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Functional characteristics of chemosensory proteins in the sawyer beetle *Monochamus alternatus* Hope

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Abstract

The Japanese pine sawyer, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), is a major pest of pines and it is also the key vector of the exotic pinewood nematode in China. In the present study, we cloned, expressed, and purified a chemosensory protein (CSP) in *M. alternatus*. We surveyed its expression in various developmental stages of male and female adult tissues and determined its binding affinities for different pine volatiles using a competitive binding fluorescence assay. A CSP known as CSP5 in *M. alternatus* was obtained from an antennal cDNA library and expressed in *Escherichia coli*. Quantitative reverse transcription polymerase chain reaction results indicated that the CSP5 gene was mainly expressed in male and female antennae. Competitive binding assays were performed to test the binding affinity of recombinant CSP5 to 13 odour molecules of pine volatiles. The results showed that CSP5 showed very strong binding abilities to myrcene, (+)-β-pinene, and (−)-isolongifolene, whereas the volatiles 2-methoxy-4-vinylphenol, p-cymene, and (+)-limonene oxide have relatively weak binding affinity at pH 5.0. Three volatiles myrcene, (+)-β-pinene, and (−)-isolongifolene may play crucial roles in CSP5 binding with ligands but this needs further study for confirmation. The sensitivity of insect to host plant volatiles can effectively be used to control and monitor the population through mass trapping as part of integrated pest management programs.

Keywords: olfactory-specific protein, qPCR, expression characteristics, competitive binding assay, volatiles

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Introduction

The Japanese pine sawyer, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), is a major pest of coniferous forests, especially pines, and it is also the key vector of the exotic pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), in eastern Asia (Kobayashi *et al*., 1984). The spread of PWN has caused tragic timber losses in pine forests during the past century (Mamiya & Enda, 1972). China is one of the regions in eastern Asia that is most highly infected by PWN (Wan *et al*., 2009), with more than 180 localities infested since 1982 (SFA, 2012). It is important to note that *M. alternatus* is native to China (Chen, 1959). The dispersal of *M. alternatus* is believed to be responsible for the spread of the PWN in East Asia given that PWN is transmitted to new hosts during maturation feeding and oviposition by nematode laden *M. alternatus* adults (Mamiya & Enda 1972). Dispersal of *M. alternatus* can be influenced by multiple factors, among which human-mediated movement of the pest is believed to be a significant component. As with many

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bark- and wood-boring insects, *M. alternatus* can commonly be moved to new locations inadvertently through the transport of infested wood, including logs, lumber, and wood packaging material (Togashi & Magira, 1981; Wang, 2004). Given the long-life cycle of *M. alternatus* and that some larvae and pupae can survive the milling process, wood products made from infested trees, especially the sapwood portion, could serve as a pathway by which *M. alternatus* is moved within China (Chen, 1959; Wang, 2004). Although *M. alternatus* could be transported long distances within China via logs and lumber, we believe that wood packaging material such as pallets and crating that are used in the transportation of commercial products are the most likely pathway for long-distance human-assisted transport of *M. alternatus* across China as well as internationally (Gu *et al.*, 2006; Haack *et al.*, 2010).

The insect olfactory system is a highly specific and sensitive sensor that can detect chemical signals from the environment and transfer information to the central nervous system in order to initiate specific behavioral reactions, such as localizing food sources and oviposition sites, mating with partners, and avoiding toxins and predators (Kaissling *et al.*, 1987; Breer *et al.*, 1994; Field *et al.*, 2000; Benton *et al.*, 2009). The olfactory perception of insects is mediated by proteins located in the sensory hairs of the antennae, including odorant binding proteins (OBPs), chemosensory proteins (CSPs), olfactory receptors (ORs), odorant degrading enzymes and sensory neuron membrane proteins (Vogt *et al.*, 1985; Wanner *et al.*, 2004; Vogt *et al.*, 2009; Zhou *et al.*, 2010). CSPs are small soluble carrier proteins that are thought to transport hydrophobic ligands (Clyne *et al.*, 1999). They were first discovered in insect sensory organs and were thought to be analogous to OBPs (Tegoni *et al.*, 2004). Subsequent research has demonstrated that CSPs are expressed ubiquitously and likely to play a role in development (Maleszka *et al.*, 2007). Several CSPs in spruce budworm were found to be upregulated during larval moult (Wanner *et al.*, 2004). The first identified member of the CSP family, olfactory-specific protein D, was purified from the antennae of *Drosophila melanogaster*. At one time, these proteins were known as sensory appendage proteins but they are now recognized as CSPs (Wanner *et al.*, 2004; Robinet *et al.*, 2009).

Thereafter, many CSPs were isolated and cloned from several insect orders, including Lepidoptera, Orthoptera, Hymenoptera, Hemiptera, and Neuroptera (Gong *et al.*, 2007; Li *et al.*, 2013; Forêt *et al.*, 2015). Compared with OBPs, which are usually expressed specifically in antennae, CSPs are broadly expressed in many organs, including antennae, probosces, legs, wings, and pheromone glands as well as other tissues (Kitabayashi *et al.*, 1998; Nagnan-Le Meillour *et al.*, 2000; Ban *et al.*, 2002; Gonzalez *et al.*, 2009; Zhang *et al.*, 2014). This broad and diverse expression pattern suggests that CSPs may play multiple roles, beyond chemosensation (Tegoni *et al.*, 2004). CSPs that are highly expressed in antenna have been suggested to have chemosensory functions in Hymenoptera and Lepidoptera (Qiao *et al.*, 2013; Zhang *et al.*, 2014). Other CSPs abundant in antennae have been implicated as having roles in the behavioral phase shift from gregarious to solitary (Guo *et al.*, 2011). In *Spodoptera exigua*, CSP3 has been implicated in ovipositioning and egg hatching (Gong *et al.*, 2012), while in *Periplaneta americana*, CSP10 seems to be a major extracellular matrix protein during limb regeneration (Kitabayashi *et al.*, 1998). Several studies indicate that CSPs may be involved in the immune response (Oduol *et al.*, 2000). CSPs are, therefore, likely to perform many diverse tasks from behavioral to various physiological processes.

In insects, the ORs mediating olfaction are most abundant in the antennae, but may also be associated with the mouthparts or external genitalia (especially near the tip of the female's ovipositor); while the taste receptors are most abundant on the mouthparts, but may also be found on the antennae, tarsi, and genitalia. However, in either case, odor molecules enter through the openings (pores) located on the sensilla cuticle where the dendrites of several (usually up to five) sensory neurons are exposed (Miles *et al.*, 2005). Diverse genes are involved in olfactory sensation (Mamidala *et al.*, 2013). In this study, quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed on chemosensory genes of *M. alternatus* that were mainly expressed in male and female antennae. The gene CSP5 was expressed and purified successfully in this study and used in competitive binding assays to test the binding affinity of its recombinant to 13 pine volatiles. The results indicate that CSP5 essentially functions in semiochemical perception and sexual maturation in *M. alternatus*.

Materials and methods

Insect rearing and tissue collection

Insects

Dead *Pinus massoniana* trees naturally infested with *M. alternatus* larvae were harvested in Yichang, Hubei, China (110°29E; 30°70N) in November and December of 2013. No specific permits were required for the field studies. The locations sampled were not privately owned or protected in any way, and this field study did not involve endangered or protected species. Trees were placed in indoor cages in April 2014 and emerging *M. alternatus* adults were collected daily until early August. Adults were reared on *P. massoniana* twigs and were kept in a cage at 25°C with a photoperiod of 14 h illumination and 10 h of dark.

RNA extraction and cDNA synthesis

Total RNA was extracted from ten antennae and heads of male and female *M. alternates* using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and for reverse transcription, the RNA concentration was determined using an ultraviolet spectrophotometer (Eppendorf Bio-Photometer Plus, Hamburg Germany). cDNA was prepared from total RNA by reverse transcription using an RT-PCR kit (Takara Code: DRR066A), (Promega, Beijing, China) according to the manufacturer's protocol.

Polymerase chain reaction

Aliquots of 1 mL crude cDNA were amplified in a Bio-Rad Gene Cycler thermocycler with gene-specific primers as follows: CSP5 F: 5-CCGGAATTCATGAAGCTTGTGGTGTTT TTGT-3 and CSP5 R: 5-CCGCTCGAGTTATATCTTGAGC CCTTCCTT-3. The *M. alternatus* gene nucleotide sequence was downloaded from the NCBI website with the gene accession number KF984162. The primers were designed using Primer Premier (Singh *et al.*, 1998). The restriction enzyme sites for *Eco*R1 and *Xho*I in the forward and reverse primers are underlined, respectively. The PCR conditions consisted of an initial 3 min step at 94°C followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min and a final 10 min step at 72°C (Li *et al.*, 2015).

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Cloning and sequencing

PCR products were ligated into a pMD-18T vector using a 1 : 5 (plasmid:insert) molar ratio and were incubated 0.5 h at 16°C. Ligation products were transformed into DH5α *Escherichia coli* competent cells and sequenced by Quintara Biosciences (Wuhan, Hubei, China). We then analyzed sequences using clustalW (Thompson *et al.*, 1994). The clones containing target PCR product were grown in lysogeny broth (LB) solid medium with 10 mg/ml ampicillin. Positive colonies were selected and grown in LB liquid medium with ampicillin. Afterwards, these were purified and sequenced (Zheng *et al.*, 2016).

Cloning of CSP5 in expression vectors

The pMD-18 T plasmid containing positive clones and pET-30a plasmid were digested with *Eco*R1 and *Xho*I restriction enzymes for 3 h at 37°C. Digested products were separated on an agarose gel. Target fragments were purified and ligated into a digested pET-30a plasmid; recombinant plasmids were transformed into DH5α *E. coli* competent cells and grown on LB solid medium with 10 mL kanamycin (50 mg ml^{-1}). Selected colonies were grown in LB liquid medium with kanamycin and then were purified and sequenced. BL21 (DE3) pLysS *E. coli* competent cells were transformed with correct recombinant plasmids. A single clone was identified and cultivated overnight in LB liquid medium that included kanamycin, on a shaker at 200 rpm and 37°C. The resulting plasmids were sequenced and were found to encode the mature proteins (Zheng *et al.*, 2016).

Recombinant protein expression and purification

A single positive clone was used to inoculate 5 ml Luria–Bertani broth containing 50 μg ml^{-1} kanamycin overnight at 37°C. The culture was diluted to 1 l^{-1} in fresh medium, and bacteria were cultured for 2–3 h at 37°C until the culture reached an optical density of 0.6–0.8 at 600 nm. Protein expression was induced by the addition of isopropyl-β D-thiogalactopyranoside to a final concentration of 0.5 mM. Cells were grown for another 4 h at 37°C after centrifugation; the pellet was collected and suspended with 0.1 mol l^{-1} phosphate-buffered saline buffer (pH = 7.4). Then after ultrasonic processing and centrifugation, the supernatant and inclusion body were analyzed by SDS-PAGE. Most of the proteins were expressed in the supernatant. The recombinant protein was purified by HisTrap affinity column (GE Healthcare, Piscataway, NJ, USA) and then desalted by HiTrap desalting column (GE Healthcare). The His-tag was removed by recombinant enterokinase (Novagen, Germany) and this was followed by a second purification process on a HisTrap affinity column and desalinated again; proteins were visualized at all stages of the purification by SDS-PAGE. The concentration of CSP5 was determined using the Bradford method (Kruger, 2002).

Fluorescence binding assays

Fluorescence binding assays were performed to determine the binding affinity of CSP5 for various volatiles using 1-NPN as a fluorescent probe (Zhang *et al.*, 2013). The 1-NPN and all pine volatiles were purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored according to manufacturer's instructions. All ligand stock solutions were prepared in spectrophotometric grade methanol. To measure the binding constants for 1-NPN, a 2 mM solution of protein in 30 mM Tris-HCl, pH 7.4 was added to aliquots of 1 mM 1-NPN, pH 7.4 at room temperature. The 1-NPN/protein mixture was excited using an excitation wavelength of 337 nm, and the fluorescence intensity was recorded between 360 and 600 nm using a RF 5301 PC fluorescence spectrophotometer (Shimadzu, Kyoto, Japan) with a 1 cm light path and a quartz cuvette. The saturation curves of the binding of 1-NPN by CSP 5 were constructed, and the dissociation constant, Kd, of the binding reaction was calculated by performing a Scatchard analysis of the data using Prism 5 software (GraphPad, La Jolla, CA, USA). The binding analyses were performed based on the assumption that the protein had 100% activity and that the stoichiometry of binding was 1 : 1 at saturation. The affinity of various volatile ligands was measured in competitive binding assays. Aliquots of competitor ligand were added to a sample containing 2 μM CSP5 and a standard concentration of 1-NPN. A reduction in the relative fluorescence intensity indicated that the competitor displaced 1-NPN from the binding site of CSP5. Binding data were collected during three independent measurements (Wei *et al.*, 2008). The Ki, which represents Kd of the competitor, was determined based on the IC_{50} value (the concentration of competitor that halved the initial fluorescence level). The Ki was calculated according to the following equation: Ki = [IC50]/1 + [1-NPN]/K1-NPN, where [1-NPN] is the free concentration of 1-NPN and K1-NPN is the Kd of the 1-NPN–CSP5 binding reaction determined in the Scatchard analysis (Campanacci *et al.*, 2001). To visualize the analysis more easily, we calculated 1/Ki × 1000, for which a bigger value indicates a stronger binding capacity (Li *et al.*, 2015).

Quantitative PCR

Total RNA from each tissue was extracted with Trizol reagent (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were measured with a NanoDrop™ 1000 spectrophotometer (ThermoScientific, Waltham, MA, USA). The cDNA synthesis was performed with 1 μg of total RNA Using PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Dalian, Liaoning, China) following the protocols (Zeng *et al.*, 2015). Briefly, genomic DNA was eliminated with gDNA eraser at 42°C for 2 min. The reverse transcription reaction was carried out at 37°C for 15 min and then the reaction was stopped at 85°C for 5 s. RACE templates (5′ and 3′) were synthesized with a SMART RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocols. The synthesized cDNA templates were stored at −20°C. Expression levels were determined using the relative $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak, 2008). Three independent biological replicates were performed for each tested item. All qPCR data were statistically analyzed using one-way analysis of variance (ANOVA) followed by LSD multiple comparison analysis in SPSS (SPSS Inc., Chicago, IL, USA). The primers are given in table S1.

Results

Gene expression pattern

We conducted qPCR analysis on antennae from different developmental stages (0-day-old antennae, 0dAt; 5-day-old

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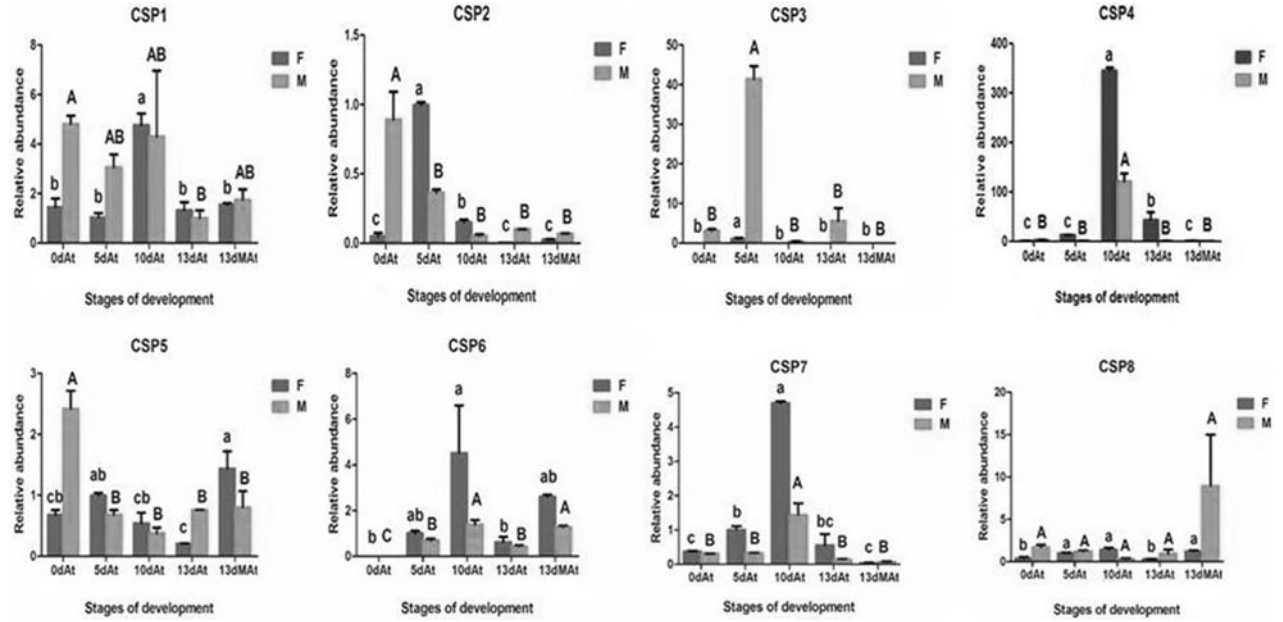

Fig. 1. The expression patterns of *Monochamus alternatus* CSP1-8. (F, female; M, male; At, antennae; Mat, mated antennae) in different development stages of *M. alternatus* adults evaluated by real-time qPCR. The column diagram is about the expression quantity at different development stages. The peak of expression levels of tested genes in development stages was marked small letters to show the difference among females ($P < 0.05$) and capital letters to show the difference among males ($P < 0.05$). β-ACTIN transcripts levels was used as an internal reference gene. The data showed that the mean values ± standard error of three biological replicates.

antennae, 5dAt; 10-day-old antennae, 10dAt; 13-day-old antennae, 13dAt, 13-day-old mated antennae; 13dMAt) of both males and females. The RT-qPCR results showed that *CSP1* had almost the same expression levels for all stages of adults in both males and females with few exceptions. *CSP2* had a higher expression level in males at 0 and 13 days old after mating but the expression was higher in females at 5 days old. *CSP5* had higher expression in males at 0 days old, and *CSP4*, *CSP6*, and *CSP7* had a higher expression in females at 10 days, while *CSP8* had higher expression in males after mating at 13 days old. The expression level of *CSP3* and *CSP4* varied a lot between male and female adults. CSP3 expression level was higher in 0-, 10-, and 13-day-old adults and highest in 5-day-old male unmated adults. However, this gene was poorly expressed in 13-day-old mated male and female adults. Expression levels of CSPs in antennae were affected by age, although without a clear pattern, analysis of gene expression differences indicated that expression of the tested genes was higher in female antennae than in male antennae. Significant differences between male and female expression levels were seen for *CSP2* at 0 days ($P < 0.01$); *CSP3* at 5 days ($P < 0.01$), *CSP4* at 10 days ($P < 0.05$), and *CSP7* ($P < 0.05$) at 10 days; *CSP1* ($P < 0.05$) and *CSP5* ($P < 0.05$) at 0 days (for unmated adults); and *CSP8* ($P < 0.01$) at 13 days (for mated adults). In this study, although each CSP showed different expression pattern during the developmental process, some of them exhibited similar expression levels in the same stage. However, relative expression was significantly affected by mating status. The expression levels of all tested genes in unmated females were higher than in mated females at different developmental stages except for *CSP3*, *CSP4*, and *CSP6* at 0 days, which showed no differences in expression between unmated and mated females (fig. 1). In our study, most of the tested genes showed the highest expression in antennae, consistent with this being the main sensory organ in *M. alternatus*, and suggesting an olfactory role for these genes. Therefore, this study only presents the expression of certain genes in main tissues especially in antennae at different developmental stages in both males and females.

Competitive binding assays

Out of all genes, the *CSP5* gene was expressed and purified successfully. There were four conserved cysteines in CSP5, which met the characteristics of a CSP family. The open reading frame (ORF) of *CSP5* was 390 bp and encoded four conserved cysteines; it was deduced that *CSP5* encodes 129 amino acids with a calculated molecular weight (MW) about 15.03 kDa, and its isoelectric point (*p*I) is 7.54.

Purified CSP5 was digested by enterokinase and used in binding assays (fig. 2). Ligand-binding affinity of the recombinant CSP5 was determined by a competitive fluorescence binding assay. First, by titrating CSP5 with increasing concentrations of 1-NPN, a saturation and linear Scatchard plot was observed, indicating that 1-NPN was a suitable fluorescent reporter. The Kd value of 1-NPN with CSP5 was 5.31 and 4.37 μM at pH 5.0 and 7.4, respectively, which was calculated from the binding curve (fig. 3). A total of 13 compounds including reported pine plant volatiles were selected to measure the binding characteristics of the CSP5. Most of the plant volatiles at concentrations up to 20 μM succeeded in displacing 50% 1-NPN from the CSP5/1-NPN complex. The results showed that CSP5 exhibited strong binding affinities to (+)-β-pinene, α-terpinolene, and (+)-α-pinene with dissociation constants 22.28, 23.8, and 23.97 μM, respectively, at pH 7.4. Whereas S-(−)-limonene, 2-methoxy-4-vinylphenol and terpinolene have relatively weak binding affinities with dissociation constants 590.92, 216.72, and 66.27 μM, respectively, at pH 7.4.

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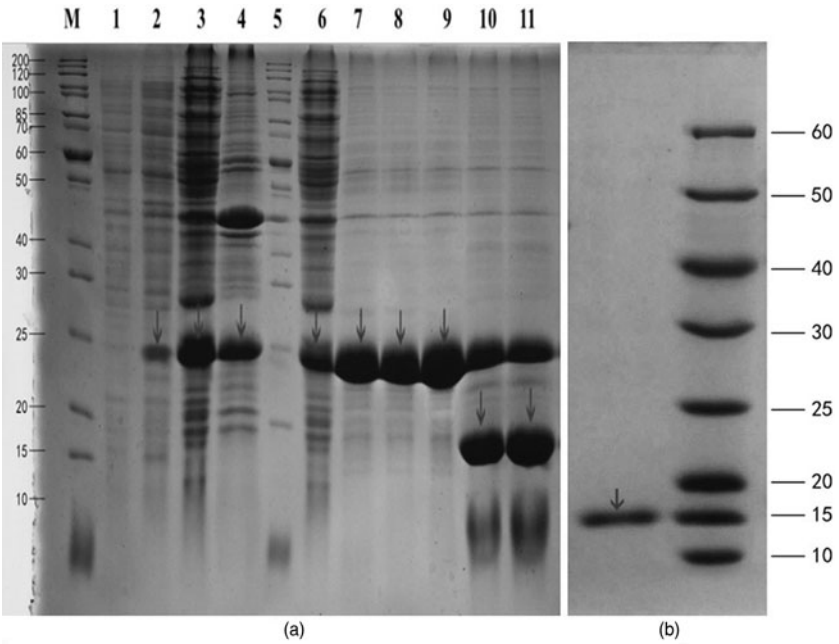

Fig. 2. (a) SDS-PAGE analysis of the sample of recombinant CSP5. The aim protein was pointed out with red arrows. In (a), M, marker; 2, the control with optical density reached to 0.6–0.8; 3, non-induced, after adding IPTG (isopropyl β-D-1-thiogalactopyranoside and shaking 4 h; 4, induced, the supernantant with protein expressed in supernatant; 5, purified, the pellet before running the elution buffer; 6–9, after running the elution buffer to remove the other extra protein in order to get clearer bands; 10–11, after the digestion with enterokinase at 23°C for 16 h. In (b), 15.03 kDa, target protein; M , marker.

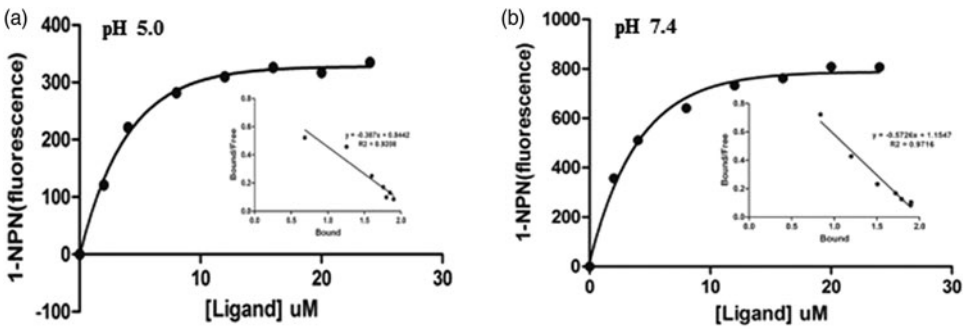

Fig. 3. Graph of the binding of the fluorescent probe 1-NPN to purified CSP5 (outer) and relative Scatchard plot analyses (inner). Protein solutions (2 μM in Tris buffer, pH 7.4 and pH 5.0) were titrated with 1 mM solution of 1-NPN in methanol to final concentrations of 2–20 μM. Calculated dissociation constants were: CSP5 (pH 7.4) 4.37 μM, CSP5 (pH 5.0) 5.31 μM.

However, three volatiles, myrcene, (+)-β-pinene, and (−)-isolongifolene, were found to have strong binding affinities with dissociation constants of 5.94, 7.03, and 8.12 μM at pH 5.0 (fig. 4). At pH 5, 2-methoxy-4-vinylphenol, p-cymene, and (+)-limonene oxide showed very weak binding affinities with a dissociation constant of 509.76, 321.73, and 38.89 μM. CSP5 had a stronger binding affinity with volatiles at pH 5.0 than at pH 7.4. All binding constants and IC_{50} values of chemicals are listed in table S2.

Discussion

This study is the first report on the expression patterns and ligand-binding properties of CSPs in *M. alternatus*. As described above, a large number of CSPs have been identified in some insect orders, e.g. Lepidoptera, Hymenoptera, Blattaria, Phasmatodea, Orthoptera, Diptera, and Hemiptera. However, no CSPs have been identified and reported in *M. alternatus* to date. In this study, we have cloned, expressed, and purified CSP5 successfully. There were four conserved cysteines in CSP5, which met the characteristics of a CSP family. The four conserved cysteines formed two disulfide bridges that can brace a narrow hydrophobic channel, which is appropriate to bind ligands with long alkyl chains (Lartigue *et al.*, 2002). The ORF of CSP5 was 390 bp and it was deduced that CSP5 encodes 129 amino acids with a calculated MW of about 15.03 kDa. CSPs play an important role in olfactory recognition. An investigation of the expression pattern of CSPs is necessary to elucidate their functions (Gu *et al.*, 2012). Therefore, in this study, *M. alternatus* CSP5 mRNA levels were monitored

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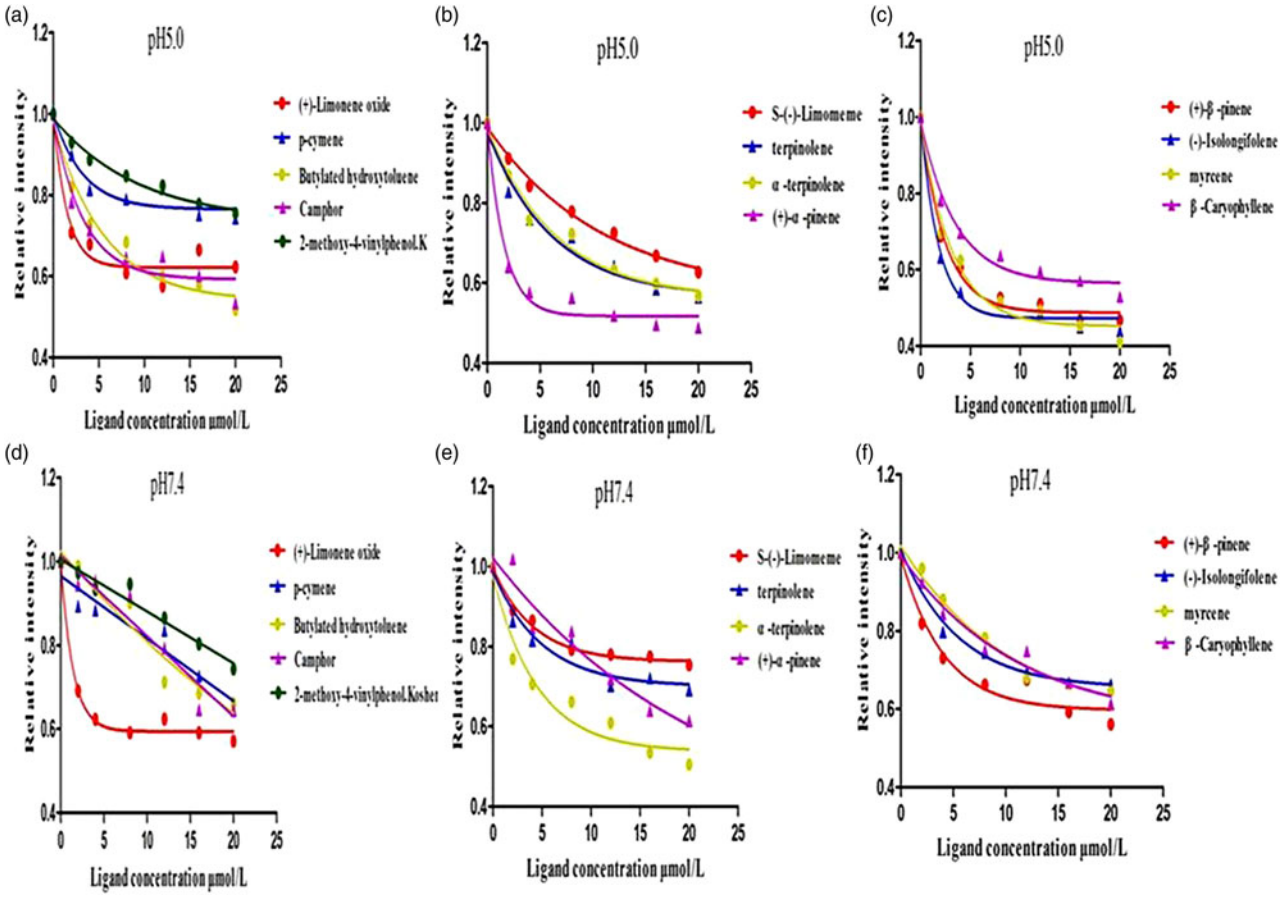

Fig. 4. Competitive binding curves of selected ligands to CSP5 at pH 5 (a), pH 5 (b), pH 5 (c), pH 7.4 (d), pH 7.4 (e), pH 7.4 (F). A mixture of protein and 1-NPN, both at 2 μM in Tris buffer, pH 7.4 and pH 5.0, was titrated with 1 mM solution of each ligand in methanol to final concentrations of 2–20 μM.

in developmental stages in antennae of males and females. CSP5 of *Helicoverpa armigera* is also typically expressed in male and female antennae (Zhang *et al.*, 2013). However, CSP5 was expressed more in males than in females. Our results are also similar to CSP19 from *Sesamia inferens* in which SinfCSP19 was predominant in antennae and significantly higher in males than in females (Zhang *et al.*, 2014).

This suggests that this gene may be involved in odorant recognition in *M. alternatus* (de Santis *et al.*, 2006). High expression levels of CSP genes in the early developmental stages of unmated *M. alternatus* females and males might help in locating suitable mates, while high expression of CSPs in mated females might help in locating plant hosts for oviposition and feeding offspring. Taken together, our results indicate that CSPs are to some extent ubiquitously expressed in adults during the entire life cycle. This suggests that, at least for some of them, are involved in functions other than chemosensing, including development. However, additional experiments, such as gene knockdown studies and site-directed mutagenesis, are required for further verification of their physiological functions.

Some previous studies have reported that the CSP gene can adjust the solitarization and gregarization of *Locusta migratoria* (Guo *et al.*, 2011). Male-based expression of CSP3 may have a broader physiological function, which needs to be confirmed in a further study. Many CSP genes have been identified so far, most of them were expressed in different parts of the insect body, and some were even expressed in non-chemosensory organs (Jacquine-Jolly *et al.*, 2001; Gong *et al.*, 2007). The binding assay is an important way to study the binding affinity of olfactory proteins with small molecules (Ban *et al.*, 2002). Olfaction is important for insect survival and reproduction, and CSPs are thought to play a crucial role in olfaction by selectively binding and transporting odorants to the surface receptors of olfactory neurons. The odorants consist of 8–9 carbon atoms in a main chain and two odorants with the same shape containing an aldehyde functional group could bind with CSPMbraA6 of *Mamestra brassicae* (Campanacci *et al.*, 2003). The special binding affinity with an aldehyde functional group suggested that the protein may have a suitable binding site for a functional group. The results suggest that the volatiles myrcene, (+)-β-pinene, and (−)-isolongifolene may play an important role in the chemoreception of *M. alternatus* with their host plants, but further study is needed to confirm this. Competitive fluorescence binding assay demonstrated that CSP5 displayed high binding affinities with plant volatile myrcene, (+)-β-pinene, and (−)-isolongifolene, suggesting that CSP5 plays olfactory roles through binding and transporting

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the plant volatiles. Our results demonstrate that *M. alternatus* CSP5 could bind to a range of ligands, depending on the nature of the ligand and the pH of CSP5. Fluorescence binding assays showed that *M. alternatus* CSP5 exhibits a high binding affinity to most pine volatiles, suggesting that volatiles may be sensed by beetles as a signal for host and oviposition location. It has been confirmed that pH-dependent conformational changes are involved in the binding and releasing of CSPs to odorants (Damberger *et al.*, 2000; Horst *et al.*, 2001; Michel *et al.*, 2011). CSP5 has a higher binding affinity with volatiles at pH 5.0 compared with pH 7.4 which further confirms the results of Li *et al.* (2013). The pH-related conformational changes indicate diverse ligand-binding and releasing mechanisms in insects. Given the sequence and functional diversity of insect CSPs, different CSPs may operate through different mechanisms in binding and releasing odorants (Pesenti *et al.*, 2009). Insects may use volatile chemicals to find mates, food, and oviposition sites. Research in identifying proteins associated with volatile chemicals will help their use in mass trapping techniques for pest control in both organic and integrated agro-eco systems. Knowledge on volatile cues can also help in the development of new innovative plant protection strategies.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485318000123.

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Author contributions

SA, LN, and MQW designed the experiments. SA and LN performed the experiments. MZA, SAIA, and MQW contributed to the date analysis. SA, MZA, and MQW wrote the manuscript.

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