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Chemosensillum immunolocalization and ligand specificity of chemosensory proteins in the alfalfa plant bug *Adelphocoris lineolatus* (Goeze)

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Insect chemosensory proteins (CSPs) are a family of small soluble proteins. To date, their physiological functions in insect olfaction remain largely controversial in comparison to odorant binding proteins (OBPs). In present study, we reported the antenna specific expression of three CSPs (AlinCSP4-6) from *Adelphocoris lineolatus*, their distinct chemosensillum distribution as well as ligand binding capability thus providing the evidence for the possible roles that they could play in semiochemical detection of the plant bug *A. lineolatus*. The results of qRT-PCR and western blot assay clearly showed that all of these three CSPs are highly expressed in the adult antennae, the olfactory organ of insects. Further cellular investigation of their immunolocalization revealed their dynamic protein expression profiles among different types of antennal sensilla. In a fluorescence competitive binding assay, the selective ligand binding was observed for AlinCSP4-6. In ad'dition, a cooperative interaction was observed between two co-expressed CSPs resulting in an increase of the binding affinities by a mixture of AlinCSP5 and AlinCSP6 to terpenoids which do not bind to individual CSPs. These findings in combination with our previous data for AlinCSP1-3 indicate a possible functional differentiation of CSPs in the *A. lineolatus* olfactory system.

Adelphocoris lineolatus (Goeze) (Hemiptera: Miridae), the alfalfa plant bug, is well known as a notoriously worldwide pest because it is extremely polyphagous and severely destroys many important crops, including cotton (*Gossypium hirsutum* L.), alfalfa (*Medicago sativa* L.), green bean (*Phaseolus vulgaris*), maize (*Zea mays* L.), and cabbage (*Brassica spp.*)[1–3]. In early June, large amount of adult *A. lineolatus* migrate into transgenic *Bacillus thuringiensis* (Bt) cotton fields in China, and cause serious destructions of crops. These bugs, as well as other mirid species, have increasingly evolved to the most important pests of crops[4,5]. These are also promoted by *A. lineolatus* remarkable reproduction ability[6], the inefficient control of predators[3], and strong dispersal capacity[7]. To date, it is not clear why and how adult *A. lineolatus* preferentially select transgenic Bt cotton as their main host plants. This may be in part due to the adequate nutrition of Bt cotton plants and significant decreases in niche competition associated with the reduction of population densities of *Helicoverpa* spp. and *Heliothis* spp. species[8]. Recent electrophysiological and behavioral studies indicate that 3-hexanone, a plant volatile emitted by Bt cotton plants, can strongly attract female adults of *A. lineolatus*, indicating an essential role of chemical cues involved in their host plant orientation[9].

The insect chemosensory system, particularly the olfactory repertoires, has been intensively studied due to its essential function in guiding insect behaviors, such as host plant selection, mate finding, oviposition site location, and predator avoidance[10–12]. The morphological characteristics of different antennal sensilla in both sexes of adult *A. lineolatus* have been characterized used scanning electron microscopy (SEM)[13], and these different antennal sensilla have different ultrastructures based on the results obtained by transmission electron microscopy (TEM) observations[14]. Such studies may help understanding molecular mechanisms and biochemistry of insect olfaction

to design new chemicals based on the chemical interaction between the pest and crop hosts for an alternative strategy of pest management.

The high sensitivity and specificity of the insect olfactory system mainly rely on the interactions between semochemicals and different proteins expressed in the olfactory sensilla, such as the membrane-bound olfactory receptors (ORs), sensory neuron membrane proteins (SNMPs), and two types of carrier proteins, odorant binding proteins (OBPs) and the chemosensory proteins (CSPs)[15,16]. The characteristics of the carrier proteins, such as their small size, solubility, and exceptional stability, have made these proteins as the research targets to interrupt the chemical communication of insect pests with their hosts[17–19]. To date, an increasing body of evidence from both *in vitro* and *in vivo* studies has strongly demonstrated that OBPs represent indispensable biochemical elements responsible for transporting, desorption, protection and detecting olfactory stimuli in the peripheral signal coding events[9,20–26]. However, the function of CSPs remains unclear. CSPs were first discovered in the antennae of *Drosophila melanogaster* by subtractive hybridization experiment[27], and their homologues were subsequently identified in insects of different orders based on sequence similarity[28–36]. The name of these proteins was initially called olfactory specific protein D (OS-D)[27] or A10[37] and then named as chemosensory proteins (CSPs) due to their specifically expression in contact chemosensilla of antennae, tarsi, and palpi[35]. Compared with OBPs, CSPs are much smaller (10–15 kDa), have only four conserved cysteines, and display higher amino acid identity across insect species. Commonly, OBPs are considered antennae-specific, whereas CSPs are found expressed in both chemosensory tissues, including antennae[13,35,38], proboscis[36], maxillary palps[30], labial palps[30,39], legs[40], wings[41], and non-chemosensory tissues, such as pheromone gland[42,43] and ejaculatory bulb[44]. In the locusts *Locusta migratoria*, CSPs were observed to be expressed in the contact chemosensilla, such as sensilla chaetica, but are mainly labelled at the outer sensillum lymph of these sensilla; in contrast, multiporous olfactory sensilla trichodea and basiconica were not stained, suggesting a non-olfactory role[36,39]. Subsequently, these proteins were demonstrated to participate in the physiological shift of *L. migratoria* from the solitary to the gregarious phase[45]. The non-olfactory functions of CSPs among other species have also been reported such as leg regeneration in *Periplaneta americana*[46,47], embryo development of honeybee, *Apis mellifera*[48], and the unknown important roles in the ejaculatory bulb of *D. melanogaster*[44]. Thus, this has raised the question whether or not these proteins are involved in chemosensory functions in insects and contribute to the sensitivity and specificity of odorant detection. However, the results of cellular immunolocalization studies, ligand binding and three-dimensional structures suggest the putative functions of CSPs in insect olfaction. In the walking stick insect, *Carausius morosus*, a CSP CSP-cmA was found strongly labelled in the sensillum lymph of the olfactory sensilla, in which dendrite branches were suspended, suggesting the CSP may play a role in odorant transduction between the odorant receptor and the external environment, as demonstrated with other OBPs[49]. Similar results were also found in *Scleroderma guani*, a species of Hymenoptera[50], and in a Dipterans species of *D. melanogaster*[27]. The studies of three-dimensional structures and putative ligand screening have provided more direct support for their roles in olfactory recognition. For example, the crystal structure of a moth CSP of *Mamestra brassicae* (CSPMbraA6) revealed a very compact and stable structure with a hydrophobic core for putative hydrophobic chemical binding, and a fluorescence binding assay demonstrated that CSPMbraA6 is able to bind compounds with C12-18 alkyl chains of brominated alkyl alcohols and fatty acids (12-bromo-dodecanol, 15-bromo-pentadecanoic acid, 9-bromo-stearic acid)[51].

Previously, three *CSP* genes (*AlinCSP1-3*) of *A. lineolatus* were isolated by screening the antennal cDNA library, and all of these *CSP* genes exhibited a high expression in adult antennae and good binding affinities to various odorants emitted from the host plants of *A. lineolatus*, indicating a potential olfactory function[13]. Considering the multiple roles of CSPs reported in other insect species and the evidence that different olfactory proteins, such as OBPs, can contribute to the detection even discrimination of different odorants or cooperatively interact with each other, in this study, we further investigated the ligand binding and the cellular co-immunolocalization of three other CSPs (AlinCSP4-6) from *A. lineolatus*. This study reports more detailed information to better understand the functional differentiation of CSPs in Hemipteran species.

Results

Full-length cDNA sequences and expression analyses. Three full-length cDNA sequences that encode for putative chemosensory proteins were identified with Blastx and CSP motif search against the antenna cDNA library of *A. lineolatus*, named as *AlinCSP4*, *AlinCSP5*, and *AlinCSP6* and deposited in GenBank with the accession numbers of GQ477017, GQ477018, GQ477019, respectively. *AlinCSP4-6* genes contain open reading frames (ORF) of 384 bp, 384 bp, and 339 bp, respectively. The predicted amino acid sequences of AlinCSP4-6 share the conserved characteristics of typical insect CSP family, such as the typical four highly conserved cysteines and spacing between them, and the predicted signal peptides of 19, 21, and 19 amino acids at the N-terminus, respectively (Figure S1). The calculated molecular masses of mature AlinCSP4-6 proteins are 12.59 kDa, 12.59 kDa, and 13.29 kDa, respectively. The predicted isoelectric points of the mature AlinCSP4-6 proteins are 4.92, 6.92, and 5.70, respectively. The amino acid identity among AlinCSP4-6 is approximately 41% overall, and their sequence alignment with 31 CSP sequences from other insect species indicates a very divergent signal peptide region and some highly conserved amino acid residues in addition to the four conserved cysteine residues (Figure 1). It is well known that olfactory genes would be expressed highly in the olfactory tissue namely antennae. Therefore, the quantitative real time RT-PCR (qRT-PCR) was conducted to investigate the tissue distribution and relative expression levels of *AlinCSP4-6* transcripts in both male and female antennae. The results revealed that all of these three *AlinCSP* genes are significantly but not exclusively expressed in the adult antennae in both sexes (Figure 2).

***In vitro* expression and purification of AlinCSP4-6.** For further functional studies the AlinCSP4-6 proteins were expressed in *E coli.* which provided good yields of the recombinant proteins (approximately 20 mg/L). All of the recombinant proteins were purified by two rounds of Ni ion affinity chromatography, and the SDS-PAGE analysis showed that the molecular weights of the final purified AlinCSP4-6 proteins are consistent with the predicted molecular masses (Figure S2).

Specific tissue distribution of AlinCSP4-6 proteins. To characterize the immunolocalization of AlinCSP4-6 proteins among different antennae sensilla of *A. lineolatus*, polyclonal antisera against each of recombinant AlinCSP4-6 proteins were produced. A western blot assay of the purified recombinant proteins suggested that each antiserum is specific and sufficient to distinguish from one another without cross reactions because anti-AlinCSP4, anti-AlinCSP5, and anti-AlinCSP6 antibody specifically reacted with recombinant AlinCSP4, AlinCSP5, and AlinCSP6, respectively (Figure S3). The tissue specific distributions of AlinCSP4-6 proteins were then analyzed by the western blot of crude extracts from different tissues. The results showed that both AlinCSP4 and AlinCSP5 were antennae-specific in male and female, whereas AlinCSP6 was highly expressed in the antennae and legs in both sexes and weakly expressed in the male wings (Figure 3). There was no detectable

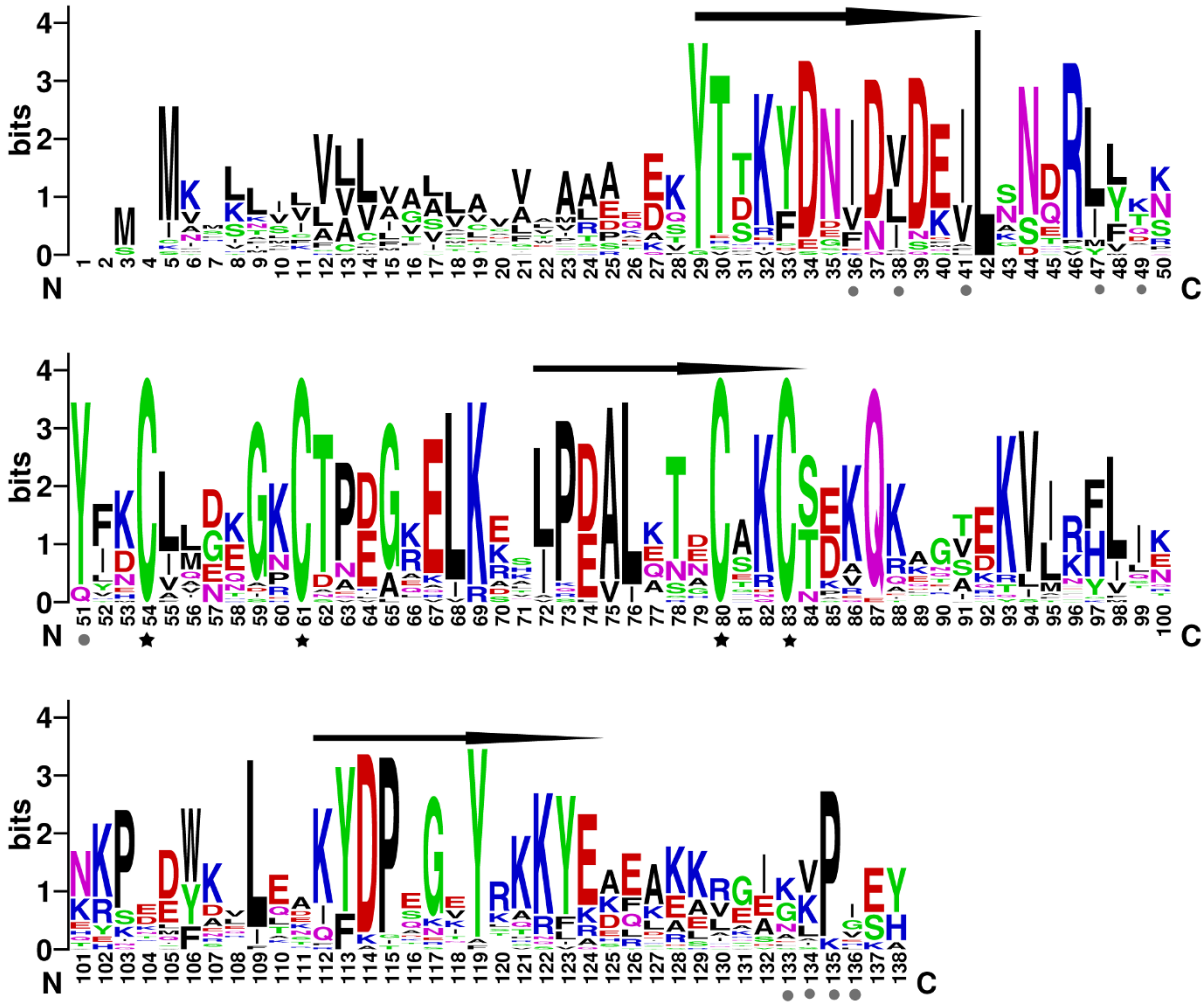

Figure 1 | Alignment of AlinCSP4-6 with CSP sequences from other insects. Each logo consists of stacks of symbols (one stack for each position in the sequence). The overall height of the stack indicates the sequence conservation at that position, and the height of the symbols within the stack suggests the relative frequency of each amino at that position. Black asterisks indicate the conserved cysteines, and gray dots show the residues involved in hydrophobic regions in MbraCSPA6[75]. The black arrows at the top of WebLogo indicate the three conserved regions among the insect CSP sequences. The insect species and GenBank accession numbers are the following: *Adelphocoris lineolatus*: AlinCSP1-6 (GQ477014-GQ477021); *Apolygus lucorum*: AlucCSP1-8 (KC136232-KC136239); *Acyrthosiphon pisum*: ApisCSP2 (CAJ01486), ApisCSP3 (CAJ01489); *Aphis gossypii*: AgosCSP2 (KC161565), AgosCSP6 (KC161568); *Nilaparvata lugens*: NlugCSP1 (HM489006), NlugCSP8 (FJ387497); *Drosophila melanogaster*: EBSPIII (U08281); *Glossina morsitans morsitans*: GmmCSP1-5 (FN432801- FN432805); *Apis mellifera*: AmelCSP2 (DQ855483), AmelCSP3 (NM_001011583), AmelCSP5 (DQ855486); *Periplaneta americana*: P10 (AF030340); *Cactoblastis cactorum*: CLP-1 (U95046); *Bombyx mori*: BmorCSP2 (AAM34275), GmorCSP3 (FN432803); *Plutella xylostella*: PxylCSP3 (EF202828).

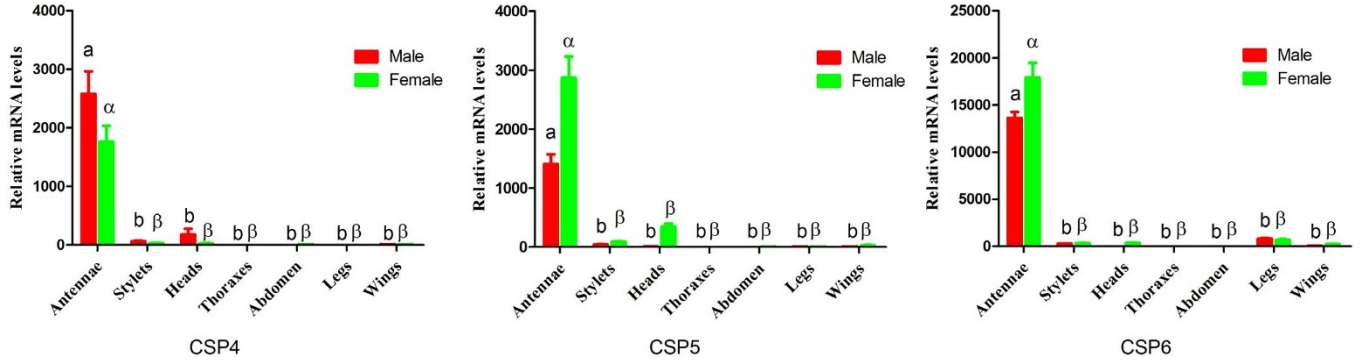

Figure 2 | Relative transcript levels of *AlinCSP4-6* among different adult tissues of both sexes analyzed by qPCR. The fold changes are relative to the transcript levels in the abdomen. The error bars represents the standard errors, and the different letters (a, b and α, β) indicate significant differences ($p < 0.05$) among different tissues in male and female, respectively.

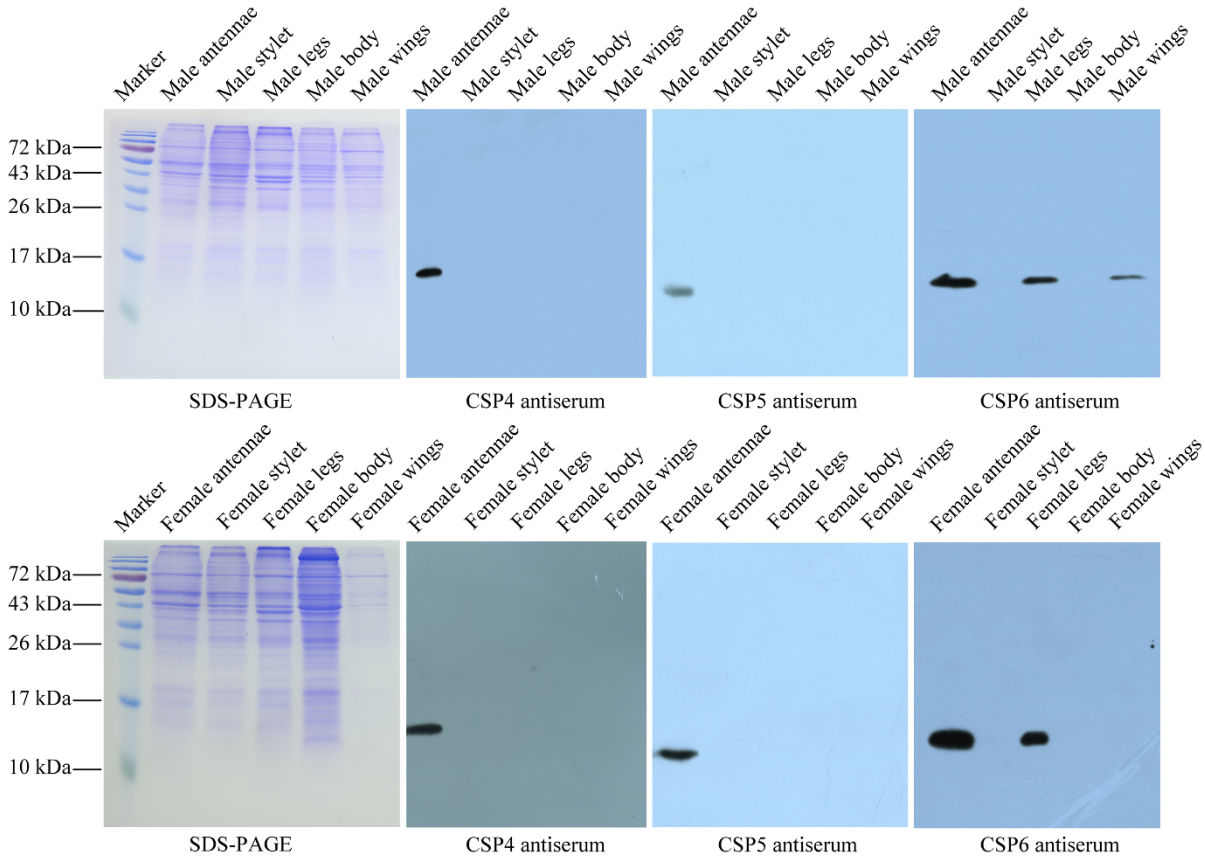

Figure 3 | SDS-PAGE and protein expression profiles among different adult tissues of both sexes measured by western blot analysis. From left to right, SDS-PAGE of crude extracts of different adult tissues of both sexes (1st panel) and western blot analysis (2nd–4th panels).

AlinCSP4-6 protein expression in the stylets and any of other non-olfactory tissues.

Specific protein expression of AlinCSP4-6 in different antennal sensilla of *A. lineolatus*. The immunolocalization of AlinCSP4-6 proteins in different antennal sensilla of both sexes was examined with ultrathin sections of the antennae subjected to colloidal gold post-embedding and immunocytochemical labelling. In general, each of AlinCSP4-6 proteins was shown to be expressed in distinct antennal sensilla of *A. lineolatus*, and similar expression patterns were observed in both sexes. AlinCSP4 was found strongly expressed in sensilla trichodea (str) and middle long sensilla basiconic (mlsba) in the sensillum lymph, the hair shaft and the cavity below the sensilla hair base (Figure 4). No positive signals were detected in short sensilla basiconica (ssba) and in two types of sensilla chaetica: long curved sensilla chaetica (lcsch) and long straight sensilla chaetica (lssch). Interestingly, not all of the str sensilla were labelled by the anti-AlinCSP4 antiserum, but nearly all of the mlsba sensilla were labelled. Anti-AlinCSP5 and anti-AlinCSP6 antiserum exhibited different labelling distributions compared with anti-AlinCSP4 antiserum. The short sensilla basiconica (ssba) were intensely stained by both anti-AlinCSP5 and anti-AlinCSP6 antisera with strong expression of both proteins in the outer sensillum lymph (Figure 5H, 5I and 6H, 6I) but not in the inner sensillum lymph where the neuronal dendrites are located[16]. Anti-AlinCSP6 also showed intensive labelling in the lcsch sensilla (Figure 6K). However, no labelling signals were observed with the neuronal dendrites for all three CSPs (Figure 4–6).

Using continuous sections, we also investigated the possible co-expression of AlinCSP4-6 in antennal sensilla of *A. lineolatus*. The results shown in Figure 7 indicate again that AlinCSP4 protein specifically expressed in the lymph of the mlsba sensillum (Figure 7B and 7D). The co-expression of AlinCSP5 and AlinCSP6 was found in the outer lymph of the ssba sensillum (Figure 7F and 7J). The labeling of anti-AlinCSP4 in the outside ring of the ssba sensillium was most likely unspecific labelling (Figure 4G and Figure 7B) as demonstrated in a repeated experiment (data not shown).

Ligand-binding of AlinCSP4-6. To further demonstrate the involvement of AlinCSP4-6 proteins in *A. lineolatus* olfaction, we explored the potential binding abilities of AlinCSP4-6 proteins to semiochemicals by performing the fluorescence competitive binding assay using N-phenyl-1-naphthylamine (1-NPN) as the fluorescence probe. The results showed that all of the three recombinant AlinCSPs could well interact with 1-NPN with dissociation constants (Kd in μM) of 2.8 ± 0.4, 4.2 ± 0.5, and 8.3 ± 1.8 for AlinCSP4/1-NPN complex, AlinCSP5/1-NPN complex, and AlinCSP6/1-NPN complex, respectively (Figure S4). This allowed us to perform a fluorescence competitive binding assay with semiochemicals using 1-NPN as the fluorescence probe.

Forty-one chemicals, including thirty-six cotton volatiles and five potential sex pheromone components of *A. lineolatus* were selected based on previous reports (Table 1) and used in the competitive binding assay. The binding affinities (Ki) of 41 chemicals are displayed in Figure 8 and Table 1. AlinCSP4 showed the binding affinity to almost all of tested cotton volatiles with Ki values less than 10 μM including putative sex pheromones, *trans*-2-hexenyl butyrate, ethyl butyrate, hexyl hexanoate and hexyl butyrate with the exception of butyl butyrate (Ki value of 13.63 μM), but no binding to one of the green leaf volatiles (GLVs), *cis*-3-hexenyl acetate (Figure 8 and Table 1). Furthermore, AlinCSP4 showed a preferential binding to terpenoids, *trans,trans*-farnesol, *trans*-β-farnesene, α-humulene, β-caryophyllene, nerolidol and β-ionone with a good affinity, but no binding to (+)-α-pinene, β-pinene, α-phellandrene, limonene and

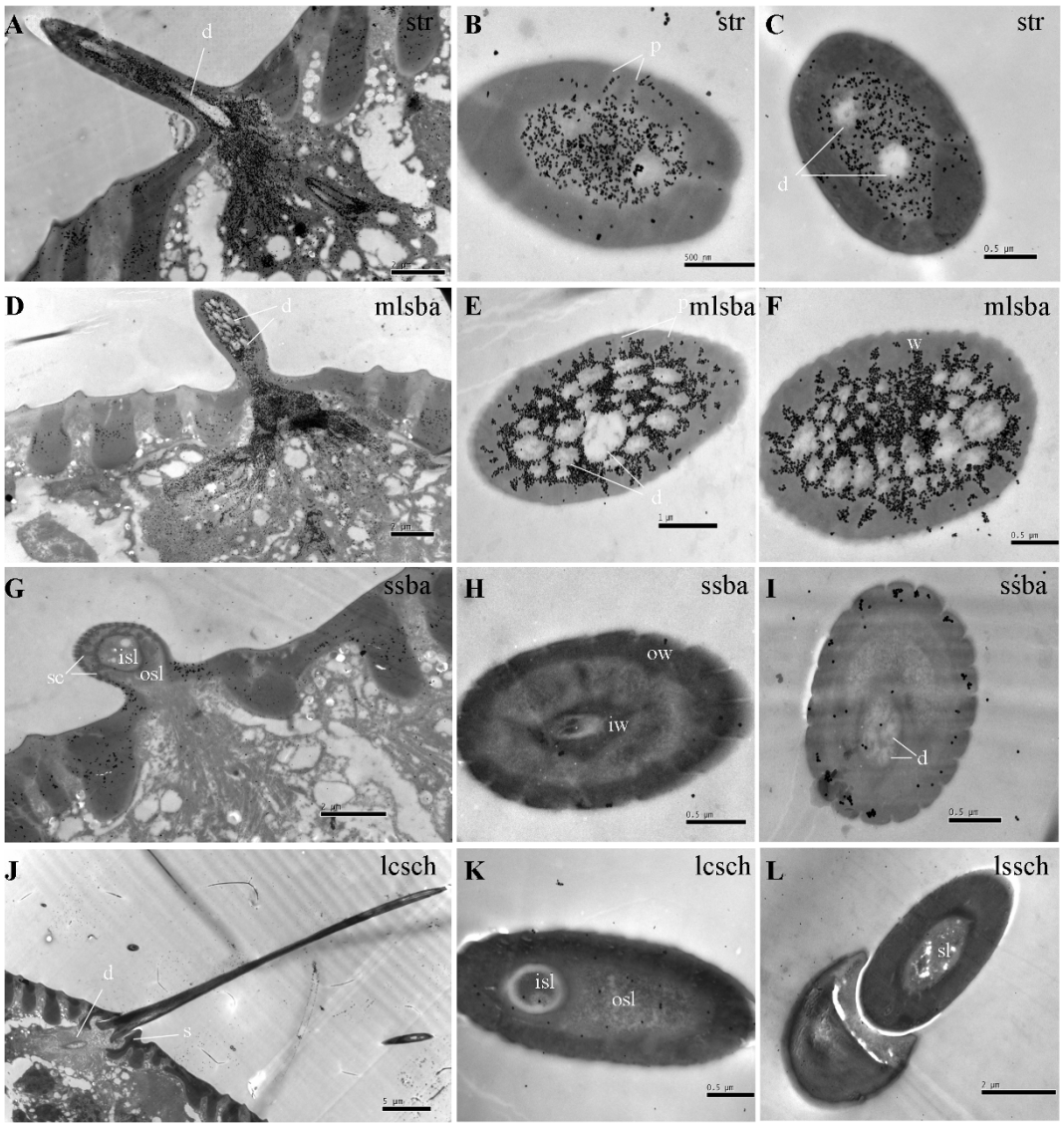

Figure 4 | Immunocytochemical localization of AlinCSP4 in different antennal sensilla of male adults. The sensilla lymph of the hair lumen and the cavities below the hair base of str and mlsba were strongly labelled by the anti-AlinCSP4 antiserum, whereas other sensilla, such as ssba, lcsch, and lssch, were not be labelled (the longitudinal sections are shown in A, D, G, and J, and the cross sections are shown in B, C, E, F, H, I, K, and L). The distribution of AlinCSP4 in the different antennal sensilla of the females was similar to that of the males. The few grains found over the cuticle and the dendrites represent the non-specific background. The dilution of the primary antibody was 1 : 2000, and the secondary antibody was anti-rabbit IgG conjugated with 10-nm colloidal gold granules at a dilution of 1 : 20. Abbreviations: str, sensilla trichodea; mlsba: middle long sensilla basiconic; ssba, short sensilla basiconica; lcsch, long curved sensilla chaetica; lssch, long straight sensilla chaetica; d, dendrites; p, pore; w, sensillum wall; isl, inner sensillum lymph; osl, outer sensillum lymph; sc, spoke channels; ow, outer sensillum wall; iw, inner, sensillum wall; s, socket; sl, sensillum lymph.

trans-β-ocimene. Unlike AlinCSP4, both AlinCSP5 and AlinCSP6 exhibited a relative narrow and weaker binding characteristic compared to AlinCSP4 (Figure 8 and Table 1). Furthermore, AlinCSP5 and AlinCSP6 have a completely different and almost non-overlapping binding profile to each other. AlinCSP6 preferentially bound to β-pinene, α-phellandrene, *trans*-β-ocimene and *trans*-β-farnesene, while AlinCSP5 preferentially bound with β-ionone, myrcene and nerolidol with low affinity, and with a higher affinity to one of sex pheromone, *trans*-2-hexenyl butyrate and one cotton volatile, methyl salicylate.

We also estimated potential binary interactions among AlinCSP4-6. The results for the binding of 1-NPN to the binary mixtures of AlinCSP4-6 are shown in Figure 9A. The equimolar mixtures of AlinCSP4/AlinCSP5 and of AlinCSP4/AlinCSP6 revealed binding curves that are not different from those of individual proteins. However, the binary mixtures of AlinCSP5 and AlinCSP6 showed an unusual binding behavior: their Scatchard plots displayed a non-linear correlation trend. We selected three terpenoids, *trans*-β-farnesene, α-humulene and β-caryophyllene, all of which exhibited weak or no binding affinities to either AlinCSP5 or AlinCSP6 but strong binding to AlinCSP4 (Figure 8), to estimate their binding possibilities to binary mixtures of AlinCSP5 and AlinCSP6. The results show that the combination of AlinCSP5 and AlinCSP6 increased the affinity to these three terpenoids with estimated IC_{50} values now less than 30 μM (Figure 9B) relative to the binding affinities of individual proteins (Table 1).

Electroantennogram (EAG) of semiochemicals. The dose-dependent EAG responses of 41 compounds included 14 previously reported[9,14] and 27 tested in current study was summarized in Figure S7. The results clearly indicated that antennal response of *A. lineolatus* was determined by both compound itself and dosage used. EAG response

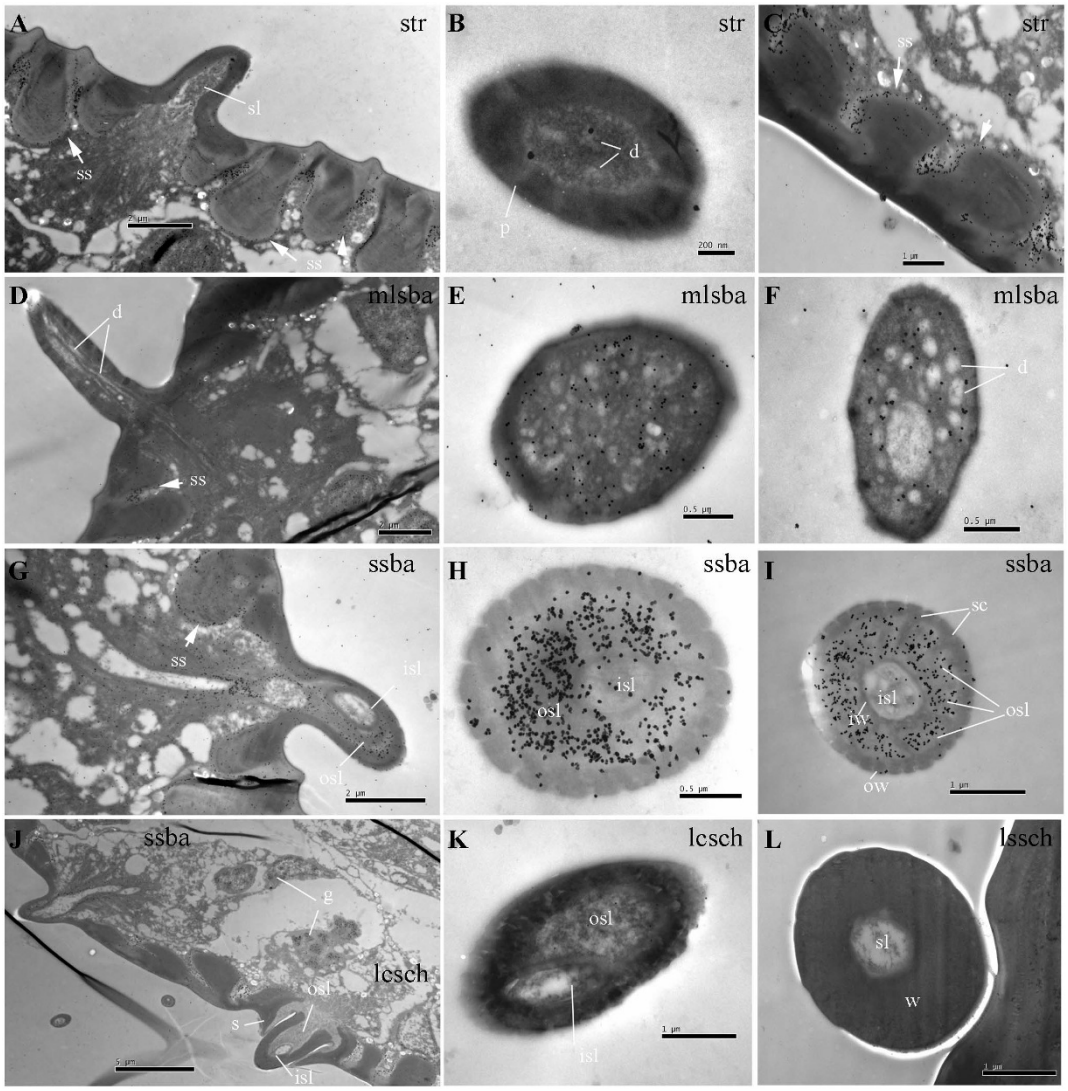

Figure 5 | Immunocytochemical localization of AlinCSP5 in different antennal sensilla of male adult. The outer sensillum lymph of ssba and the subcuticular spaces (ss) were heavily labelled, whereas the sensillum lymph of mlsba was weakly labelled. No obvious labelling was observed at the sensillum lymph of str, lcsch, or lssch (the longitudinal sections are shown in A, D, G, and J, and the cross sections are shown in B, C, E, F, H, I, K, and L). The distribution of AlinCSP5 in the different antennal sensilla of the females was similar to that of the males. The few grains found over the cuticle and the dendrites represent the non-specific background. The dilution of the primary antibody was 1 : 2000, and the secondary antibody was anti-rabbit IgG conjugated with 10-nm colloidal gold granules at a dilution of 1 : 20. The abbreviations are similar to those used in Figure 4.

of most chemicals increased as the tested dose enhanced by ten-folds, and different compounds had distinct EAG saturations. Both male and female bug can strongly respond to almost all the general cotton volatiles and green leaf volatiles. Among five potential sex pheromone components, hexyl butyrate and *trans*-2-hexenyl butyrate elicited more sensitive EAG responses. Interestingly, most of the terpenoids failed to elicit good EAG responses except of limonene, α-phellandrene and nerolidol at 10% level.

Discussion

In present study, we reported the antenna specific expression of three CSPs (AlinCSP4-6) from *A. lineolatus* at transcript level as well as protein level, their distinct chemosensillum distribution as well as ligand binding capability thus providing the evidence for the possible roles that they could play in *A. lineolatus* semiochemical detection. Our finding resembles the distributions of CSPs in chemosensory tissues, including antennae[13,35], proboscis[36], maxillary palps[30], labial palps[30,39], legs[40], wings[37] and the chemosensory organs of *Schistocerca gregaria*[35] and *Locusta migratoria*[39], thus indicates a conserved chemosensory role of CSPs among different insect species. However, we cannot rule out other physiological functions that insect CSPs may play due to their wide tissue distributions, including non-chemosensory tissues such as the pheromone gland[42,43] and ejaculatory bulb[44].

Three CSP genes (*AlinCSP1-3*) were previously identified in *A. lineolatus*, and their ligand binding and sensillum distribution were also characterized[13], in addition, the antennal responses (EAG) to some of compounds used in the binding assay were also assessed[9,14], these data together with those of the current study on immumolocalization and binding of AlinCSP4-6 (Figure S5 and S6) and EAG responses of the additional 27 chemicals (Figure S7) provide an unique information to elucidate the physiological function and the interaction among *A. lineolatus* CSPs. Among six CSPs (AlinCSP1-6) AlinCSP4 showed a better and preferential binding to biologically active compounds; including all potential sex pheromone components, particularly hexyl hexanoate; three green leaf volatiles and some general cotton volatiles (undecane, nonyl acetate and amyl acetate) (Figure 8, Figure S7). This reflects the specific expression of AlinCSP4 in the str and mlsba sensilla which have been demon-

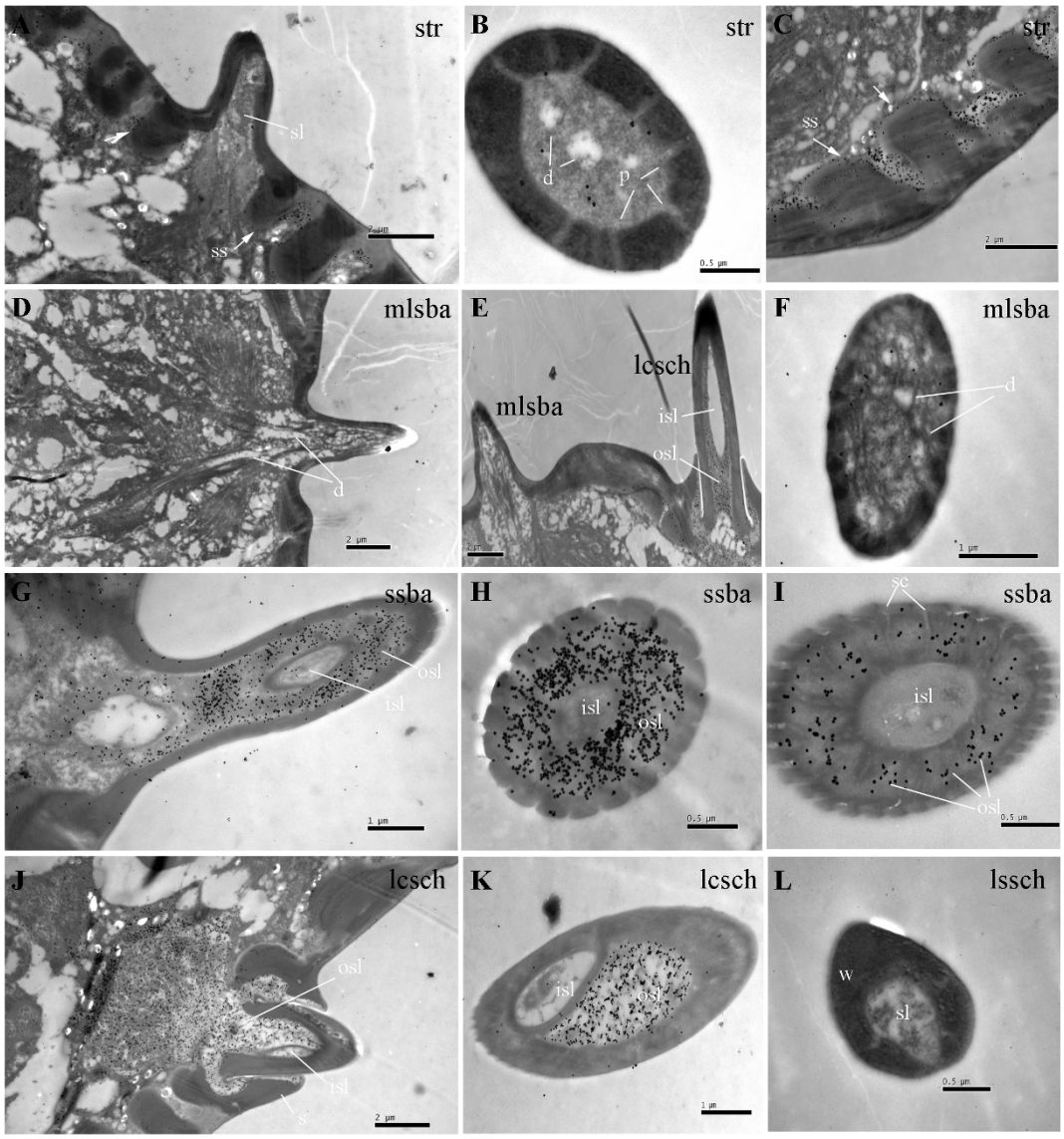

Figure 6 | Immunocytochemical localization of AlinCSP6 in different antennal sensilla of male adults. The outer sensillum lymph of ssba and lcsch and the sub-cuticular spaces (ss) were heavily labelled, whereas other sensilla, such as str, mlsba, and lssch, were not labelled (the longitudinal sections are shown in A, D, G, and J, and the cross sections are shown in B, C, E, F, H, I, K, and L). The distribution of AlinCSP6 in the different antennal sensilla of the females was similar in that found in the males. The few grains found over the cuticle and the dendrites represent the non-specific background. The dilution of the primary antibody was 1 : 2000, and the secondary antibody was anti-rabbit IgG conjugated with 10-nm colloidal gold granules at a dilution of 1 : 20. The abbreviations are similar to those used in Figure 4.

strated to be the sex pheromone and general odorant sensitive sensilla in lepidoperan insects, respectively[52]. However, one of the EAG active compounds, *cis*-3-hexenyl acetate showed no or very weak binding to AlinCSP4, while previous reported olfactory sensilla-biased AlinCSP1-3 in particular AlinCSP1 showed very good binding affinity to this compound[13], suggesting a different contribution of AlinCSPs in response to this odorant. In contrast, AlinCSP1-4 all exhibited good binding affinities to some strong EAG biologically active compounds such as *cis*-3-hexen-1-ol, and valeraldehyde, implying a possible cooperation in the perception of these plant bug-sensitive volatiles. Interestingly, AlinCSP4 has much high binding affinity to some low EAG active compounds (β-caryophyllene, α-humulene, *trans*-β-farnesene, *trans*, *trans*-farnesene) (Figure 8, Figure. S7). It is commonly found that some terpenoids in comparison to other compounds elicit lower EAG activity but have stronger behavioral response to mirid bugs[9,53-55], AlinCSP4 could be a suitable candidate protein to explore the molecular basis of this unusual olfactory coding event. Nevertheless, with its specific expression in the str sensilla, the high affinity to almost all of sex pheromone components and the lack of the binding of other CSPs to these pheromones, AlinCSP4 could be involved in the detection of these compounds and regulation the plant bug's sexual behaviors. To date, no confirmed pheromone-binding proteins (PBPs) have been identified in the Hemipteran species; thus, the significant binding (K_i far less than 10 μM) of AlinCSP4 relative to AlinCSP1-3[13] and AlinCSP5-6 (Figure 8) to putative male-sensitive sex pheromones of *A. lineolatus* provide the first crucial information for the identification of putative *PBP* genes in Hemipteran species.

For the first time at cellular level we showed two Hemipteran CSPs, AlinCSP5 and AlinCSP6 proteins are co-expressed only in the outer sensillum lymph of the short sensilla basiconica (ssba), where no neuron dendrites are observed. This result suggested that they are unlikely involved in helping odorants to interact with their receptors localized on the membrane of neuron dendrites in inner sensillum of ssba. This finding showed a good correspondence with localization of one OBP, PBPRP2, in *D. melanogaster*, which was

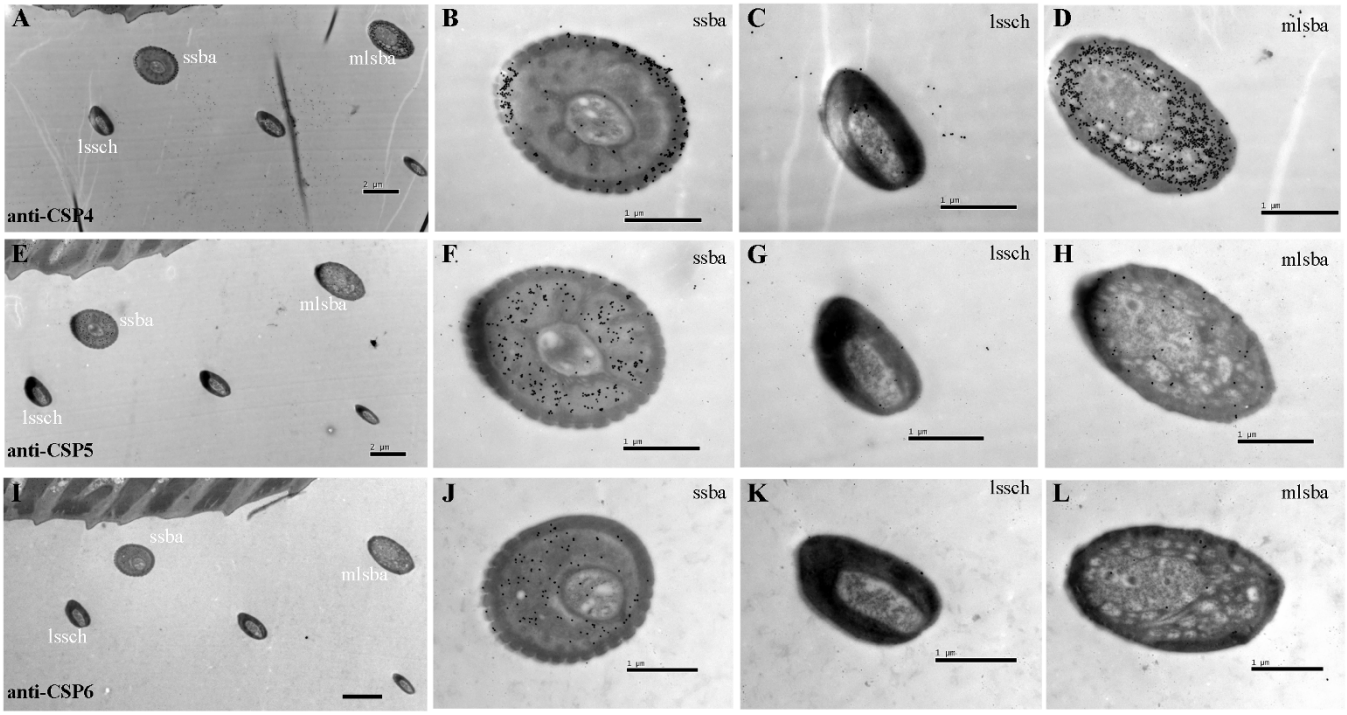

Figure 7 | Immunocytochemistry analysis demonstrates the co-localization of AlinCSP4-6 in the different antennal sensilla of *A. lineolatus*. A, E, and I show the serial sections that were incubated with anti-AlinCSP4, anti-AlinCSP5, and anti-AlinCSP6 antiserum, respectively. The images shown in B, C, D; F, G, H; and J, K, L are higher-magnification images of the ssba, lssch, and mlsba shown in A, E, and I, respectively. The results clearly demonstrate that AlinCSP5 and AlinCSP6 can be co-expressed in the outer sensilla lymph of ssba. The few grains found over the cuticles and the dendrites represent the non-specific background. The dilution of each primary antibody was 1 : 2000, and the secondary antibody was anti-rabbit IgG conjugated with 10-nm colloidal gold granules at a dilution of 1 : 20.

proposed to work as a sink for odorant capture to limit the interaction of odorants with correlative receptors for prevention desensitization of olfactory[56]. Interestingly, both AlinCSP6 and AlinCSP5 can selectively bind to tested compounds, and have a clear complementary binding profile; most of compounds that bound to AlinCSP5 did not bind to AlinCSP6 and *viceversa* (Figure 8), suggesting the complementary contributions to the semiochemical transport to downstream chemosensory proteins and additional selective layer in ligand recognition in chemosensillum. Methyl salicylate, β-ionone, nerolide and *trans*-2-hexenyl butyrate are likely the ligands to which AlinCSP5-associated molecular elements would respond, and octanal, nonanal and *trans*-β-farsense are the ligands that AlinCSP6-associated molecular elements would respond to. One odorant binding protein, AlinOBP13, is expressed strongly in the inner cavity of ssba sensilla and may function as a ligament between odorants from AlinCSP5 and AlinCSP6 and their associated receptors[14].

Our results of the binding profiles of the binary mixtures of either AlinCSP4/AlinCSP5 or AlinCSP4/AlinCSP6 with 1-NPN showed no significantly difference with the binding of the single proteins. The combination of AlinCSP5 with AlinCSP6 increased the ligand binding affinity (Figure 9). It is the first report for insect CSPs although the complementary interaction between OBPs have been reported previously[57,58]. These further indicate possible differential contributions of co-localized binding proteins in semiochemical signal transductionin insects. In odorant recognition process, a semiochemical could be captured by one binding protein, and passed to others then transported to the odorant sensitive receptor or be captured and ferried alone by a binding protein to the odorant sensitive receptors. The former mechanism could be more efficient, faster and selective than individual binding proteins work independently because a larger protein molecule will have a much slower diffusion coefficient than ligand molecules about 100 times smaller in the lymph even if the ligands are hydrophilic. Similarly, elimination of redundant olfactory stimulus could require higher and faster reaction efficiency, our result of co-expression and cooperative interaction of AlinCSP5 and AlinCSP6 nicely meet the needs of high efficiency, and indicate they may involve in potential odorant elimination in ssba. Further studies in the identification of olfactory receptors and their co-localization of these binding proteins in the ssba sensilla as well as single sensillum electrophysiological recording are needed to confirm possible existence of associated receptors and their interactions thus the molecular mechanisms of ligand specificity and recognition of insects.

In summary, our study revealed three CSPs (AlinCSP4-6) may exhibit different roles important in the *A. lineolatus* olfactory system. Further comparisons of the results with the previously reported data of AlinCSP1-3 suggest that CSPs may have a functional differentiation based on their expression sites in chemosensilla. Because the selected compounds used in the ligand binding assay are important host volatiles and the putative sex pheromones of *A. lineolatus*, this study not only broadens the theoretical research of CSP in the mechanism of olfactory peripheral recognition but also lays the foundation for an investigation to devise strategies to disrupt *A. lineolatus* behaviors in both host plant location and mate searching.

Methods

Insects. The *A. lineolatus* adults were collected from the cotton fields at the Langfang Experimental Station of the Chinese Academy of Agricultural Sciences, Hebei Province, China. The colony at the laboratory was established as previously reported method[59]. Briefly, the adult bugs were reared on green beans and 10% honey under a 14-h light/10-h dark cycle, and the temperature and relative humidity (RH) were maintained at 29 ± 1°C and 60 ± 5%, respectively.

Full-length cDNA sequences analysis. The putative CSP genes of *A. lineolatus* were identified from our previously constructed cDNA library by BlastX and the "CSP Motif" search of C_1-X_{6-8}-C_2-X_{16-21}-C_3-X_2-C_4 as reported previously[60,61]. The putative N-terminal signal peptides and the most likely cleavage site were predicted by the SignalP 3.0 program[62] (http://www.cbs.dtu.dk/services/SignalP/). The alignments of

Table 1 | Binding data of all of the selected compounds to the recombinant AlinCSP4-6 proteins. A solution of two proteins in Tris-buffer (pH 7.4), both at the concentration of 2 μM, was titrated with 1 mM solution of 1-NPN in methanol to final concentrations ranging from 2 to 30 μM. The dissociation constants (Ki) were measured from the values of the ligands that halve the 1-NPN fluorescence (IC_{50}). U.d. means that the IC_{50} value exceeds 30 μM and thus that the binding affinities (Ki) of the candidate competitive ligand were deemed to lose directly calculated rationality from the test ligand concentration. The compounds used in this binding assay were classed into several groups, including general cotton volatiles, GLVs, terpenoids, putative sex pheromone components of *A. lineolatus*. a, b, c, d, e, f and g represent references[68–74], respectively

Ligand			CSP4		CSP5		CSP6	
Number	Name	CAS Number	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
General cotton volatiles								
1	2-Hexanol [a]	626-93-7	9.63 ± 0.30	6.22 ± 0.18	u.d.	u.d.	u.d.	u.d.
2	Pentanol [a]	71-41-0	11.34 ± 2.29	7.36 ± 1.50	27.57 ± 0.89	20.40 ± 0.68	u.d.	u.d.
3	Valeraldehyde [a]	110-62-3	10.86 ± 1.06	6.96 ± 0.68	17.66 ± 1.52	12.57 ± 1.04	u.d.	u.d.
4	Hexanal [a]	66-25-1	10.52 ± 2.11	6.77 ± 1.35	16.16 ± 1.64	11.59 ± 2.23	u.d.	u.d.
5	Heptanal [a, b]	111-71-7	11.41 ± 0.47	7.43 ± 0.29	u.d.	u.d.	26.65 ± 1.74	21.78 ± 1.41
6	Octanal [a, b]	124-13-0	10.93 ± 0.86	7.04 ± 0.50	u.d.	u.d.	20.44 ± 1.83	16.71 ± 1.51
7	Nonanal [a, b]	124-19-6	12.99 ± 1.43	8.32 ± 0.90	u.d.	u.d.	14.87 ± 2.11	12.25 ± 1.72
8	2-Hexanone [a, b]	591-78-6	25.71 ± 0.53	16.11 ± 0.32	18.41 ± 1.76	13.20 ± 1.38	20.57 ± 2.98	16.94 ± 2.43
9	2-Heptanone [a, b]	110-43-0	27.25 ± 0.60	17.51 ± 0.36	18.38 ± 4.08	13.08 ± 2.85	u.d.	u.d.
10	2-Octanone [a, b]	111-13-7	25.23 ± 2.39	16.06 ± 1.46	23.29 ± 1.65	16.65 ± 1.24	u.d.	u.d.
11	3-Hexanone [a, b]	589-38-8	24.15 ± 0.84	15.53 ± 0.51	15.11 ± 2.30	10.78 ± 1.65	u.d.	u.d.
12	6-Methyl-5-hepten-2-one [a, b]	110-93-0	20.24 ± 2.62	12.96 ± 1.65	u.d.	u.d.	u.d.	u.d.
13	Amyl acetate [a, b]	628-637-7	11.44 ± 2.25	7.33 ± 1.45	28.30 ± 1.40	20.23 ± 1.00	u.d.	u.d.
14	Nonyl acetate [a, b]	1143-13-5	5.83 ± 0.44	3.73 ± 0.28	24.21 ± 2.66	17.24 ± 1.84	u.d.	u.d.
15	Undecane [a, b]	1120-21-4	7.13 ± 1.29	4.55 ± 0.83	u.d.	u.d.	u.d.	u.d.
16	Indole [a, b, c, d]	120-72-9	23.35 ± 1.60	14.50 ± 0.94	18.42 ± 1.25	13.13 ± 0.88	u.d.	u.d.
17	Benzaldehyde [a, b,]	100-52-7	14.28 ± 1.86	9.10 ± 1.18	30.19 ± 0.49	21.53 ± 0.40	u.d.	u.d.
18	3,4-Dimethyl-benzaldehyde [b]	5973-71-7	10.62 ± 0.75	6.83 ± 0.47	16.50 ± 0.87	11.84 ± 0.61	u.d.	u.d.
19	Acetophenone [a, b]	98-86-2	27.46 ± 1.73	15.58 ± 1.00	20.59 ± 1.96	14.81 ± 1.38	u.d.	u.d.
20	Methyl salicylate [b]	119-36-8	12.67 ± 2.01	8.17 ± 1.30	13.49 ± 1.22	9.68 ± 0.86	u.d.	u.d.
Green leaf Volatiles								
21	1-Hexanol [c]	111-27-3	12.47 ± 0.88	7.87 ± 0.52	23.99 ± 0.76	17.31 ± 0.64	22.06 ± 3.06	18.05 ± 2.53
22	*cis*-3-Hexen-1-ol [c, d]	928-96-1	21.66 ± 0.88	13.31 ± 0.51	18.75 ± 2.95	13.58 ± 2.23	u.d.	u.d.
23	*trans*-2-Hexenal [c, d]	6278-26-3	16.26 ± 0.51	10.69 ± 0.59	21.28 ± 1.65	15.24 ± 1.14	u.d.	u.d.
24	*cis*-3-hexenyl acetate [d]	3681-71-8	u.d.	u.d.	u.d.	u.d.	u.d.	u.d.
Terpenoids								
25	*trans* -β-Ocimene [c, d]	3016-19-1	u.d.	u.d.	u.d.	u.d.	12.75 ± 2.49	10.53 ± 2.04
26	Limonene [c, d]	5989-27-5	u.d.	u.d.	u.d.	u.d.	u.d.	u.d.
27	α-Phellandrene [a]	99-83-2	u.d.	u.d.	u.d.	u.d.	15.78 ± 2.85	12.99 ± 2.31
28	β-Pinene [a, c, d]	18172-67-3	u.d.	u.d.	u.d.	u.d.	25.51 ± 0.41	20.93 ± 0.34
29	(+)-α-Pinene [a, c, d]	7785-70-8	u.d.	u.d.	26.51 ± 0.93	19.05 ± 0.64	19.76 ± 3.97	16.25 ± 3.24
30	β-Ionone [b]	79-77-6	10.13 ± 1.24	6.47 ± 0.78	25.63 ± 2.21	18.02 ± 1.67	u.d.	u.d.
31	Myrcene [c, d]	123-35-3	20.14 ± 0.81	12.79 ± 0.49	20.19 ± 0.54	14.45 ± 0.38	u.d.	u.d.
32	Nerolidol [e]	7212-44-4	2.00 ± 0.17	1.3 ± 0.1	23.97 ± 0.27	17.23 ± 0.20	u.d.	u.d.
33	β-Caryophyllene [c, d]	87-44-5	2.26 ± 0.26	1.46 ± 0.16	u.d.	u.d.	u.d.	u.d.
34	α-Humulene [c, d]	6753-98-6	1.97 ± 0.13	1.30 ± 0.08	u.d.	u.d.	u.d.	u.d.
35	*trans*-β-Farnesene [c, d, e]	18794-84-8	1.87 ± 0.13	1.20 ± 0.08	u.d.	u.d.	17.75 ± 4.65	14.57 ± 3.80
36	*trans,trans*-Farnesol [c, d, e]	106-28-5	1.73 ± 0.06	1.13 ± 0.04	17.26 ± 0.64	12.33 ± 0.39	18.31 ± 2.73	15.06 ± 2.22
Putative sex pheromones								
37	Hexyl butyrate [f]	2639-63-6	9.22 ± 1.77	6 ± 1.12	27.97 ± 0.31	20.23 ± 0.19	28.06 ± 1.58	22.91 ± 1.30
38	Hexyl hexanoate [g]	6378-65-0	2.36 ± 0.21	1.54 ± 0.13	u.d.	u.d.	27.55 ± 1.02	22.55 ± 0.81
39	Butyl butyrate [f]	109-21-7	21.13 ± 0.51	13.63 ± 0.32	16.33 ± 1.50	11.89 ± 1.12	u.d.	u.d.
40	Ethyl butyrate [f]	105-54-4	9.81 ± 1.55	6.12 ± 0.89	25.11 ± 0.37	18.12 ± 0.34	u.d.	u.d.
41	*trans*-2-hexenyl butyrate [f]	53398-83-7	10.99 ± 2.09	7.02 ± 1.35	12.13 ± 0.79	8.72 ± 0.61	u.d.	u.d.

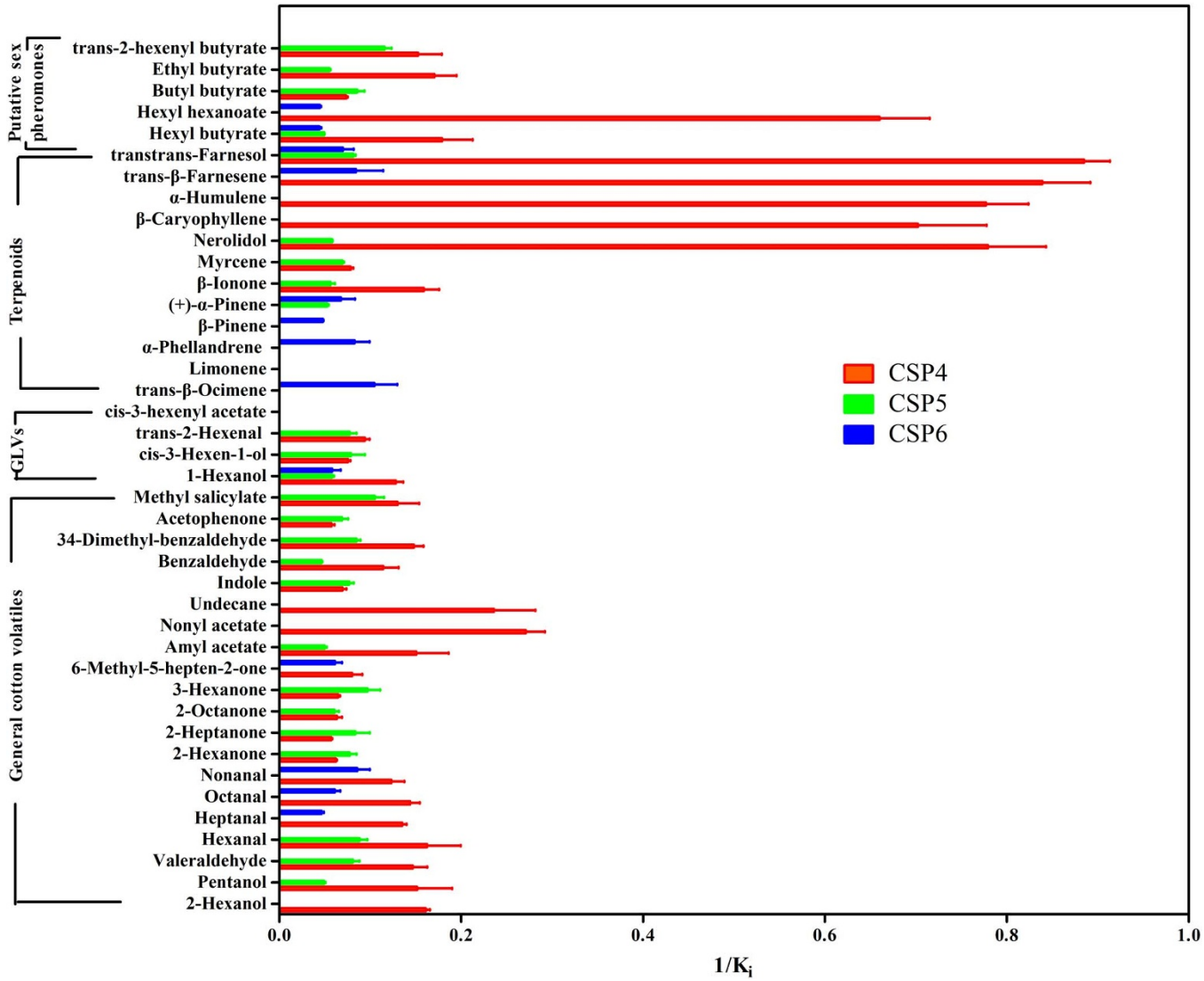

Figure 8 | Reverse values of the dissociation constants (K_i) measured with all 41 compounds and three recombinant AlinCSP4-6 proteins. A mixture of the protein and 1-NPN in Tris-buffer (pH 7.4), both at a concentration of 2 μM, was titrated with 1 mM solutions of each competitive ligand to final concentrations ranging from 2 to 30 μM. The dissociation constants (Ki) were measured from the values of the ligands that halve the 1-NPN fluorescence (IC_{50}). The compounds with the IC_{50} value more than 30 μM were deemed to have no binding and the binding affinities (K_i) were not calculated in this study. The chemical names in corresponding to the numbers on Y-axis are listed in Table 1. The calculated dissociation constants and the binding data relative to all of the ligands tested are reported in Table 1.

the amino acid sequences of these AlinCSPs were made using ClustalX 1.83 with the default gap penalty parameters of a gap opening of 10 and an extension of 0.2 and subsequently edited using ESPript (http://espript.ibcp.fr/ESPript/ESPript/)[63] and WebLogo (http://weblogo.berkeley.edu/)[64].

Quantitative real-time PCR (qRT-PCR). To assess the *AlinCSP* gene tissue expression, different tissues, including the antennae, stylets, heads (without antennae and stylets), thorax, abdomen, legs, and wings, were excised from adults of both sexes, immediately frozen in liquid nitrogen, and then stored at −80°C until use. Total RNA of each sample was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), and the first-strand cDNA was synthesized by FastQuant RT-kit with gDNA Eraser (TianGen, Beijing, China) according to the manufacturer's instructions. The cDNA was diluted to 200 ng/μl for the subsequent qRT-PCR reaction. The specific primer pairs of *AlinCSP* genes and a *β-actin* gene (GenBank accession No.GQ477013) of *A. lineolatus*, which was used as a reference gene, were designed by Beacon Designer 7.90 (PREMIER Biosoft International) and are listed in Table S1. The qRT-PCR was conducted using an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA), and the reaction volume of 25 μL contained 12.5 μL of SuperReal PreMix Plus (TianGen, Beijing, China), 0.75 μL of each primer (10 μM), 0.5 μL of Rox Reference Dye, 9.5 μL of sterilized H_2O, and 1 μL of the sample cDNA (200 ng). The parameters of the qRT-PCR were as following: 95°C for 15 min and 40 cycles of 95°C for 10 s and 60°C for 32 s. To measure the dissociation curves, the PCR products were then heated to 95°C for 15 s, cooled to 60°C for 1 min, heated to 95°C for 30 s, and cooled to 60°C for 15 s. To check the reproducibility, each qRT-PCR reaction for each sample included three technical replicates and three biological replicates for each transcript.

Raw Ct values were converted to quantities representing relative expression levels using a modified comparative Ct method[65], with correction for different amplification efficiencies[66]. Briefly, after qRT-PCR, Ct values were exported into the LinRegPCR program to correct the amplification efficiencies for each reaction. The relative expression levels (Pfaffl ratio) of AlinCSP genes to the reference gene was then calculated for each sample as:

$E_{CSP}^{\Delta Ct,\ CSP}/E_{\beta\text{-}actin}^{\Delta Ct,\ \beta\text{-}actin}$. Where E_{CSP} and $E_{\beta\text{-}actin}$ are corrected amplification efficiencies for *AlinCSP* and *Alinβ-actin*, respectively, and in different tissues, ΔCt, *CSP* is calculated as: $Ct_{CSP\ of\ abdomen} - Ct_{CSP\ of\ X}$, and ΔCt, *β-actin* is calculated as: $Ct_{\beta\text{-}actin\ of\ abdomen} - Ct_{\beta\text{-}actin\ of\ X}$. Where X represents different tissues. To estimate the relative fold change in different tissues, the abdomen sample was used as the calibrator for the comparison between tissues. The differences in the expression levels between the tissues were assessed statistically through one-way analysis of variance (ANOVA) at a significance level of $\alpha = 0.05$ using the Stata 9.0 software (Stata Crop LP, Texas, USA).

Heterologous expression and purification of AlinCSP proteins. Specific primers with restriction enzyme sites *Nco I* in the sense primer and *Xho I* in the antisense primer (Table S1) were used to clone cDNAs encoding the mature AlinCSP proteins under the following PCR conditions: 94°C for 4 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final elongation step at 72°C for 10 min. The

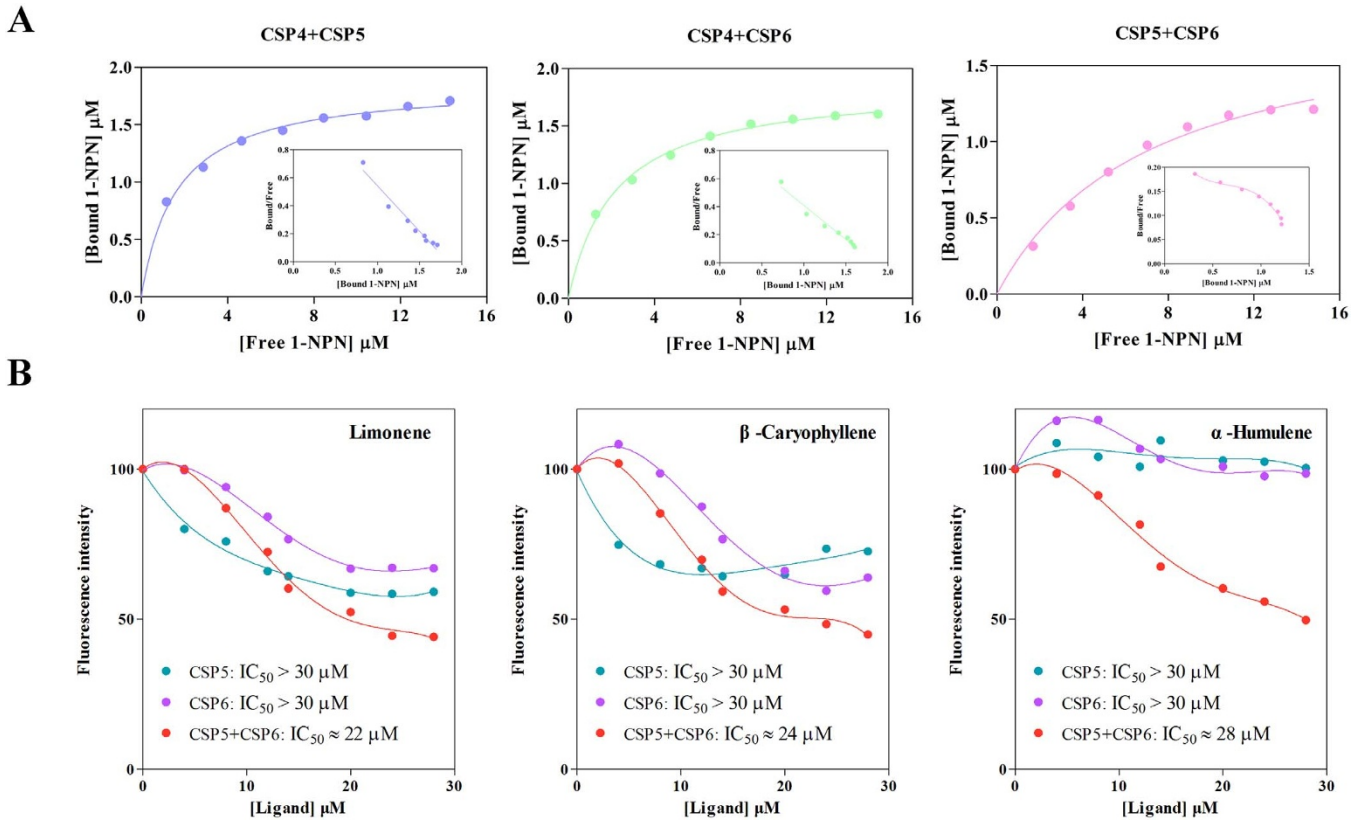

Figure 9 | **A. Binding curves of 1-NPN to binary mixtures of AlinCSP4-6 and relative Scatchard plot analysis.** A solution of two proteins in Tris-buffer (pH 7.4), both at the concentration of 2 μM, was titrated with 1 mM solution of 1-NPN in methanol to final concentrations ranging from 2 to 16 μM. The binary mixtures of AlinCSP4 and AlinCSP5 and of AlinCSP4 and AlinCSP6 showed a regular binding behavior in agreement with that obtained for each single protein. AlinCSP5 and AlinCSP6 indicated a different binding behavior, and the Scatchard plot of this combination resembled an inclined letter "J". B. Binding curves of selected terpenoids to binary mixtures of AlinCSP5 and AlinCSP6. A solution of two proteins in Tris-buffer (pH 7.4), both at the concentration of 2 μM, was titrated with 1 mM solution of 1-NPN in methanol to final concentrations ranging from 2 to 30 μM. The results showed an increasing binding ability compared with the previous data.

correct product, as confirmed by sequencing, was sub-cloned into the bacterial expression vector pET30a (+) (Novagen, Madison, WI), which was previously digested with the same restriction enzymes. The correct plasmid containing *AlinCSP* gene was transformed into *Escherichia coli* BL21 (DE3)-competent cells for AlinCSP protein expression. A verified single colony was grown overnight in 5 mL of LB broth with 100 mg/mL kanamycin. The culture was diluted to 1 : 100 with fresh medium and then continued at 37°C for approximately 2 h until the OD_{600} value reached 0.6. The productions of the recombinant proteins were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 3–6 h. The bacterial cells were harvested by centrifugation at 7,000 g for 20 min, resuspended in lysis buffer (80 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 4% glycerol, pH 7.2, and 0.5 mM phenylmethanesulfonyl fluoride), and then sonicated in ice (10 s, five passes). After another round of centrifugation at 16,000 g for 20 min, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the recombinant protein of AlinCSP6 was obtained in soluble form, whereas the AlinCSP4 and AlinCSP5 proteins were found in inclusion bodies. The proteins as inclusion bodies were solubilized and refolded according to a previous protocol by Prestwich[67]. Briefly, the inclusion bodies were washed with 0.2% Triton X-100 in 50 mM Tris buffer (pH 6.8) and dissolved in 5 ml of 6 N guanidinium hydrochloride followed by the addition of 5 ml of 10 mM DTT in 200 mM Tris-HC1 (pH 8.0). After incubation at room temperature for 30–60 min, 1 mL of 100 mM cystine (in 0.5 N NaOH) was added to oxidize remaining DTT. Finally, the mixture was diluted 1 : 9 with 5 mM cysteine in 100 mM Tris-HCI (pH 8.0) and dialyzed overnight at room temperature with 100 mM Tris-HCI (pH 8.0).

The recombinant proteins were purified through two rounds of Ni ion affinity chromatography (GE-Healthcare), and the His-tag was removed with recombinant enterokinase (Novagen). The highly purified proteins were desalted through extensive dialysis. The size and purity of the recombinant proteins were verified by 15% SDS-PAGE.

AlinCSP4-6 antisera production. Polyclonal antiserum against recombinant AlinCSP4-6 was obtained by injecting robust adult rabbits subcutaneously and intramuscularly with the highly purified recombinant AlinCSP4, AlinCSP5, and AlinCSP6 proteins. Each recombinant AlinCSP protein was emulsified with an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO, USA) for the first injection (500 μg of recombinant protein) and then with incomplete adjuvant for the three additional injections (300 μg each time). The interval between each injection was approximately half a month, and rabbit blood was collected 7 days after the last injection and centrifuged at 6000 rpm for 20 min. The serum was purified using a MAb Trap kit (GE Healthcare) following the manufacturer's instructions. The rabbits were maintained in large cages at room temperature, and all of the operations were performed according to ethical guidelines to minimize the pain and discomfort of the animals.

Western blot analysis. To check the specificity of each AlinCSP antiserum and investigate the protein expression profile among different tissues of both female and male adults of *A. lineolatus*, the purified recombinant AlinCSP4-6 proteins and the crude extracts from different tissues of adult female and male bugs, including the antennae, stylets, legs, wings and other body parts including heads, thoraxes and abdomen, were separated on 15% SDS-PAGE, respectively. After the samples were transferred to a polyvinylidene fluoride membrane (PVDF, Millipore, Carrigtwohill, Ireland) at 200 mA for 50 min, the membrane was blocked with 5% dry skimmed milk (BD Biosciences, San Jose, CA, USA) in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST) for 2 h at room temperature. After washing three times with PBST (10 min each time), the blocked membrane was incubated with purified rabbit anti-AlinCSP antiserum (dilution 1 : 2,000) for 1 h. After washings three times with PBST, the membrane was incubated with anti-rabbit IgG horseradish peroxidase (HRP) conjugate and HRP-streptavidin complex (Promega, Madison, WI, USA) at a dilution of 1 : 10000 for 1 h. The membrane was then incubated with the western blot substrates of the enhanced chemiluminescence western blot kit (CoWinbiotech, China), and the bands were visualized by exposing to X-OMATBT films (Kodak, New York, USA).

Immunocytochemical localization. The antennae of both female and male adult bugs were fixed separately in a mixture of paraformaldehyde (4%) and glutaraldehyde (2%) in 0.1 M PBS (pH 7.4) at room temperature for 24 h, dehydrated in an ethanol series (30%, 50%, 70%, 80%, 90%, 95% and 100%), and embedded in the LR white resin (Taab, Aldermaston, Berks, UK) for polymerization at 60°C. Ultrathin sections (60–80 nm), including both cross and longitudinal sections, were cut using a diamond knife on a Reichert Ultracut ultramicrotome (Reichert Company, Vienna,

Austria). For immunocytochemical assay, the grids were floated in 25 μL droplets of PBSG (PBS containing 50 mM glycine) and then PBGT (PBS containing 0.2% gelatin, 1% bovine serum albumin, and 0.02% Tween-20), and incubated with purified rabbit anti-AlinCSP antiserum (dilution 1 : 2,000) at 4°C overnight. After washing six times with PBGT, the sections were incubated with secondary antibody (anti-rabbit IgG) coupled with 10-nm colloidal gold granules (Sigma, St. Louis, MO, USA) at a dilution of 1 : 20 at room temperature for 90 min. Before being observed with a HITACHI H-7500 TEM (Hitachi Ltd., Tokyo, Japan), the sections were subjected to optional silver intensification for 15 min and stained with 2% uranyl acetate to increase the contrast. The serum supernatant from an uninjected healthy rabbit at the same dilution rate acted as the negative control. The immunocytochemical assay was conducted on three male and female adult antennae.

Fluorescence competitive binding assays. To confirm whether AlinCSPs are responsible for odorant binding, we conducted competitive fluorescence binding assays. The binding assays were performed on an F-380 fluorescence spectrophotometer (Tianjin, China) at room temperature (25°C) with a 1-cm light path quartz cuvette and 10-nm slits for both excitation and emission. The excitation wavelength was 337 nm, and the emission spectrum was recorded between 390 and 460 nm. To measure the affinity of the fluorescent probe N-phenyl-1-naphthylamine (1-NPN) to each recombinant CSP, a 2 μM solution of the protein in 50 mM Tris-HCl (pH 7.4) was titrated with aliquots of 1 mM 1-NPN dissolved in methanol to final concentrations ranging from 1 μM to 16 μM. The affinity of other ligands was measured through competitive binding assays using 1-NPN as the fluorescent reporter at a concentration of 2 μM, and the concentration of each competitor ranged from 2 μM to 30 μM. The fluorescence intensities at the maximum fluorescence emission between 390 and 460 nm were plotted against the free ligand concentration to determine the binding constants. The bound chemical was evaluated based on its fluorescence intensity with the assumption that the protein was 100% active with a stoichiometry of 1 : 1 (protein: ligand) saturation. The binding curves were linearized using a Scatchard plot, and the dissociation constants of the competitors were calculated from the corresponding IC_{50} values based on the following equation: $K_i = [IC_{50}]/(1 + [1\text{-NPN}]/K_{1\text{-NPN}})$, where [1-NPN] is the free concentration of 1-NPN and $K_{1\text{-NPN}}$ is the dissociation constant of the complex protein/1-NPN. In this study, if the IC_{50} value was less than 20 μM, the candidate competitive ligand was considered to have a good binding ability with the recombinant AlinCSP proteins; in contrast, if the IC_{50} value of the candidate competitive ligand exceeded 30 μM, the further calculation of the binding affinity (Ki) was not considered.

Electroantennogram (EAG) recordings. Dose-response was performed with EAG recording to assess the physiological relevance of AlinCSPs putative ligands in *A. lineolatus*. Previously, EAG responses of 14 chemical included 3-hexanone, two green leaf volatiles and eleven terpenoids have been reported[9,14]. In the present study, we focus on the rest of 27 compounds. Dose-response EAG recording was conducted in Syntech EAG 2000 program (Syntech), the detail protocol has been described previously[9,14]. Briefly, the antennae of newly emerged female and male adults were cut from head and immediately attached to two electrode holders with nondrying clay (Spectra 360 Electrode Gel). An air stimulus controller CS-55 (Syntech, Netherlands) was used for control and stimulant delivery with a constant flow of 10 ml/sec. Signals were recorded for 5 s, beginning at 1 s before the onset of the stimulus pulse and passed through a high-impedance amplifier (CS-05 model; Syntech,). Four different concentrations (0.01%, 0.1%, 1%, 10% v/v) were employed to estimated effect of the stimulant dose on antennal responses. Paraffin oil (Fluka, Buchs, Switzerland) was used as control, while 10% (v/v) 3-hexanone acted as a reference to normalize all responses. A 10 μL aliquot of each tested compounds was used and each compound was tested on six female and male adult antennae, respectively. All results are presented as normalized mean (± SE) EAG responses. Significant difference among doses were assessed statistically with a one-way analysis of variance (ANOVA) at a significance level of $\alpha = 0.05$ using Stata 9.0 software (Stata Crop LP, Texas, USA).

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Author contributions

L.S., J.J.Z., Y.J.Z. and Z.W.L. designed the experiments. L.S. performed the experiments and S.H.G. assisted in the gene identification. H.J.X., Y.J.Z. and Y.Y.G. contributed to statistical analysis and reagents/materials preparation. L.S. and J.J.Z. wrote and revised the manuscript.

Additional information

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