

Serpin7 controls egg diapause of migratory locust (*Locusta migratoria*) by regulating polyphenol oxidase

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Keywords

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Diapause is a state of arrested growth, which allows insects to adapt to diverse environments. Serine protease inhibitors (serpins) play an important role in various physiological processes, including blood coagulation, fibrinolysis, development, complement activation and extracellular matrix remodeling. We hypothesized that serpin may affect energy metabolism and thereby control diapause of migratory locust (*Locusta migratoria*) embryos by regulating protease cascades. A total of seven nonredundant serpin genes (named *serpin1–serpin7*) of *L. migratoria* were obtained through transcriptomic analysis. We further performed label-free proteomic sequencing and analysis of diapause and nondiapause eggs of *L. migratoria*, revealing significant differences in *serpin7* expression. A significant reduction in diapause rate under the short photoperiod was observed in insects treated with *serpin7* double-stranded RNA. Furthermore, knock-down of the *serpin7* gene resulted in significant upregulation of the activity of polyphenol oxidase. We therefore propose that the observed *serpin7* gene plays a crucial role in diapause, suggesting that control of energy metabolism may have potential as a future strategy for the reproductive control of insect pests.

Diapause is an important behavior of insects that allows the insects to adapt to diversified environments. Migratory locust, *Locusta migratoria* L., belongs to the facultative diapause type of embryonic diapause insect [1,2]. Temperature and photoperiod are the key factors affecting diapause of *L. migratoria*. The maternal *L. migratoria* can sense the environmental information and transmit this information in the form of signals to the offspring to cut off the developmental signal, which induces diapause of the eggs in the locusts [3].

Serine protease inhibitors (serpins) belong to the widely distributed protein family protease inhibitors

[4,5]. More than 1500 serpins have been extensively studied in animals, plants, bacteria and viruses [6]. Generally, the serpin could be 350–500 amino acids found in typical serpin, and irreversible inhibition occurs whenever serpin binds to its substrates [4,7]. Studies showed that the serpins play an important role in blood coagulation, fibrinolysis, complement activation, inflammatory reactions, immunity, physiology, digestion, development and extracellular matrix remodeling [8–12]. Seven serpins were purified from hemolymph of tobacco moth, *Ephestia elutella*, that regulated the activation of phenol oxidase (PO) and played a role in immune defense [13–15]. In

Abbreviations

A, absorbance; ddH₂O, double-distilled water; dsRNA, double-stranded RNA; LB, lysogeny broth; NJ, neighbor-joining; ORF, open reading frame; pI, isoelectric point; PO, phenol oxidase; PPO, polyphenol oxidase; proPO, prophenoloxidase; RH, relative humidity; RNAi, RNA interference; RT-qPCR, real-time quantitative PCR.

addition, serpins also play a crucial role in tissue synthesis and embryonic development of animals [16]. *Spn27A* regulates the formation of dorsal ventral axis of drosophila embryos in the early developmental stage by inhibiting the Toll signaling pathway [17,18]. In addition, *Spn88Ea* is necessary for wing development in fruit flies [19]. Balance of *SRP-2* (serpin), a cross-class inhibitor, is also important for postembryonic development of nematodes, *Caenorhabditis elegans* [20]. However, no extensive and comprehensive studies have been made on the effects of serpin in relation with insect diapause. Keeping in view the importance of serpins in other plants and animals, this novel study was designed to carry out the transcriptome analysis of diapause and nondiapause eggs of migratory locust, *L. migratoria*, especially for the serpin genes [21,22]. We further performed label-free proteomic sequencing on diapause and nondiapause eggs of migratory locust to understand the expression of serpin-related genes [21,22]. We hypothesized that serpin may affect energy metabolism and could control diapause of migratory locust embryos by regulating protease cascade reaction. To explore the role of serpin in diapause regulation, we performed RNA interference (RNAi)-mediated silencing of serpin gene. We further planned to study the content of polyphenol oxidase (PPO) by applying RNAi of specific serpin gene. This study would provide a theoretical basis for further study on the diapause mechanism of the migratory locust through serpins.

Materials and methods

Insect materials

The *L. migratoria* L. colony used in this study was originally collected from the field at Cangzhou, Hebei, China (39°37'N, 98°30'E, 40 m above sea level) and was maintained by the State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences for successive years. Locust eggs were hatched in an artificial climate box (PRX-250B-30; Haishu Saifu Experimental Instrument Factory, Ningbo, China) at a temperature of around 30 °C with relative humidity of 60%. The photoperiodic regimen used for nondiapause locusts in the experiment was 16 h light : 8 h darkness. Similarly, to induce diapause, we reared locusts under a short photoperiod at 10 h light : 14 h darkness, 27 °C and 60% relative humidity [23,24]. Freshly grown wheat seedlings were fed to the locusts in the laboratory.

Identification of serpin genes in the migratory locust

The transcriptome sequencing and analysis were performed on diapause and nondiapause eggs of migratory locusts in the State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences. A total of seven serpin genes (*serpin1* to *serpin7*) were obtained. Molecular formula, molecular weight and isoelectric points of serpin proteins were analyzed by EXPASY software (Swiss Institute of Bioinformatics, Geneva, Switzerland). Meanwhile, we used WOLF PSORT software (<http://wolfsort.org/>) to predict the subcellular localization of serpins (Data S1).

Amino acid sequence alignment and construction of phylogenetic tree

DNAMAN software (version 7.212; Lynnon Corp., Quebec, QC, Canada) was used to translate the serpin sequence of migratory locust. We obtained the amino acid sequences of serpins' open reading frame (ORF). Sequence alignment was performed between serpin amino acid sequences of the migratory locust (Data S2) and other serpins sequences of silkworm, *Bombyx mori*, and fruit fly, *Drosophila melanogaster*, published by Universal Protein Knowledgebase (UniProt). The neighbor-joining (NJ) method was used to construct phylogenetic trees by MEGA 6.0 software (Molecular Evolutionary Genetics Analysis Version 6.0), and 1000 bootstrap tests were performed [25].

Advanced structure analysis of serpin proteins

Using the Self-Optimized Prediction method With Alignment (SOPMA) online server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html), we analyzed the secondary structure of serpin proteins and predicted the tertiary structure of serpin proteins by EXPASY software (Swiss Institute of Bioinformatics).

Clone of *serpin7* gene

Third-instar nymph of locusts was dissected, and the digestive tract was clinically removed. The remaining tissues were used for the extraction of total RNA. TRIzol Reagent (Tianmo, Huailai, China) was used to extract RNA. Total RNA was isolated according to the manufacturer's protocol. The quality was checked on a spectrophotometer with A_{260}/A_{280} between 1.9 and 2.0, whereas the reliability of RNA was confirmed on 1% agarose gel, which gave three clear bands. cDNA was synthesized according to the PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa, Dalian, China). By analyzing transcriptome of the migratory locust, we obtained the sequence of *serpin7* gene, and

primers were then designed by DNAMAN software (version 7.212; Lynnon Corp.). Using cDNA of migratory locust as a template, we amplified the *serpin7* gene by primers *serpin7*-1F/*serpin7*-1R. The PCR-amplified fragment was 894 bp (Fig. 1). The obtained PCR product was purified by TIANgel Midi Purification Kit (Tiagen, Beijing, China) and was connected to the 1- μ L pMD19-T vector (TaKaRa, Dalian, China), 6 μ L solution (TaKaRa, Japan) and 3 μ L DNA to incubate at room temperature for 6 h. Later, the recombinant was transformed into *Escherichia coli* Trans1-T1 strain, and 500 μ L LB (lysogeny broth) liquid medium was added. Notably, no restriction enzymes were used. The obtained product was allowed to shake at 200 r.p.m. at 37 °C for 2 h. A total of 100 μ L bacterial solution was applied to LB solid medium, including 1% of ampicillin. The medium was incubated at 37 °C for 12 h. The recombinant colonies were transferred into liquid LB culture medium containing 1% ampicillin and were shaken for 3–6 h at 37 °C. Finally, the medium for PCR template was prepared. Primers for this particular study were synthesized by Sangon Biotech Co. Ltd. (Beijing, China) (Table 1).

Synthesis and injection of double-stranded RNA of *serpin7* gene

Recombinant plasmid including *serpin7* gene fragment was extracted by using EZgene™ Plasmid Miniprep Kit (Bio-miga, San Diego, CA, USA). Using the recombinant plasmid as template, the *serpin7* gene was amplified by primers *serpin7*-2F/*serpin7*-2R (Table 1). The amplified PCR products were then purified with TIANgel Midi Purification Kit (Tiagen), followed by quantification through NanoPhotometer™ (Implen GmbH, Munchen, Germany). *Serpin7* double-stranded RNA (dsRNA) was synthesized using the T7 RiboMAX™ Express RNAi System Kit (Promega, Madison, WI, USA). The expected size of *serpin7*'s dsRNA

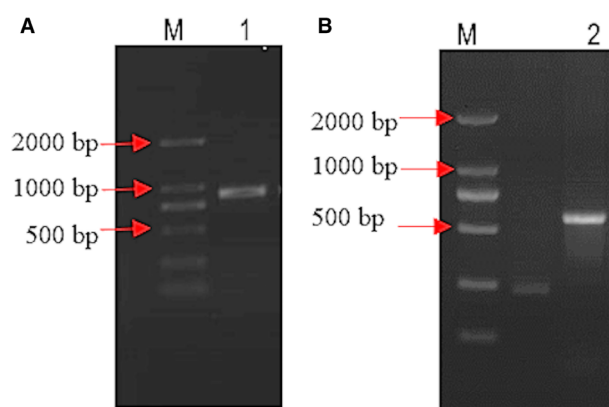


Fig. 1. (A, B) Electrophoresis pattern of *serpin7* by PCR and *serpin7* dsRNA. 1, PCR fragment of *serpin7* gene; 2, *Serpin7* dsRNA; M, marker.

Table 1. List of specific primers used and synthesized for this study.

Primers	Primer sequences (5'-3')	Intention
<i>Serpin7</i> -1F	TTTCTCTCCAGCCAGCAT	Clone of <i>serpin7</i> gene
<i>Serpin7</i> -1R	GCTTCAGTGCCTTCCTCAT	
<i>Serpin7</i> -2F	TAATACGACTCACTATAGG CTGCTCAGGAGATTGTAGAGG	Synthesis of <i>serpin7</i> dsRNA
<i>Serpin7</i> -2R	TAATACGACTCACTATAGG GACCATCATCTCTTCTTTGGC	
<i>Serpin7</i> -3F	GAAGGGAAGCTGATGACACGG	<i>Serpin7</i> primer for RT-qPCR
<i>Serpin7</i> -3R	TCTTGCTCTCAACCCATTCA	
<i>Easter</i> -F	CGCATCGGATACATCGGGT	
<i>Easter</i> -R	TTCTTGAAGGCGGGCTTG	
<i>Toll</i> -F	GGCTGTAATGAATGGGGAA	<i>Toll</i> primer for RT-qPCR
<i>Toll</i> -R	GTAACCTGGAACCTGGTGCG	
<i>Pelle</i> -F	CCAACACGGAGAATAGATAGT	<i>Pelle</i> primer for RT-qPCR
<i>Pelle</i> -R	TGGTAAAATTCCAAGGTAGA	
<i>MyD88</i> -F	GGCTTCCTCCTCAGCATCT	<i>MyD88</i> primer for RT-qPCR
<i>MyD88</i> -R	GACCTCCAACCAATCAGC	
<i>Cactus</i> -F	CAGCGGTGCTTGCCCTCTAC	<i>Cactus</i> primer for RT-qPCR
<i>Cactus</i> -R	TTTTCTCCAACCTGTCTCT	
<i>Actin</i> -F	GTTACAAACTGGGACGACAT	Reference gene of RT-qPCR
<i>Actin</i> -R	AGAAAGCACAGCCTGAATAG	

was 602 bp (Fig. 1). dsRNA concentration of *serpin7* was detected by a NanoPhotometer™ (Implen, GmbH, München, Germany), and the final concentration was adjusted to 1 μ g· μ L⁻¹ for further analysis.

Twenty-five female *L. migratoria* were selected from each photoperiod within 24 h after adults were injected with 10 μ L *serpin7* dsRNA (μ g· μ L⁻¹) between the third and fourth abdominal segments. Double-distilled water (ddH₂O) as control was injected in a similar manner to the selected females. Dissecting the whole bodies of dsRNA-injected and control group's adult locusts after 36 h, we obtained hind leg, ovary and fat body. The efficiency of RNAi-mediated knockdown was determined with real-time quantitative PCR (RT-qPCR).

RT-qPCR

To check the efficiency of RNAi-mediated knockdown in different tissues of *L. migratoria*, we dissected out the hind leg, ovary and fat body from each treatment. RNA was extracted from each sample using TRIcom Reagent

(Tianmo) followed by estimating the RNA concentration through a NanoPhotometer™ (Implen, GmbH). For reverse transcription, 5 µL of total RNA was reverse transcribed with PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa, Dalian, China). To evaluate RNAi efficiency, we used primers *serpin7*-3F/*serpin7*-3R to amplify endogenous *serpin7* gene on the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed with the Bester® SYBR Green qPCR MasterMix (DBI® Bioscience, Berlin, Germany). *β-actin* was used as a reference gene in the study. A total of three technical replicates were set up. The relative mRNA level was calculated by $2^{-\Delta\Delta C_t}$ method [26], where

$$\Delta\Delta C_t = [(C_t(\text{Target}) - C_t(\text{actin}))_{\text{Treatment}} - (C_t(\text{Target}) - C_t(\text{actin}))_{\text{control}}].$$

ELISA

PPO activity was detected using Insect PPO ELISA Kit (Colodi Biotechnology Co., Ltd., Quanzhou, China) according to the manufacturer's protocol. The standard curve was generated by plotting the average absorbance (A_{450} nm) obtained for each of the six standard concentrations on the vertical (x) axis versus the corresponding concentration on the horizontal (y) axis. First, we calculated the mean A_{450} value for each standard and sample. Later, all of the A_{450} values were subtracted by the mean value of the blank well before interpretation of results. We constructed the standard curve using graph paper or statistical software. The A_{450} value of the sample was substituted into the equation, and the concentration of the sample was then calculated.

Diapause rate

Remaining locusts were reared at 28 °C, until eggs laying. The numbers of hatched nymph of locusts (D_1) and the unhatched eggs (D_2) were counted, and the diapause rate (%) was calculated accordingly:

$$\text{Diapause rate(\%)} = \frac{D_2}{(D_1 + D_2)} \times 100.$$

Statistical analysis

Independent samples t -test was used for measuring mRNA levels, enzyme activities and diapause rate. Statistically significant differences were considered on an error probability of $P < 0.05$. Data are presented as means \pm SE. Asterisks on the bars in the figures represent significant differences among the treatments and control. Statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA), whereas GRAPHPAD PRISM software version

6.01 (GraphPad Software Inc., San Diego, CA, USA) was used for constructing the graphs.

Results

Identification of the serpin genes

Seven nonredundant serpin protein sequences were identified by transcriptome sequencing of diapause and nondiapause eggs in the migratory locust and were respectively named from *serpin1* to *serpin7* (Table 2). The encoding gene of serpin proteins ranged from 987 bp (*serpin1*) to 1500 bp (*serpin5*). The isoelectric point (pI) was between 5.13 (*serpin3*) and 8.36 (*serpin4*). Similarly, prediction of subcellular localization was performed by WOLF PSORT software (<http://wolffpsort.org/>). Higher value means accurate prediction. After analysis, *serpin1* was mainly distributed in the endoplasmic reticulum, nucleus and cytoplasm followed by a small amount of distribution in mitochondria and peroxisome. Among them, four proteins were predicted to be endoplasmic reticulum localization, including *serpin2*, *serpin3*, *serpin5* and *serpin6*. Moreover, two of the serpin proteins were predicted to be localized in cytoplasm, including *serpin4* and *serpin7* (Table 3).

Structure and phylogenetic tree of serpin protein

Amino acid sequence alignment of seven serpin proteins was performed using DNAMAN software (version 7.212; Lynnon Corp.). High similarity among seven serpin proteins was found (Fig. 2). The phylogenetic tree was constructed by comparing the amino acid sequences of seven serpin proteins of migratory locust (Data S2) using the NJ method (Fig. 3). Two of the serpin proteins matched with fruit fly, *D. melanogaster*, and two with silkworm, *B. mori*. Through phylogenetic analysis, it was found that *serpin5* in the migratory locust is closely related to *serpin27A* of *D. melanogaster*, whereas the relationship between *serpin1*, *serpin7*, *serpin3* and *serpin4* was relatively close; however, among them, the relationship between *serpin1* and *serpin3* was much closer. *Serpin1* was mainly distributed in the endoplasmic reticulum, nucleus and cytoplasm followed by a small amount of distribution in mitochondria and peroxisome predicted by WOLF PSORT software (<http://wolffpsort.org/>).

Analysis of the advanced structure of serpin proteins in the migratory locust

The secondary and tertiary structures of the seven serpin proteins (*serpin1*–*serpin7*) were analyzed by the

Table 2. Characteristics and features of serpins in *L. migratoria*.

Protein name	ORF	Amino acids	Molecular formula	Molecular weight	Theoretical pI
Serpin1	987	328	C ₁₆₅₅ H ₂₆₂₃ N ₄₄₅ O ₄₉₂ S ₉	36.92	5.9
Serpin2	1164	387	C ₁₉₄₅ H ₃₀₇₈ N ₄₉₀ O ₅₅₉ S ₁₈	42.85	6.73
Serpin3	1155	384	C ₁₉₆₆ H ₃₀₅₆ N ₄₉₄ O ₅₆₉ S ₁₃	43.13	5.13
Serpin4	1176	391	C ₁₉₉₇ H ₃₁₁₇ N ₅₁₉ O ₅₆₅ S ₁₄	43.89	8.36
Serpin5	1500	499	C ₂₅₄₃ H ₃₉₆₀ N ₆₆₄ O ₇₃₉ S ₈	55.92	5.91
Serpin6	1446	481	C ₂₄₀₂ H ₃₇₉₄ N ₆₄₀ O ₇₂₈ S ₁₁	53.64	5.86
Serpin7	1161	386	C ₁₉₃₃ H ₃₁₀₆ N ₄₉₄ O ₅₆₇ S ₂₀	42.98	6.08

Table 3. Subcellular localization and prediction of serpins using WOLF PSORT software.

Protein name	Plasma membrane	Endoplasmic reticulum	Nucleus	Cytoplasm	Mitochondria	Peroxisome	Lysosome	Secreted
Serpin1	–	8	9	6.5	3	2.5	–	2
Serpin2	1	16	3	–	–	4	–	2
Serpin3	2	10	4	5	1	–	–	6
Serpin4	4	5	–	14	2	5	–	2
Serpin5	7	11	–	–	–	1	2	11
Serpin6	7	12	–	–	–	1	3	9
Serpin7	–	–	–	22.5	8	–	–	–

SOPMA online server and EXPASY software (Table 4; Fig. 4). There was a slight difference in the ratio of four secondary structures (α -helix, extended strand, β -turn and random coil) among the serpin proteins, in which the amino acids proportion of α -helix was 34.79–54.27%, whereas β -turn was 4.58–7.16%. Similarly, the amino acids proportion of extended chains was 10.37–16.80%. Moreover, the ratio of random coils ranged from 28.96% to 46.67%. Furthermore, secondary structures of α -helix and random coils were dominant in serpin proteins. The tertiary structure of serpin was similar with three β -turn and eight to nine α -helices (Fig. 4).

RNAi efficiency

To verify the function of *serpin7* on regulating locust diapause, we synthesized and subsequently injected dsRNA of *serpin7* into female adults of *L. migratoria* to RNAi the *serpin7* under long and short photoperiods. RNAi efficiency of *serpin7* was confirmed through RT-qPCR. The results showed that the mRNA level of *serpin7* gene in hind leg and fat body was significantly different as compared with control ($P < 0.05$) under both long (relative humidity [RH] 60%; 30 °C; 16 h light:8 h darkness) and short (RH 60%; 27 °C; 10 h light:14 h darkness) photoperiods (Fig. 5). The results further confirmed that the *serpin7* gene was successfully interfered under two photoperiods. Under the long photoperiod, mRNA level of *serpin7* gene in the

hind leg decreased by 95.98% compared with that of the control, whereas mRNA level of *serpin7* gene in fat body decreased by 99.08%. Furthermore, under the short photoperiod, mRNA level of *serpin7* gene in hind leg decreased by 99.14% compared with that of the control, whereas mRNA level of *serpin7* gene in fat body decreased by 93.85%.

Effects of *serpin7* RNAi on the Toll pathway

ds*serpin7* and ddH₂O were injected into the female locusts within 24 h after adulthood under both long and short photoperiods. Locusts were dissected after 36 h to collect the fat body. The mRNA relative level of *Easter*, *Toll*, *Pelle*, *MyD88* and *Cactus* genes in the Toll pathway was checked (Fig. 6). Under the long photoperiod, the mRNA level of *Easter* in fat body was significantly up-regulated ($P < 0.05$), whereas the mRNA levels of *Pelle*, *MyD88* and *Cactus* in fat body were significantly down-regulated ($P < 0.05$). Similarly, the mRNA level of *Toll* gene in the treatment group was down-regulated as compared with the control group, but with no significant difference. Under the short photoperiod, the variation trend of gene levels was similar to that of the long photoperiod. The mRNA relative levels of *Toll*, *Pelle*, *MyD88* and *Cactus* genes were significantly lower than those of the control group ($P < 0.05$), whereas the mRNA level of *Easter* gene was increased but did not reach a level of significant difference.

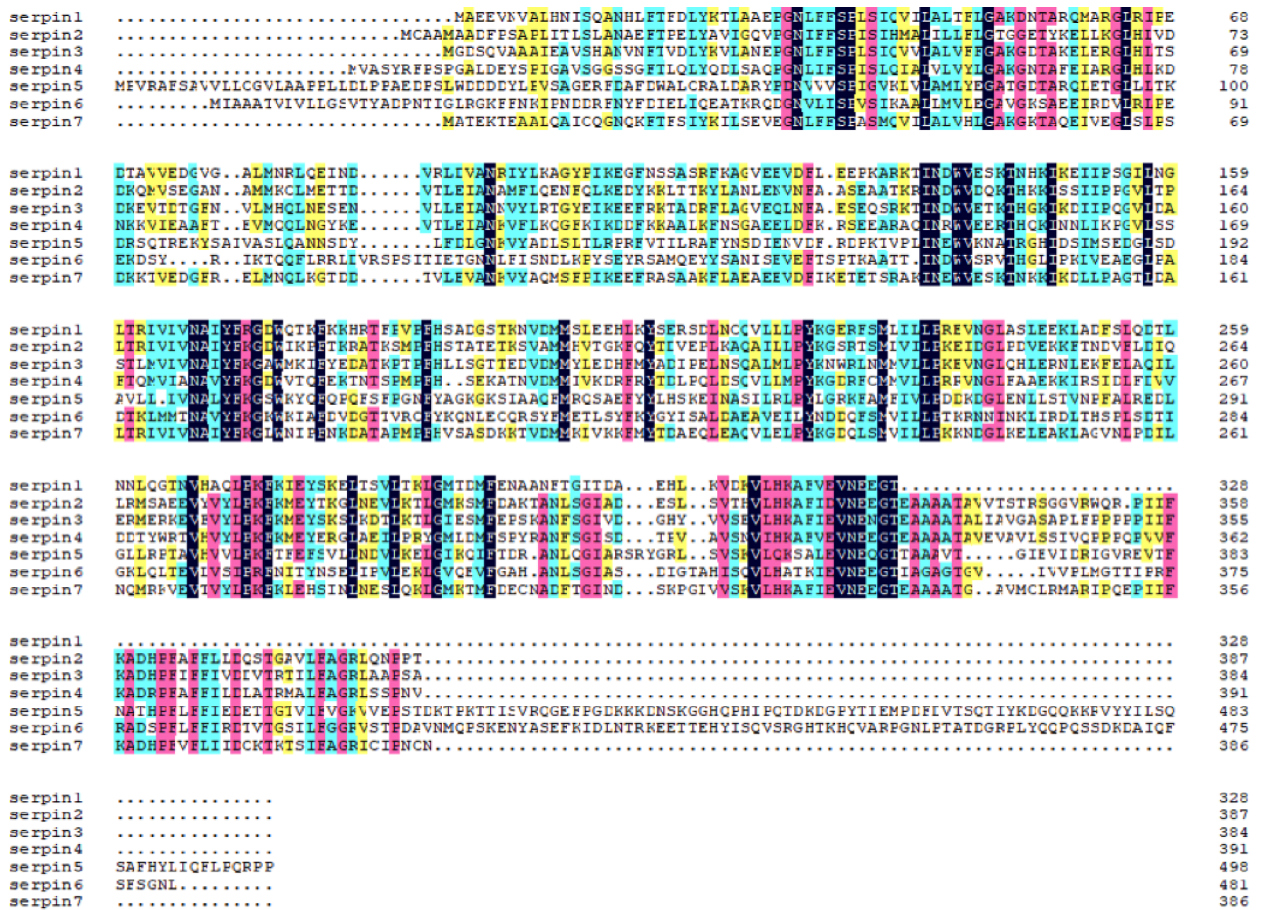


Fig. 2. Homology of serpin proteins in *L. migratoria* constructed by using DNAMAN software.

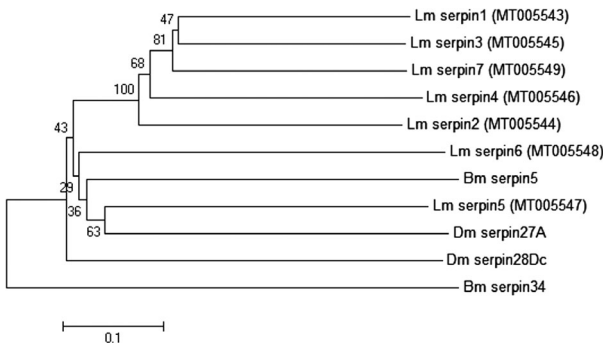


Fig. 3. Phylogenetic tree of insect serpin proteins constructed by MEGA 6.0 and the NJ method. A species acronym is added before the name of each serpin protein, whereas Dm, Bm and Lm, respectively, represent *D. melanogaster*, *B. mori* and *L. migratoria*. GenBank accession numbers were provided for our nucleotide sequences, where *serpin1*, *serpin2*, *serpin3*, *serpin4*, *serpin5*, *serpin6* and *serpin7* are respectively numbered MT005543, MT005544, MT005545, MT005546, MT005547, MT005548 and MT005549.

Table 4. Secondary structure of the serpin proteins in *L. migratoria*.

	Number/Percentage			
	Random coil	α -Helix	Extended strand	β -Turn
Serpin1	101/30.79	168/51.22	39/11.89	20/6.10
Serpin2	131/33.85	175/45.22	60/15.50	21/5.43
Serpin3	128/33.33	164/42.71	69/17.97	23/5.99
Serpin4	138/35.29	182/46.55	56/14.32	15/3.84
Serpin5	217/43.49	177/35.47	75/15.03	30/6.01
Serpin6	215/44.70	180/37.42	67/13.93	19/3.95
Serpin7	138/35.75	170/44.04	59/15.28	19/4.92

Effect of RNAi-mediated silencing of *serpin7* gene on activity of PPO

Under long and short photoperiods, the female adults were injected with dsRNA of *serpin7* and ddH₂O, respectively. After 36 h, the female locusts were

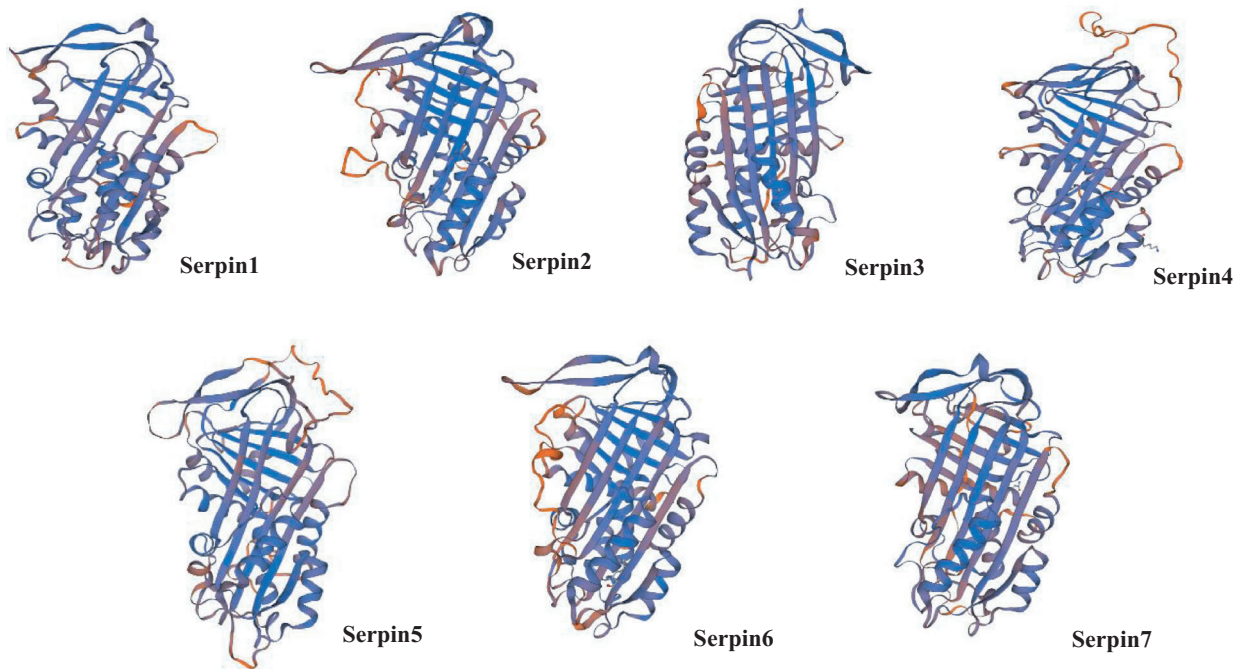


Fig. 4. Tertiary structures of the serpin proteins obtained through EXPASY. Serpin1: α -helix, 54.27%; extended strand, 10.37%; β -turn, 6.40%; random coils, 28.96%. Serpin2: α -helix, 45.99%; extended strand, 16.80%; β -turn, 5.43%; random coils, 31.78%. Serpin3: α -helix, 45.57%; extended strand, 15.36%; β -turn, 6.25%; random coils, 32.81%. Serpin4: α -helix, 44.50%; extended strand, 14.07%; β -turn, 7.16%; random coils, 34.27%. Serpin5: α -helix, 35.47%; extended strand, 16.23%; β -turn, 6.01%; random coils, 42.28%. Serpin6: α -helix, 34.79%; extended strand, 13.96%; β -turn, 4.58%; random coils, 46.67%. Serpin7: α -helix, 46.11%; extended strand, 16.32%; β -turn, 5.96%; random coils, 31.61%.

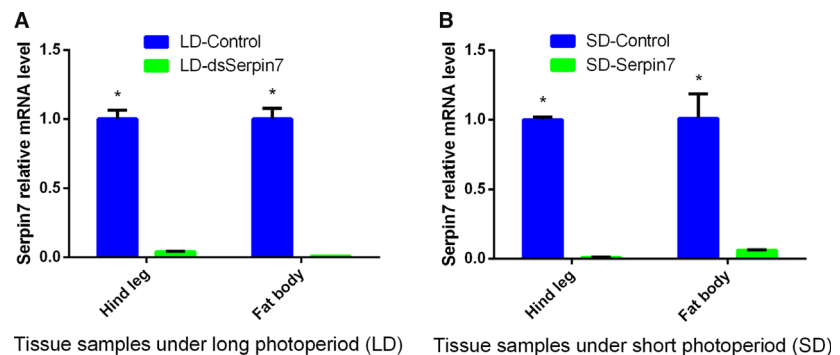


Fig. 5. Relative mRNA levels of *serpin7* after RNAi under two photoperiodic regimens: (A) long photoperiod and (B) short photoperiod. Three replicates are used for each treatment. Asterisks (*) indicate a standard error probability of $P < 0.05$ by Student's *t*-test.

dissected to obtain the hind legs, fat bodies and ovaries. The PPO content was detected (Fig. 7). Results showed that the content of PPO in the hind leg, fat body and ovary significantly increased ($P < 0.05$) after *serpin7* RNAi under both long and short photoperiods.

Diapause rate

Under the long photoperiod, RNAi-mediated silencing by injecting *serpin7* dsRNA had no effect on diapause rate. However, under the short photoperiod, diapause

rate (%) of the individuals injected with *serpin7* dsRNA was significantly reduced ($P < 0.05$). Diapause rate in the dsRNA-injected group was 84.19%, which was 13.25% lower than that of the control group. The result suggests that *serpin7* may play a modulatory role in egg diapause of the migratory locust (Fig. 8).

Discussion

Serpins are a superfamily of proteases found in serine protease inhibitors, usually consisting of 350–400

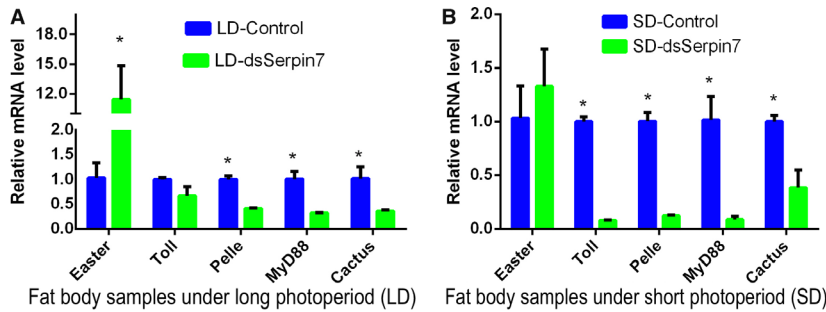


Fig. 6. Data acquired from fat body samples and relative mRNA level of genes in the Toll pathway after *serpin7* RNAi under two photoperiodic regimens: (A) long photoperiod and (B) short photoperiod. Three replicates are used for each treatment. Asterisks (*) indicate a standard error (SE) probability of $P < 0.05$ by Student's *t*-test.

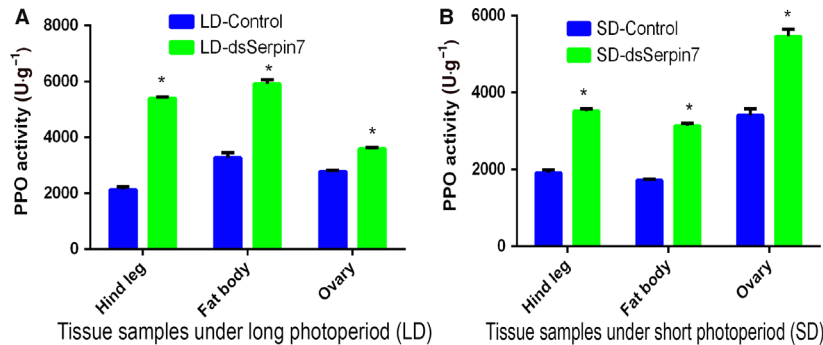


Fig. 7. PPO activity in the migratory locust after *serpin7* RNAi under two photoperiodic regimens: (A) long photoperiod and (B) short photoperiod. Three replicates are used for each treatment. Asterisks (*) indicate a standard error probability of $P < 0.05$ by Student's *t*-test.

amino acids [4]. At the C terminus of serpin, there is a reactive central ring exposed outside the main body [27]. Sequence of serpin is highly conserved, but its function is highly differentiated; that is why serpin has an irreversible inhibitory effect with a suicidal nature [7], just like trypsin inhibitors, which affect the growth, development and survival of insects [28]. Serpins play an important role in innate immunity of insects [19]. Currently, a negatively correlated serpin was found to be associated with the Toll signaling pathway of fruit flies [29,30], and other serpins were mostly involved in melanization caused by PO cascade [31,32]. In addition, a specific group of serpins is involved in tissue synthesis and embryonic development [17-18,20].

Serpins regulate some innate immune responses of insects by inhibiting endogenous protease [33]. In insects, serpins have been identified in several species, but extensive studies of insect serpins are mainly focused on fruit fly, *D. melanogaster*, and tobacco horn worm, *Manduca sexta*. So far, six *M. sexta* serpins have been characterized and shown to be inhibitory [13,34-37]. To investigate the effect of *serpin* gene on diapause of migratory locust, we subsequently cloned *serpin7* of the migratory locust in this particular study. The ORF of *serpin7* gene was 1161 bp, encoding 386 amino acid residues, and had the molecular formula of $C_{1933}H_{3106}N_{494}O_{567}S_{20}$. The subcellular localization of *serpin7* was predicted to be cytoplasmic and mitochondrial. Meanwhile, the

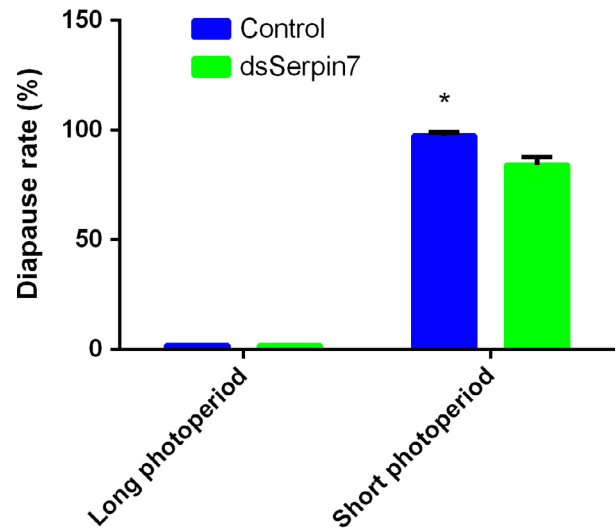


Fig. 8. Diapause rate of migratory locusts by injecting *dsSerp7* along with control under both long and short photoperiods. Three replicates are used for each treatment. Asterisk (*) indicates a standard error probability of $P < 0.05$ by Student's *t*-test.

molecular mass of the protein was 42.98 kDa, which was similar to that of serpin's superfamily protein [38]. The theoretical pI of *serpin7* was 6.08, and there was no signal peptide. It belongs to the serpin superfamily and was a typical inhibitory serpin. Multiple sequence alignment showed that seven serpin

amino acid sequences of the migratory locust showed high similarity (Fig. 2). Phylogenetic analysis was performed on serpin amino acid sequences by the NJ method, including seven serpin proteins in the migratory locust, two serpin proteins in fruit flies and two serpin proteins in silkworms. We observed a close relationship between *serpin1* and *serpin7* of migratory locusts. Moreover, *serpin5* of migratory locusts was closely related to that of *serpin27A* of *Drosophila* [39,40]. In addition, *serpin6* of the migratory locust was closely related to *serpin28Dc* of *Drosophila* [41]. Furthermore, *serpin1*, *serpin7*, *serpin3* and *serpin4* were closely related with each other (Fig. 3).

Down-regulation of the expression of specific genes through RNAi has been widely used in entomological research for functional genomics in a variety of insects, and its potential for RNAi-based pest control has been increasingly emphasized mainly because of its high specificity [42,43]. We used the RNAi technology to mediate silencing of *serpin7* gene of migratory locust for elaborating the effect of serpin on diapause of locust [23-24,44]. The RNAi efficiency of *serpin7* was checked by RT-qPCR, and the results revealed successful knocking down of *serpin7* gene. Our results showed the 100% hatching of all eggs under the long photoperiod, for both treated and untreated (control) groups with zero diapause. In contrast, under the short photoperiod, diapause rate of the individuals injected with *serpin7* dsRNA was significantly reduced as compared with the control group. Results suggested that maternal *serpin7* promotes the egg diapause process of *L. migratoria*. Serpins are an important regulator in PO cascade reaction [32,39,45]. To further understand the regulatory mechanism, we checked the effect of *serpin7* RNAi on PPO activity in *L. migratoria*. Results showed that PPO activity increased significantly in the hind leg, fat body and ovary after *serpin7* RNAi. PO plays an important role in melanization [31,32] and synthesis of antimicrobial peptides, where the proPO mainly exists as an inactive precursor [46,47]. proPO regulates various downstream factors, such as protease, protease inhibitors in *Drosophila* and *Manduca sexta* [48,49]. We speculated that *serpin7* negatively regulates PPO and affects diapause of migratory locust eggs by PO cascade reaction. In addition, we examined the effect of *serpin7* gene RNAi on Toll pathway gene of the migratory locust. The results showed that after *serpin7* gene RNAi under long and short photoperiods, the mRNA level of *Easter* increased in fat body, whereas mRNA levels of *Toll*, *Pelle*, *MyD88* and *Cactus* genes decreased compared with the control group in fat body.

Conclusions

RNAi of *serpin7* affected PPO activities in fat, hind leg and ovary of *L. migratoria* that ultimately revealed the possible role of *serpin7* in locust diapause. *Serpin7* may also be involved in the cascade reaction of the Toll signaling pathway, which needs to be further verified.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

JC, DC and HU conceptualized the study. KH performed formal analysis. JC, DC and KH were involved in the investigation. KH and DC contributed to the methodology of the study. XT and ZZ were involved in project administration. DC and HU wrote the original draft. KH and HU contributed to the writing, reviewing and editing of the manuscript.

Data accessibility

The sequences of *Serpin1*, *Serpin2*, *Serpin3*, *Serpin4*, *Serpin5*, *Serpin6* and *Serpin7* were deposited in GenBank with the accession numbers MT005543, MT005544, MT005545, MT005546, MT005547, MT005548 and MT005549, respectively.

References

- 1 Uvarov BP (1966) *Grasshoppers and Locusts*, Vol. 1. Cambridge University Press, Cambridge, London.
- 2 Uvarov BP (1977) *Grasshoppers and Locusts*, Vol. 2. Centre for Overseas Pest Research, Cambridge University Press, Cambridge, London.

- 3 Jarwar AR, Hao K, Bitume EV, Ullah H, Cui D, Nong X, Wang G, Tu X and Zhang Z (2019) Comparative transcriptomic analysis reveals molecular profiles of central nervous system in maternal diapause induction of *Locusta migratoria*. *G3 (Bethesda)* **9**, 3287–3296.
- 4 Irving JA, Pike RN, Lesk AM and Whisstock JC (2000) Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. *Genome Res* **10**, 1845–1864.
- 5 Rawlings ND, Tolle DP and Barrett AJ (2004) MEROPS: the peptidase database. *Nucleic Acids Res* **32**, D160–D164.
- 6 Law RH, Zhang Q, McGowan S, Buckle AM, Silverman GA, Wong W, Rosado CJ, Langendorf CG, Pike RN, Bird PI *et al.* (2006) An overview of the serpin superfamily. *Genome Biol* **7**, 216.
- 7 Gettins PG (2002) Serpin structure, mechanism, and function. *Chem Rev* **102**, 4751–4804.
- 8 Abraham EG, Pinto SB, Ghosh A, Vanlandingham DL, Budd A, Higgs S, Kafatos FC, Jacobs-Lorena M and Michel K (2005) An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites. *Proc Natl Acad Sci USA* **102**, 16327–16332.
- 9 Gulley MM, Zhang X and Michel K (2013) The roles of serpins in mosquito immunology and physiology. *J Insect Physiol* **59**, 138–147.
- 10 Silverman GA, Whisstock JC, Bottomley SP, Huntington JA, Kaiserman D, Luke CJ, Pak SC, Reichhart JM and Bird PI (2010) Serpins flex their muscle I. Putting the clamps on proteolysis in diverse biological systems. *J Biol Chem* **285**, 24299–24305.
- 11 Pauchet Y, Wilkinson P, Vogel H, Nelson DR, Reynolds SE, Heckel DG and Ffrench-Constant RH (2010) Pyrosequencing the *Manduca sexta* larval midgut transcriptome: messages for digestion, detoxification and defence. *Insect Mol Biol* **19**, 61–75.
- 12 Richardson J, Viswanathan K and Lucas A (2006) Serpins, the vasculature, and viral therapeutics. *Front Biosci* **11**, 1042–1056.
- 13 Tong Y, Jiang H and Kanost MR (2005) Identification of plasma proteases inhibited by *Manduca sexta* serpin-4 and serpin-5 and their association with components of the prophenol oxidase activation pathway. *J Biol Chem* **280**, 14932–14942.
- 14 Kanost MR (2007) Serpins in a lepidopteran insect, *Manduca sexta*. In *Molecular and Cellular Aspects of the Serpinopathies and Disorders in Serpin Activity* (Silverman GA and Lomas DA, eds), pp. 229–241. World Scientific Publishing Co., Hackensack, NJ.
- 15 Jiang H (2008) The biochemical basis of antimicrobial responses in *Manduca sexta*. *Insect Sci* **15**, 53–66.
- 16 Jenkins G (2008) The role of proteases in transforming growth factor- β activation. *Int J Biochem Cell Biol* **40**, 1068–1078.
- 17 Hashimoto C, Kim DR, Weiss LA, Miller JW and Morisato D (2003) Spatial regulation of developmental signaling by a serpin. *Dev Cell* **5**, 945–950.
- 18 Ligoxygakis P, Roth S and Reichhart JM (2003) A serpin regulates dorsal-ventral axis formation in the *Drosophila* embryo. *Curr Biol* **13**, 2097–2102.
- 19 Reichhart JM, Gubb D and Leclerc V (2011) The *Drosophila* serpins: multiple functions in immunity and morphogenesis. In *Methods in Enzymology* (Whisstock JC and Bird PI, eds), Vol. **499**, pp. 205–225. Academic Press, Cambridge, MA.
- 20 Pak SC, Kumar V, Tsu C, Luke CJ, Askew YS, Askew DJ, Mills DR, Brömme D and Silverman GA (2004) SRP-2 is a cross-class inhibitor that participates in postembryonic development of the nematode *Caenorhabditis elegans* initial characterization of the clade I serpins. *J Biol Chem* **279**, 15448–15459.
- 21 Tu X, Wang J, Hao K, Whitman DW, Fan Y, Cao G and Zhang Z (2015) Transcriptomic and proteomic analysis of pre-diapause and non-diapause eggs of migratory locust, *Locusta migratoria* L. (orthoptera: acridoidea). *Sci Rep* **5**, 11402.
- 22 Hao K, Wang J, Tu X, Whitman DW and Zang Z (2017) Transcriptomic and proteomic analysis of, *Locusta migratoria*, eggs at different embryonic stages: comparison for diapause and non-diapause regimes. *J Integr Agric* **16**, 1777–1788.
- 23 Hao K, Jarwar AR, Ullah H, Tu X, Nong X and Zhang Z (2019) Transcriptome sequencing reveals potential mechanisms of the maternal effect on egg diapause induction of *Locusta migratoria*. *Int J Mol Sci* **20**, 1974.
- 24 Hao K, Tu X, Ullah H, McNeill MR and Zhang Z (2019) Novel Lom-dh genes play potential role in promoting egg diapause of *Locusta migratoria* L. *Front Physiol* **10**, 767.
- 25 Tamura K, Stecher G, Peterson D, Filipowski A and Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* **30**, 2725–2729.
- 26 Rau JC, Beaulieu LM, Huntington JA and Church FC (2007) Serpins in thrombosis, hemostasis and fibrinolysis. *J Thromb Haemost* **5**, 102–115.
- 27 Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.
- 28 Zhao H, Ullah H, McNeill MR, Du G, Hao K, Tu X and Zhang Z (2019) Inhibitory effects of plant trypsin inhibitors Msti-94 and Msti-16 on *Therioaphis trifolii* (Monell) (Homoptera: Aphididae) in Alfalfa. *Insects* **10**, 154.
- 29 Levashina EA, Langley E, Green C, Gubb D, Ashburner M, Hoffmann JA and Reichhart JM (1999) Constitutive activation of toll-mediated antifungal

- defense in serpin-deficient *Drosophila*. *Science* **285**, 1917–1919.
- 30 Reichhart JM (2005) Tip of another iceberg: *Drosophila* serpins. *Trends Cell Biol* **15**, 659–665.
 - 31 Jiang R, Kim EH, Gong JH, Kwon HM, Kim CH, Ryu KH, Park JW, Kurokawa K, Zhang J, Gubb D *et al.* (2009) Three pairs of protease-serpin complexes cooperatively regulate the insect innate immune responses. *J Biol Chem* **284**, 35652–35658.
 - 32 Tang H (2009) Regulation and function of the melanization reaction in *Drosophila*. *Fly* **3**, 105–111.
 - 33 Söderhäll I, Wu C, Novotny M, Lee BL and Söderhäll K (2009) A novel protein acts as a negative regulator of prophenoloxidase activation and melanization in the freshwater crayfish *Pacifastacus leniusculus*. *J Biol Chem* **284**, 6301–6310.
 - 34 Jiang H and Kanost MR (1997) Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-1 from *Manduca sexta*. *J Biol Chem* **272**, 1082–1087.
 - 35 Gan H, Wang Y, Jiang H, Mita K and Kanost MR (2001) A bacteria-induced, intracellular serpin in granular hemocytes of *Manduca sexta*. *Insect Biochem Mol Biol* **31**, 887–898.
 - 36 Wang Y and Jiang H (2004) Purification and characterization of *Manduca sexta* serpin-6: a serine proteinase inhibitor that selectively inhibits prophenoloxidase-activating proteinase-3. *Insect Biochem Mol Biol* **34**, 387–395.
 - 37 Zhu Y, Wang Y, Gorman MJ, Jiang H and Kanost MR (2003) *Manduca sexta* serpin-3 regulates prophenoloxidase activation in response to infection by inhibiting prophenoloxidase-activating proteinases. *J Biol Chem* **278**, 46556–46564.
 - 38 Silverman GA, Bird PI, Carrell RW, Church FC, Coughlin PB, Gettins PG, Irving JA, Lomas DA, Luke CJ, Moyer RW *et al.* (2001) The serpins are an expanding superfamily of structurally similar but functionally diverse proteins evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J Biol Chem* **276**, 33293–33296.
 - 39 De Gregorio E, Han SJ, Lee WJ, Baek MJ, Osaki T, Kawabata SI, Lee BL, Iwanaga S, Lemaitre B and Brey PT (2002) An immune-responsive serpin regulates the melanization cascade in *Drosophila*. *Dev Cell* **3**, 581–592.
 - 40 Moussian B and Roth S (2005) Dorsoventral axis formation in the *Drosophila* embryo-shaping and transducing a morphogen gradient. *Curr Biol* **15**, R887–R899.
 - 41 Shakeel M, Xu X, De Mandal S and Jin F (2019) Role of serine protease inhibitors in insect-host-pathogen interactions. *Arch Insect Biochem Physiol* **102**, e21556.
 - 42 Price DR and Gatehouse JA (2008) RNAi-mediated crop protection against insects. *Trends Biotechnol* **26**, 393–400.
 - 43 Yu N, Christiaens O, Liu J, Niu J, Cappelle K, Caccia S, Huvenne H and Smaghe G (2013) Delivery of dsRNA for RNAi in insects: an overview and future directions. *Insect Sci* **20**, 4–14.
 - 44 Han P, Fan J, Liu Y, Cuthbertson AG, Yan S, Qiu BL and Ren S (2014) RNAi-mediated knockdown of serine protease inhibitor genes increases the mortality of *Plutella xylostella* challenged by destruxin A. *PLoS ONE* **9**, e97863.
 - 45 Tang H, Kambris Z, Lemaitre B and Hashimoto C (2008) A serpin that regulates immune melanization in the respiratory system of *Drosophila*. *Dev Cell* **15**, 617–626.
 - 46 Kan H, Kim CH, Kwon HM, Park JW, Roh KB, Lee H, Park BJ, Zhang R, Zhang J, Söderhäll K *et al.* (2008) Molecular control of phenoloxidase-induced melanin synthesis in an insect. *J Biol Chem* **283**, 25316–25323.
 - 47 Buchon N, Poidevin M, Kwon HM, Guillou A, Sottas V, Lee BL and Lemaitre B (2009) A single modular serine protease integrates signals from pattern-recognition receptors upstream of the *Drosophila* Toll pathway. *Proc Natl Acad Sci USA* **106**, 12442–12447.
 - 48 Lemaitre B and Hoffmann J (2007) The host defense of *Drosophila melanogaster*. *Annu Rev Immunol* **25**, 697–743.
 - 49 Ling E, Rao XJ, Ao JQ and Yu XQ (2009) Purification and characterization of a small cationic protein from the tobacco hornworm *Manduca sexta*. *Insect Biochem Mol Biol* **39**, 263–271.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Selected serpins nucleic acid sequences.

Data S2. Selected serpins amino acid sequences.