Cloning and Expression Analysis of Chemosensory Protein AzanCSP4 from *Agrilus zanthoxylumi* (Coleoptera: Buprestidae)[1]

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Abstract Chemosensory proteins (CSPs) are widely distributed in insect tissues and are involved in olfactory and non-olfactory functions. In this study, based on the transcriptome data of *Agrilus zanthoxylumi* Hou (Coleoptera: Buprestidae), the *AzanCSP4* of was cloned by RT-PCR and bioinformatically analyzed, and RT-qPCR was conducted to analyze their expression levels of *AzanCSP4* in different genders and tissues (head, thorax, abdomen, leg and wing). Sequence analysis showed that *AzanCSP4* had an open reading frame (ORF) length of 366 bp, encoding 121 amino acids with an estimated molecular weight 13.96 kD. The encoded protein had no transmembrane domain, and the signal peptide was located in the position 1–15 at the N-terminal of the amino acid sequence. Sequence alignment revealed that AzanCSP4 had four conserved cysteines. Phylogenetic analysis revealed that the AzanCSP4 and AmalCSP6 from *Agrilus mali* Matsumura (Coleoptera: Buprestidae) were closely clustered into the same clade. RT-qPCR results showed that *AzanCSP4* of *A. zanthoxylumi* was expressed in different tissues of both male and female adults, and the expression in the same tissue was greater in female adults than in male adults. The expression of *AzanCSP4* in the head of female adults was significantly higher than that in other tissues of male and female adults. This study provides a theoretical basis for further research on the function of AzanCSP4, especially on the chemical communication mechanism in *A. zanthoxylumi*.

Key Words *Agrilus zanthoxylumi*, chemosensory protein, gene cloning, bioinformatics analysis, RT-qPCR

Agrilus zanthoxylumi Hou (Coleoptera: Buprestidae) is an important invasive pest that can cause severe damage to Chinese prickly ash (*Zanthoxylum bungeanum* Maxim) in northern China (Dang et al. 1988). Insect-infested trees often suffer from desiccation of leaves and weakening of the trees, resulting in death of the trees in severe cases (Li et al. 1990). The larvae prevent the tree from transferring nutrients and water by feeding on the base of the trunk, and the adults form pupal orifices by feeding on the xylem, thus destroying the conductive tissues of the trunk. (Xu et al. 2020). The damage caused by *A. zanthoxylumi* not only causes ecological losses in Chinese prickly ash fields, but also exposes growers to serious economic losses

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(Dang et al. 2017). Therefore, there is an urgent need to develop a new approach for sustainable green control of *A. zanthoxylumi*.

The sensitive sensory systems of insects play an important role in insect behaviors, such as feeding, mating, and oviposition (Jacquin-Joly and Merlin 2004). The process of odor perception in insects involves interactions between odor molecules in the environment and several families of chemically compensated related proteins (Su et al. 2009). Odor molecules bind to chemosensory proteins (CSPs) or odorant binding proteins (OBPs) (Leal 2013) to form complexes, and then are transported to sensory neuron membrane proteins (SNMPs) (Zhang et al. 2015), ionotropic receptors (IRs) (Benton et al. 2009), or odorant receptors (ORs) (Trible et al. 2017). Subsequently, chemical signals are converted into electrical signals, which are transmitted to the central nervous system and regulate the behavioral responses of insects (Kaissling 1986). At the same time, in order to prevent odor molecules from repeatedly stimulating the olfactory system, odor degrading enzyme (ODE) (He et al. 2014) rapidly degrades odor molecules.

CSPs were first discovered and named as olfactory-specific protein D (OS-D) in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) by McKenna et al. (1994). In recent years, with the rapid development of sequence genome and transcriptome of various organisms, the identification of CSPs has been applied to various insects. The CSPs are acidic water-soluble proteins with a molecular weight of 10–15 kD, and encode 100–120 amino acids (Picimbon et al. 2000). The vast majority of insect CSPs have 4 conserved cysteines (Cys), with C_1-X_6-C_2-X_{18}-C_3-X_2-C_4 for Lepidoptera, Diptera, and Coleoptera (Gong et al. 2009). For example, based on *Xylotrechus quadripes* Chevrolat (Coleoptera: Cerambycidae) transcriptome data, 14 *CSPs* genes were identified, and the amino acid sequences all conformed to the C_1-X_6-C_2-X_{18}-C_3-X_2-C_4 arrangement (Zhuang et al. 2020).

CSPs are widely distributed in olfactory and non-olfactory organs of insects, and the distribution characteristics imply different biological functions. Clarifying the distribution characteristics of these genes can help to explore the functions of insect CSPs. The expression of *AmalCSP1* was significantly higher in the antennae of *Agrilus mali* Matsumura (Coleoptera: Buprestidae) males than females, implying that *AmalCSP1* may participate in recognizing gender pheromone of female *A. mali* (Sun 2018). *BtabCSP11* was highly expressed in the abdomen of female *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae), and the female reproduction was significantly reduced after RNA interference, suggesting that *BtabCSP11* played a role in regulating reproduction (Zeng et al. 2020). The expression of *PameP10* was significantly up-regulated during regeneration of leg truncation in *Periplaneta americana* L. (Blattaria: Blattoidea) larvae and down-regulated to normal levels after the end of regeneration, indicating that *PameP10* was associated with the limb regeneration process (Kitabayashi et al. 1998). The highest expression of *NlugCSP6* was found in the epidermis of *Nilaparvata lugens* Stal (Hemiptera: Delphacidae) adults, after RNAi reduced the expression of *NlugCSP6*, the wings of adults developed abnormally, with deformed female wings and ineffective wing closure in males, suggesting that *NlugCSP6* played an important role in wing formation of *N. lugens* (Gao et al. 2022).

In this study, *AzanCSP4* gene was further identified from its transcriptome data assembled in our laboratory, and we cloned *AzanCSP4* gene by RT-PCR and analyzed it bioinformatically. The expression of *AzanCSP4* gene in the head, thorax,

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abdomen, leg, and wing of male and female adults was examined by RT-qPCR. Our ultimate objective was to increase the understanding of AzanCSP4, but also provide a theoretical basis for exploring the functions of AzanCSP4.

Materials and Methods

Insects. The *A. zanthoxylumi* adults used in this study were collected from May to August 2021 in Xi Lijiagou Village, Puhua Town, Lantian County, Xi'an City, Shaanxi Province in China. The collected *A. zanthoxylumi* adults were placed in a beaker with fresh Chinese prickly ash leaves and fruits, covered with a gauze net at the mouth of the beaker for air permeation. After transported to the laboratory, male and female adults were separated according to morphological characteristics, and the head, thorax, abdomen, legs, and wings of the *A. zanthoxylumi* adults were dissected for RNA extraction. For each treatment, we had 3 biological replicates. All collected tissues were placed in 1.5-ml Eppendorf tubes, frozen immediately with liquid nitrogen, and then stored at −80°C until use.

RNA extraction and cDNA synthesis. RNA was extracted from 5 tissues of male and female adults using TRIzol Reagent (Biolab, Beijing) according to the manufacturer instructions. The integrity of RNA samples was detected by 1% agarose gel electrophoresis, and the purity and concentration of RNA samples were assessed with a Spectrophotometer (NanoDrop™ 2000 Thermo Fisher Scientific, Waltham, MA). The qualified RNA samples were synthesized by a first strand Reverse Transcription Kit (Vazyme, Nanjing) and placed in a −20°C refrigerator.

Primer design and gene cloning. The *AzanCSP4* sequence was screened from *A. zanthoxylumi* transcriptome data (SUB6796283). The primers were designed by Primer|SGD (https://www.yeastgenome.org/primer3) with AzanCSP4-F (GTGCGT CCGTGAAGTGTAC) as forward primers and AzanCSP4-R (AGCATTACTTAGGTTG GATCT) as reverse primers. Amplification of the coding sequence of *AzanCSP4* gene using cDNA as template. PCR reactions were performed in a total volume of 25 μl containing 1 μl of cDNA, 12.5 μl of 2× Es *Taq* Master Mix, 1 μl of each primer, and 9.5 μl sterilized ultrapure water. Reactions were as follows: 94°C for 3 min, 94°C for 30 s, 59°C for 30 s, 35 cycles of 72°C for 1 min, and 72°C for 5 min. The amplified products were detected by 2% agarose gel electrophoresis, and the target bands were purified by MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa, DaLian). The purified target fragments were ligated into the cloning vector pMD18-T (TaKaRa, Beijing), transformed into DH5α *Escherichia coli* cell (TaKaRa, Dalian) by thermal excitation, cultured with LB medium containing Amp resistance, and screened for positive colonies. The positive clone strains were sent to Shanghai Biotechnology Services Co. Ltd. and sequenced using universal primers: (M13-47: CGCCAGGGTTTTCCCAGTCACGAC; RV-M: AGCGGATAACAATTTCACACAGGA).

Bioinformatics analysis. The open reading frame (ORF) of *AzanCSP4* gene was predicted by ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The molecular weight and theoretical isoelectric point (PI) of protein AzanCSP4 were calculated online with the ExPASy tool "ProtParam" (https://web.expasy.org/protparam). Signal peptide of protein AzanCSP4 was predicted using SignalP-4.1 (https://services.healthtech.dtu.dk/ser-vice.php?SignalP-4.1) and the conserved structural domain of protein AzanCSP4 was searched by the NCBI CD-Search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Transmembrane domain of protein AzanCSP4

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was searched using TMHMM-2.0 (https://services.healthtech.dtu.dk/service. php? TMHMM2.0) for analysis.

The BLASTX program in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for CSPs with high similarity to AzanCSP4, and amino acid homologous sequences were performed with the software DNAMAN (version 6.0). After sequence alignment, a phylogenetic tree was constructed using the neighbor-joining (NJ) method (Bootstrap: 1,000 times) to analyze the affinities among the CSPs of other coleopterans.

Reverse transcription quantitative real-time PCR (RT-qPCR). The transcript levels of *AzanCSP4* gene in different tissues and genders of *A. zanthoxylumi* were analyzed with RT-qPCR. Based on the *AzanCSP4* gene sequence, gene-specific primers for RT-qPCR were designed using Primer|SGD. The forward primer (CSP4-qF: TGCTTTTACAACGCACATCAA) and the reverse primer (CSP4-qR: GGCAAGTTCCT CTCAAAACCA) were synthesized by Shanghai

Biotechnology Services Co. Ltd. The expression of *AzanCSP4* gene was detected by PCR using *28S* as the internal reference gene and cDNA as the template. Reactions were performed in a total volume of 25 μl containing 1 μl of sample cDNA, 2.5 μl of ROX Dye I, 12.5 μl of 2×*Taq* SYBR Green qPCR Mix, 1 μl of each primer (10 μM), and 8 μl sterilized ultrapure water. Reactions were first kept at 94°C for 3 min, and then allowed to run 40 cycles of 60°C for 1 min. The reactions of each sample were performed with 3 technical replicates and 3 independent biological replicates. The relative expression levels of *AzanCSP4* gene were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Analysis of variance (ANOVA) was used to analyze the significant differences in the relative expression of *AzanCSP4* gene (mean ± standard error) between the same gender and different tissues, and the LSD method was applied for multiple comparisons and significance of differences tests. *T*-test ($\alpha = 0.05$) was used to compare the relative expression of *AzanCSP4* gene between the same tissue and different genders. All analyses were conducted in SPSS Statistics 26.0. The GraphPad Prism 9 was used to plot the bar chart.

Results

Gene cloning and sequence analysis of AzanCSP4. The *AzanCSP4* gene sequence was cloned using RT-PCR and 1% agarose gel electrophoresis showed a distinct band at around 400 bp (Fig. 1), as expected (403 bp). After sequencing and comparison, the sequence of *AzanCSP4* gene obtained by cloning was consistent with the sequence of *AzanCSP4* (GenBank No. MT291821.1) in NCBI, and the *AzanCSP4* gene contained a complete ORF of 366 bp in length.

Sequence analysis showed that AzanCSP4 had the molecular formula $C_{612}H_{1015}N_{175}O_{182}S_7$. The *AzanCSP4* gene encoded 121 amino acids (Fig. 2), which consisted of 20 kinds of amino acids, with the highest amino acid content being Lys and the lowest being Trp and His. There were 15 negatively charged amino acid residues (Asp + Glu) and 24 positively charged amino acid residues (Arg + Lys). Physicochemical properties of protein AzanCSP4 were as follows: the molecular weight was 13.96 kDa and the PI was 9.49. The hydrophilic mean coefficient was −0.713 indicating that it was a hydrophilic protein. The instability coefficient was 34.75 (<40), indicating that it was in a stable state. As shown in

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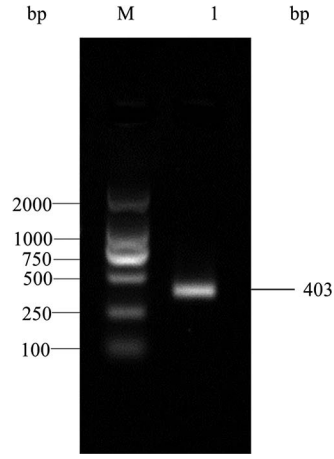

Fig. 1. PCR products of *AzanCSP4*. M represents DL 2000 DNA Marker; 1 represents PCR result of *AzanCSP4*.

Fig. 2, the N-terminal of the AzanCSP4 amino acid sequence contained a signal peptide, which was present at the positions of amino acids 1–15. AzanCSP4 contained an OS-D super family, which was located between amino acid residues 18–110, and TMHMM result showed AzanCSP4 was not found to contain a transmembrane domain.

Sequence alignment and phylogenetic analysis of AzanCSP4. An alignment of AzanCSP4 (GenBank No. QTJ02340.1) with AzanCSP7 (GenBank No. UTE95282.1), AmalCSP6 (GenBank No. AXG21599.1), AmalCSP8 (GenBank No. AXG21601.1) compared by DNAMAN V6 is shown in Fig. 3. The sequence identity between AzanCSP4 and AmalCSP6 was 85.94%. AzanCSP4 was found to be a

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                418       428       438       448       458       468       478       488       498       508
409  GTGCGTCCGTGAAGTGTACGGGTTCCTTAAAAGTTAAACTATAACAATAACTTAACAATAGAATAATGAAACTACTATTATTGCTTCTCGTAATTGCTGTTG
1                                                                                 M  K  L  L  L  L  L  L  V  I  A  V

                520       530       540       550       560       570       580       590       600       610
511  TAGCTGCAGACAAATATACAACAAAATACGACAATGTCGACATCGATAGGATTCTCTCAAACCAAAGAGTTCTCACAAATTACATCAAGTGTTTGATGGAAG
13   V  A  A  D  K  Y  T  T  K  Y  D  N  V  D  I  D  R  I  L  S  N  Q  R  V  L  T  N  Y  I  K  C  L  M  E

                622       632       642       652       662       672       682       692       702       712
613  AAGGACCTTGCACTTCAGAAGGAAGAGAACTCAAAAAAACCCTTCCTGACGCTTTATCAACCGGTTGTACAAAATGCAATTTGAAACAGAAACAAACTGCAG
47   E  G  P  C  T  S  E  G  R  E  L  K  K  T  L  P  D  A  L  S  T  G  C  T  K  C  N  L  K  Q  K  Q  T  A

                724       734       744       754       764       774       784       794       804       814
715  AAAAAGTTATTAGGCATTTAATGAAGAACCGACCATCTGACTGGGAAAGACTCACTGCAAAATATGACCCCAAAGGAGAATACAAGAAGCGTTTCCAACCTC
81   E  K  V  I  R  H  L  M  K  N  R  P  S  D  W  E  R  L  T  A  K  Y  D  P  K  G  E  Y  K  K  R  F  Q  P

                826       836       846       856       866       876       886       896       906       916
817  AATCGAGAGATGGCAAAAACTAAATAAACAATAGATCCAACCTAAGTAATGCTTATCTTCAAGCGTATATTTTAAGTTTTATAAGACTATACGATTATTGTC
115  Q  S  R  D  G  K  N
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Fig. 2. The nucleic acid sequence of *AzanCSP4* and its corresponding amino acids sequence. Signal peptides are underlined in blue; Four conserved cysteines are marked with red boxes.

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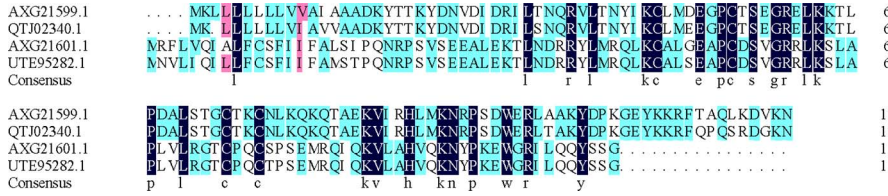

Fig. 3. Sequence alignment among AzanCSP4 with CSPs from other Coleoptera insects. Black represents completely identity; Pink represents identity above 75%; Blue represents identity above 50%; White represents identity below 30%.

classic CSP of Coleoptera, showing a common characteristic of 4 Cys with the following pattern: C_1-X_6-C_2-X_{18}-C_3-X_2-C_4.

The phylogenetic tree based on CSPs from *A. zanthoxylumi* and other coleopterans was constructed by the neighbor-joining method. As can be seen from the Fig. 4, AzanCSP4 and AzanCSP7 were dispersed to 2 large clades. AzanCSP4 and AmalCSP6 from *A. mali* were closely clustered into the same clade in the phylogenetic tree with a bootstrap support of 95%, indicating that AzanCSP4 had the closest evolutionary relationship with AmalCSP6.

Expression of *AzanCSP4* gene in tissues (head, thorax, abdomen, leg, and wing) of male and female adults. The RT-qPCR results (Fig. 5) showed that the *AzanCSP4* gene was expressed in the head, thorax, abdomen, leg, and wing of both male and female adults. The relative expression of *AzanCSP4* gene in female tissues was from high to low in the order of head, wing, abdomen, thorax and leg, with significantly higher expression in the head than in the other tissues ($P < 0.05$) and no significant difference in the expression in the remaining 4 tissues. The relative expression in male tissues was from high to low in the order of abdomen, head, wing, thorax, and leg. The relative expression of the *AzanCSP4* gene was significantly higher in the head, thorax, and wings of female insects than in their male insects ($P < 0.05$), with no significant gender differences in the relative expression of the leg and abdomen ($P > 0.05$).

Discussion

In this study, the complete ORF sequence of *AzanCSP4* was cloned for the first time, encoding 121 amino acids; the molecular weight was 13.96 kDa. The AzanCSP4 protein was a newly identified CSP of *A. zanthoxylumi* following AzanCSP3 (Yang et al. 2020) and AzanCSP7 (Gao et al. 2023). Amino acid sequence analysis revealed that AzanCSP4 had no transmembrane domain, and the AzanCSP4 signal peptide was located in amino acids 1-15 at the N-terminal of the protein, which was presumed to be a secreted protein (Peng et al. 2011).

The alignment of CSP sequences from coleopteran species showed that AzanCSP4 had 4 Cys (C_1-X_6-C_2-X_{18}-C_3-X_2-C_4) which conformed to the common characteristic of CSPs in Coleoptera (Wanner et al. 2004). Sequence identity between AzanCSP4 and AmalCSP6 was high, and a neighbor-joining tree of AzanCSP4 and homologous CSPs from other coleopterans indicated that AzanCSP4 and AmalCSP6

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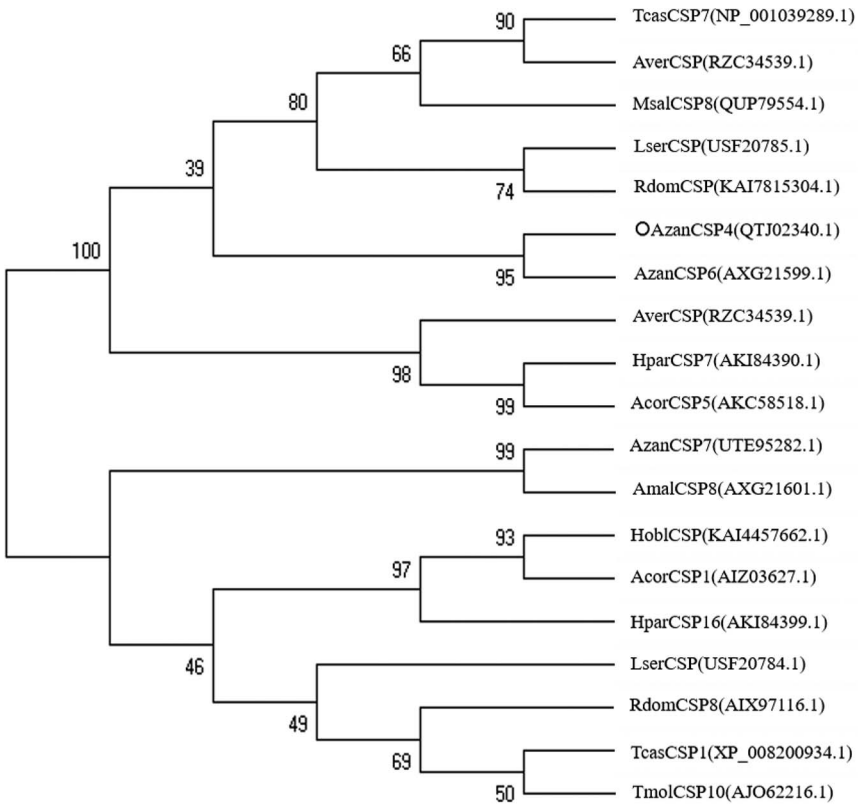

Fig. 4. The phylogenetic analysis of AzanCSP4 and CSPs of other coleopteran insects. GenBank ID and its corresponding CSP: NP_001039289.1 (*Tribolium castaneum* chemosensory protein 7), RZC34539.1 (*Asbolus verrucosus* chemosensory protein), QUP79554.1 (*Monochamus saltuarius* chemosensory protein 8), USF20785.1 (*Lasioderma serricorne* chemosensory protein), KAI7815304.1 (*Rhyzopertha dominica* chemosensory protein), QTJ02340.1 (*Agrilus zanthoxylumi* chemosensory protein 4), AXG21599.1 (*Agrilus mali* chemosensory protein 6), RZC34539.1 (*Asbolus verrucosus* chemosensory protein), AKI84390.1 (*Holotrichia parallela* chemosensory protein 7), AKC58518.1 (*Anomala corpulenta* chemosensory protein 5), UTE95282.1 (*Agrilus zanthoxylumi* chemosensory protein 7), AXG21601.1 (*Agrilus mali* chemosensory protein 8), KAI4457662.1 (*Holotrichia oblita* chemosensory protein), AIZ03627.1 (*Anomala corpulenta* chemosensory protein 1), AKI84399.1 (*Holotrichia parallela* chemosensory protein 16), USF20784.1 (*Lasioderma serricorne* chemosensory protein), AIX97116.1 (*Rhyzopertha dominica* chemosensory protein 8), XP_008200934.1 (*Tribolium castaneum* chemosensory protein 1), AJO62216.1 (*Tenebrio molitor* chemosensory protein 10).

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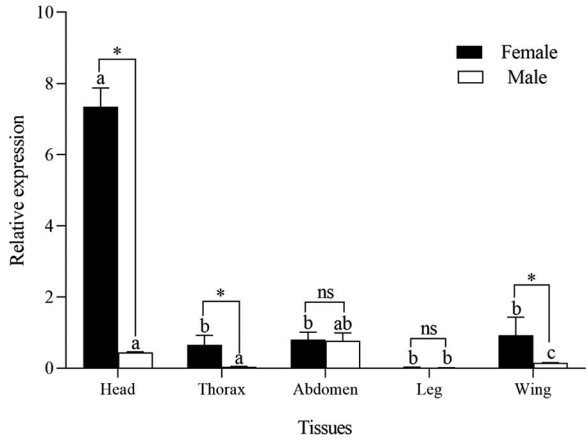

Fig. 5. The expression of *AzanCSP4* in tissues (head, thorax, abdomen, leg, wing) of male and female adults. Data are means ± SEM. The relative expression of *AzanCSP4* in the same tissue of different genders was analyzed by independent sample *t* test; ns means no significant difference ($P > 0.05$); * means significant difference ($P < 0.05$); ** means extremely significant difference ($P < 0.01$). The relative expression of *AzanCSP4* in different tissues of the same gender was analyzed by one-way ANOVA, and there was a significant difference between the relative expression of *AzanCSP4* expressed by different letters ($P < 0.05$).

were closely clustered into the same clade with a bootstrap support of 95%. These results indicated that AzanCSP4 had the closest evolutionary relationship with AmalCSP6. The interspecific similarity of OBPs is 10–15%, and CSPs are significantly more evolutionarily conserved compared to OBPs, with CSPs having a higher similarity between different species (Zhang et al. 2019). CSPs have an earlier origin than OBPs (Sánchez-Gracia et al. 2009) and are considered to be one of the proteins involved in the ancient mechanism of biorecognition of chemical stimulation but, in higher animals, genes with higher specificity in OBPs and PBP families have replaced the corresponding functions of CSPs (Xu et al. 2015). Although CSPs are rarely found in vertebrates, they are still widespread in arthropods, and insects generally have both OBPs and CSPs, indicating that CSPs still perform their corresponding physiological functions (Vieira et al. 2011).

Physiological and biochemical functions of genes are closely linked to their distribution and expression in insects. *AzanCSP4* gene was expressed in the head, thorax, abdomen, leg, and wing of both male and female adults in this study, reflecting the wide distribution of CSPs in the insect, such as the *EgriCSP8* was expressed in the head, midgut, epididymis, and fat body of *Ectropis grisescens* Warren (Lepidoptera: Geometridae) (Yan et al. 2022), and the *AipsCSP2* was expressed in the head, thorax, abdomen, leg, wing, gonad, antennae, and rostrum of *Agrotis ipsilon* Rottemberg (Lepidoptera: Noctuidae) (Rao et al. 2021). The RT-qPCR results showed that the expression level of *AzanCSP4* in the same tissue was higher in females than

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in males, which was similar to the tissue expression of *AmalCSP2* from *A. mali* (Cui 2018), indicating that *AzanCSP4* was likely to have gender-biased expression. The expression of *AzanCSP4* in the head of female adults was significantly higher than that in other tissues of both male and female adults, this may be related to the presence of more olfactory sensilla in the antennae, lower labial whiskers, and mandibular whiskers in the head of insects, such as the trichome sensilla on the antennae of female *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae), which can sense volatile compounds of the host plant, floral odors, and female pheromones. In addition, the rod sensilla in the LPO sensilla on the lower labial palpi were capable of sensing the changes in CO_2 concentration (Liu et al. 2023). *Drosophila melanogaster* had 60 conical sensilla distributed on the surface of its mandibular palpi, which were capable of sensing a wide range of odorants (De Bruyne et al. 1999).

This study clarifies the distribution characteristics of *AzanCSP4* gene in different genders and tissues of *A. zanthoxylumi* adults, and lays the foundation for further investigation of the chemical communication mechanism of *A. zanthoxylumi*. However, this study only speculates on the functions of *AzanCSP4* gene. We can not only observe the chemosensory sensilla in the head of the *A. zanthoxylumi* adults by scanning electron microscopy, but also can study the distribution of AzanCSP4 in the head chemosensory sensilla of *A. zanthoxylumi* using immunofluorescence localization. In addition, the binding ability of AzanCSP4 with host volatiles can also be further investigated by fluorescence competition binding experiment in the future.

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