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Expression profile and function analysis of *MsCSP17* and *MsCSP18* in the larval development of *Mythimna separata*

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Research Paper

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Abstract

Chemosensory proteins (CSPs) were necessary for insect sensory system to perform important processes such as feeding, mating, spawning, and avoiding natural enemies. However, their functions in non-olfactory organs have been poorly studied. To clarify the function of CSPs in the development of *Mythimna separata* (Walker) larvae, two CSP genes, *MsCSP17* and *MsCSP18*, were identified from larval integument transcriptome dataset. Both of MsCSP17 and MsCSP18 contained four conserved cysteine sites (C × (6)-C × (18)-C × (2)-C), with a signal peptide at the N-terminal. RT-qPCR analysis showed that *MsCSP17* and *MsCSP18* have different expression patterns among different developmental stages and tissues. *MsCSP17* was highly expressed in 1st–4th instar larvae, and *MsCSP18* had high expression in adults. Both genes were expressed highly in larval head, thorax, integument and mandible. Moreover, both of *MsCSP17* and *MsCSP18* were lowly expressed in larval integuments when larvae molted for 6 h and 9 h from 3rd to 4th instar, but highly at the beginning and end phase during molting. After injection of dsMsCSP17 and dsMsCSP18, the expression levels of two genes decreased significantly, with the body weight of larvae decreased, the mortality increased, and the eclosion rate decreased. It was suggested that *MsCSP17* and *MsCSP18* contributed to the development of *M. separata* larvae.

Introduction

Mythimna separata (Walker) is a polyphagous pest damaging corn and other cereal crops, with the characteristics of wide host range, strong reproduction capacity, and long-distance migration ability (Jiang *et al.*, 2011, 2014; Liu *et al.*, 2016). Chemical pesticides are mainly used to control *M. separata* to prevent serious crop losses caused by larval feeding (Liu *et al.*, 2016, 2017; Duan *et al.*, 2017). However, the use of chemical pesticides in a large area can easily lead to serious consequences such as insecticide resistance and residues, and damaging beneficial insects and natural enemies (Lv *et al.*, 2014; Younas *et al.*, 2016; Duan *et al.*, 2017). Therefore, it is of great practical significance to explore new green and efficient pest control measures.

Chemosensory proteins (CSPs) are important for insect sensory system to sense external stimuli to obtain information, and complete important processes such as feeding, mating, spawning, and avoiding natural enemy (Kaupp, 2010; Leal, 2013; Wang *et al.*, 2015; Antony *et al.*, 2016; Pelosi *et al.*, 2018). CSPs mainly bind and dissolve small hydrophobic compounds, and are widely distributed in various chemical receptors of insects (Gong *et al.*, 2012; Pelosi *et al.*, 2014). In addition to olfactory perception, they also play the functions of pheromone carrier, nutrient absorption, visual pigment carrier, insecticide resistance, regeneration, and development in insects (Pelosi *et al.*, 2018). Although a great number of researches on CSPs have been reported in insects these years, most of them focused on the roles of CSPs in insect olfactory system. Other functions exercised in non-olfactory organs have been poorly studied.

In this study, two highly expressed CSPs were identified from the larval integument transcriptome of *M. separata*. The spatio-temporal expression patterns and functions of these two CSPs during larval development were analyzed, expecting to provide new insights of CSP function in insects. The results will lay a foundation for deeply analyzing the molecular regulation mechanism of *M. separata* larva growth, which is helpful to explore novel molecular targets for pest control.

Materials and methods

Insects

Mythimna separata was collected in the field of Yu'an District, Lu'an City, Anhui Province, and a stable population was established in the laboratory in Anhui Agricultural University.

The larvae were reared with fresh corn leaves after hatching, and the adults were fed with 10% honey water after eclosion. Insects were maintained under the conditions of temperature 25 ± 1℃, relative humidity 70%, and photoperiod 12 L:12 D.

Cloning of MsCSP17 *and* MsCSP18

MsCSP17 and *MsCSP18* were identified from the larval transcriptome dataset of *M. separata* (Huang *et al.*, 2021), with their open reading frame (ORF) sequences predicted using the ORF Finder (http://www.ncbi.nlm.nih.gov/orffinder/). The online tool Primer3web 4.1.0 (https://primer3.ut.ee/, Untergasser *et al.*, 2012) was used to design specific primers MsCSP17-(S/A) and MsCSP18-(S/A) for the ORF cloning (Table S1). Genes were amplified by PCR with the cDNA template synthesized from the RNA of 4th instar larval integuments using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time, Takara, Dalian, China) according to the manufacturer's protocol. The 25 μl PCR reaction contained 12.5 μl Prime STAR max Premix 2×, 1 μl each primer, 1 μl cDNA template, and 9.5 μl RNase-free H_2O. The reaction procedure was: pre-denaturation at 98°C for 3 min; 35 cycles of denaturation at 98°C for 10 s, annealing at 53°C for 15 s, and extension at 72°C for 2 s; and extension at 72°C for 5 min. The PCR products were detected by 1% agarose gel electrophoresis, and the target bands were purified. After T-vector ligation and verification, monoclone was sent to Tsingke Biotechnology Co., Ltd (Nanjing, Jiangsu) for sequencing. The plasmids with right ORF sequences were extracted and stored at −20°C until use.

Sequences analysis and phylogenetic tree construction

The amino acid sequences of *MsCSP17* and *MsCSP18* were obtained by A plasmid Editor (ApE, Davis and Jorgensen, 2022), with their physicochemical properties such as relative molecular mass and isoelectric point predicted using ExPASy (https://web.expasy.org/protparam/, Gasteiger *et al.*, 2005). The protein tertiary structures of CSPs were predicted by SWISS-MODEL (https://swissmodel.expasy.org/interactive, Waterhouse *et al.*, 2018). The signal peptides were predicted by SignalP4.1 (http://www.cbs.dtu.dk/services/SignalP/, Almagro Armenteros *et al.*, 2019). The similarity of MsCSP17 and MsCSP18 was compared using the Blastp from the National Center for Biotechnology Information (NCBI), and multiple sequence comparisons of amino acids were performed using DNAMAN 9.0 (Lynnon Biosoft, San Ramon, CA). The phylogenetic tree was constructed using the Maximum-likelihood method in MEGA7 with Bootstrap of 1000 replicates as previous report (Kumar *et al.*, 2016; Huang *et al.*, 2018), with orthologs from other insect species collected from the NCBI database.

Spatio-temporal expression analysis of MsCSP17 *and* MsCSP18

The cDNAs of different developmental stages of *M. separata* (eggs, 1st–6th instar larvae, pupae, male adults, and female adults), different tissues of 4th instar larvae (head, thorax, abdomen, midgut, Malpighian tubule, fat body, integument, ventral nerve cord, brain, and mandible), integuments of 1st–6th instar larvae, mandibles of 3rd–6th instar larvae, and integuments of 3rd–4th instar larvae during molting were synthesized as templates for RT-qPCR analysis of two CSP genes. *Ribosomal protein 49* (*rp49*) was used as the internal reference gene (Table S1, Huang *et al.*, 2021; Zhao *et al.*, 2022). RT-qPCR reactions contained 5 μl of TB Green Premix Ex Taq II (Tli RNaseH Plus) (2×), 0.5 μl cDNA sample, 0.5 μl each primer (10 μM), and 3.5 μl RNase-free water. The reaction procedure was: pre-denaturation at 95℃ for 30 s; 40 cycles of denaturation at 95℃ for 5 s, annealing and extension at 60℃ for 30 s; and a simultaneous solubility curve from 60 to 95℃ to verify the specificity of each primer pair. Three biological replicates were set for each genes and samples. The results were analyzed by the comparative cycle threshold (CT) method referring to the reference gene (Huang *et al.*, 2020).

Microinjection of dsRNA

Primers with a T7 promoter sequence at the 5' end were designed to amplify PCR products to synthesize dsRNA for *MsCSP17* and *MsCSP18* using the online website https://primer3.ut.ee/, with green fluorescent protein (GFP) gene as a negative control (Table S1). PCR amplification was performed with *MsCSP17*-, *MsCSP18*-, and *GFP*-containing plasmids. The correctly sequenced products were used as templates for dsRNA synthesis in vitro according to the instructions of the T7 RiboMAX™ Express Large Scale RNA Production System kit (Takara, Dalian). DsMsCSP17, dsMsCSP18 and dsGFP were injected into healthy third instar larvae by Nanoliter2010 microinjection system (World Precision Instruments, Sarasota, FL, USA). The injection site was at the front of the abdomen (1st–2nd abdominal segments). Each larva was injected with 2 μl of dsRNA at a concentration of 1000 ng/μl, and 24 larvae were injected as a group, with three replicates for each gene.

Phenotypes of larvae after RNAi

After injection, the larvae were fed with fresh corn leaves, weighing daily. The abnormal phenotypes were counted and recorded by a QImaging Micropublisher 3.3 digital camera mounted on an Olympus BS41 stereo microscope (Tokyo, Japan). Larvae injected with dsMsCSP17, dsMsCSP18 and dsGFP for 24 hours were randomly selected for RNA extraction and cDNA synthesis, with three biological replicates and two larvae in each sample. The silencing efficiency was detected by RT-qPCR technique.

Data statistics and analysis

Data were processed using Microsoft Excel 2019, with data plots produced using GraphPad Prism 8 (GraphPad Software, Boston, MA). One-way analysis of variance (ANOVA) was performed on gene spatiotemporal expression using SPSS 16.0 (IBM, New York, NY), followed by Duncan's test ($p < 0.05$). The significance of RNAi silencing efficiency was analyzed by independent sample *t*-test with $p < 0.05$.

Results

Sequences characterizations of MsCSP17 *and* MsCSP18

Two CSP genes of *M. separata* were cloned based on the sequences from the transcriptome dataset, which were named as *MsCSP17* and *MsCSP18*. The ORF of *MsCSP17* was 378 bp which encoded 125 amino acids (aa), containing a 15 aa of signal peptide at the N-terminal (fig. 1a, c). The relative molecular weight of MsCSP17 was 14.00 kD, and the isoelectric point was 6.42. Similarly, the ORF of *MsCSP18* was 387 bp which encoded

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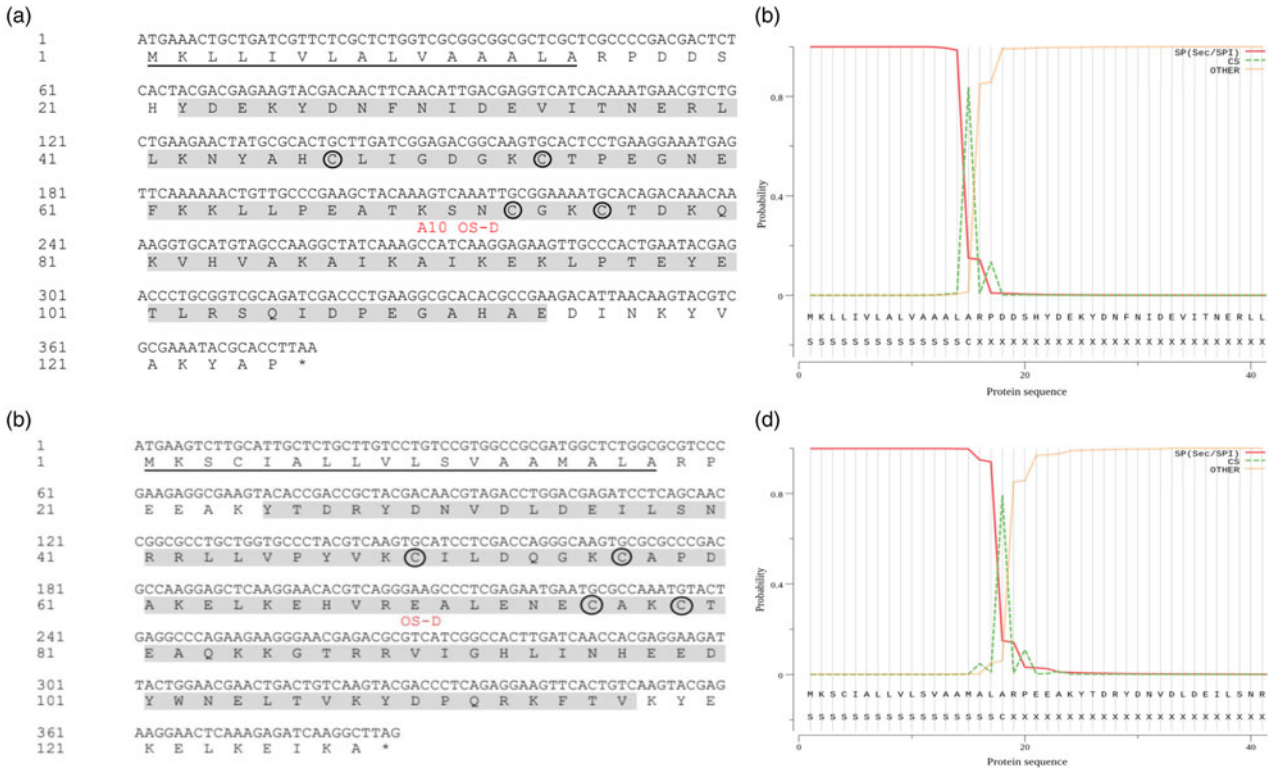

Figure 1. Sequence analysis of *MsCSP17* and *MsCSP18*. The nucleotide sequence and putative amino acid sequence of *MsCSP17* (a) and *MsCSP18* (b), where the circles indicate the conserved cysteine sites. Signal peptide prediction of MsCSP17 (c) and MsCSP18 (d).

128 aa, containing an 18 aa of signal peptide at the N-terminal (fig. 1b, d). The relative molecular weight of MsCSP18 was 14.84 kD, and the isoelectric point was 6.83. Both of MsCSP17 and MsCSP18 contained four conserved cysteine sites (C × (6)-C × (18)-C × (2)-C), which were involved in the formation of two pairs of disulfide bonds (fig. 1a, b).

Alignment and phylogenetic analysis of MsCSP17 and MsCSP18

Alignment analysis showed that sequences of MsCSP17 and MsCSP18 shared 32% similarity with each other (fig. 2a). Using SWISS-MODEL, the structures of MsCSP17 and MsCSP18 were constructed based on CSPsg4 from *Schistocerca gregaria*, which showed that MsCSP17 and MsCSP18 were both α-helix-rich globular proteins consisting of six α-helices and containing four typical cysteine sites (fig. 2b, c). Phylogenetic analysis showed that MsCSP17 had high homology with HaCSP23 and SeCSP12, while MsCSP18 was clustered into the different clade which was close to AiCSP3 and MsCSP2 (fig. 2D).

Expressions of MsCSP17 *and* MsCSP18 *in different developmental stages and tissues*

The expression of *MsCSP17* and *MsCSP18* at different developmental stages of *M. separata* was detected by RT-qPCR. The results showed that *MsCSP17* was expressed in all 1st–6th instar larvae, with the highest expression in 2nd and 1st instar larvae but low in non-larva stages (fig. 3a). Differently, *MsCSP18* was significantly highly expressed in female and male adults, followed by the expression in 1st instar larvae (fig. 3b). Among different tissues of 4th instar larvae, *MsCSP17* was highly expressed in the head, thorax and integument, followed by the mandible (fig. 3c), while *MsCSP18* was significantly highly expressed in the head and mandible, followed by the thorax and integument (fig. 3d).

Expressions of MsCSP17 *and* MsCSP18 *in larval integuments and mandibles*

Since the high expression of *MsCSP17* and *MsCSP18* in larval integuments and mandibles, the expression of two genes in integuments of 1st–6th instar larvae, larval integuments during molting, and mandibles of 3rd–6th instar larvae were detected by RT-qPCR. The results showed that the expression of *MsCSP17* was the highest in the integument of 5th instar larvae and the lowest in the integument of 2nd instar larvae (fig. 4A), while *MsCSP18* was significantly highly expressed in the integument of 3rd instar larvae but lowly in the integument of 6th instar larvae (fig. 4B). Interestingly, both of *MsCSP17* and *MsCSP18* lowly expressed in larval integuments when larvae molted for 6 h and 9 h from 3rd to 4th instar, but highly at the beginning and end phase during molting (fig. 4C, D). Moreover, the expression of *MsCSP17* was high in the mandible of 3rd instar larvae, while the expression of *MsCSP18* was high in the mandible of 5th instar larvae and low in 6th instar larval mandibles (fig. 4E, F).

Silencing efficiency of MsCSP17 *and* MsCSP18

A total of 2 μg of dsMsCSP17, dsMsCSP18, and dsGFP were injected into a 3rd instar larva, respectively. Compared with control groups injected with dsGFP, the mRNA expressions of *MsCSP17* and *MsCSP18* were significantly down-regulated in

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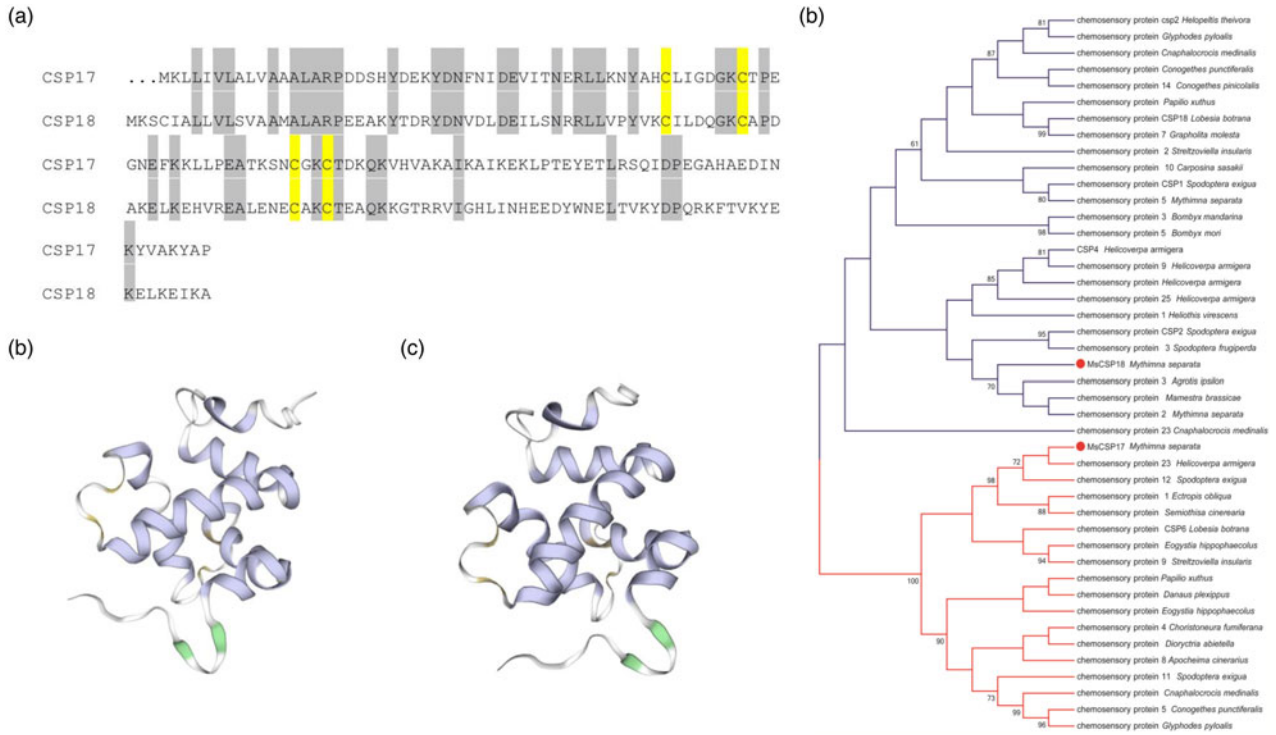

Figure 2. Characteristics of MsCSP17 and MsCSP18. (a) Sequence similarity alignment of MsCSP17 and MsCSP18. Predicted protein structures of MsCSP17 (b) and MsCSP18 (c). The purple and yellow regions indicate the α-helix and cysteine in the structure, respectively. (d) The phylogenetic tree of CSPs. Proteins marked with red dots are MsCSP17 and MsCSP18.

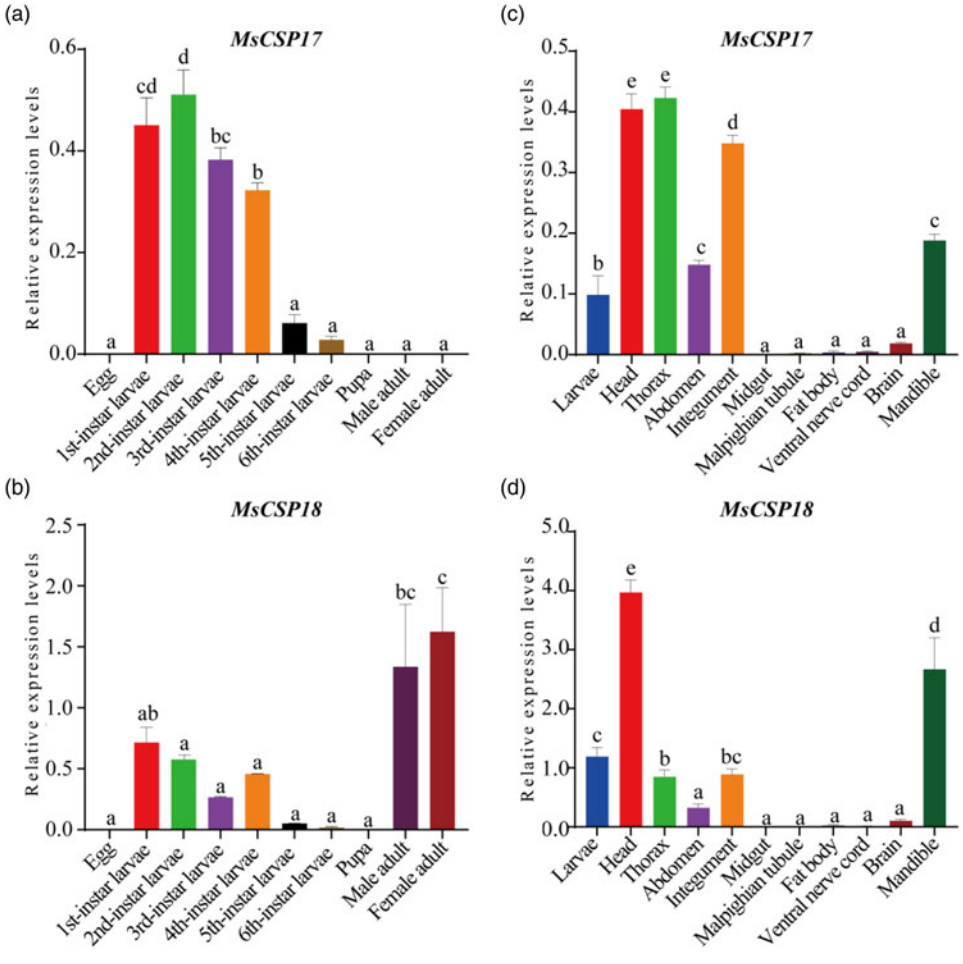

Figure 3. Expression profiles of *MsCSP17* and *MsCSP18* among different developmental stages and tissues. The relative expression levels of *MsCSP17* (a) and *MsCSP18* (b) at different developmental stages. Relative expression levels of *MsCSP17* (c) and *MsCSP18* (d) in different tissues of 4th instar larvae. Data represent means of three replicates. One-way ANOVA was performed followed by Duncan's test ($p < 0.05$).

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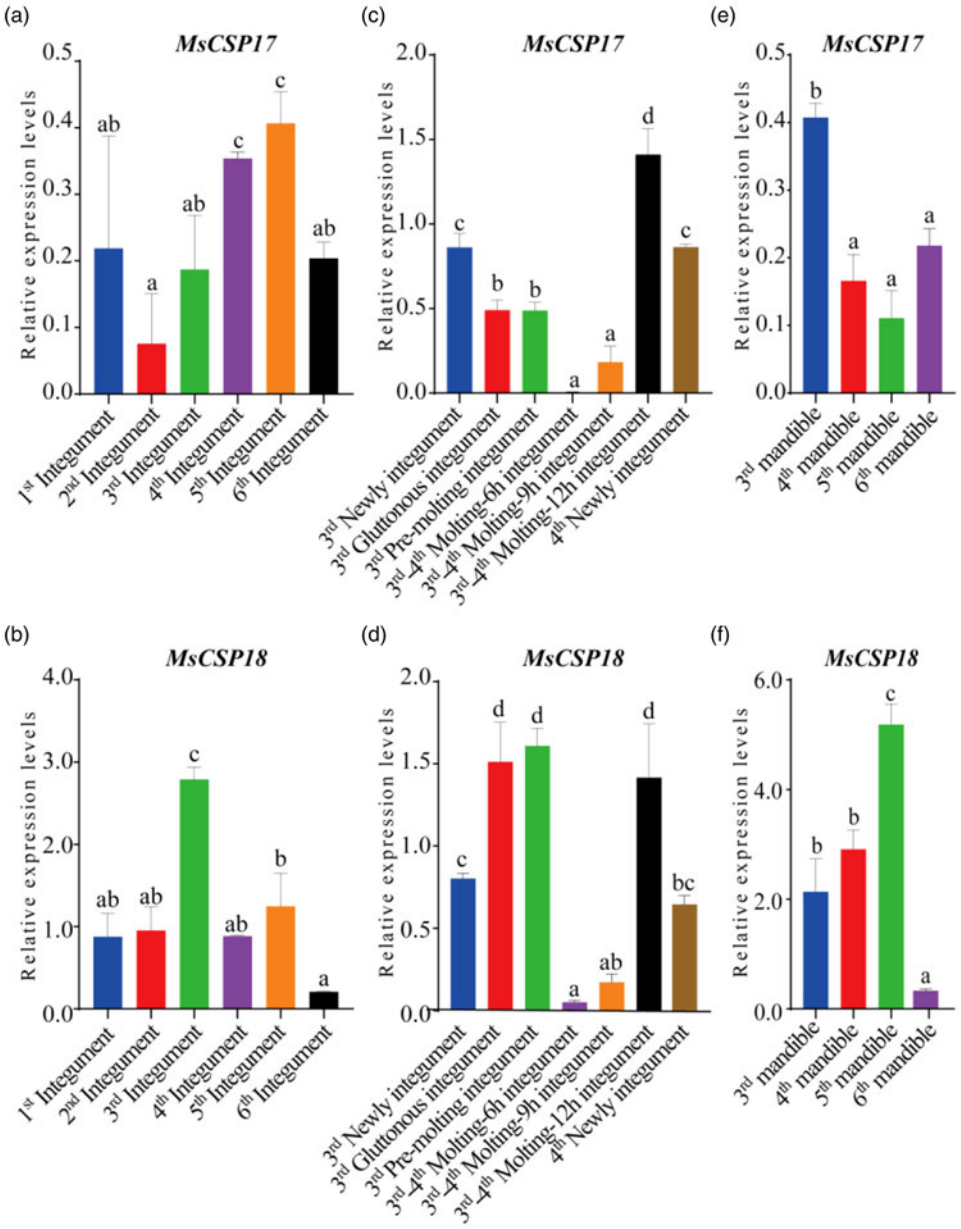

Figure 4. Expressions of *MsCSP17* and *MsCSP18* in larval integuments and mandibles. The relative expression of *MsCSP17* (a) and *MsCSP18* (b) in larval integuments at different developmental stages. The relative expression of *MsCSP17* (c) and *MsCSP18* (d) in larval integuments during molting from 3rd to 4th instar. The relative expression of *MsCSP17* (e) and *MsCSP18* (f) in mandibles of 3rd–6th instar larvae. Data represent means of three replicates. One-way ANOVA was performed followed by Duncan's test ($p < 0.05$).

larvae injected with dsMsCSP17 and dsMsCSP18, with a decrease of 66 and 56%, respectively (fig. 5A, B), indicating the high silencing efficiency of dsRNA microinjection on CSPs in *M. separata*.

Effects of RNAi on the development of M. separata

The dsRNA-injected larvae were fed with fresh maize leaves to analyze their phenotype and growth. The larvae injected with dsGFP showed normal growth and development, while 15.0 and 17.5% of the larvae injected with dsMsCSP17 and dsMsCSP18 showed retarded growth and smaller size compared to the control, respectively, with old integument stuck between the head and thorax (fig. 6a). Most of the abnormal larvae died due to not feeding (fig. 6b), and a few ones gradually returned to normal at the end of 5th instar or the beginning of 6th instar, but failed to implement eclosion after pupation (fig. 6c). According to the results, the mortalities were 40.5 and 36.0% for dsMsCSP17- and dsMsCSP18- injected larvae after 12 days, respectively, while the mortality of the control was 4.2%. There was no significant difference in the pupation rate among different groups, while the emergence rates were 87.5, 75.0 and 95.7% for dsMsCSP17-, dsMsCSP18- and dsGFP-injected groups, respectively. The RNAi larvae without obvious phenotype were weighed every 24 h after injection. Compared with the control group, the body weight of the larvae injected with dsMsCSP17 and dsMsCSP18 was significantly reduced before pupation, respectively (fig. 6d).

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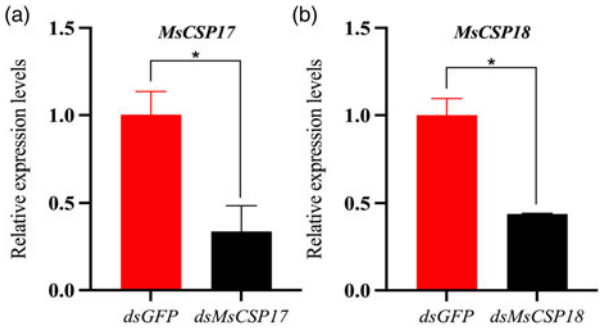

Figure 5. Knockdown efficiency of *MsCSP17* and *MsCSP18* in *M. separata* larvae. Relative mRNA expression levels of *MsCSP17* (a) and *MsCSP18* (b) after microinjection of dsRNA for 24 h. Data represent means of three replicates. Black bar and the asterisk represent the significant difference analyzed by Student's *t*-test ($p < 0.05$).

Discussion

CSPs play a crucial role in insect survival and reproduction (Pelosi *et al.*, 2014), which have a stable and compact structure that binds and solubilizes hydrophobic small molecules, such as sex pheromones, nutrients, visual pigments, growth and development-related hormones. In this study, two CSP genes, *MsCSP17* and *MsCSP18* were cloned from *M. separata,* both of which have four conserved cysteine sites that are typical characteristics of CSPs in insects (Cao *et al.*, 2014; Wang *et al.*, 2016; Xue *et al.*, 2016). MsCSP17 and MsCSP18 shared 32% sequence similarity with each other, while they have 70–88% sequence similarity with their homologs of other insects such as *Agrotis ipsilon* and *Mamestra brassicae*, which was consistent with the high conservation of CSPs among different species (Wanner *et al.*, 2004). The functional importance of conserved amino acid residues can

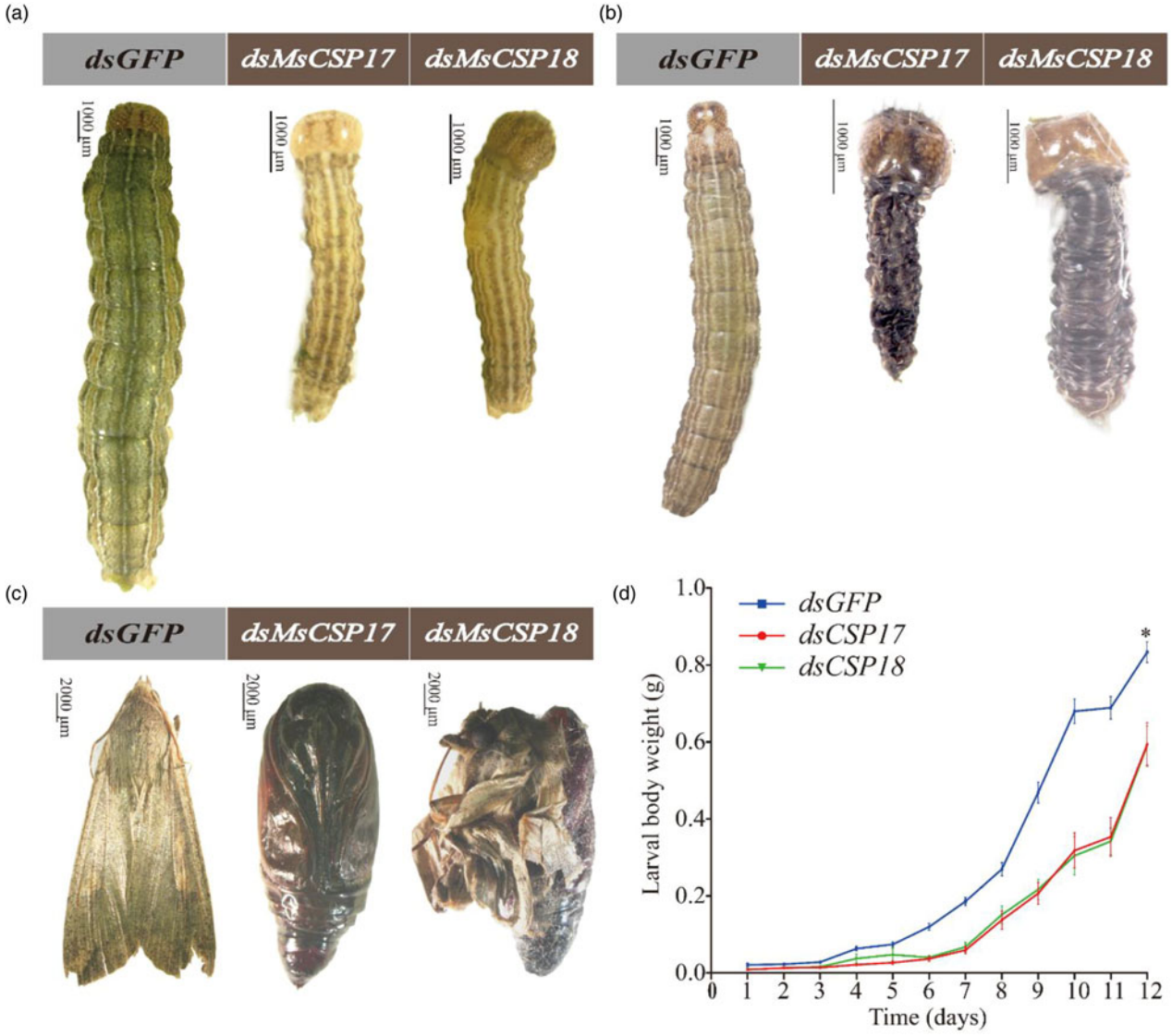

Figure 6. Effects of RNAi on the development of *M. separata* larvae. (a) Changes of larval size after injection of dsRNA. (b) Larvae with abnormal phenotypes died after injection of dsMsCSP17 and dsMsCSP18. (c) *M. separata* failed eclosion after injection of dsMsCSP17 and dsMsCSP18. (d) Changes in larval body weight after injection of dsRNA.

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infer that CSPs probably play an important role in insect physiology (Gu *et al.*, 2012).

Gene spatio-temporal expression pattern is helpful for grasping the dynamic changes of gene expression levels and predicting their functions in insects. In this study, *MsCSP17* was significantly highly expressed in the larval stage, especially in head, thorax, integument, and mandible, while *MsCSP18* was expressed highly in adults and larvae, and the head and mandible of larvae. This was consistent with reports that CSPs were not only expressed in the antennae, but also in non-olfactory organs such as the head, thorax, feet, wings, and gonads of insects (Wanner *et al.*, 2004; Zhang and Lei, 2015). The high levels of *MsCSP18* in adults indicated that it may have the potential role of finding host or mating location. The broad-spectrum expression of *MsCSP17* and *MsCSP18* in *M. separata* larvae suggested that CSP genes may have important functions other than chemosensory (Guo *et al.*, 2011, Gu *et al.*, 2012; Zhang *et al.*, 2013).

With the high expression levels in larval integuments, the expressions of two genes in larval integuments at different stages were compared. *MsCSP17* had higher expression in 5th instar larval integuments, while *MsCSP18* expressed highly in 3rd instar larval integuments. Combined with the variations of mRNA levels during molting, *MsCSP17* and *MsCSP18* might be involved in the formation of new integument in *M. separata*. Moreover, with the high expression levels in larval mandibles, the relative expression levels of two genes were compared among mandibles of 3rd–6th instar larvae. The results showed that *MsCSP18* highly expressed in the mandibles of 3rd–5th instar but lowly in 6th instar, which indicated its potential role in the development of mandibles (Zhao *et al.*, 2022).

RNAi is widely used in the field of insect functional genomics, especially in screening lethal target genes, which are involved in important physiological and biochemical processes such as reproductive development, energy metabolism, immunity, and neuromodulation in insects. In this experiment, RNAi was effectively applied to explore the functions of two CSPs in *M. separata* larvae, with the expression of *MsCSP17* and *MsCSP18* significantly silenced after injection of dsRNA. Compared with the control, CSP-injected larvae showed stunted growth, smaller size, increased mortality, and reduced eclosion rate. The abundant expression of CSPs in metabolically active tissues and organs was closely related to the development of insects, hence the reduced expressions of CSPs can affect growth and reproduction negatively (Gong *et al.*, 2012; Ma *et al.*, 2019). For instance, CSPs were involved in the foot regeneration in *Periplaneta americana* (Kitabayashi *et al.*, 1998), molting process in *Pheidole megacephala* larvae (Pelosi *et al.*, 2018), and reproduction in *Spodoptera exigua* (Gong *et al.*, 2012). Moreover, inhibition of SiCSP9 expression has been reported to affect fatty acid biosynthesis and inhibit cuticle development and molting in *Solenopsis invicta* (Cheng *et al.*, 2015). Thus, *MsCSP18* and *MsCSP19* were functional in the development of *M. separata*.

In conclusion, *MsCSP17* and *MsCSP18* were important for the development of *M. separata* larvae, with high expressions in larvae and their heads, integuments, and mandibles. Our findings expanded our current understanding of the expression patterns of CSPs in non-olfactory tissues and their functions in *M. separata* larvae, providing potential targets to develop effective pest control strategies.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485323000354.

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Competing interests. None.

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