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Chemosensory proteins of the eastern honeybee, *Apis cerana*: Identification, tissue distribution and olfactory related functional characterization

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ABSTRACT

Chemosensory proteins (CSPs), a class of small soluble proteins, are thought to be involved in insect chemoreceptive behavior. Here, six CSP genes, AcerCSP1–6 from *Apis cerana*, were cloned and characterized from worker bees' antennae. Results revealed that the AcerCSPs' amino acid sequences shared high similarity with the homologous genes of *Apis mellifera*, but low similarity with other insect species. Compared with corresponding CSPs of *A. mellifera*, AcerCSPs (1, 3, 4, and 6) exhibit quite similar gene expression profiling. On the contrary, AcerCSP2 showed a higher expression level in the forager antennae and legs than CSP2 of *A. mellifera*. Furthermore, AcerCSP5 was not specifically expressed in larvae, unlike CSP5 of *A. mellifera*. In a ligand-binding assay, AcerCSP1 and AcerCSP2, which exhibited the highest expression in antennae of *A. cerana*, had a stronger affinity with candidate floral chemicals and pheromones than AcerCSP4, the results of which was supported by docking analysis, suggesting that the relevance of them with *A. cerana* olfactory functions. Taken together, these results suggest that despite the quasi-similarity of protein sequences between *A. cerana* and *A. mellifera*, differences in tissue expression and functional characteristics between the two species still exist, indicating that homologous proteins potentially perform different tasks even in related species.

1. Introduction

Insects can recognize volatile cues through chemical communication that confer the capacity to detect food, predators, and mates. The understanding of the biochemical mechanisms that mediate this chemoreception in insects can be greatly improved with the discovery of olfactory and taste related proteins (Pelosi et al., 2006). In the hemolymph of the chemosensilla of insects there exist two known classes of small soluble proteins: odorant-binding proteins (OBPs) and chemosensory proteins (CSPs). Except that some involved in taste and a few in neither (Galindo and Smith, 2001), OBPs are always specially expressed in olfactory tissues and thought to sense the special odors like pheromones and plant volatiles underlying the olfactory function (Leal, 2013). Various CSPs are known to be ubiquitously expressed in insects and shown to be related to larval development, carbon dioxide detection, tissue regeneration, and so on (Pelosi et al., 2006). In spite of this, some CSPs are highly distributed in the chemosensory organs like antennae and legs in a wide range of insect species, and are believed to be involved in odor recognition and chemical communication (Sanchez-Gracia et al., 2009), while their exact physiological function and mechanism still remains elusive.

Olfactory specific-D (OS-D) related proteins, as CSPs were called in early parlance, were first identified from the antennae of *Drosophila melanogaster* (McKenna et al., 1994). With the development of comprehensive surveys of the genome, in addition to transcriptome and expressed sequence tag (EST) databases (Zhou et al., 2006), more and more CSP families and their expressions and biochemical functionality in numerous insect species have been described (Sanchez-Gracia et al., 2009; Xu et al., 2009; Yi et al., 2014). Data has revealed that some CSPs or CSP-like genes are expressed not only in the main olfactory organs, the antennae (Zhang et al., 2009), but also in the legs (Picimbon et al., 2001), proboscis (Liu et al., 2014; Nagnan-Le Meillour et al., 2000), pheromone gland (Dani et al., 2011), and wings (Zhou et al., 2008), as well as in all other components of the insect body (Gong et al., 2007). On the other hand, the ligand-binding assays are usually used to characterize the binding affinity between CSPs and various ligands from different sources, such as plant volatiles (Yi et al., 2014), cuticular hydrocarbon (Ozaki et al., 2005), cuticular lipids (Gonzalez et al., 2009) and brood pheromone (Briand et al., 2002). These extraordinarily complex expression profiles and binding functionality suggest that CSPs might play an intricate role in the chemosensory systems of insects.

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In the western honeybee (*Apis mellifera*) genome (The Honeybee Genome Sequencing Consortium, 2006), all CSP gene families (CSP1–6) have been identified and their gene expression profiles have been characterized by using semi-quantitative approaches (Forêt et al., 2007). Moreover, a number of CSPs have been found to have physiological functions, such as CSP3 in brood pheromone transportation (Briand et al., 2002) and CSP5 in embryonic development (Maleszka et al., 2007). The binding characteristics of CSP1–4 with candidate ligands such as β-ionone have also been identified via competitive fluorescence assays (Dani et al., 2010). It revealed that the binding functions of *A. mellifera* CSP family, and provided the precondition for the comparisons of binding functional CSP homology between two bee species to some extent in our studies.

As another unique honeybee species that developed in East Asia, *Apis cerana* is an indigenous and economically important bee species in Chinese apiculture. *A. cerana* has many unique characteristics comparing to *A. mellifera*. For instance, it was thought to exhibit a sensitive chemosensory capability that is able to sense a ectoparasitic mites, *Varroa destructor* (Peng et al., 1987), which shifted to the new host *A. mellifera* and have resulted in the great economic loss in the apiculture of the world in recent decades (Cook et al., 2007). In addition, *A. cerana* can pollinate the flowering plants blooming in early winter in Chinese mountainous areas, relying on its sensitive olfactory selection to those floral volatiles and adaptation to the low temperature (Tan et al., 2012).

In previous studies, only one CSP gene, AcerCSP3 (also named ASP3), has been cloned from the antenna of *A. cerana* (Li et al., 2007) and subsequently identified to have floral volatile organic compound binding capabilities, to some extent (Li et al., 2013). However, the functional characteristics of the other CSPs in *A. cerana*, are still unknown. Here, we identified the entire *A. cerana* CSP gene family and compared their similarity and difference on development and tissue distributions between *A. cerana* and *A. mellifera*. The olfactory related functions of three recombinant AcerCSPs proteins were further studied by a competitive fluorescence assay and then verified by molecular docking analysis. Our studies will be helpful for the increasing knowledge about the comparison of chemosensory systems between the two similar bee species.

2. Material and methods

2.1. Experimental insects

Local *A. cerana* colonies were maintained in Hangzhou (eastern China) Langstroth hives. Bees of known ages were identified by marking newly emerged bees from a frame of capped brood kept in an incubator, with the bees being returned to the hive and collected when required. The developmental stages of workers were classified according to the methods of (Michelette and Soares, 1993; Rachinsky et al., 1990). For the transcriptional pattern, the material of 100 worker eggs, 3 larvae and 3 pupae and the antennae, head, thorax, abdomen, legs, and wings from 50 18-day-old adult worker bees of *A. cerana* were immersed in liquid nitrogen and prepared for the following total RNA isolation.

2.2. Chemicals and odors

All of the chemicals were purchased from diverse supplied chemical companies and the details are listed in Supplementary File 1. These include the fluorescent reporter, N-phenyl-1-naphthylamine (1-NPN); the floral volatiles, β-ionone, 3,4-dimethylbenzaldehyde, phenylacetaldehyde, methyl salicylate, methyl eugenol, geraniol, linalool, L-(−)menthol; the pheromones, mthyl-p-hydroxylbenzoate (HOB) and homovanillyl alcohol (HVA), farnesol, nerol, 2-heptanone and isoamyl acetate; the brood pheromone components, methyl palmitate, ethyl palmitate, ethyl oleate, ethyl stearate, methyl oleate, methyl stearate; and other organic acids: myristic acid and palmitic acid. Milli-Q water (18.2 MΩ) was purchased from Millipore (Bedford, MA) and methanol (HPLC grade) from TEDIA (TEDIA, US). All other chemicals used were of analytical grade or better.

2.3. Amplification of cDNA of genes encoding CSP families of A. cerana

AcerCSP1, 2, 4, and 6 were discovered from the unigene annotation of the transcriptome of *A. cerana* (Wang et al., 2012). Adding known AcerCSP3 discovered by us before (Li et al., 2007), the full-length open reading frame primer pairs of AcerCSPs families were designed based on CSP1–6 of *A. mellifera* (GenBank accession No. DQ855482~7). Full-length sequences of CSPs were amplified and cloned into pGEM-T vector (Promega), then was transformed into *Escherichia coli* JM109 competent cells. Positive colonies, screened for the presence of the insert by restriction analysis by using BamH I and EcoR I, were sequenced by

BL21(DE3) (Novagen, DE) competent cells. Three positive clones of each target gene were sequenced by the sequencing department of Invitrogen Company (Shanghai, China). Recombinant proteins in competent cells were induced upon addition of 1 mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG). After sonication of the bacterial pellet and centrifugation, all the proteins were present in the supernatant. The recombinant proteins with Hig-tag in the N-terminal were purified by Ni^{2+}-NTA affinity chromatography, then digested by enterokinase, and purified the 2nd round by Ni^{2+}-NTA affinity chromatography according to the GE Healthcare manual, and then all the eluent including untagged recombinant proteins were repeatedly dialyzed in triplicate by the PBS buffer (pH 7.4) until all the imidazole from the chromatography was eliminated. Finally, the protein solutions were quantified using the standard Bradford measuring method for the following functional process.

2.6. Fluorescence binding assays

N-phenyl-1-naphthylamine (1-NPN) was dissolved in methanol to yield a 1 mM stock solution. Spectra were recorded with a scan speed of 300 nm/min and three accumulations. The slit width used for excitation and emission was 10 nm. All ligands used in competition experiments were dissolved in HPLC purity grade methanol. Binding data were collected as three independent measurements. Concentrations of competitors that caused a reduction of fluorescence to half-maximal intensity (IC_{50} values) were taken as a measure of binding dissociation constants were calculated from the corresponding IC_{50} values using the formula: $K_D = [IC_{50}] / (1 / [1\text{-NPN}] / K_{1\text{-NPN}})$ (Gu et al., 2012), [1-NPN] being the free concentration of 1-NPN and $K_{1\text{-NPN}}$ being the dissociation constant of the complex AcerCSPs/1-NPN, which was calculated from the binding curve using the computer program software Origin 8.0 Trial (OriginLab Inc.). The differences between K_D values of all candidate chemicals were analyzed by One-way ANOVA tests (post hoc multiple comparisons applied as equal variances assumed of LSD and S-N-K test, $p < 0.01$ is shown as two stars, $0.01 < p < 0.05$ is shown as one star). The calculated K_D values are listed in Supplementary File 3. K_D.

2.7. Molecular docking

The predicted 3D crystal structure of AcerCSPs (1, 2, 4) was generated by homology modeling on SWISS-MODEL Workspace (Guex et al., 2009). Based on the QMENA4 value retrieved from the modeling results, we used CSPMbraA6 of *Mamestra brassicae* (PDB ID: 1N8V) (Campanacci et al., 2003) as template for homology modeling. The 3D structure of methyl oleate was downloaded from PubChem database (CID 5364509, http://pubchem.ncbi.nlm.nih.gov/). To study the binding mode of three AcerCSPs and methyl oleate, Molegro Virtual Docker 4.2 software was used for molecular docking analysis. The best binding pose was obtained according to the searching algorithm of MolDock optimizer and energetic evaluation of the complex with MolDock. The binding pose was also analyzed and displayed by MVD software.

3. Results

3.1. cDNA sequence of AcerCSPs

Blast analysis and unigene annotation of *A. cerana* transcriptome (Wang et al., 2012) revealed that AcerCSPs share a high similarity with the CSPs of *A. mellifera*, based on the low E-value between the corresponding CSPs of the two bee species. Therefore, we directly designed specific full-length primers to amplify the open reading frames of the cDNA sequences. After cloning and sequencing, the cDNA sequences were obtained and submitted to GenBank (accession no. seen in Table 1). Their signal peptides and the mature amino acid sequences were then predicted by SignalP 4.1 software (Fig. 1). Multiple sequence alignments of the deduced AcerCSP sequences were performed against homology sequences of other insects by ClustalW program. As can be seen from Fig. 1, all AcerCSPs exhibit conserved insect CSPs' features, such as four cysteine residues forming two disulfide bridges and three alpha-helix domains. Using the proteomics server ExPASy, the theoretical isoelectric point and molar weight of mature AcerCSPs were separately computed (Table 1). These characteristics also revealed the similarity of AcerCSPs to other members of the insect CSP gene family.

3.2. Phylogeny of AcerCSPs

Based on the analysis of the evolutionary tree by using the neighbor-joining method (NJ, condensed tree, cutoff value is 50%) of deduced AcerCSPs, we divided six AcerCSPs into two distinct branches of the evolutionary tree; AcerCSP2 and AcerCSP5 were grouped together, while the other four AcerCSPs, AcerCSP1, 3, 4, and 6, were grouped together (Fig. 2). As seen from Table 1, AcerCSPs demonstrate high similarities to Amel-CSPs (94.5–99.2%), whereas they share low similarities with CSPs from other insect species. For example, compared with most of the other homologies, AcerCSP1–6 was only similar to MrotEBSP1 (57.8%), SexiCSP2 (11.1%), SexiCSP4 (41.4%), TcasCSP10 (50.0%),

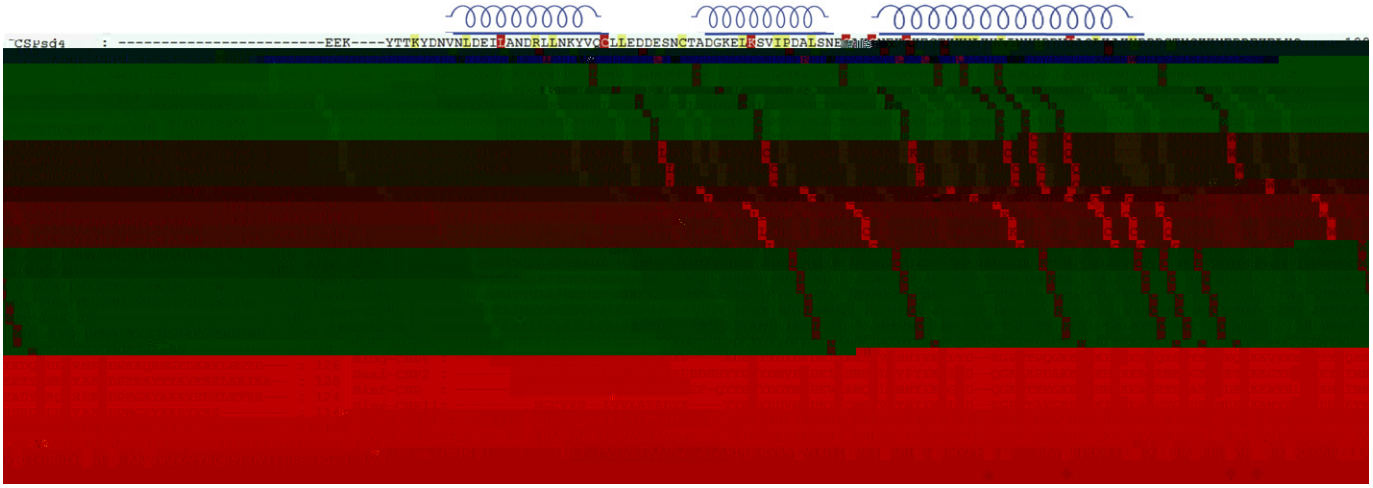

Fig. 1. Sequence alignments of AcerCSPs with similar sequences of CSPs from other insect species. Shadowed amino acid residues represent the N-terminal signal peptide sequence. Residues common to most sequences are highlighted. Three predicted alpha-helix motifs are separately shown as helical curves above the corresponding amino acid sequences. The 4 conservative Cys are labeled below by the blue rhombus.

GmorCSP5 (12.5%), and DpleCSP (39.8%), implying that AcerCSP family sequences are quite unique and further indicating an evolutional conservatism of CSPs in Apidae.

3.3. Variant transcriptional pattern of AcerCSPs in different organs

To identify the transcriptional patterns of AcerCSP1–6 in 10 different tissues of *A. cerana*, real time PCR assays were performed and analyzed using the method of relative ($2^{-\Delta\Delta Ct}$) quantitative, using β-actin cDNA of *A. cerana* as positive control. As seen from Fig. 3, in all developmental

binding characteristics of candidate ligands to each CSP. When excited at 282 nm, all AcerCSPs displayed a maximum emission peak at about 340 nm (Fig. 5-A). The corresponding Scatchard plots of the three AcerCSPs binding with 1-NPN showed perfect linear gradients with increasing concentrations of 1-NPN (Fig. 5-A, inset). The dissociation constants of AcerCSP1, 2, and 4 with 1-NPN were calculated as 1.24, 1.25, and 2.99 μM, respectively.

Competitive binding assays were used to measure 22 potential synthetic competitors, including floral volatiles, various honeybee pheromones, and a number of compounds associated with olfactory behavior in honeybees (Borg-Karlson et al., 2003; Laska and Galizia, 2001; Le Conte et al., 2001; Pankiw et al., 2000). The competitive binding curves of a few of the representative ligands to the complex of recombinant AcerCSP1, 2, and 4 with 1-NPN were recorded (Fig. S1). Results revealed that the ligands with a high affinity for the corresponding protein always exhibited a much stronger quenching ability to 1-NPN in the complex. The correlation between the number of carbons in each ligand and the K_D is shown in Fig. 5-B. From this data, it is evident that the tested C8–C9 esters, aldehydes, alcohols, and ketones had a high affinity with all three AcerCSPs.

Furthermore, based on the IC_{50} values (the concentration of ligand halving the initial fluorescence value), the dissociation constants (K_D) for each AcerCSPs/ligand combination were calculated and compared (Fig. 6). Although the three AcerCSPs showed variation in their binding profiles, most of the chemicals tested effectively displaced 1-NPN from the AcerCSPs/1-NPN complex at concentrations up to 50 μM. In total, seven ligands bound with all three AcerCSPs: four plant volatiles (β-ionone, 3, 4-dimethylbenzaldehyde, phenylacetaldehyde, and methyl salicylate), one queen pheromone (HOB), one alarm pheromone (isoamyl acetate), and one worker pheromone (nerol). In addition, one

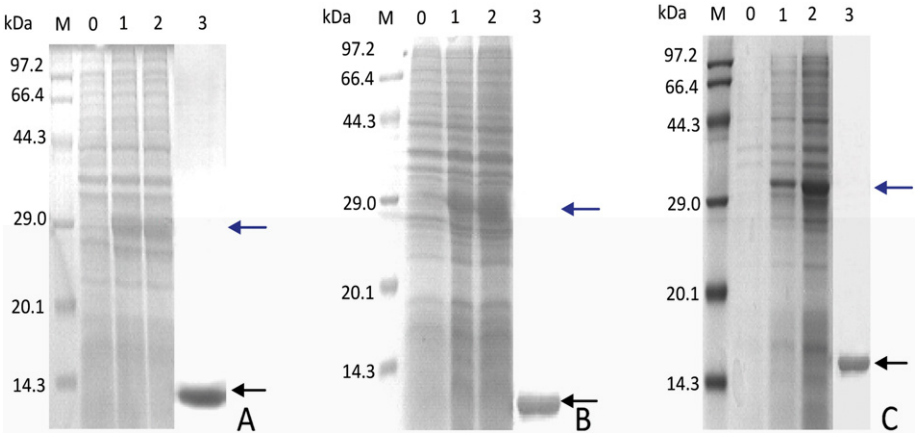

Fig. 4. SDS-PAGE analysis of expressed and purified AcerCSP1 (A), AcerCSP2 (B), and AcerCSP4 (C). M, protein molecular marker; 0, before induction with IPTG; 1 and 2, induction with IPTG (the recombinant protein bands with a His-tag at the N-terminal are identified by blue arrows); 3, purified proteins and His-tag digested target bands (as indicated with black arrows).

while one brood pheromone (methyl stearate) and one plant volatile (geraniol) bound specifically to AcerCSP2 and AcerCSP4, respectively.

AcerCSP1 had the highest affinity for the 22 synthetic competitors among the three AcerCSPs, while AcerCSP2 had a higher affinity compared to AcerCSP4 (except HOB). Taken together, this data clearly suggests that AcerCSP1 exhibits the strongest binding capability. Considering the high expression of AcerCSP1 and 2 in the antennae of *A. cerana*, it is very likely that they are involved in the chemoreceptive process of antennae in *A. cerana*.

3.6. Molecular docking analysis

Based on the separately calculated QMEAN4 values of −1.29, −1.34, and −0.24 from SWISS-MODEL Workspace, the 3D crystal structure of CSPMbraA6 of *M. brassicae* (PDB ID: 1N8V) was suggested to be optimally homologous to AcerCSPs (1, 2, 4) of *A. cerana*, with sequence identities of 37.23%, 18.39%, and 52.43%, respectively (Fig. 7D). According to the ligand binding results, we chose methyl oleate for testing the molecular docking of the three AcerCSPs. Results showed that the solid surface appearances of the three AcerCSPs were obviously different. AcerCSP1 and 2 were both tightly bound within the binding cavity (Fig. 7A, B), whereas AcerCSP4 barely bound with the ligand, owing to its half open binding cavity (Fig. 7C). Alternatively, two residues, Asn66 in AcerCSP1 and Ser63 in AcerCSP2, were predicted to exist in the ligand binding cavity to produce distinct hydrogen bonds between the amino acids and methyl oleate. By comparison, AcerCSP4 and methyl oleate lacked hydrogen bonds. These docking results help support the functional binding assays showing that AcerCSP1 and 2 have a higher affinity with more ligands than AcerCSP4.

4. Discussion

In the present study, base on the previous studies (Li et al., 2007) and the unigene annotation of transcriptomic data (Wang et al., 2012), we cloned all six of the CSP family genes from the Eastern honeybee, *A. cerana*. All the predicted amino acid sequences exhibited the typical characteristics of insect CSPs, such as the conserved four-cysteine signature (Pelosi et al., 2006). The amino acid sequences of AcerCSPs were very similar to the homologous CSPs of *A. mellifera* (94.5 to 99.2%), but less similar to other insect species (11.1 to 57.8%) (Fig. 1). The similar results are also shown as the evolutionary tree (Fig. 2), suggesting that the CSPs family of the two bee species exhibit the high sequences homology and specificity among genus. These evidences imply that the sequence of CSPs in the two kindred bee species is much conserved.

However, it was not as similar as expected that the results of tissue distribution of CSPs in the two bee species. According to the integrated comparison of CSPs gene between the two bee species (Table 2), the three CSPs (1, 2, and 3) in *A. mellifera* exhibited a complex pattern in

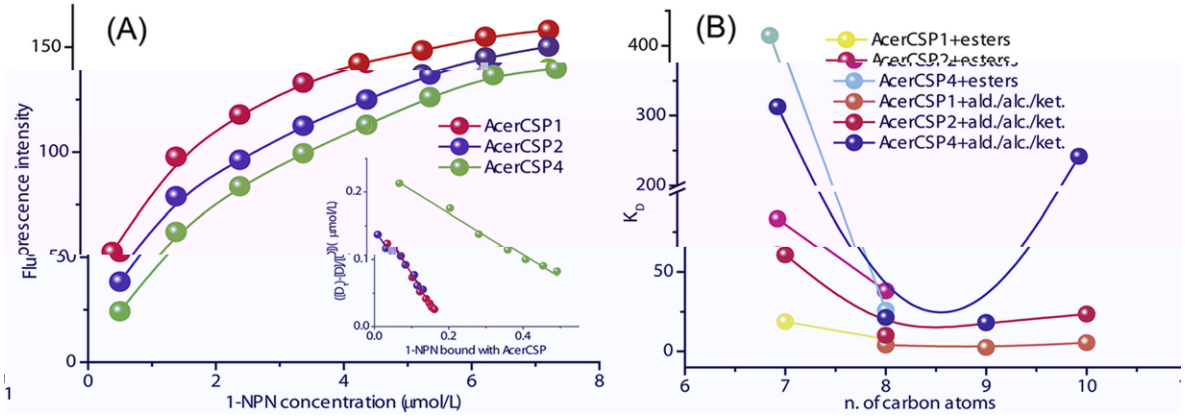

Fig. 5. Binding characteristics of recombinant AcerCSPs with candidate odors. (A). Binding curves of 1-NPN and Scatchard plots (inset). Dissociation constants (average of three replicates) were AcerCSP1, 1.24 μM (SD 0.25); AcerCSP2, 1.25 μM (SD 0.21); and AcerCSP4, 2.99 μM (SD 0.31). The chemical structures of the ligands are shown on the right. Fluorescence intensities are reported as percent of the values in the absence of a competitor. (B). The right bottom panel reports the dissociation constants with respect to the number of carbon atoms of homologous series of linear aldehydes (ald.), alcohols (alc.), and ketones (ket.) to AcerCSP1, AcerCSP2 and AcerCSP4, respectively. The candidate ligands with strongest binding affinity were found at the 8 and 9 carbon atoms.

diverse tissues throughout development by means of relative ribosomal S8 expression (Forêt et al., 2007). Alternatively, based on the more precise analysis of qPCR, it shows that all six AcerCSPs express ubiquitously in all adult tissues and throughout most of developmental stages (Fig. 3). The low sensitivity of relative quantitative method in expressional study of CSPs in *A. mellifera* might result in non-detected of CSPs in some tissues, such as CSP5 and CSP6 expressed in antennae.

The tissue distributions of other four CSPs (CSP1, 3, 4, and 6) were similar between the two bee species (Table 2). Nevertheless, there are evidently difference of two CSPs, CSP2 and CSP5 between *A. cerana* and *A. mellifera*. AcerCSP2 exhibited the highest expression levels in the two main chemosensory tissues, antennae and legs (Fig. 3, Table 2), while CSP2 in *A. mellifera* only expressed in many tissues, such as antennae, newly emerged workers, queens, and drones at low level (Calvello et al., 2005; Forêt et al., 2007). Combing this result with the ligand binding assay and docking analysis, it supports the viewpoint that AcerCSP2 is likely involved in the chemoreceptive process of *A. cerana* (Figure S1, 6, 7B).

Some CSPs in diverse insects have been characterized as having the semiochemical binding capabilities, such as CSP1, 2, and 3 (Gu et al., 2012), as well as CSP4, 5, and 6 in the alfalfa bug, *Adelphocoris lineolatus* (Sun et al., 2015); CSP1 in the oriental fruit fly, *Bactrocera dorsalis* (Yi et al., 2014); CSP4 in two moth species, *Helicoverpa armigera* and *Helicoverpa assulta* (Liu et al., 2014), etc. In this study, except that CSP3 in *A. cerana* has been characterized before (Li et al., 2011), three CSPs, CSP1, 2, and 4 in *A. cerana*, were characterized the diverse ligands binding capacities to candidate plant volatiles and bee pheromones. For

expression in antennae, the main chemoreceptive tissue (Fig. 3), and showed the strongest binding affinity with candidate chemical ligands (Fig. 5). This is similar to CSP1 in *A. mellifera*, which also has the highest expression level in CSP family (Table 2) and exhibited the capability of binding straight-chain primary alcohols and esters (Dani et al., 2010), though those compounds did not include pheromone components of *A. mellifera*.

AcerCSP1, 2 and 4 showed the regular curves between the dissociation constants and the number of carbon atoms of homologous series of linear aldehydes (ald.), alcohols (alc.), and ketones (ket.), respectively. The best candidate ligands were found at the 8 and 9 carbon atoms (Fig. 5-B). The similar regular curves were also shown in CSPs in *A. mellifera*, whereas it was different with 13 carbon atoms in the best ligands found in CSP1 and 3 in *A. mellifera* (Dani et al., 2010).

Among the CSPs family in *A. cerana*, AcerCSP1 exhibited the strongest affinity to most candidate ligands, especially to a brood pheromone, methyl oleate (Fig. 6; S3), which is thought to serve as a capping signal when emitted by larvae (Le Conte et al., 1990), but is also a synergistic component of queen retinue pheromone (QRP) (Keeling et al., 2003; Slessor et al., 2005). From the docking results between methyl oleate and three CSPs, AcerCSP1 showed one deeper cavity than AcerCSP2, and 4 (Fig. 7), which was in agreement with the results of the fluorescence reporter (Fig. 5, inset) and semiochemicals binding assay (Fig. 6). The underlying physiological role of CSP1 involved in the behavior of *A. cerana* still remains further investigation. Taken together, these data imply that AcerCSP1, as well as AcerCSP2, may be involved in the sensitive chemosensory process responding to the diverse bee pheromones of *A. cerana*.

CSP3 in both bee species exhibits similarities in antennae, heads, abdomens, and legs at high expressional level, except lower expression in larvae of *A. cerana* (Fig. 3, Table 2). In the honeybee, CSP3 is one ubiquitous protein totally expressed in most tissues at high level (Table 2). In *A. mellifera*, CSP3 has been identified to be able to specifically bind to large fatty acids and ester derivatives, which are brood pheromone components, while not to tested general odorants and other tested pheromones (sexual and nonsexual) (Briand et al., 2002). On the contrary, in our previous studies, CSP3 in *A. cerana* was able to bind to some candidate floral volatiles rather than brood pheromones (Li et al., 2011; Li et al., 2013).

Our results revealed that CSP4 from both *A. cerana* and *A. mellifera* was not expressed before eclosion. In adults, of o

bee species may not be involved in the chemoreceptive process and play a distinctly different physiological role in both honey bee species. Additional experiments related to the physiological function of AcerCSP5 are required to fully understand these differences. Interestingly, we found AcerCSP2 and AcerCSP5 to be in the same molecular phylogenic branch, whereas AcerCSP1, 3, 4, and 6 were grouped into a separate branch (Fig. 2). These findings imply that the differentiation in chemoreception between *A. cerana* and *A. mellifera* might result from CSPs genes with a closer phylogenic relationship than others.

Finally, CSP6 of *A. cerana* showed similarities with CSP6 of *A. mellifera* in that it was highly expressed in pupae (Table 2). These results indicate that CSP6 is upregulated before the imaginal molt, which implies that it may be involved in the process of eclosion in the development of honey bees. The similar upregulation of several CSPs before molting has been reported in the moth *Choristoneura fumiferana* (Wanner et al., 2005) and *Bombyx mori* (Gong et al., 2007).

To summarize, although six amino acid sequences of the CSP family of *A. cerana* exhibit high similarity with *A. mellifera*, their respective expression profiles and functional ligand-binding experiments still exhibit obvious differences between the two honeybee species. This work will be beneficial for helping to interpret the differences in the chemosensory systems of *A. cerana* and *A. mellifera*, especially in relation to their respective characteristic chemoreceptive behavior.

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