's multiple comparison method to analyse the relative expression of genes at different time points, as well as the significance of enzyme content and the significance of the differences in enzyme activities. The significance of the difference in mortality and RNAi on the growth and development-related parameters of *M. separata* was analysed by the *t*-test ($P < 0.05$). The numerical calculation was carried out in Excel 2010 and chart production was by GraphPad Prism 8.

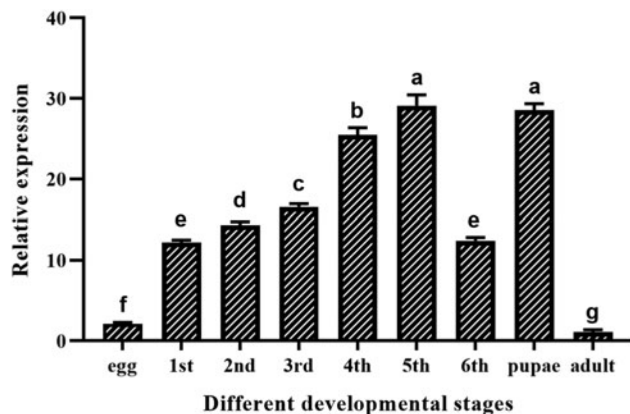

Figure 3. Expression levels of *MsKaSPI* at different developmental stages of *M. separata.* Three biological replicates were conducted. The relative expression levels were measured using the $2^{-\Delta\Delta CT}$ method and mean ± SE were calculated. Different lowercase letters above bars indicate significant difference ($P < 0.05$, Duncan's multiple range test).

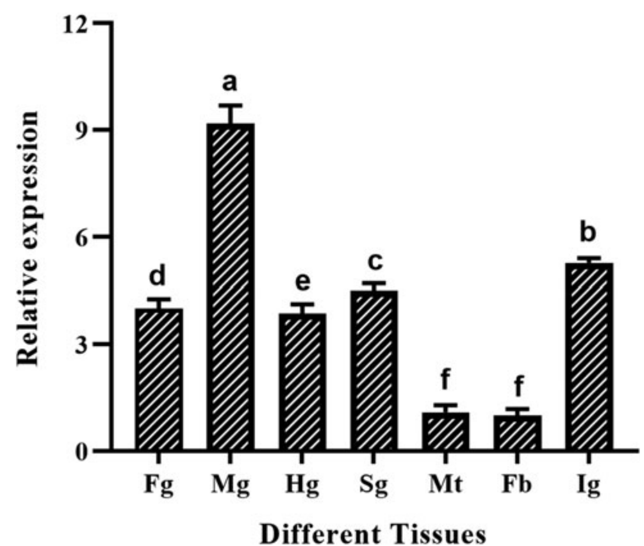

Figure 4. Expression levels of *MsKaSPI* in different tissues of *M. separata.* Fg: foreguts; Mg: midguts; Hg: hindguts; Sg: salivary glands; Mt: malpighian tubules; Fb: fat bodies; Ig: integuments. The relative transcript levels were measured using the $2^{-\Delta\Delta CT}$ method and mean ± SE were calculated. Different lowercase letters above bars indicate significant difference in the gene expression level at different developmental stages ($P < 0.05$, Duncan's multiple range test).

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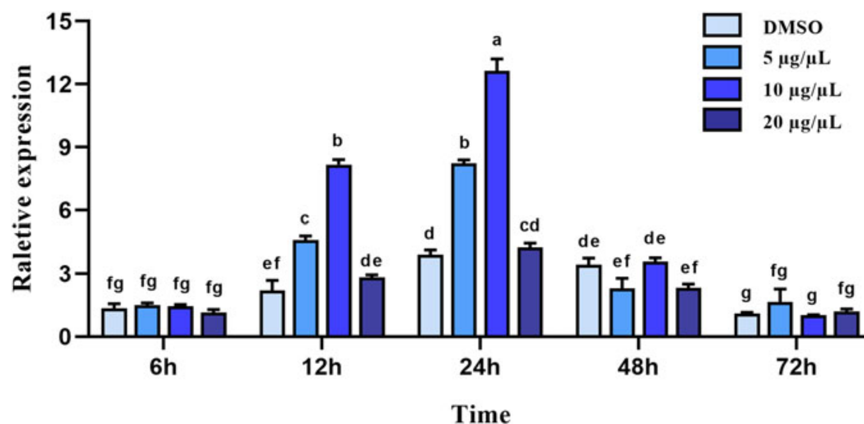
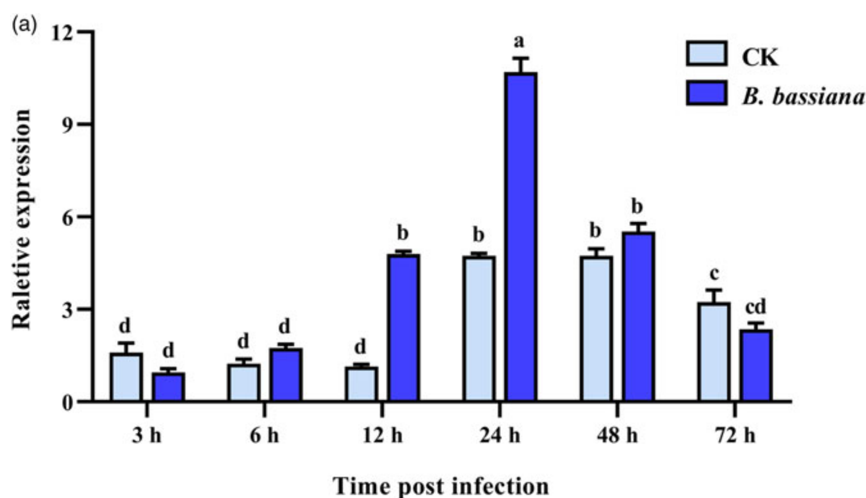


Figure 5. Effect of different doses of 20-hydroxyecdysone (20E) on the relative *MsKaSPI* expression. Total RNA was extracted from third-day third-instar larvae injected with 20E concentrations of 5, 10 and 20 µg µl^{-1}. The time points on the *x*-axis indicate hours after the injection. DMSO is the control group. The relative transcript levels were measured using the $2^{-\Delta\Delta CT}$ method and mean ± SE were calculated. Different lowercase letters above bars indicate significant difference in the gene expression level among different treatment time points ($P < 0.05$, Duncan's multiple range test).



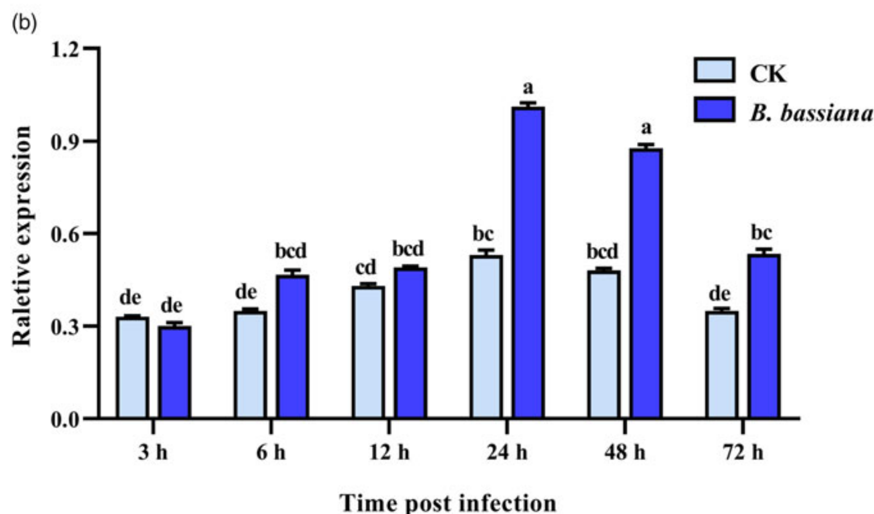


Figure 6. Effect of MsKaSPI expression pattern in *M. separata* after infection by *B. bassiana*. The expression of *MsKaSPI* at the mRNA level (a) and the MsKaSPI content (b) at different times after the larvae were treated with *B. bassiana* or blank control (CK). The spore suspension with the LC_{50} value of 4.75×10^8 spores ml^{-1} was used for infection, and sterile water containing 0.1% Tween 80 was used as the CK. The relative transcript levels were measured using the $2^{-\Delta\Delta CT}$ method and mean ± SE were calculated. Different lowercase letters above bars indicate significant difference among different treatment time points ($P < 0.05$, Duncan's multiple range test).

Results

Identification and analysis of MsKaSPI

We cloned and identified a novel KaSPI cDNA sequence from *M. separata*, referred to as *MsKaSPI* (GenBank accession number MN931651). The full-length cDNA sequence is 869 nucleotides long and contains a 540-nucleotide open reading frame and 3′, 5′ untranslated regions. The cDNA encodes an amino acid sequence with 179 residues and contains conserved GXDXXTYXNXC motif and six non-conserved cysteine sequences (fig. 1), which forms the disulphide bonds and is a characteristic of the KaSPI family. A 20 amino acid putative signal peptide was detected using the Signal P 4.1Server (http://www.cbs.dtu.dk/services/SignalP/) and the molecular weight of the gene is about 19.30 kDa and the isoelectric point is

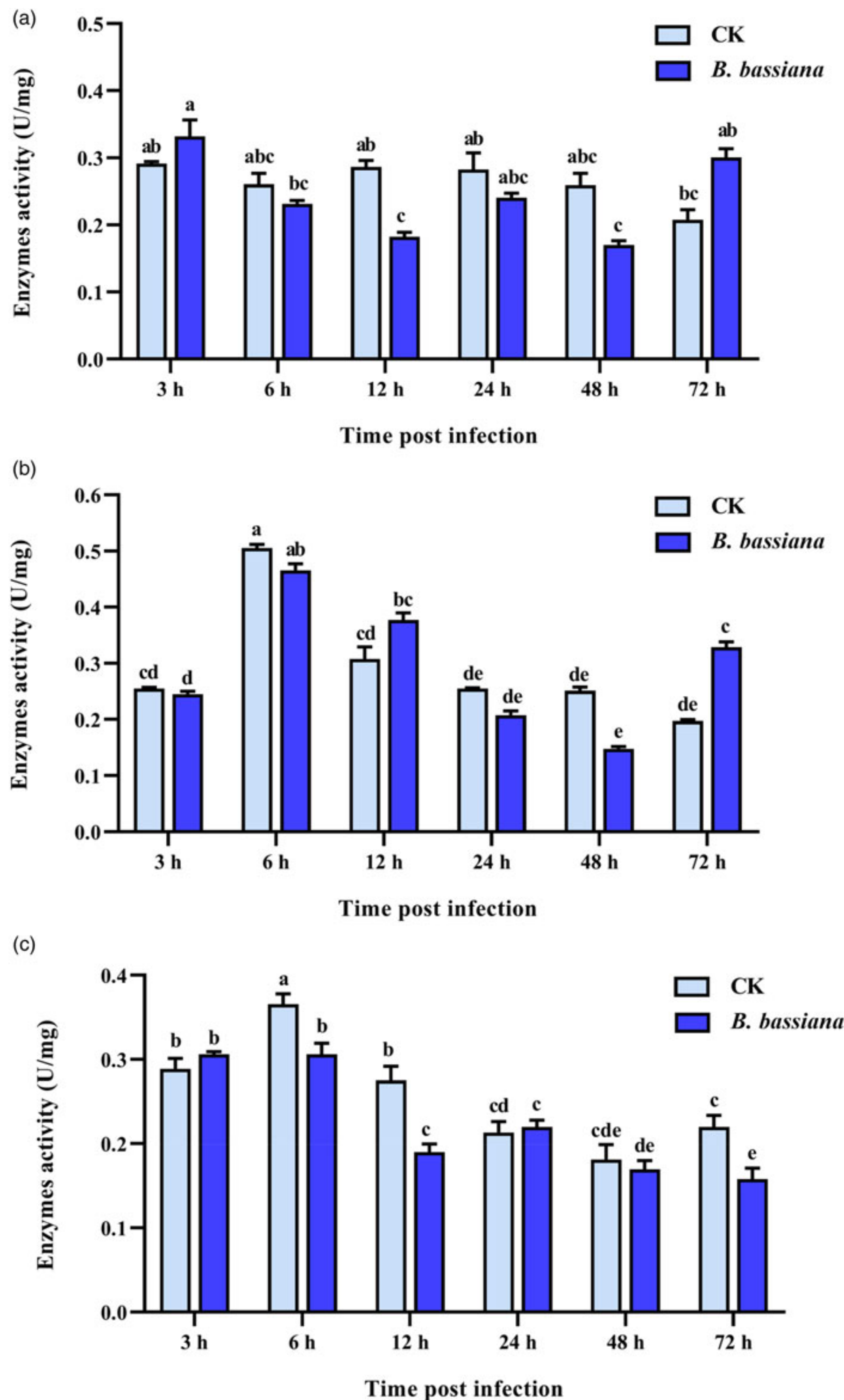


Figure 7. Effects of *B. bassiana* infection on the activities of serine proteinase (a), trypsin (b) and chymotrypsin (c) in *M. separata.*

4.95 by the compute pI/Mw tool software of ExPASy (http://ca.expasy.org/tools/). The phylogenetic tree showed that the MsKaSPI was closely related to *Spodoptera litura* (XP_022823076), *Spodoptera frugiperda* (XP_035444185) and *Helicoverpa armigera* (XP_021186066), but far from *Nasonia vitripennis* (NM001170879) and *A. aegypti* (XP_001658905) (fig. 2). There is a certain correlation between the phylogenetic relationship of KaSPIs.

Stage and tissue expression patterns of MsKaSPI

The *MsKaSPI* was expressed at different developmental stages of *M. separata*, and the expression level was the highest in fifth-instar larvae, which was 13.77, 2.39, 1.99, 1.76, 1.14, 2.34 and 26.79-fold higher than that in egg, first to sixth-instar larvae and adult, respectively ($P < 0.05$), but there was no significant difference from the pupal stage (fig. 3).

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Expression pattern analysis of *MsKaSPI* in third-instar larvae showed that the expression level in the midguts was significantly higher than in the other tested tissues and was 2.30, 2.38, 2.04, 8.40, 9.19 and 1.74-fold higher than in foreguts, hindguts, Malpighian tubules, fat bodies and integuments, respectively ($P < 0.05$). As shown in fig. 4.

Effect of ecdysone on MsKaSPI

Different concentrations of 20E were used to induce the expression of *MsKaSPI*. We found that the trend in *MsKaSPI* expression variations was consistent among different concentrations of 20E treatment groups at the different time points. As the 20E concentration increased from 5 to 10 $\mu g\,\mu l^{-1}$, the *MsKaSPI* expression level showed an upward trend. *MsKaSPI* expression increased from 3 to 24 h reaching its highest level at 24 h, decreased at 48 h.The expression level of MsKaSPI was highest 24 h after injection of 10μg μl^{-1} 20E, which was 3.24-fold than control. The 5 and 10 $\mu g\,\mu l^{-1}$ 20E significantly up-regulated the expression of *MsKaSPI* gene after 12 and 24 h, while the high concentration 20E (20 $\mu g\,\mu l^{-1}$) did not show any effect on up-regulation of the expression of *MsKaSPI* (fig. 5).

Effects of *B. bassiana* infection on the MsKaSPI in *M. separata*

The expression level of *MsKaSPI* within 72 h in *M. separata* after being infected with LC_{50} *B. bassiana* peaked at 24 h compared with the control group ($P < 0.05$), which is 4.18-fold of the control group (fig. 6a). As shown in fig. 6b, the protein content of MsKaSPI was significantly different from that of the control group after 12, 24 and 48 h after infection with *B. bassiana* ($P < 0.05$), which were 1.94, 3.11 and 2.53-fold, respectively. However, there was no significant difference in protein content at 72 h after infection. It can be seen that *B. bassiana* induced the expression of MsKaSPI.

Effect of *B. bassiana* infection on serine protease activity in *M. separata*

The activity of SP (fig. 7a) in *M. separata* infected by *B. bassiana* compared with the control significantly decreased by 36.39 and

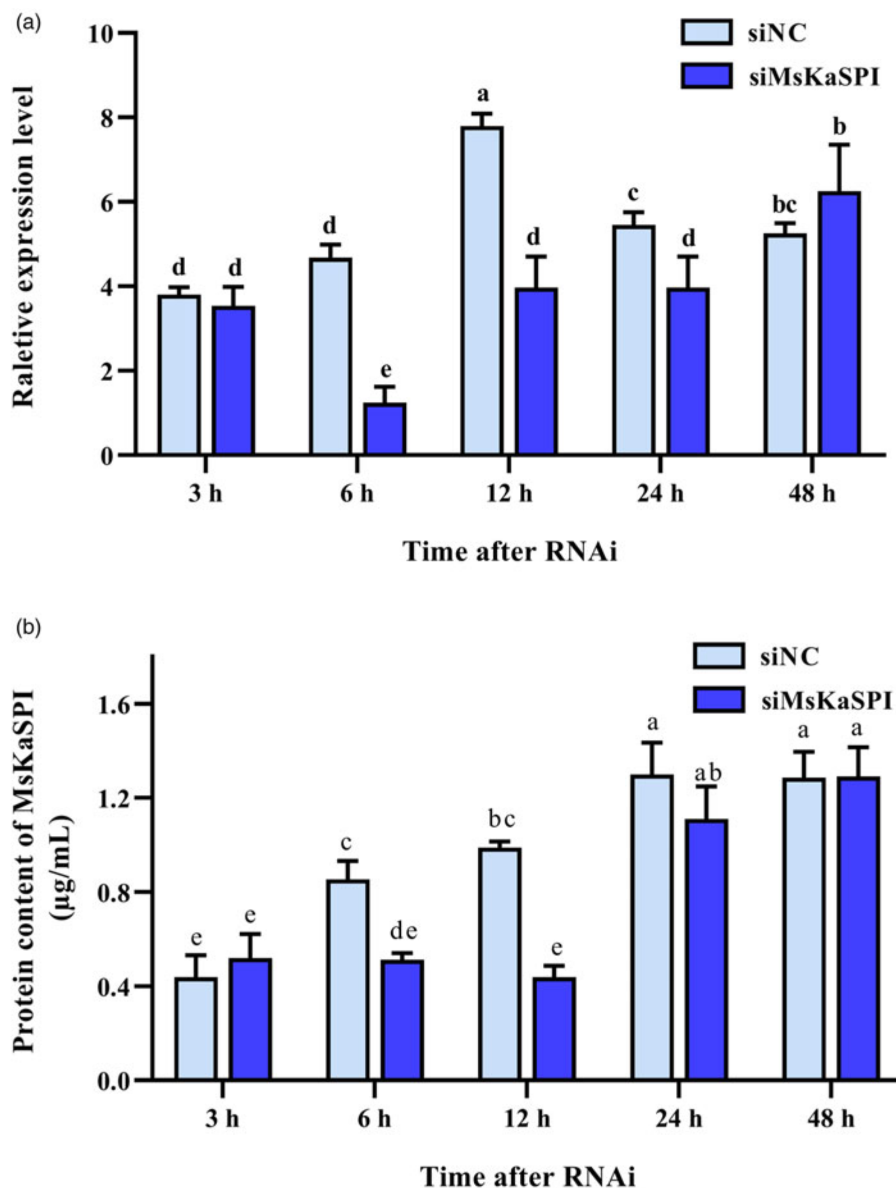

Figure 8. Effects of RNAi on *MsKaSPI* expression levels. The expression of *MsKaSPI* at the mRNA level (a) and the protein content of MsKaSPI (b) at different times after the larvae were injected with siRNA(siMsKaSPI) or negative control (siNC). Total RNA was extracted at 3, 6, 12, 24 and 48 h after injection with siRNA. The relative transcript levels were measured using the $2^{-\Delta\Delta CT}$ method and mean ± SE were calculated. Different lowercase letters above bars indicate significant difference among different treatment time points ($P < 0.05$, Duncan's multiple range test).

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34.31% after treatment for 12 and 48 h. While the trypsin activity (fig. 7b) was only significantly decreased from the control group at 48 h by 41.18% ($P < 0.05$), the chymotrypsin (fig. 7c) activity was significantly different from that of the control group at 12 and 72 h ($P < 0.05$), and the enzyme activity decreased by 30.95 and 28.21%, respectively.

Influence of RNA interference on MsKaSPI

The efficiency of *MsKaSPI* knowdown was calculated using qRT-PCR. At 6, 12 and 24 h point of injection of siMsKaSPI, the expression levels in third-day third-instar larvae were repressed by 73.44, 49.06 and 27.23% compared to the expression levels after injection of siNC, respectively (fig. 8a).

In order to further study the function of *MsKaSPI* in the third-day third-instar larvae in response to *B. bassiana* infection, the changes of MsKaSPI protein content during 3, 6, 12, 24 and 48 h were detected by the double-antibody sandwich method after being treated with siRNA. As shown in fig. 8b, at different time points of RNAi, the content of MsKaSPI decreased in varying degrees. At 6 and 12 h, the MsKaSPI content was significantly different from that of the control ($P < 0.05$), and decreased by 40.02 and 55.66%, respectively.

In addition, the *MsKaSPI* gene-silenced larvae showed slow growth, and the larval and pupal stages were prolonged ($P < 0.05$), but it had no effect on the pre-oviposition and adults lifespan. Compared with the control group, the oviposition of females was reduced by 181 ($P < 0.05$) (table 1), but there was no significant difference in pupation rate and emergence rate. The silence of *MsKaSPI* slightly increased the mortality of *M. separata* after 48 and 96 h, but the differences were not significant (fig. 9). While the insect mortality rate has significantly increased in *MsKaSPI*-silenced larvae after 24, 48 and 96 h infection with *B. bassiana* ($P < 0.05$), which increased by 8.33, 13.56 and 18.96%, respectively (fig. 9). It indicated that the silence of MsKaSPI could help to increase the infection rate of *B. bassiana* to *M. separata*.

Effects of RNAi of MsKaSPI on enzyme activities in M. separata

After RNAi treatment, the SP (fig. 10a) and trypsin (fig. 10b) activities showed an overall trend of initial increase and then decrease. At 6 h after treatment, the SP activity began to increase significantly ($P < 0.05$). The trypsin activity showed a significant increase from 3 to 24 h after treatment ($P < 0.05$); chymotrypsin (fig. 10c) activity increased slightly after treatment, but not significantly different from the control group. By silencing of *MsKaSPI*, the activity of SP and trypsin in *M. separata* was enhanced, indicating that MsKaSPI may effectively regulate related SPs by adjusting the content of SPIs.

Discussion

SPIs play an important role in insect growth and development, metabolism and immune defence. In order to further study the mechanism of SPIs in insects, a cDNA sequence MsKaSPI of SPI gene with Kazal domain was obtained by transcriptome sequencing. The sequence deduced by *MsKaSPI* contains three Kazal-type domains formed by three disulphide bonds and six cysteine residues in a specific pattern (CysI-CysV, CysII-CysIV and CysIII-CysVI). Sequence analysis shows that this MsKaSPI protein belongs to group II of the non-classical KaSPI (Cabrera-Muñoz *et al.*, 2019).

Table 1. Growth and development parameters of *M. separata* under different treatments

Treatment	4–6th larval duration (d)	Pupal duration (d)	Preoviposition duration (d)	Female moth life (d)	Male moth life (d)	Larval weight (g)	Pupal weight (g)	Pupation rate (%)	Emergence rate (%)	Number of eggs (per female)
siNC	9.12 ± 0.75	13.04 ± 0.71	4.80 ± 0.11	9.94 ± 0.35	10.94 ± 0.67	0.21 ± 0.01	0.28 ± 0.01	94.11 ± 0.75	91.66 ± 0.95	942.60 ± 53.77
siMsKaSPI	10.06 ± 0.69*	13.78 ± 0.68*	4.92 ± 0.18	9.73 ± 1.23	10.74 ± 0.72	0.19 ± 0.01	0.26 ± 0.01	95.27 ± 0.52	92.50 ± 0.59	761.30 ± 43.02*

Data in the table are means ± SEs. Asterisk following the data in a column indicates significant difference from the control ($P < 0.05$, *t*-test). 30 worms were observed (including 14 females and 16 males) and conducted growth parameter data statistics every 24 h. The number of eggs (per female) were counted for 14 female adults.

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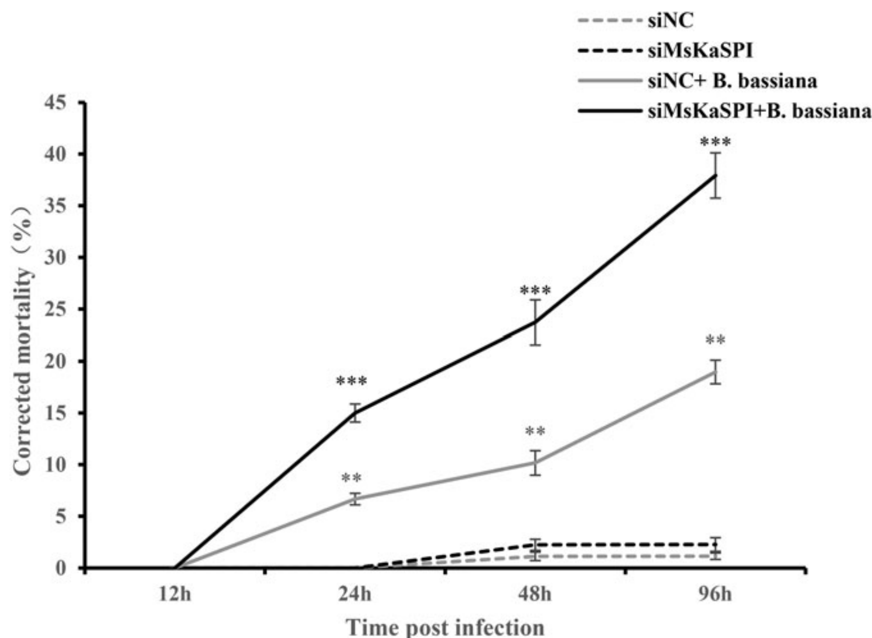

Figure 9. Corrected mortality of MsKaSPI RNAi treated *M. separata* and infection by *B. bassiana*. Corrected mortality (%) = [(mortality of the treatment group–mortality of the control group)/(1–mortality of the control group)] × 100. Data in the figure are means ± SEs. Asterisk following the data in a column indicates significant difference from the control (**$P < 0.05$, ***$P < 0.01$, Duncan's multiple range test).

The *MsKaSPI* had the highest expression in fifth-instar larvae and the midgut of *M. separata*. This expression pattern indicates it may be related to food digestion and absorption in *M. separata*. The expression of BmSPI3 was the highest both in the fifth-instar larvae and midguts of the fifth-instar larvae (Zheng *et al*., 2010). ApKTSPI was specifically highly expressed in the fat body of fifth-instar larvae in *A. pernyi* (Wang *et al*., 2014). In addition, it was also found that the expression of insect KaSPI was positively regulated by ecdysone 20E which participated in the regulation of insect innate immunity (Tian *et al*., 2010; Rus *et al*., 2013; Sun *et al*., 2016). The CG7906 and CG7924 genes in *D. melanogaster* were successfully induced after ecdysone 20E treatment, the expression levels were 3.49 and 2.41 times higher than control (Beckstead *et al*., 2007), respectively. In this experiment, the expression of *MsKaSPI* in the third-instar larvae was the highest 24 h after injection of 10 $\mu g \mu l^{-1}$ 20E. It was speculated that 20E could induce *MsKaSPI* expression. When the concentration of 20E increased from 5 to 10 $\mu g \mu l^{-1}$, the expression of *MsKaSPI* increased gradually, but it decreased when injected with the concentration of 20 μg μl 20E, which may be because although the growth and development of larvae were regulated by ecdysone and juvenile hormone, excessive concentrations would affect the normal physiological process of insects. There was a similar situation in *B. mori*, the expression of *BmKaSPI* was significantly up-regulated in silk glands of both female and male silkworms 12 and 24 h after treatment with low concentration of 20E, while there was no up-regulation of *BmKaSPI* after treatment with high concentration of 20E (Gan *et al*., 2016). However, 20E regulates insect innate immunity in a variety of ways, and the specific mechanism is not clear (Wu *et al*., 2016).

After treatment with *B. bassiana*, the expression of *MsKaSPI* was significantly up-regulated, and the activities of SP, trypsin and chymotrypsin were significantly inhibited, indicating that *MsKaSPI* may participate in the resistance of armyworm to the invasion of pathogens. ApKaSPI was up-regulated after nuclear polyhedrosis virus, *E. coli* and *B. bassiana* (Wang *et al*., 2014). *AaKaSPI* was up-regulated after immune stimulation by virus DENV (Soares *et al*., 2018). After leafhoppers were stimulated by the bacteria and *E. coli*, the expression of *KaSPI* in the leafhoppers midgut, blood cells and whole insects were collectively up-regulated (Gonella *et al*., 2019). The results of these studies showed that insect protease inhibitors are involved in the immune response, but the gene expression patterns are different under the immune stimulation of different pathogens. In addition, it was also found that after treatment with *B. bassiana*, the content of MsKaSPI protein increased significantly (fig. 6b). However, it has decreased from 24 h, which was not consistent with the expected results. According to the trend that ecdysone first increased and then decreased before and after moulting in insects and the results of *MsKaSPI* expression induced by 20E *in vitro* (fig. 5), it was speculated that the increase of MsKaSPI protein content might be caused by keeping a certain concentration of ecdysone for a short time after 24 h treatment of third-instar larvae.

RNAi technology was taken to further study the function of MsKaSPI in the induced metabolism of *M. separata* by *B. bassiana*. The results showed that the expression of *MsKaSPI* and the protein content of KaSPI were significantly decreased, and the SP and trypsin activities were significantly enhanced, indicating that MsKaSPI silencing would cause changes in related proteins and enzyme activities. After silence of *MsKaSPI*, the mortality of *M. separata* increased after *B. bassiana* infection. It is inferred that MsKaSPI functions in the resistance to *B. bassiana*. The bacteriostatic effect of KaSPI has also been reported in other insects. For example, it was found that over-expressed *BmKaSPI* in silkworms can effectively inhibit subtilisin which plays an important role in resisting pathogen invasion in silkworms (Guo *et al*., 2015; Chen and Lu, 2017); AmKaSPI can bind to the surface of bacteria and fungi, and exhibits antimicrobial activity against fungi and Gram-positive and -negative bacteria (Qian *et al*., 2015; Yang *et al*., 2017), it can be seen that KaSPI plays an important role in resisting pathogen invasion in insects.

Meanwhile, it was also found that the number of eggs per female was significantly reduced after the RNAi expression of *MsKaSPI*. A KaSPI gene named Greglin related to reproduction

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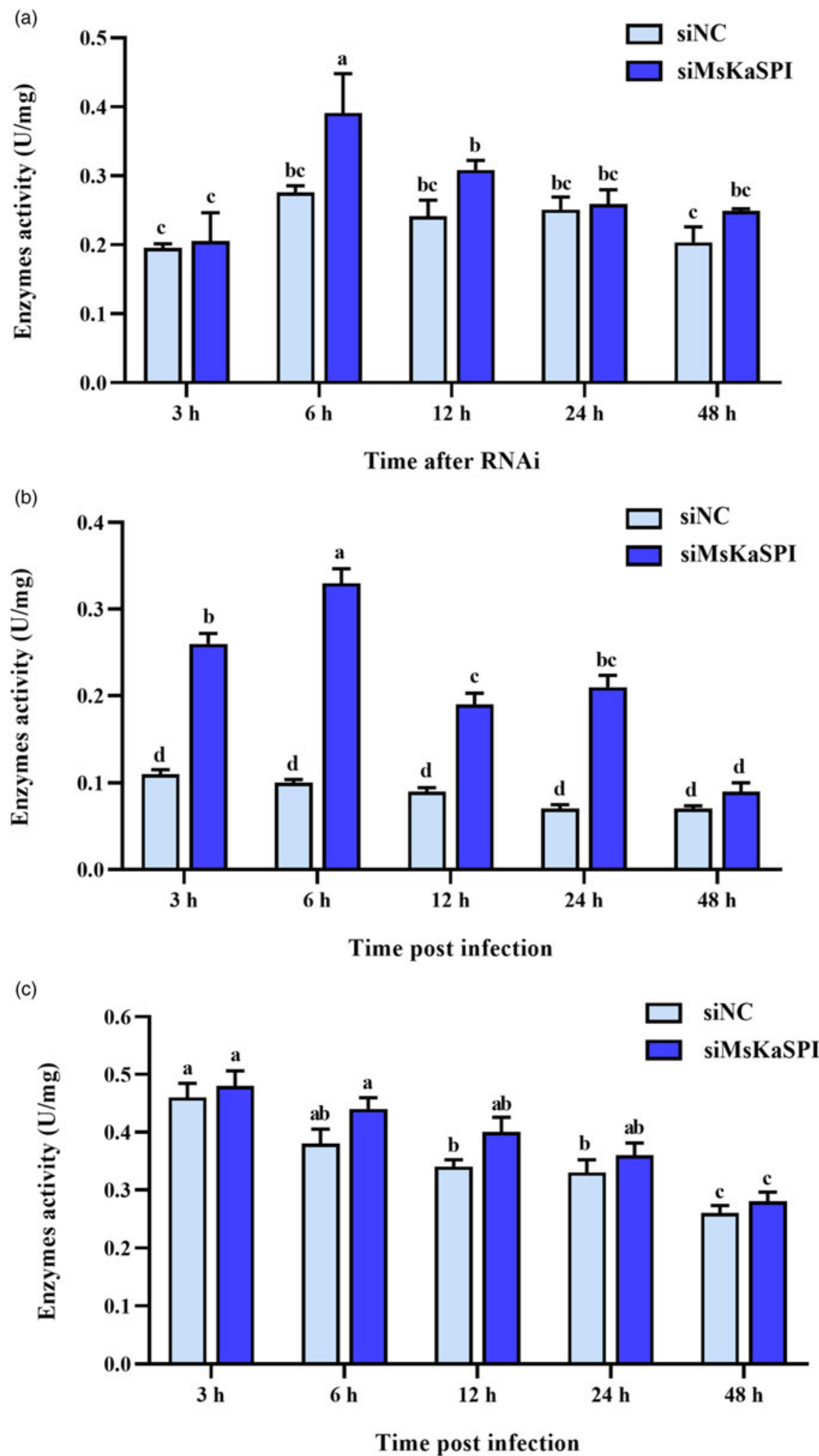

Figure 10. Effects of RNAi of *MsKaSPI* on the activities of serine proteinase (a), trypsin (b) and chymotrypsin (c) in *M. separa*.

was also found in migratory locusts. Knockdown of *Greglin* in adult female locusts results in a significant reduction in Greglin content, oocyte maturation was blocked, ovarian growth stagnated and follicular epithelial cells atrophied in *L. migratoria*, and the number of eggs laid and hatching rate decreased, indicating that the gene was involved in the reproductive process of locusts (Guo *et al*., 2015). It can be seen that MsKaSPI not only plays an important role in resisting the invasion of pathogens, but also has a great impact on the reproduction of *M. separata*.

Conclusions

In summary, we described the molecular characteristics of *MsKaSPI* as well as its structure properties and the spatio

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temporal expression profiles. The *MsKaSPI* gene expression was induced by 20E treatment and the infection of *B. bassiana*. *MsKaSPI* gene knockdown could significantly increase the activities of SP, trypsin and the mortality by the infection of *B. bassiana*, and reduced the number of eggs in *M. separata*. Our results provide further insights into the role of *MsKaSPI* on reproduction and immune defence, and potential target for pest control.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S000748532300041X

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Competing interests. None.

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