agriculture

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Molecular Characterization of Three Chemosensory Proteins from *Carposina sasakii*

Liu Liu, Guisheng Qiu, Huaijiang Zhang, Qiang Yue, Wentao Yan and Lina Sun *

Research Institute of Pomology, Chinese Academy of Agricultural Sciences, 98 South Street, Xinghai, Huludao 125100, China; liuliu040907@163.com (L.L.); gszbpest@163.com (G.Q.); zhanghuaijiang@caas.cn (H.Z.); yueqiang@caas.cn (Q.Y.); yanwentao@caas.cn (W.Y.)
* Correspondence: slncaas@163.com; Tel.: +86-429-3598266

Abstract: The peach fruit moth, *Carposina sasakii*, is part of the Carposinidae, and is harmful to the families Rosaceae and Rhamnaceae. *C. sasakii* lays eggs on the hairy surface of the fruit's stalk cavity and calyx end. After hatching, the moth can bore into the fruits and feed on the flesh inside. Chemosensory proteins (CSPs) are a class of low-molecular-weight soluble carrier proteins that are highly evolutionarily conserved. To enhance our understanding of the recognition of host plant volatiles by CSPs of *C. sasakii*, the expression patterns and binding characteristics of CsasCSP7, CsasCSP9 and CsasCSP11 in *C. sasakii* were investigated. In our study, the results of real-time quantitative polymerase chain reaction (qPCR) assays demonstrate that *CsasCSP7* and *CsasCSP9* transcripts were abundantly expressed in the antennae of males, and CsasCSP11 was highly expressed in the wings of females. Fluorescence competitive binding assays with 38 candidate ligands showed that CsasCSP7 could bind to benzaldehyde and dodecanal, whereas CsasCSP9 bound to butyl octanoate, decanal and (-)-beta-pinene. CsasCSP11 could also bind to1-hexanol, beta-ocimene and 6-methyl-5-hepten-2-one. Our results suggest that CsasCSP7, CsasCSP9 and CsasCSP11 may play a crucial role in locating the host plant of *C. sasakii*.

Keywords: *Carposina sasakii*; chemosensory proteins; fluorescence competitive binding; plant volatile organic compounds

Citation: Liu, L.; Qiu, G.; Zhang, H.; Yue, Q.; Yan, W.; Sun, L. Molecular Characterization of Three Chemosensory Proteins from *Carposina sasakii*. *Agriculture* **2023**, *13*, 2066. https://doi.org/10.3390/agriculture13112066

Academic Editor: David João Horta Lopes

Received: 5 September 2023
Revised: 24 October 2023
Accepted: 25 October 2023
Published: 27 October 2023

1. Introduction

The peach fruit moth, *Carposina sasakii*, is one of the Carposinidae, and is widely distributed in North Korea, South Korea, Japan, the Russian Far East and China. It is harmful to apple, jujube, pear and other fruit trees from the families Rosaceae and Rhamnaceae [1]. Female adult *C. sasakii* prefers to lay eggs on the surface of the fruit, especially in the calyx pits of apples. In the case of peach fruits, which are covered with fine hairs, the insect lays eggs all over the fruit surface [2]. After hatching, *C. sasakii* can bore into the fruits and feed on the flesh inside, making it difficult to reduce larvae numbers using chemicals and causing losses in fruit production [3]. Preventing oviposition could be one of the most effective methods of controlling *C. sasakii*. The adult females release a sex pheromone composed of two odorants, (Z)-7-eicosen-11-one and (Z)-7-nonadecen-11-one, which attracts adult males for mating, and the pheromone has been used to trap adult males for controlling and monitoring the *C. sasakii* population. However, it is not sufficiently effective for mating disruption [4]. Overall, more efficacious management processes are urgently required.

Insects communicate with their environment through olfaction when detecting food and habitats, as well as when finding mates and escaping predators. To successfully perform such behaviors, insects must respond to chemical stimulation at the right moment. Insects' olfactory systems are modified depending on age, feeding state, circadian rhythm, and mating status. Disrupting the chemical communication system as a method to disrupt mating is a possible novel, ecologically friendly, and environmentally friendly approach for pest control [5].

The sex pheromone of *Bombyx mori* was identified first, and from then on, research into pest olfactory systems has developed rapidly. Pest behavior regulation techniques based on the olfactory system are internationally recognized as a green plant protection technology. Compared to other techniques, olfactory behavior regulation technology has five characteristics: safety, selectivity, efficiency, effectiveness and compatibility [6]. There are two important types of compounds used as part of the technology: one relates to the volatile organic compounds released by plants, and the other is the sex pheromones released by insects. Both of them have several advantages. First, pheromones are natural compounds that are generally used in very small quantities (billionth of a unit). Second, pheromones break down relatively quickly in the environment (they do not leave residues). Third, they are highly specific—they do not act on non-target organisms. Fourth, they allow the rapid detection of insects in the field, aiding agricultural decision-making [7].

Plant volatile organic compounds (VOCs) are major vehicles of information transfer between organisms, and they mediate many ecological interactions. Several herbivorous insects utilize plant chemical cues to identify hosts for feeding. Given the multifunctional nature of induced VOC emissions, it is safe to assume that, with changes in the selective environment, the major function of an information-mediating trait, such as VOC emission, will shift [8]. Several insects employ plant volatile organic compounds as olfactory cues when seeking a host plant, enabling them to discriminate between host plants and nonhost genotypes. Herbivorous insects can search for their hosts by plant volatiles, and the natural enemies of herbivores can find their prey by plant volatiles. Fruit-feeding insects are, logically, attracted to fruit volatiles. For these insects, fruits are not only a nutritional resource but also ideal mating sites. Blends of fruit volatiles indicate mating opportunities and may stimulate calling (pheromone release) and mating behaviors [9]. From an evolutionary perspective, there is a strong selection pressure on insects to develop sophisticated means for detecting and locating food resources upon which survival and reproduction depend [10].

In most species of moths, the sex pheromone is a blend of several components comprising fatty acid derivatives, usually alcohols, aldehydes, or acetates connected by a C_{10}-C_{18} straight chain [11], and is produced and secreted by the female pheromone glands (PGs) located between the eighth and ninth abdominal segments. Sex pheromones induce sexual behavior in insects. A male insect may smell the sex pheromone released by the female and then attempt to copulate with her [12]. To date, sex pheromones, especially female-produced sex attractants, have been the most widely used semiochemicals in attract-and-kill techniques. They are selective, often attracting only the target species [13].

The perception of these compounds depends on the olfactory receptor neurones (ORNs) in sensillae, mostly found on the insects' antennae, which can recognize individual molecular structures [14]. After odor compounds enter the sensilla via the numerous pores on its surface, these molecules encounter an aqueous barrier, the sensillum lymph [11]. In the lymph, there are many protein families that determine the operation of the odor compounds. Chemosensory proteins (CSPs) are one of them.

CSPs were first reported in *Drosophila melanogaster* and were identified as olfactory segment D (OS-D) at the time [15]. Antennal protein 10 was also found in *Drosophila melanogaster*. Subsequently, proteins with close similarities to OS-D were also found on the antennae of several stick insects (identity 30–45%) [16–18]. A protein with a 47% structural similarity to OS-D, named CLP-1, was found on the lips of *Cactoblastis cactorum*, and this protein is expressed in females and not in males [19]. Similar proteins have also been found in *Anopheles gambiae*, referred to as sensory appendage proteins (SAPs) [20,21]. CSPs were officially named in 1999, and seven soluble proteins with structural similarities to OS-D were discovered in the antennae of *Schistocerca gregaria* [22].

CSP is highly conserved in evolution, with 40–50% identical residues between species with more orthologous phylogenetic development compared to odorant-binding proteins (OBPs), which are only conserved at 10–15% [23]. The cause may be related to the disulfide bond. In OBP, where three disulfide bonds bind to each other to keep the protein stable,

the effect of residue substitution is very limited, while in CSP, two disulfide bonds are connected to adjacent cysteines, and conservative residue sequences are folded to form hydrophobic pockets and maintain protein stability [24].

We have evaluated the relative expression levels of *CSPs* from different tissues in *C. sasakii*, and the results showed that CsasCSP7 and CsasCSP9 are highly expressed in the antennae of the male and female moth, while CsasCSP11 is extremely highly expressed in the wings. In order to better understand the function of CsasCSPs in the olfactory communication system, we analyzed the characteristics of the CsasCSPs sequence, constructed the recombinant expression vector of CsasCSPs in a prokaryotic expression system, and tested the binding affinities of CsasCSPs to volatile molecules through fluorescence competitive binding assays. Our study enriches our understanding of the functions of CSPs from *C. sasakii* and complex olfactory mechanisms in insects.

2. Materials and Methods

2.1. Insect Rearing and Sample Collection

The larvae of *C. sasakii* were collected from the apple orchard of the Institute of Pomology, Chinese Academy of Agricultural Sciences (CAAS), Liaoning province (latitude 40.61° N, longitude 120.73° E), China. The pests were maintained under constant conditions of 25 ± 1 °C, 70 ± 5% relative humidity, and a 15:9 (L:D) h photoperiod. They were fed a 10% honey solution. *C sasakii* commenced eclosion after 4:00 p.m., and the virgin moths mated after 9:00 p.m. on the same day. Therefore, we infer that *C. sasakii* reached sexual maturity on the day of the eclosion [25]. We separately dissected 400, 100, 100, 100, 100, and 100 1-day-old adults to get different tissues of antennae (200 males and 200 females), heads without antennae (20 males and 20 females, respectively), thoraxes and abdomens (10 males and 10 females, respectively), legs, and wings (50 males and 50 females, respectively). The samples were placed in RNase-free centrifuge tubes under an optical microscope. Three biological replicates were prepared. All samples were immediately immersed in liquid nitrogen and stored at −80 °C.

2.2. Total RNA Extraction and cDNA Synthesis

The total RNA of different tissue samples was extracted using a Trizol Reagent handbook (TAKARA BIO Inc., Shiga, Japan). The integrity and concentration of RNA were assessed via 1.5% agarose gel electrophoresis and an ultra-microspectrophotometer, and the first-strand cDNA was synthesized with 1 μg of total RNA using a PrimeScript RT reagent Kit with a gDNA eraser (TAKARA BIO Inc., Shiga, Japan) according to the manufacturer's instructions. This was then subjected to PCR and q-PCR.

2.3. cDNA Cloning and Sequence Analysis

PCR assays were performed in a mixture of 40 μL containing 4 μL of antennae cDNA (100 ng), 20 μL of Primer fix Kit, 2 μL of each primer (10 μM), and 12 μL of RNase-Free Water. The PCR amplification procedure was as follows: predenaturation at 95 °C for 3 min; 35 cycles of 95 °C for 30 s; 55 °C for 30 s; 72 °C for 45 s; and final extension at 72 °C for 10 min. The PCR product was visualized using 1% agarose gel electrophoresis; the recovered target gene was then ligated into the pMD19-T vector (TAKARA BIO Inc., Shiga, Japan) and then transformed into DH5α competent cells (TAKARA BIO Inc., Shiga, Japan) for splicing sequencing (BGI Genomics, Beijing, China).

The open reading frames (ORFs) for these sequences were found using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html, accessed on 9 March 2022). The signal peptides of *CsasCSPs* were predicted using the SignalP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP/, accessed on 9 March 2022) [26]. The molecular weight was computed using ProtParam (http://web.expasy.org/protparam/, accessed on 1 May 2022). The isoelectric point was also calculated using the same software. The *C. sasakii* CSPs were chosen for phylogenetic analysis along with CSPs from Lepidoptera, Diptera, Hemiptera and Coleoptera, which were downloaded from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.

cgi, accessed on 1 October 2021). DNAMAN (Lynnon Biosoft, San Ramon, CA, USA) was used for multiple sequence alignment. A phylogenetic tree was constructed in MEGA7.0 using the neighbor-joining method with 1000 bootstrap replicates and clarified with iTOL (https://itol.embl.de/, accessed on 10 October 2021).

2.4. Expression Levels of Three CSPs mRNA from C. sasakii

The RT-qPCR assay was carried out using the CFX96 Connect Real-Time Detection System produced by BioRad in the USA using β-actin and elongation factor 1α (EF-1α) as the endogenous gene, the cDNA obtained as above as the template, and the female head (without antennae) as a control. In the experiment, RT-qPCR primers were designed using Beacon Design 8.0 (Table 1), and the amplification efficiencies were 90–110%. Three biological replicates were performed, and two technical replicates were also performed. The specific operation is detailed below.

Table 1. Primers used in this study.

Primer Name	Primer Sequence (5′-3′)	Length (bp)	Purpose
CsasCSP7-F	AGGTTATTGAGCATCTGATTAAG	95	Fluorescence quantification
CsasCSP7-R	TTCATACTTCTTTCTCCACTTG		
CsasCSP9-F	GTTATGGAGTACATCATAGATC	102	
CsasCSP9-R	TTTCTTCTCTTCATACTTACTC		
CsasCSP11-F	CAAGTAGTCCGATACATTAGG	125	
CsasCSP11-R	TAATCATCAGAAGCGAAGAAT		
CsasCSP7-F	CGGGATCCATGGAAGAAAAGTATTCGGACAAATA	342	Prokaryotic expression
CsasCSP7-R	TGGAATTCCTATTTTTCAGGTATTTCAATACCCCT		
CsasCSP9-F	CGGGATCCATGCGCCCCGAAGAGCACT	432	
CsasCSP9-R	TGGAATTCTTATGGCCTTGACGGTGCG		
CsasCSP11-F	CGGGATCCATGGATGAGGAGCAGTATACAGATAGAT	333	
CsasCSP11-R	TGGAATTCTTAATCATCAGAAGCGAAGAATG		

Note: The restriction sites are underlined.

Reaction system: Each RT-qPCR reaction was conducted in a 20 μL reaction mixture containing 10 μL of SYBR Master Mix, 1 μL of sample cDNA, 0.5 μL of each primer (10 μmol/L), and 8 μL of sterilized ddH_2O. Reaction procedure: denaturation at 95 °C for 3 min; 40 cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s.

After the program, the Ct values of the endogenous and target genes were obtained. According to the endogenous gene, the relative expression of the target gene was calculated from the $2^{-\Delta\Delta Ct}$. A significant difference analysis of the target genes between different tissues was undertaken using a one-way analysis of variance (ANOVA), and then Tukey's honestly significant difference ($p < 0.05$) method was employed for multiple comparisons. These were implemented through the SPSS Statistics 19.0 software.

2.5. Expression and Purification of Recombinant CsasCSPs Proteins

The target gene was amplified through adult antennae cDNA, and primers were designed using Primer Premier 5 (Table 1). The PCR product was ligated to the pMD-19T vector, then to the pET-28a vector (EcoRI, XhoI). It was then transformed into BL21 (DE3) competent cells [3]. After positive verification had been performed, the bacterial solution was cultured in 500 mL of liquid Luria–Bertani (LB) medium containing 50 μg/mL of kanamycin at 37 °C. When the OD_{600} reached 0.6–0.8, the recombinant protein was induced at 37 °C for 10 h using isopropyl β-d-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. The cells were harvested via centrifugation at 12,000 rpm for 10 min, suspended in lysis buffer (80 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 4% glycerol, pH 7.2 and 0.5 mM PMSF), sonicated in ice for 3 s, over five passes, and then centrifuged again. The 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) assay showed that these proteins persisted in the inclusion body. A refolding inclusion body assessment was conducted via the redox method. The proteins were concentrated in the 10 kDa and

30 kDa ultrafiltration cube. Before the 15% SDS-PAGE analysis, the solutions containing the proteins were dialyzed using 500 mL of Tris-HCl buffer at a final concentration of 20 mM three times at 4 °C.

2.6. Fluorescence Competitive Binding Assay

Thirty-six common volatile components and two sex pheromones from *C. sasakii* were chosen as ligands for use in the fluorescence competitive binding assays (Table 2). Fluorescence binding assays were performed using a multimode reader. The fluorescent emission spectra of the probe, 1-Aminoanthraquinone (1-AMA, Sigma–Aldrich, St. Louis, MO, USA), were recorded in the range of 400 to 750 nm with an excitation wavelength of 260 nm. To measure the affinity of 1-AMA to each protein, 2 μM of protein in 50 mM Tris–HCl (pH 7.2) was titrated with 1 mM 1-AMA to final concentrations ranging from 2 to 80 μM. Dissociation constants for the protein/1-AMA complex were inferred from Scatchard plots of the binding data. Ligands with concentrations ranging from 1 to 30 μM were added to the mixtures of protein and probe at the same final concentration of 2 mM. The dissociation constants of the competitors were calculated from their corresponding IC_{50} values (concentrations of ligands halving the initial fluorescence value of 1-AMA), using the equation $Ki = [IC_{50}]/ (1 + [1\text{-AMA}]/K_{1\text{-AMA}})$, where [1-AMA] is the free concentration of 1-AMA, $[IC_{50}]$ is the ligand concentration displacing 50% of the fluorescent reporter, and $K_{1\text{-AMA}}$ is the dissociation constant of the complex protein/1-AMA. All values were obtained from three independent measurements. The protein with 100% activity was assumed, inferred via the stoichiometry assessment of a 1:1 ratio of protein/ligand, and the data were analyzed as above.

Table 2. Plant volatiles and sex compounds for this experiment.

Code	Prospective Ligand	CAS	Molecular Weight /g·mol^{-1}	Purity/%
L1	2-Methylbutyl acetate	624-41-9	130.18	99
L2	Butyl butyrate	109-21-7	144.21	>99
L3	Butyl heptanoate	5454-28-4	186.29	>99
L4	Butyl octanoate	589-75-3	200.32	>99
L5	Ethyl butyrate	105-54-4	116.16	99
L6	Ethyl heptanoate	106-30-9	158.24	>98
L7	Ethyl hexanoate	123-66-0	144.21	99
L8	Hexyl hexanoate	6378-65-0	200.32	98
L9	Isoamyl acetate	123-92-2	130.19	99
L10	Methyl jasmonate	39,924-52-2	224.3	95
L11	Propyl octanoate	624-13-5	186.29	98
L12	Tert-butyl acetate	540-88-5	116.15	99
L13	(Z)-3-Hexenyl acetate	3681-71-8	142.2	98
L14	Benzaldehyde	100-52-7	106.12	>99
L15	Decanal	112-31-2	156.27	97
L16	Dodecanal	112-54-9	184.32	>95
L17	(E)-Hex-2-enal	6728-26-3	98.14	98
L18	Hexanal	66-25-1	100.16	97
L19	Honanal	124-19-6	142.24	96
L20	Octanal	124-13-0	128.215	97
L21	1-Hexanol	111-27-3	102.18	>98
L22	2-Ethylhexanol	104-76-7	130.22	99
L23	3-Methyl-1-butanol	123-51-3	88.15	99
L24	(E)-2-Hexen-1-ol	928-95-0	100.16	97
L25	(Z)-Hex-3-en-1-ol	928-96-1	100.16	≥98
L26	Decane	124-18-5	142.29	>99
L27	Hexadecane	544-76-3	226.45	>98
L28	Octadecane	593-45-3	254.49	98
L29	Pentadecane	629-62-9	212.41	98
L30	Tetradecane	629-59-4	198.39	≥99

Table 2. *Cont.*

Code	Prospective Ligand	CAS	Molecular Weight /g·mol^{-1}	Purity/%
L31	alpha-Farnesene	502-61-4	204.25	>99
L32	Beta-Ocimene	13,877-91-3	136.23	>90
L33	(-)-beta-Pinene	18,172-67-3	136.24	>94
L34	Myrcene	123-35-3	136.236	>80
L35	6-Methyl-5-hepten-2-one	110-93-0	126.2	>98
L36	Benzonitrile	100-47-0	103.12	>99
L37	Z-7-Eicosene-11-one	63,408-44-6	294.5	>99
L38	Z-7-Nonadecen-11-one	63,408-45-7	280.5	>99

3. Results

3.1. Sequence Analysis of CsasCSPs

The length of the open reading frame (ORF) of *CsasCSP7*, *CsasCSP9* and *CsasCSP11* was 387-bp, 432-bp and 402-bp, respectively. This encoded 128 amino acids, 143 amino acids and 133 amino acids, respectively. At the N-terminus of the polypeptide chain, *CsasCSP7* was predicted to contain a signal peptide consisting of 19 amino acid residues. *CsasCSP9* was predicted to contain a signal peptide consisting of 15 amino acid residues. *CsasCSP11* was predicted to contain a signal peptide consisting of 24 amino acid residues. As the signal peptide was cleaved off, the MW of the mature protein *CsasCSP7* was 13.25 kDa with an isoelectric point of 5.36. The predicted MW of the *CsasCSP9* protein was 14.54 kDa, and the isoelectric point was 4.93. The predicted MV of the *CsasCSP11* protein was 13.15kDa, and the isoelectric point was 8.53. The results of multiple alignments show that *CsasCSP7*, *CsasCSP9* and *CsasCSP11* presented a typical four-cysteine signature, forming two pairs of disulfide bonds (Figure 1). To analyze the phylogenetic relationships of *CsasCSPs* with other insects, including Lepidoptera, Diptera, Hemiptera and Coleoptera, a phylogenetic tree was constructed. The results clearly show that *CsasCSPs* were located in the Lepidoptera group. Compared to other orders, Lepidoptera were explicitly clustered together (Figure 2).

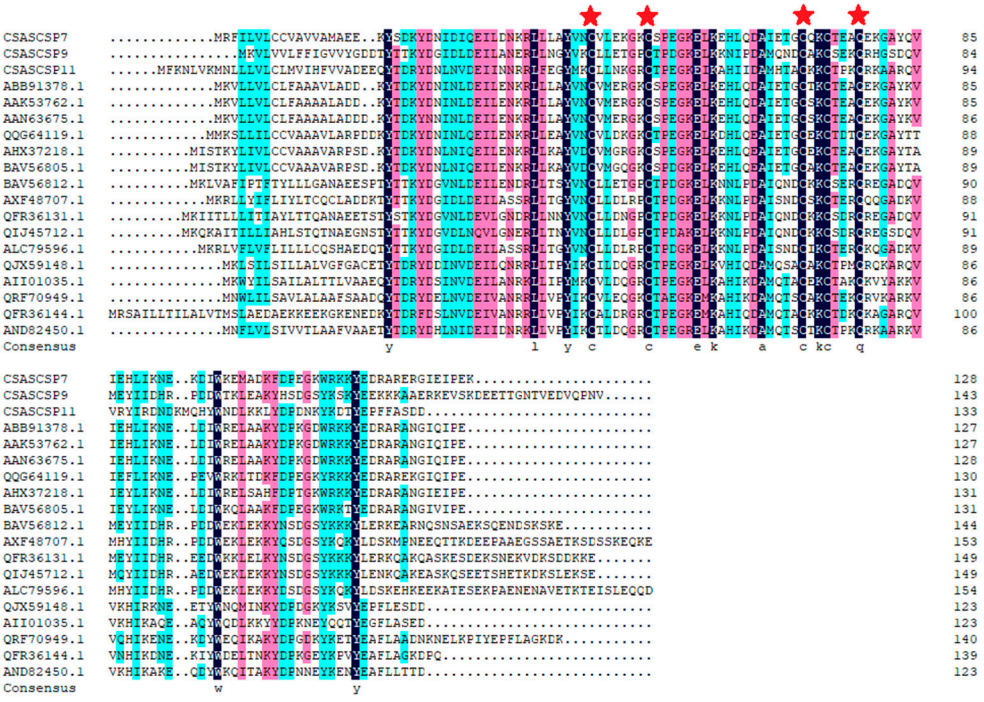

Figure 1. Multiple sequence comparison between CasaCSPs and Lepidoptera. Lepidoptera included *Athetis dissimilis* (AND82450.1), *Conogethes pinicolalis* (QFR36131.1, QFR36144.1), *Conogethes punctiferalis*

(AHX37218.1), *Dendrolimus kikuchii* (AII01035.1), *Dioryctria abietella* (QQG64119.1, QJX59148.1), *Glyphodes pyloalis* (QIJ45712.1), *Grapholita molesta* (ALC79596.1), *Helicoverpa armigera* (AAK53762.1), *Helicoverpa assulta* (ABB91378.1), *Helicoverpa zea* (AAN63675.1), *Lobesia botrana* (AXF48707.1), *Ostrinia furnacalis* (BAV56805.1, BAV56812.1) and *Semiothisa cinerearia* (QRF70949.1). The red stars explain four highly conserved cysteine residues of the CSP family.

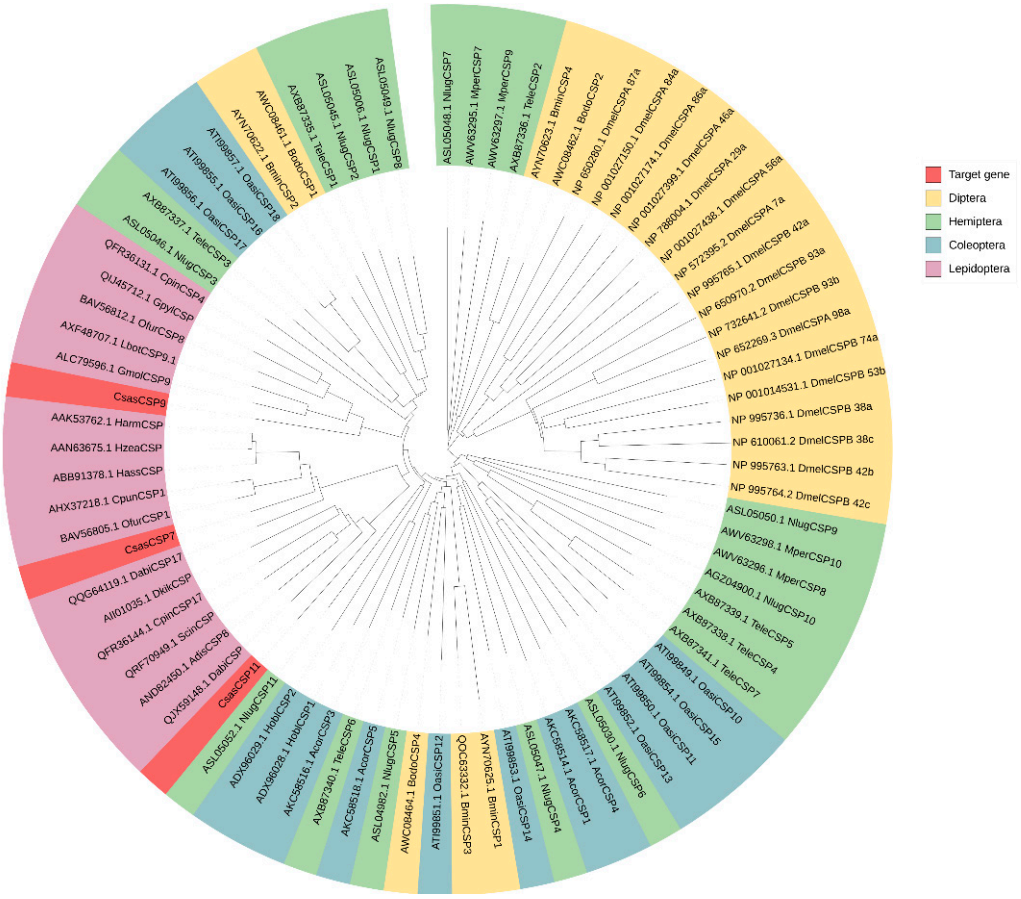

Figure 2. Phylogenetic tree of CsasCSPs and other organisms. Diptera included *Bactrocera minax* and *Drosophila melanogaster*; Hemiptera included *Myzus persicae*, *Nilaparvata lugens* and *Tropidothorax elegans*; Coleoptera included *Holotrichia oblita*, *Anomala corpulenta* and *Oedaleus asiaticus*; Lepidoptera included *Athetis dissimilis*, *Conogethes pinicolalis*, *Conogethes punctiferalis*, *Dendrolimus kikuchii*, *Dioryctria abietella*, *Glyphodes pyloalis*, *Grapholita molesta*, *Helicoverpa armigera*, *Helicoverpa assulta*, *Helicoverpa zea*, *Lobesia botrana*, *Ostrinia furnacalis* and *Semiothisa cinerearia*.

3.2. Tissue Expression Patterns of CsasCSPs

To better understand the function of *CsasCSPs*, their expression patterns in different tissues were measured via RT-qPCR. The expression analysis revealed similar expression patterns of *CsasCSP9* in both males and females. *CsasCSP9* was predominantly expressed in the antennae of both sexes, with the lowest expression observed in the other tissues. *CsasCSP7* and *CsasCSP11* showed broad expression profiles in the whole body of male and female adults, but *CsasCSP7* was predominantly expressed in the male antennae, and *CsasCSP11* was expressed at a significantly higher level in female wings (Figure 3).

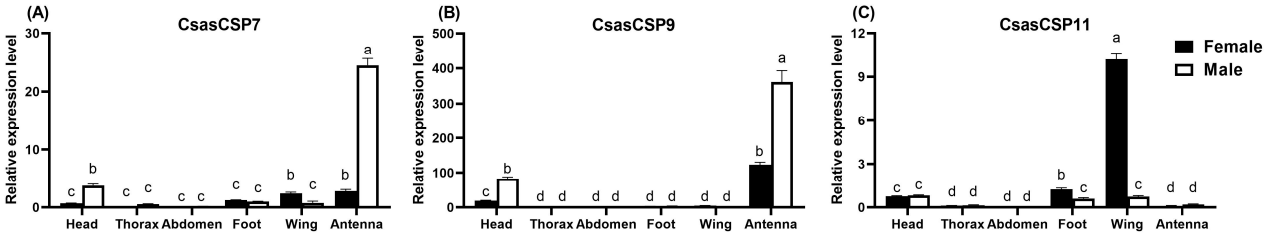

Figure 3. Expression levels of the *CsasCSP7*, *CsasCSP9* and *CsasCSP11* genes between male and female *C. sasakii*. (**A**). *CsasCSP7*; (**B**) *CsasCSP9*; (**C**) *CsasCSP11*. The letters on the top of each bar means the statistic differences ($p < 0.05$).

3.3. Bacterial Expression and Purification of CsasCSPs

The pET-28a (+)/CsasCSPs proteins were assessed for expression in vitro (*Escherichia coli* BL21 (DE3) cells), and the target proteins were found more abundantly in the insoluble fraction containing inclusion bodies compared to the supernatant when induced with IPTG via 15% SDS-PAGE. The 15% SDS-PAGE analysis revealed that the final purified proteins were present in single bands of approximately 13.25 kDa, 14.54 kDa and 13.15 kDa, as is consistent with the MW of the recombinant proteins (Figure 4).

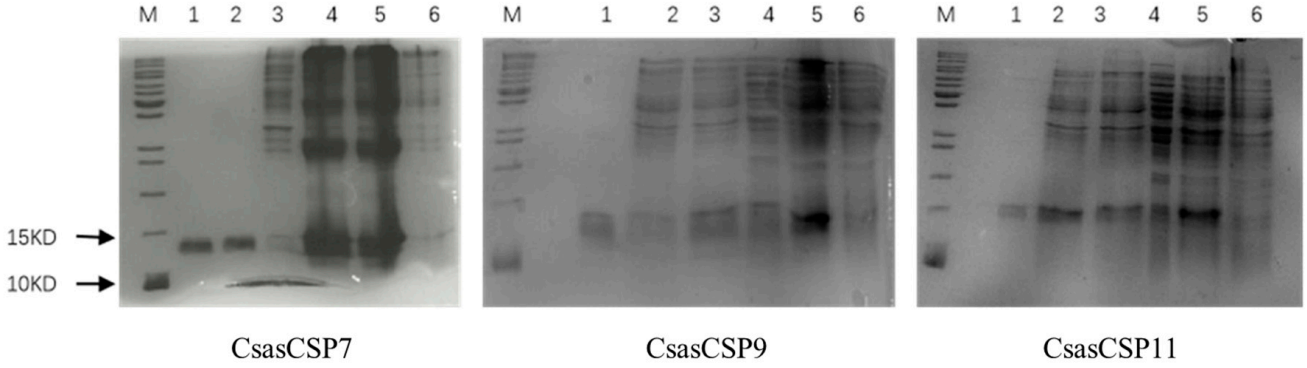

Figure 4. SDS-PAGE analysis of expressed CsasCSP7, CsasCSP9 and CsasCSP11 proteins. 1: The purified protein; 2: The refolding protein; 3: Supernatant of the induced; 4: Inclusion body of the induced; 5: Expressed product induced with IPTG; 6: Expressed product without induction with IPTG; M: Standard protein marker.

3.4. Binding Properties of Recombinant CsasCSPs

For the further characterization of CsasCSPs ligand-binding affinity to host volatiles, 1-AMA was used as a competitive fluorescent reporter. When 1-AMA was added dropwise to the protein solution, the maximum emission peak at 420 nm was shifted to approximately 560 nm. CsasCSP7, CsasCSP9 and CsasCSP11 bind to the fluorescent probe 1-AMA rather weakly, with dissociation constants of 20.10, 12.39 and 13.83 μM, respectively, yielded by the Scatchard equation (Figure 5). Therefore, the Ki (the ratio of the dissociation constant of the competitor to the partition coefficient of the competitor in the aqueous phase) and IC_{50} (the concentration of the competitor when the fluorescence intensity of the complex protein/1-AMA declined to 50% of the initial fluorescence) values of the ligands with CsasCSPs are presented in Tables 3–5. Compounds that we tested that reduced the fluorescence intensity of the complex systems to 50% or lower were regarded as binding ligands. This group of chemicals includes two sex pheromone components and 36 plant volatiles. Two volatiles, benzaldehyde (Ki = 7.25 ± 0.23 μM) and dodecanal (Ki = 13.61 ± 0.54 μM), were tested for use as potential ligands for CsasCSP7. CsasCSP9 showed high capacities for binding to three chemical volatiles, namely, butyl octanoate (Ki = 1.47 ± 0.13 μM), decanal (Ki = 1.65 ± 0.31 μM) and (-)-beta-pinene (Ki = 14.26 ± 0.62 μM). Three of the ligands tested, 1-hexanol (Ki = 8.13 ± 0.78 μM), beta-ocimene (Ki = 1.83 ± 0.66 μM) and

6-methyl-5-hepten-2-one (Ki = 0.71 ± 0.07 μM), exhibited relatively strong binding affinities for CsasCSP11. Besides this, the CsasCSPs showed no affinity for the components of sex pheromones (Figure 6).

Table 3. Binding affinities of CsasCSP7 to all tested ligands.

Ligand	K_i	IC50	Ligand	K_i	IC50	Ligand	K_i	IC50
2-Methylbutyl acetate	-	>50	Benzaldehyde	7.25 ± 0.23	8.00 ± 0.59	Hexadecane	-	>50
Butyl butyrate	-	>50	Decanal	-	>50	Octadecane	-	>50
Butyl heptanoate	-	>50	Dodecanal	13.61 ± 0.54	14.97 ± 0.60	Pentadecane	-	>50
Butyl octanoate	-	>50	(E)-Hex-2-enal	-	>50	Tetradecane	-	>50
Ethyl butyrate	-	>50	Hexanal	-	>50	alpha-Farnesene	-	>50
Ethyl heptanoate	-	>50	Honanal	-	>50	Beta-Ocimene	-	>50
Ethyl hexanoate	-	>50	Octanal	-	>50	(-)-beta-Pinene	-	>50
Hexyl hexanoate	-	>50	1-Hexanol	-	>50	Myrcene	-	>50
Isoamyl acetate	-	>50	2-Ethylhexanol	-	>50	6-Methyl-5-hepten-2-one	-	>50
Methyl jasmonate	-	>50	3-Methyl-1-butanol	-	>50	Benzonitrile	-	>50
Propyl octanoate	-	>50	(E)-2-Hexen-1-ol	-	>50	Z-7-Eicosene-11-one	-	>50
Tert-butyl acetate	-	>50	(Z)-Hex-3-en-1-ol	-	>50	Z-7-Nonadecen-11-one	-	>50
(Z)-3-Hexenyl acetate	-	>50	Decane	-	>50			

IC50 labeled ">50" indicates that binding affinities could not be calculated with the tested ligand concentrations, so K_i of the ligands is represented as "-".

Table 4. Binding affinities of CsasCSP9 to all tested ligands.

Ligand	K_i	IC50	Ligand	K_i	IC50	Ligand	K_i	IC50
2-Methylbutyl acetate	-	>50	Benzaldehyde	-	>50	Hexadecane	-	>50
Butyl butyrate	-	>50	Decanal	1.65 ± 0.31	1.92 ± 0.34	Octadecane	-	>50
Butyl heptanoate	-	>50	Dodecanal	-	>50	Pentadecane	-	>50
Butyl octanoate	1.47 ± 0.13	1.71 ± 0.16	(E)-Hex-2-enal	-	>50	Tetradecane	-	>50
Ethyl butyrate	-	>50	Hexanal	-	>50	alpha-Farnesene	-	>50
Ethyl heptanoate	-	>50	Honanal	-	>50	Beta-Ocimene	-	>50
Ethyl hexanoate	-	>50	Octanal	-	>50	(-)-beta-Pinene	14.26 ± 0.62	16.56 ± 0.24
Hexyl hexanoate	-	>50	1-Hexanol	-	>50	Myrcene	-	>50
Isoamyl acetate	-	>50	2-Ethylhexanol	-	>50	6-Methyl-5-hepten-2-one	-	>50
Methyl jasmonate	-	>50	3-Methyl-1-butanol	-	>50	Benzonitrile	-	>50
Propyl octanoate	-	>50	(E)-2-Hexen-1-ol	-	>50	Z-7-Eicosene-11-one	-	>50
Tert-butyl acetate	-	>50	(Z)-Hex-3-en-1-ol	-	>50	Z-7-Nonadecen-11-one	-	>50
(Z)-3-Hexenyl acetate	-	>50	Decane	-	>50			

IC50 labeled ">50" indicates that binding affinities could not be calculated with the tested ligand concentrations, so K_i of the ligands is represented as "-".

Table 5. Binding affinities of CsasCSP11 to all tested ligands.

Ligand	Ki	IC50	Ligand	Ki	IC50	Ligand	Ki	IC50
2-Methylbutyl acetate	-	>50	Benzaldehyde	-	>50	Hexadecane	-	>50
Butyl butyrate	-	>50	Decanal	-	>50	Octadecane	-	>50
Butyl heptanoate	-	>50	Dodecanal	-	>50	Pentadecane	-	>50
Butyl octanoate	-	>50	(E)-Hex-2-enal	-	>50	Tetradecane	-	>50
Ethyl butyrate	-	>50	Hexanal	-	>50	alpha-Farnesene	-	>50
Ethyl heptanoate	-	>50	Honanal	-	>50	Beta-Ocimene	1.83 ± 0.66	2.09 ± 0.76
Ethyl hexanoate	-	>50	Octanal	-	>50	(-)-beta-Pinene	-	>50
Hexyl hexanoate	-	>50	1-Hexanol	8.13 ± 0.78	9.31 ± 0.39	Myrcene	-	>50
Isoamyl acetate	-	>50	2-Ethylhexanol	-	>50	6-Methyl-5-hepten-2-one	0.71 ± 0.07	0.81 ± 0.08
Methyl jasmonate	-	>50	3-Methyl-1-butanol	-	>50	Benzonitrile	-	>50
Propyl octanoate	-	>50	(E)-2-Hexen-1-ol	-	>50	Z-7-Eicosene-11-one	-	>50
Tert-butyl acetate	-	>50	(Z)-Hex-3-en-1-ol	-	>50	Z-7-Nonadecen-11-one	-	>50
(Z)-3-Hexenyl acetate	-	>50	Decane	-	>50			

IC50 labeled ">50" indicates that binding affinities could not be calculated with the tested ligand concentrations, so K_i of the ligands is represented as "-".

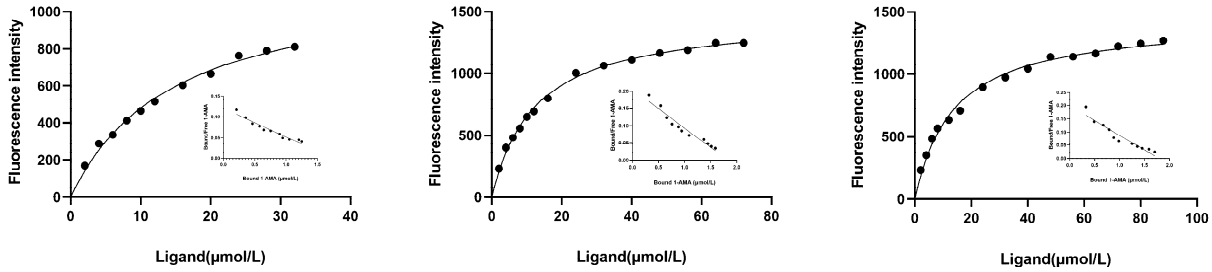

Figure 5. Binding curves of 1-AMA to CsasCSPs.

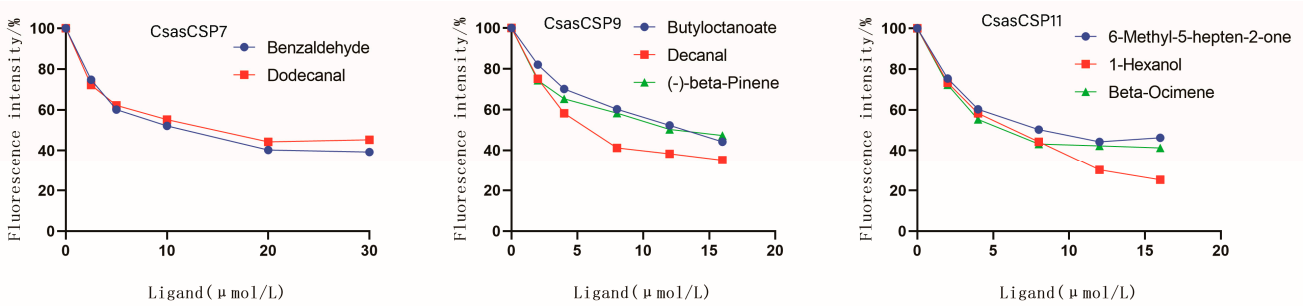

Figure 6. Competitive binding curves of CsasCSPs with compounds.

4. Discussion

In this work, we cloned and sequenced the cDNA of *CsasCSP7*, *CasaCSP9* and *CasaCSP11*, and analyzed the signal peptides, isoelectric points, protein molecular weights and protein properties of the CsasCSP7, CasaCSP9, and CasaCSP11 proteins. They all showed four conserved cysteines and two disulfide bonds, as is typical of the CSP family.

CSP is distributed in various parts of an insect's body, such as the antennae [27,28], legs [29], abdomen [30], midguts [31], sensillum lymph [32] and wings [33]. There have also been reports showing that CSP is expressed differently at different times [34]. Before now, many studies have shown that CSPs have many different functions.

CSPMbraA and CSPMbraB, expressed in the antennae and pheromone glands, were thought to be involved in the transport of hydrophobic molecules through different aqueous media, such as the sensillar lymph and the pheromonal gland cytosol, in *Mamestra brassicae* [35,36]. LmigCSP91 was found to be expressed in the male organs of *Locusta migratoria*, but after mating, it was also found in female organs [37]. This was also the case for *Apis mellifera* AmelCSP3 [38]. It can be speculated that these proteins are key to the transportation of pheromones.

CSP is also thought to be closely related to the reproduction, regeneration and development of insects. Protein p10 was found in the regenerated legs of *Periplaneta americana* larvae at levels 30 times higher than in normal legs. Fluorescent immunology showed that p10 was present only in a newly formed epidermis [39,40]. *Apis mellifera* AmelCSP5 was specifically expressed only in the gonads of female adults. When the gene encoding AmelCSP5 was silenced, eggs did not fully develop and did not hatch. This protein has been shown to play a vital role in the development of eggs [41,42]. *Solenopsis invicta* SinvCSP9 and AmelCSP5 belong to the same evolutionary branch. Gene silencing not only affected the synthesis of fatty acids, but also the development and molting of the stratum corneum [43]. The HamCSP4 of *Helicoverpa armigera* was expressed in large quantities in the mouthparts, and has been verified as a surfactant, suggesting that it may be involved in dissolving the terpenoids in nectar, given the affinity of HarmCSP4 with terpenoids [44]. LmigCSP IIII (Lmig EST6) in *Locusta migratoria* exhibited a strong binding capacity with the non-host plant compound α-amyl cinnamaldehyde (AMCAL). AMCAL has been shown to be an effective food repellent for use against locusts. Locusts were less sensitive to this compound after LmicsP IIII had been knocked out [45]. *Bemisia tabaci* BtabCSP11 was

highly expressed in the abdomen. After it was silenced, the fertility of female whiteflies decreased significantly, indicating that it may be involved in their reproduction [46].

In our study, the results show that CasaCSP7 and CasaCSP9 were highly expressed in antennae, and CasaCSP11 was highly expressed in the wings. *Mythimna separata* MsepCSP5 was mainly expressed in the antennae compared to other tissues, and combined with some rice volatiles, suggesting its potential major role in chemodetection [47]. *Monochamus alternatus* MaltCSP5 was also reported to be expressed in the antennae of both male and female adults, and the protein showed a high binding activity to most pine volatiles [48]. *Apolygus lucorum* AlucCSP2 and AlucCSP3 were specifically expressed in female wings. AlucCSP4 was expressed relatively highly in female wings but was also expressed in other tissues. Interestingly, the capacity of the three proteins to bind to cotton secondary metabolites is better than their capacity to bind to plant volatiles [33]. Therefore, it is hypothesized that not only the antennae-predominant CasaCSP7 and CasaCSP9, but also the wing-predominant CasaCSP11, may both bind to host volatiles during olfactory recognition.

Ligand-binding experiments have demonstrated that CsasCSPs undergo highly selective binding to volatile compounds, including benzaldehyde, dodecanal, butyl octanoate, decanal, (-)-beta-pinene, 1-hexanol, beta-ocimene and 6-methyl-5-hepten-2-one. In previous studies, benzaldehyde, decanal, 6-methyl-5-hepten-2-one and β-pinene were identified as promoting aggregation behavior in insects [49–52].

Benzaldehyde is not only very common, but also a predominant component of the floral scents of many species [53]. In single sensillum recording experiments on both male and female *Sitona humeralis* antennae, olfactory sensory neurons (OSN) were stimulated successfully using benzaldehyde in a clear dose-dependent manner. Field experiments revealed that traps baited with benzaldehyde caught significantly more *S. humeralis* than unbaited traps, indicating an attractant effect. There were no apparent differences between the sexes [54]. Meanwhile, behavioral experiments were conducted on *Spodoptera littoralis*, and the results showed that the larvae were attracted to benzaldehyde at 100 and 10 μg/μL [55]. *Rhopalosiphum padi* males responded positively to benzaldehyde. The release of benzaldehyde with a conspecific sex pheromone increased the catch rates of both species of aphid [56]. Trapping tests undertaken in the field also showed that benzaldehyde has a lure effect on *Conotrachelus nenuphar* [57]. In 2015, an article confirmed that benzaldehyde has insecticidal activity against *Galleria mellonella* (100% insect mortality) [58]. All this suggests that benzaldehyde can be used in the development of new insecticides.

A strong binding affinity between CsasCSP7 and dodecanal was also shown. It has been shown that MsepCSP5 is abundantly expressed in the antennae of adult females and selectively recognizes dodecanal [47]. The dodecanal identified in the headspace of acidified chicken feces elicited electroantennogram responses from the antennae of *Culex quinquefasciatus* females [59]. The dodecanal of 50 and 100 ppm exhibited oviposition-deterring activities against gravid *Aedes aegypti* females. [60]. Olfactometer experiments with synthetic chemical compounds revealed the significant attraction of *Bactrocera dorsalis* female flies to dodecanal [61]. In addition, dodecanal was considered a sex pheromone because it was identified on extracts of filter paper contaminated by young *Cephalonomia tarsalis* females. It also had an arresting effect on males, but not on females [62]. CsasCSP7 showed sensitivities to benzaldehyde and dodecanal, suggesting that CsasCSP7 plays an important role in the identification of host plants in the olfactory communication system.

CsasCSP9 showed strong capacities for binding to three volatiles, namely, butyl octanoate, decanal and (-)-beta-pinene. The electroantennographic results demonstrate that decanal elicited significant antennal responses in *Quadrastichus mendeli*. Bioassays confirmed that *Q. mendeli* was repelled by decanal [63]. On the other hand, wind tunnel experiments showed that decanal attracted male *Lutzomyia longipalpis* [64]. A pure compound of decanal and 0.03 ppm decanal were also reported as attractants for *Heterorhabditis bacteriophora* and *Steinernema kraussei*, respectively [65]. Compared with normal *Sitobion avenae* SaveOBP9, in experiments with SaveOBP9 knockdown, wheat aphids showed sig-

nificantly decreased attractive responses toward decanal [66]. Similarly, after silencing LmigOBP1 in *Locusta migratoria*, the nymphs showed significantly decreased electroantennography (EAG) responses to decanal [67]. In GC-EAD, the antennae of gravid female *Ostrinia nubilalis* responded to decanal, which is an established maize volatile [68]. Interestingly, Morawo et al. found that decanal elicited 0.82 mV EAG response in *Microplitis croceipes*, which was the highest response value. However, decanal accounts for only 1% of cotton-fed host volatiles [69].

β-pinene is a ubiquitous plant terpenoid and elicits host recognition behaviors in female *Conogethes punctiferalis* [70]. Under four-arm olfactometer bioassays, the *Porphyrophora sophorae* larvae showed a clear preference for β-pinene, and β-pinene proved to be the major chemical cue used by *P. sphorae* neonates when searching for the roots of their host plant [71]. In a series of novel hydronopylformamides derivatives synthesized from a naturally occurring compound, (-)-β-pinene, four exhibited repellency against *Blattella germanica* at a concentration of 20 mg/mL [72]. CsasCSP9 had a binding affinity to butyl octanoate, decanal and (-)-beta-pinene, suggesting that CsasCSP9 can transport host volatiles in the olfactory communication system.

The compound 6-methyl-5-hepten-2-one is released by animals, is also a major component identified from flower scents and may play an important role outside animal-host seeking [73]. In *Triatoma dimidiate*, both females and males were attracted to 6-methyl-5-hepten-2-one [74]. *Diaziella yangi* and *Lipothymus* sp enter *Ficus curtipes* in response to the body odors of obligate wasps and one of the main compounds emitted by figs—6-methyl-5-hepten-2-one [75]. 6-methyl-5-hepten-2-one, which has been identified in zebra skin, was one of the three ketones (acetophenone and geranylacetone) that reduced the catch levels of *Glossina pallidipes* in field trials [76]. The mixture of 6-methyl-5-hepten-2-one and geranylacetone in a 1:1 ratio increased the repellency of DEET to *Anopheles gambiae*, *Culex quinquefasciatus* and *Aedes aegypti* when present at low concentrations. In olfactometer trials, 6-methyl-5-hepten-2-one interfered with the attraction of mosquitoes to a host [77]. A mixture of two compounds, 6-methyl-5-hepten-2-one and geranylacetone, showed significant repellency towards *Culicoides impunctatus* in the field [78]. The electrophysiological testing of 6-methyl-5-hepten-2-one using five fly species (*Musca autumnalis*, *Haematobia irritans*, *Hydrotaea irritans*, *Stomoxys calcitrans* and *Wohlfahrtia magnifica*) showed that it was physiologically active towards the flies tested. At certain concentrations, 6-methyl-5-hepten-2-one increased upwind flight in flies and reduced fly loads [79]. As such, 6-methyl-5-hepten-2-one may have the potential to be developed into novel repellents. Meanwhile, volatile characterization with gas chromatography–mass spectrometry identified the contents of *Gelis agilis* emissions as 6-methyl-5-hepten-2-one, a known insect defense semiochemical that acts as an alarm pheromone in ants [80].

β-ocimene is a key plant volatile with multiple relevant functions in plants, depending on the organ and the time of emission. AmelCSP4 has the best affinity with β-ocimene. In AmelCSP4, Tyr98 and Asp67 are involved in β-ocimene binding [81]. Tea plants infested with *Ectropis obliqua* larvae triggered neighboring plants to release β-ocimene, which repels *E. obliqua* adults, especially mated females. The levels of β-ocimene released by infested tea plants increased rapidly [82]. Pest-infested plants (*Silene latifolia*) emitted higher amounts of β-ocimene [83], as did *Morus alba* [84]. Compared with healthy plants, the treatment of Chinese cabbage with β-ocimene inhibited the growth and reproduction of *Myzus persicae*, and we saw that winged aphids preferred to stay on the healthy plants. *Aphidius gifuensis*, however, was shown to prefer cabbage treated with β-ocimene [85]. Similar results have been reported elsewhere [86]. In male *Hyphantria cunea*, when β-ocimene was added to a sex pheromone, the efficiency of trapping male moths increased [84]. Subtractive bioassays performed in a dual-choice olfactometer showed that a 3-component terpenoid plant-derived blend comprising (E)-linalool oxide, β-pinene and β-ocimene was more attractive to female *Anopheles gambiae* [87].

In a study on *Adelphocoris lineolatus*, 1-hexanol was identified as the sex pheromone antagonist [88]. However, further research found that AlinOBP10 had a higher binding

affinity for 1-hexanol. In Y-tube olfactometer trials, 1-hexanol was repellent against female adults [89]. In a feeding test of *Ips typographus*, high concentrations of 1-hexanol solution were shown to cause food rejection (AFI = 1.00), and males were found to be more sensitive than females [90]. In *Grapholita molesta*, recombinant GmolCSP8 (rGmolCSP8) also showed the strongest binding affinity to 1-hexanol. In our research, CsasCSP11 showed sensitivities to these three compounds, providing evidence that CsasCSP11 may be involved in the detection of plant volatiles.

5. Conclusions

Two of the three genes selected were highly expressed in male antennae. Unfortunately, the two genes showed nonsignificant binding affinities for sex pheromones of *C. sasakii*, but were able to bind to plant volatiles. Different compounds will produce different results in different insects. The results for some of these compounds are consistent with our experimental results, while others are in opposition, and these compounds have attractive/repellent effects on insects. In conclusion, our results show that CsasCSP7, CsasCSP9 and CsasCSP11 may have a dual role in host-seeking. The characterization and function of CsasCSP7, CsasCSP9 and CsasCSP11 from *C. sasakii* contributes to our understanding of the underlying mechanisms of olfactory communication in insects.

Author Contributions: Conceptualization, L.S. and G.Q.; methodology, L.S. and G.Q.; software, L.L. and Q.Y.; validation, W.Y. and L.S.; formal analysis, L.L.; investigation, L.S. and G.Q.; resources, L.S. and G.Q.; data curation, L.S. and L.L.; writing—original draft preparation, L.L.; writing—review and editing, L.L. and L.S.; visualization, L.L., H.Z. and Q.Y.; supervision, L.S.; project administration, L.S. and G.Q.; funding acquisition, L.S. and G.Q. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Applied Basic Research Project of Liaoning Province (2022JH2/101300192), the Agricultural Science and Technology Innovation Program (CAAS-ASTIP-2021-RIP-05) and the National Natural Science Funds (31601643).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

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