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ORIGINAL ARTICLE

# Involvement of chemosensory proteins in host plant searching in the bird cherry-oat aphid

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**Abstract** Chemosensory systems are considered to play an important role in host plant selection in herbivorous insects. However, few studies have focused on chemosensory proteins (CSPs) for aphid host-location mechanisms. The roles of CSPs in searching for different Poaceae species (wheat, barley, triticale, maize and sorghum) were tested in *Rhopalosiphum padi*, an important cereal pest. The olfactometer assays showed that *R. padi* responds to plant odors. Seven *R. padi CSP* genes were identified. Influence of aphid morph, tissue and starvation state on expression patterns of *CSPs* was evaluated. Expression levels of *CSP1*, *CSP4*, *CSP5* and *CSP6* in winged aphids were significantly higher than those in wingless ones. Transcription levels of four genes (*CSP1*, *CSP4*, *CSP5* and *CSP6*) were relatively higher in the head with antennae, and the four genes tended to be upregulated following starvation. Silencing of three *CSP*s (*CSP4*, *CSP5* and *CSP6*) altered aphid host-location behavior in response to the five different host plants tested. Three volatile compounds of host plants (octanal, [*E*]-2-hexenol and linalool) have significant attraction to winged *R. padi* according to the four-arm olfactometer tests. Molecular docking predicted hydrogen bonding sites which played key roles in the binding of CSP4, CSP5 and CSP6 with volatile compounds. Knockdown of *CSP4* or *CSP5* significantly decreased the staying time of *R. padi* in the arms with octanal. However, knockdown of *CSP6* could not affect the response of *R. padi* to octanal. These results bring evidence for the involvement of three CSPs in *R. padi* host-location behavior.

**Key words** host choice experiment; olfactometer; *Rhopalosiphum padi*; RNAi; starvation; wing form

## Introduction

The bird cherry-oat aphid, *Rhopalosiphum padi* (L.), is an agricultural pest which causes severe economic damage on Poaceae crops (e.g., wheat, oat, barley, maize) and has a worldwide distribution. The aphid not only damages plants by sucking plant liquid and nutrients directly and secreting honeydew, which causes sooty mold, but also transmits the barley yellow dwarf virus (BYDV), which is a global and economically devastating cereal crop virus (Krueger *et al.*, 2013; Leybourne *et al.*, 2020). *R. padi* feeds on a variety of host plants, including cereal crops and wild grasses (Leather & Dixon., 1982; Peng *et al.*, 2020). Populations of *R. padi* typically show winged and wingless individuals. Wingless individuals develop faster, show high fecundity and are produced when food

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source is abundant. In contrast, winged aphids are formed in adverse environmental conditions (e.g., high density, limited or low-quality food source) and possess a series of behavioral, morphological and physiological traits adapted to flight, plant location and selection, and reproduction on a new host plant (Braendle *et al.*, 2006). However, molecular mechanisms of such host searching behavior have not been explored so far.

To accurately perceive volatile compounds under complex natural conditions, insects have evolved a complicated, highly specific and sensitive chemosensory system (Field *et al.*, 2000). Numerous studies have shown that the chemosensory system is indispensable for many insect species, playing an important role in searching for host plants, locating mates and oviposition sites, identifying conspecifics and avoiding predators (Pelosi *et al.*, 2006; Hansson & Stensmyr 2011; Leal *et al.*, 2013; Gadenne *et al.*, 2016; Pelosi *et al.*, 2017). The high sensitivity and specificity of the chemosensory system mostly depend on interactions between chemical signals and various olfactory proteins, including the two types of transport proteins, chemosensory proteins (CSPs) and odorant-binding proteins (OBPs), three types of receptors, olfactory receptors (ORs), gustatory receptors (GRs), ionotropic receptors (IRs), as well as sensory neuron membrane proteins (SNMPs) and odorant-degrading enzymes (ODEs) (Kaupp, 2010; Leal, 2013; Pelosi *et al.*, 2017). CSPs encompass a family of small soluble proteins that possess four conserved cysteines, forming two independent loops and a characteristic N-terminal signature motif (Angeli *et al.*, 1999; Tegoni *et al.*, 2004). CSPs can distinguish and selectively bind hydrophobic chemicals from external environments and transport these molecules across the sensillum lymph to ORs or GRs, activating olfactory-related signal transduction (Laughlin *et al.*, 2008; Sánchez-Gracia *et al.*, 2009; Pelosi *et al.*, 2017).

To date, a large number of CSP sequences have been identified from different insect orders, and the number of CSP in different insects varies (Pelosi *et al.*, 2006; Xu *et al.*, 2009; Fan *et al.*, 2011; Pelosi *et al.*, 2017). In Aphididae, nine to 13 CSPs were identified from the genomic data of *Acyrthosiphon pisum* (13 CSPs), *Aphis glycines* (nine) and *Myzus persicae* (nine) (Richards *et al.*, 2010; Mathers *et al.*, 2017; Wenger *et al.*, 2017). Five to nine CSPs were identified from transcriptomic data in *A. gossypii* (nine), *Sitobion avenae* (five) and *Daktulosphaira vitifoliae* (seven) (Gu *et al.*, 2013; Xue *et al.*, 2016; Zhao *et al.*, 2017). Although Kang *et al.* (2018) found there were nine *CSP* genes in *R. padi* through the transcriptome analysis, the nucleotide sequence of these genes was not uploaded, and the expression of *CSP* genes has not been detected under different conditions. Functional studies of aphid *CSP* genes have mainly focused on recognizing and detecting the alarm pheromone (*E*)-beta-farnesene in *S. avenae* (Fan *et al.*, 2015), *A. pisum* (Zhang *et al.*, 2017) and *R. padi* (Fan *et al.*, 2017).

Since their first discovery in *Drosophila melanogaster*, CSPs have attracted increased attention (McKenna *et al.*, 1994). CSPs have multiple functions and can bind small molecules including nutrients, chemical signals, toxic compounds and hormones (Pelosi *et al.*, 2017). Compelling evidence has been provided that CSPs have chemosensory functions in *Bactrocera dorsalis* (Diptera: Tephritidae) (Yi *et al.*, 2013), *Microplitis mediator* (Hymenoptera: Braconidae) (Peng *et al.*, 2017), *Dendroctonus armandi* (Coleoptera: Curculionidae: Scolytinae) (Li *et al.*, 2018), *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae) (Zeng *et al.*, 2018), *Nilaparvata lugens* (Hemiptera: Delphacidae) (Waris *et al.*, 2018, 2020), *Mythimna separata* (Lepidoptera: Noctuidae) (Younas *et al.*, 2018) and *Grapholita molesta* (Lepidoptera: Tortricidae) (Li *et al.*, 2019). Recently, it was found that CSPs have several miscellaneous functions beyond the chemosensory system (Pelosi *et al.*, 2017). For example, CSP genes can play significant roles in modulating behavioral phase change of *Locusta migratoria* (Orthoptera: Acrididae) (Guo *et al.*, 2011); silencing of SexiCSP3 can influence female survival and reproduction in *Spodoptera exigua* (Lepidoptera: Noctuidae) (Gong *et al.*, 2012); in *Solenopsis invicta* (Hymenoptera: Formicidae), the *CSP9* gene has essential functions in integument and molting process of larvae (Cheng *et al.*, 2015). It was reported that CSPs can play important roles in insect host search behavior (Hansson & Stensmyr, 2011; Gadenne *et al.*, 2016; Pelosi *et al.*, 2017); however, the functions of *CSP* genes in host plant searching are still unknown in aphids.

Wheat (*Triticum aestivum*), sorghum (*Sorghum bicolour*), maize (*Zea mays*), triticale (x *Triticosecale* Wittmack) and barley (*Hordeum vulgare*) are common widely grown Poaceae crops. *R. padi* can feed well on these plant species, and these plants are main hosts of *R. padi* (Leather & Dixon., 1982). There were many volatile compounds released by host plants, for example, aldehydes, alcohols, esters, alkenes. Some volatile compounds, such as (*E*)-2-hexenol, (*E*)-2-hexenyl acetate, (*E*)-$\beta$-ocimene, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, benzaldehyde, $\beta$-myrcene, decanal, heptanal, linalool, linalool oxide, methyl salicylate, nonanal and octanal, were commonly present in *R. padi* host plants including wheat, sorghum, maize and barley (Quiroz & Niemeyer, 1998; Padmaja *et al.*, 2010; Dong *et al.*, 2015; Schröder *et al.*, 2015). Thus, we chose these volatiles for behavioral responses tests of *R. padi*. In this study, we explored

the olfactory response of *R. padi* and measured the expression profiles of *CSP* genes on both winged and wingless forms, in different tissues/organs and at different starvation states. We hypothesized that aphid morph and starvation status could affect olfactory response of the aphid (Braendle *et al.*, 2006; Fan *et al.*, 2011; Pelosi *et al.*, 2017). Then, we investigated the RNA interference (RNAi) efficiency of *CSP* genes and the effect of *CSP* gene*s* on aphid olfactory response. Finally, behavioral responses of winged *R. padi* to 14 synthetic compounds were determined. Binding of CSP proteins and the compounds were predicted using molecular docking. Our objective was to characterize the role of *CSP* gene*s* in host plant searching of *R. padi*, and by doing so, to contribute to a better understanding of the molecular mechanisms of aphid olfactory response to host plants.

## Materials and methods

### *Aphids and host plants*

For all the experiments we used a *R. padi* colony originally collected from *Triticum aestivum* (cultivar "Xiaoyan 22") in Yangling, Shaanxi Province, China, in May 2016, and subsequently maintained on wheat seedlings of the same *T. aestivum* cultivar using cages (42 × 42 × 42 cm) covered with mesh gauze (100 mesh) in climate chambers at 24 ± 1 °C and 70% ± 5% relative humidity (RH) with a 16 : 8 L : D photoperiod. Wheat seeds were planted in plastic pots (10 cm in diameter) containing nutritive medium and sterile soil (1 : 1, v : v). The seedlings were watered, and were not used until they reach two- or three-leaf stage (BBCH-scale 12–13). To eliminate maternal effects from the field environmental conditions, the *R. padi* clone was kept in the climate chambers for >3 generations before experiments.

Seeds of wheat, sorghum, maize, triticale and barley were obtained from a commercial supplier (Jindao Company, Yangling, China). The five plants were established individually in plastic pots (10 cm in diameter). To minimize the effects of nutrition, the same volume of nutritive medium and sterile soil was used for all the plants. The seedlings of different plant species with similar sizes (20 cm tall) were randomly selected for each experiment. All tested plants and aphids were free from pathogens.

### *Identification and phylogenetic analysis of CSP genes in R. padi*

Because we started this study before the release of the *R. padi* genome (Thorpe *et al.*, 2018) and the current assembly may not be complete, we conducted the identification of *CSP* genes in *R. padi* by cloning method. Total RNA was extracted from 10 adult aphids that were randomly selected from the clone described earlier using a TRIzol kit (Invitrogen, Carlsbad, CA, USA), and it was reverse transcribed into complementary DNA (cDNA) using a reverse transcriptase (Promega, Madison, WI, USA). The gene-specific primers (Table S1) were designed to clone the chemosensory protein genes using the transcript fragments of each gene in *R. padi* transcriptome data (Duan *et al.*, 2017). The SMARTer RACE cDNA amplification kit was used to obtain the 5'-UTR (untranslated region) and 3'-UTR region sequences of these genes.

The identified gene sequences were further checked for sequence similarity and open reading frame (ORF) identification using the National Center for Biotechnology Information Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and ORF Finder software (https://www.ncbi.nlm.nih.gov/orffinder/). The signal peptide was identified by SignalP-5.0 Server program (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignment and phylogenetic analysis were performed using the maximum likelihood algorithm in MEGA v7 (Kumar *et al.*, 2016). The tree branches were generated by 1000 bootstrap replications.

### *Morph and tissue specificity of CSP expression patterns in R. padi*

To investigate the relative expression levels of the seven *CSP* genes in winged and wingless parthenogenetic females, total RNA was extracted from 10 winged and wingless parthenogenetic females (newly emerged adults) from the clone rearing on wheat seedlings described earlier. The DNA-free Kit (Applied Biosystems, Foster City, CA, USA) was used to eliminate DNA contamination. The 2 μg total RNA of each sample was reverse transcribed into the first strand cDNA using the reverse transcriptase described earlier. The quantitative primers were designed by Primer Premier 6 software and are shown in Table S1. The quantitative polymerase chain reaction (qPCR) (95 °C for 3 min, 40 cycles of 95 °C for 10 s, 58 °C for 20 s and 72 °C for 20 s; and one cycle at 72 °C for 10 min) was carried out on the Rotor Q thermocycler (Qiagen, Hilden, Germany) using the FastStart Essential DNA Green Master (Roche, Basel, Switzerland). To eliminate the possibility of reagent contamination, a blank (no-template) control was conducted for each run and each gene. The $\beta$-actin and $\alpha$-tubulin genes were used as the reference genes for normalization (Kang

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*et al.*, 2016; Zhang *et al.*, 2018). All analyses were performed with three technical and biological replicates. The melting curves were used to further confirm the specificity of qPCR primers. The qPCR efficiency of these primers was determined using a pool of cDNA samples in a 5 fold serial dilution. The relative expression patterns of seven *CSP* genes were calculated by the relative quantitative method ($2^{-\Delta\Delta Ct}$) (Livak & Schmittgen, 2001).

The relative transcriptional levels of *CSP* genes in different tissues of winged parthenogenetic females were assessed by using reverse transcription qPCR (qRT-PCR). The head with antennae, thorax with legs, and abdomen of aphids were dissected in the RNA storage solution. Each treatment consisted of 100 winged newly emerged adult females from the clone rearing on wheat seedlings, and three technical and biological replicates were used for each treatment. The tissues were frozen in liquid nitrogen for RNA extraction, and qPCR was performed according to the method described above.

### *Effects of starvation and refeeding on expression patterns of CSP genes in R. padi*

The starvation could significantly affect the olfactory response of insects to their host plants (Reisenman *et al.*, 2013; Pelosi *et al.*, 2014). Expression level change of *CSP* genes may influence the olfactory sensitivity of insects. To examine whether starvation and refeeding after starvation influenced the expression of the seven *CSP* genes in *R. padi*, wingless adults from the clone described earlier were managed separately with starvation for 24 h (S1), starvation for 24 h and feeding for 24 h (S1F1), starvation for 48 h (S2) and starvation for 48 h and feeding for 24 h (S2F1). The wingless adults with no starvation were used as controls. Aphids placed on a plastic Petri dish (one aphid per Petri dish) were checked daily, and newborn nymphs were removed. Each treatment consisted of 60 wingless adults, and three biological replicates were used for each treatment. The surviving aphids were frozen in liquid nitrogen for RNA extraction, and qPCR was performed according to the method described above.

### *CSP silencing by RNAi*

Sequence-specific primers of seven *CSP* genes (Table S1) were synthesized to amplify target fragments for RNAi. PCR fragments were gel-purified with the Gel Extraction Kit (Promega, Madison, WI, USA) and used to synthesize double-stranded RNA (dsRNA) using the T7 RiboMAX$^{TM}$ Express RNAi System (Promega, Madison, WI, USA) according to the manufacturer's instructions. ds*GFP* was synthesized and served as a control. The concentrations of purified dsRNA were examined using a biophotometer (Eppendorf BioPhotometer Plus, Eppendorf, Germany), and the purity and integrity were verified by agarose gel electrophoresis. The dsRNAs were stored at −80 °C until use.

To evaluate the RNAi efficiency, we selected newly emerged wingless adult aphids for RNAi experiments, and 50 nL dsRNAs with four different concentrations (4.45, 6.67, 8.89 and 11.11 $\mu$g/$\mu$L) were respectively injected into the suture joining the ventral mesothorax and metathorax using an automatic nanoliter injector (Märzhäuser, Wetzlar, Germany) equipped with a microglass needle prepared using a P-97 Micropipette Puller (Sutter Instrument Co., Novato, CA, USA). Controls consisted of newly emerged adult aphids injected with nuclease-free water (hereafter referred to as "$H_2O$"). After injection, 10 surviving adults that were reared in climate chambers (24 ± 1 °C; 70% ± 5% RH; 16 : 8 h L : D) were randomly collected at 24 h, 48 h and 72 h in each treatment. qPCR was used to measure the transcriptional level of the targeted genes, and three replications were carried out per treatment.

### *Effects of RNAi targeting CSP genes on host plant searching*

The host plant searching behavior of *R. padi* wingless adult was investigated using a Y-tube olfactometer bioassay. The winged aphids are active and easily escape from the Y-tube, thus we chose wingless aphids to conduct olfactometer bioassay (Quiroz & Niemeyer, 1998). In the Y-tube olfactometer, the length of the stem is 10.0 cm, the two arms are both 10.0 cm in length and are at an angle of 60 degrees, and the internal diameter is 2.0 cm. The system consisted of two 2.5 L glass containers, and the whole plant was placed into one of the glass containers. The other container did not contain anything and served as the control. A Y-tube olfactometer bioassay with an air flow of 150 mL/min was carried out in the observation room. To eliminate lighting bias, cool white fluorescent lights were placed on top of the observation room (Zhang *et al.*, 2019b). *R. padi* individuals used for the choice experiment were chosen from the clone described earlier, which was reared on wheat. These aphids were starved for 6 h before being used in the olfactometer bioassay (Moayeri *et al.*, 2014; Germinara *et al.*, 2016). One *R. padi* wingless adult was placed at the entrance of the stem tube. The initial choice of an *R. padi* that responded by walking into one of the arms, crossing 5 cm of the

arm and remaining there for at least 1 min was recorded. If an *R. padi* made no choice 3 min after release, it was recorded as "no choice". Each tested aphid was used only once, and a total of 240 aphid individuals were recorded in each choice experiment. The Y-tube was wiped with absolute ethanol after each test. After 10 individuals had been observed, the Y-tubes were replaced by clean tubes, and the plant was also changed. We presented aphids with the following choices: wheat versus air; sorghum versus air; maize versus air; triticale versus air; barley versus air.

Seven-d-old wingless adult aphids were randomly collected and divided into five groups. $H_2O$, ds*GFP*, ds*CSP5*, ds*CSP6* and ds*CSP4* were injected into the aphids of five groups, respectively. Two d after dsRNA injection, a choice experiment was performed with the surviving aphids to analyze the effect of RNAi of *CSP5*, *CSP6* or *CSP4* genes on the host search ability of *R. padi* using a Y-tube olfactometer. The method of the choice experiment was as described above. Each tested aphid was used only once, and choice situations of *R. padi* were recorded in each host searching behavior experiment. Different host plants (wheat, sorghum, maize, triticale and barley) were used in different groups. A total of 50 individuals were recorded in each treatment ($H_2O$, ds*GFP*, ds*CSP5*, *CSP6* and ds*CSP4*) and each host plant. Three replicates were performed.

### *Behavioral responses of R. padi to synthetic compounds*

The chemicals used in the test included (*E*)-2-hexenol, (*E*)-2-hexenyl acetate, (*E*)-$\beta$-ocimene, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, benzaldehyde, $\beta$-myrcene, decanal, heptanal, linalool, linalool oxide, methyl salicylate, nonanal and octanal. All the chemical were obtained from Sigma-Aldrich (St Louis, MO, USA) and the purities were >98%. Each compound was dissolved in n-hexane when being used for the behavioral response test (Sigma-Aldrich), and the concentration of each compound was 0.05 μg/μL, which has been reported as active at behavioral level for *R. padi* (Schröder *et al.*, 2015).

Behavioral responses of winged *R. padi* to the compounds were determined using a Perspex four-arm olfactometer (Pettersson, 1970). To eliminate lighting bias, cool white fluorescent lights were placed on top of the observation room. The filter paper 1 cm × 1 cm was placed on the bottom of the small glass tubes. A 20 μL of compound was added to the filter papers in two opposing arms, and the remaining two arms containing the same volume of n-hexane solution were used as control. A single winged *R. padi* was introduced into the center of the chamber and was observed for 15 min. Staying time of the aphid in each of the four arms was recorded. The aphid was considered no response and discarded when it did not choose an arm within 3 min. The filter paper was replaced after observing the behavioral response of one aphid. Thirty replications were carried out per compounds, and the chamber was rotated 90° clockwise in each replication. The four-arm olfactometer was cleaned with 80% ethanol and $ddH_2O$ after testing of each compound. The Perspex chambers were left to air dry. The glass tubes were baked at 180 °C overnight.

### *Molecular modeling and docking*

The three-dimensional (3D) structures of the CSPs were built by homology modeling, and the modeled structures of the CSPs from *R. padi* were obtained employing online Swiss-model software (https://swissmodel.expasy.org/). The optimal templates obtained for the modeling targets were selected based on a low E-value, a high sequence identity (>30%) and query coverage. The 3D structures of octanal, (*E*)-2-hexenol and linalool were obtained using the online ZINC website (http://zinc.docking.org). The ligands were energy optimized for molecular docking using PyMOL software (DeLano, 2002). For docking validation, simulation of protein-ligand interactions were conducted within 10 independent runs with $2.5 \times 10^7$ numbers of evaluations for each docking experiment, and Lamarckian genetic algorithm (GA) of AutoDockTools-1.5.6 was used as the search method. The dimensions of the grids were set at 100 × 100 × 100 points in the x-, y- and z-dimensions based on the protein volume. The binding poses of the CSP-octanal complex, CSP-(*E*)-2-hexenol complex or CSP-linalool complex were obtained with the searching algorithm of AutoDock Tools Optimizer and the energetic evaluation of AutoDock Tools Score. The optimized binding pose and hydrogen bonds were displayed by PyMOL software.

### *Role of CSP genes in the response of R. padi to volatiles*

Winged adult aphids injected with $H_2O$, ds*GFP*, ds*CSP4*, ds*CSP5* or ds*CSP6* were used for choice experiment using a Perspex four-arm olfactometer. The method of the choice experiment was described earlier. The staying time of the aphid in each of the four arms was recorded, and 30 replications were carried out per synthetic compound. The chamber was rotated 90° clockwise in each replication.

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*Statistical analysis*

The expression levels of seven *CSP* genes in different treatments were subjected to one-way analysis of variance ($P < 0.05$). Data from choice experiments were expressed as percentages and were log-transformed to meet the assumptions of normality and homoscedasticity required for these analyses. Means were compared using Tukey's honestly significant difference (HSD) test ($P < 0.05$). All statistical analyses were executed with SPSS v.20 (IBM-SPSS, Armonk, NY, USA).

## Results

*Identification and characteristics of CSP genes*

Seven full-length *CSP* genes were cloned from *R. padi*. Generally, *CSP* ORFs of *R. padi* contained 393–495 nucleotides, encoding 131–165 amino acids. The predicted molecular weights of RpCSPs were 15.08–19.20 kDa. The theoretical isoelectric points of RpCSPs ranged from 7.53 to 9.67. Seven RpCSPs contained a putative signal peptide at the N-terminus and a common conserved domain (insect pheromone-binding family) (Fig. 1A). The alignment results indicated low nucleotide sequence similarity among the conserved domains of the seven *CSP* genes. Full-length BLASTp searches showed the sequence identities between *CSP1* and *CSP2*, *CSP4*, *CSP5*, *CSP6*, *CSP7* and *CSP8* were 24.24%, 26.67%, 21.21%, 29.17%, 13.41% and 22.47%, respectively (Fig. 1A). Seven CSP amino acid sequence alignments revealed a typical four-cysteine motif in the conserved domain (Fig. 1B). In addition, seven RpCSPs shared one conserved amino acid proline between the second and third "C". The seven *CSP* genes sequences were further confirmed by analyzing the *R. padi* genome published by Thorpe *et al.* (2018) and Morales-Hojas *et al.* (2020).

Phylogenetic analysis indicated that the seven RpCSPs clustered in different clades (Fig. 2). The different CSPs clustered with homologous CSPs of other aphids (*Myzus persicae*, *Acyrthosiphon pisum*, *Aphis gossypii*, *Diuraphis noxia*, *Daktulosphaira vitifoliae*, *Melanaphis sacchari*, *Pseudoregma bambucicola*, *R. maidis* and *Sipha flava*).

*Selection of candidate CSP genes related to host plant searching for gene silencing*

The relative mRNA expression levels of the seven *CSP* genes were compared between wingless and winged parthenogenetic females. The transcriptional levels of

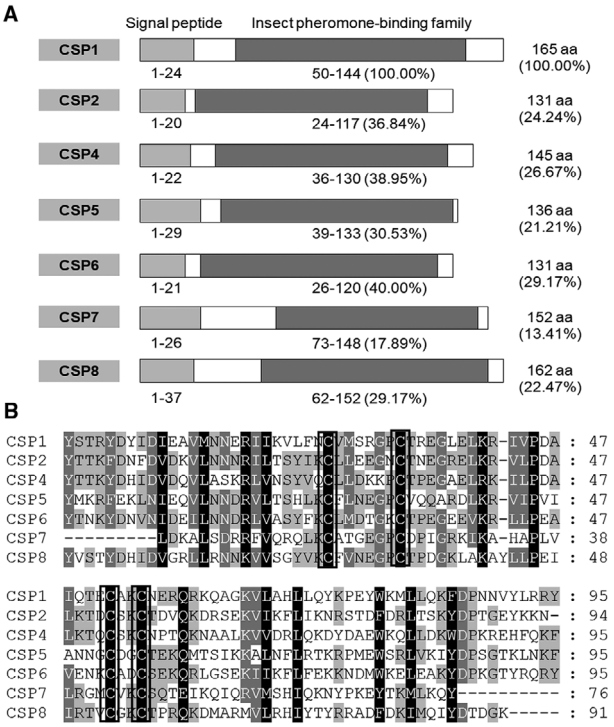


**Fig. 1** The characterization and sequence similarity of seven chemosensory proteins cloned from *Rhopalosiphum padi* (A) and alignment of the common domain sequence of the seven chemosensory proteins (CSPs) in *R. padi* (B).

*CSP1* ($P < 0.01$), *CSP4* ($P < 0.001$), *CSP5* ($P < 0.01$) and *CSP6* ($P < 0.01$) in winged parthenogenetic females were significantly higher than those in wingless females (Fig. 3A). The expression of *CSP7* ($P < 0.01$) gene in wingless parthenogenetic females was significantly higher than those in winged females. The *CSP2* and *CSP8* genes did not show expressional difference between winged and wingless individuals.

The qRT-PCR results showed that *CSP1* was highly expressed in head with antennae ($44.43 \pm 9.43$) compared to the other *CSPs*, but lowly expressed in thorax with legs, and abdomen of winged individuals (Fig. 3B). In addition, the expression levels of *CSP4* ($F = 11.03$; df = 2, 6; $P = 0.010$), *CSP5* ($F = 17.32$; df = 2, 6; $P < 0.01$) and *CSP6* ($F = 8.26$; df = 2, 6; $P = 0.019$) in head with antennae were significantly higher than those in thorax with legs, and abdomen (Fig. 3B). The *CSP7* gene was not expressed in the head with antennae. The *CSP2* and *CSP8* genes showed no expressional difference between organs.

Three genes (*CSP4*, *CSP5* and *CSP6*) tended to be upregulated following starvation for 24 h, and significant differences were found in the expression levels of these

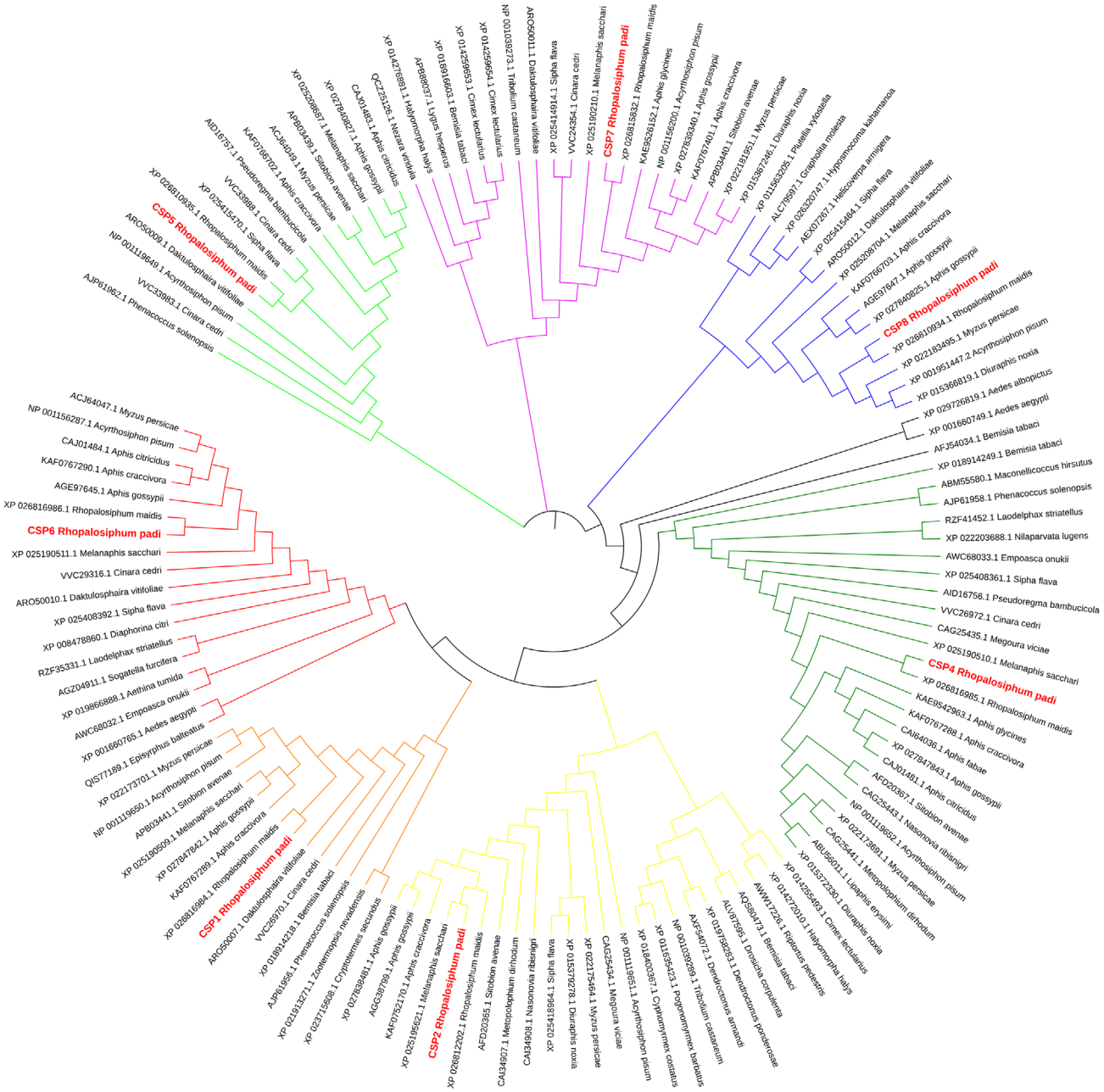


**Fig. 2** The phylogenetic relationships of the seven *CSP* genes of *Rhopalosiphum padi* with those of other insect species. The phylogenetic tree is based on aligned amino acid sequences using MEGA v7. The Latin names are shown behind the GenBank accession numbers, and *R. padi* sequences are in bold red typeface.

genes between the control and the S1 treatment. The transcript levels of four genes (*CSP1*, *CSP4*, *CSP5* and *CSP6*) in aphids starved for 48 h were significantly higher than those in control aphids. The expression levels of *CSP5* and *CSP6* decreased after subsequent feeding compared with those after starvation (*CSP1*: $F = 12.74$; df = 4, 10; $P < 0.001$; *CSP4*: $F = 110.59$; df = 4, 10; $P < 0.001$; *CSP5*: $F = 47.36$; df = 4, 10; $P < 0.001$; *CSP6*: $F = 16.76$; df = 4, 10; $P < 0.001$) (Fig. 4). Surprisingly, three genes (*CSP2*, *CSP7* and *CSP8*) tended to be downregulated following starvation for 24 h and 48 h, and the expression of these three genes significantly increased

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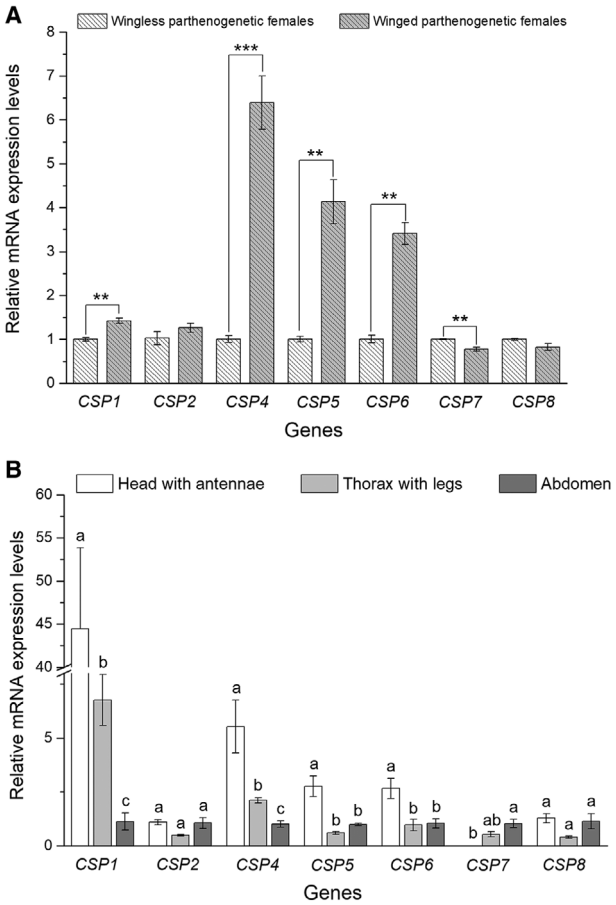


**Fig. 3** Expression profiles of the seven *CSP* genes from wingless and winged parthenogenetic females in *Rhopalosiphum padi* (A). Significant differences between two groups were assayed by *t*-test using a threshold *P*-value < 0.05. Expression profiles of the seven *CSP* genes in different tissues of winged parthenogenetic females in *R. padi* (B). Total RNA was extracted from head with antennae, thorax with legs, and abdomen. Significant differences between tissues were assayed by Tukey's honestly significant difference (HSD) test using a threshold *P*-value < 0.05.

after subsequent refeeding compared with after starvation treatment (*CSP2*: $F = 151.67$; df = 4, 10; $P < 0.001$; *CSP7*: $F = 290.08$; df = 4, 10; $P < 0.001$; *CSP8*: $F = 34.14$; df = 4, 10; $P < 0.001$) (Fig. 4).

### *Assessment of RNAi efficiency on CSP genes*

To examine the role of *CSP* genes in the search for host plants, we selected *CSP1*, *CSP4*, *CSP5* and *CSP6* for the RNAi experiments. These four *CSP* genes were highly expressed in winged parthenogenetic females, and these genes were upregulated following starvation. The expression of the *CSP4* gene differed significantly between the ds*GFP* and ds*CSP4* treatments on d 2 ($P < 0.01$, Fig. 5A). The expression levels of *CSP5* gene decreased dramatically (63.90% reduction) on d 1 after injection of ds*CSP5* compared with the levels after the ds*GFP* injection, and there were significant differences between the ds*GFP* and ds*CSP5* treatments on d 2 ($P < 0.01$, Fig. 5A). The RNAi effect of ds*CSP5* was greatly reduced on d 3. Injection of ds*CSP6* also induced a reduction in the transcript levels of the *CSP6* gene in *R. padi* compared to those after the ds*GFP* injection, and the transcript levels of the *CSP6* gene were significantly reduced on d 2 ($P = 0.016$, Fig. 5A). After injection of dsRNA, *CSP6* mRNA levels were similar to those in the control on d 1 and 3, respectively (Fig. 5A). However, the interference experiment on *CSP1* failed because the RNAi efficiency was too low.

In comparison with the control (injection of ds*GFP*) group, the expression levels of *CSP4* were significantly decreased 2 d post-injection of 8.89 $\mu$g/$\mu$L ($P < 0.01$) and 11.11 $\mu$g/$\mu$L ($P = 0.036$) ds*CSP4*; the expression levels of *CSP5* were significantly reduced 1 d post-injection of 6.67 $\mu$g/$\mu$L ($P = 0.017$), 8.89 $\mu$g/$\mu$L ($P < 0.01$) and 11.11 $\mu$g/$\mu$L ($P = 0.012$) ds*CSP5*; after injection with four different concentrations of ds*CSP6*, the expression levels of *CSP6* were significantly decreased (4.45 $\mu$g/$\mu$L: $P = 0.012$; 6.67 $\mu$g/$\mu$L: $P = 0.047$; 8.89 $\mu$g/$\mu$L: $P = 0.036$; 11.11 $\mu$g/$\mu$L: $P = 0.016$) (Fig. 5B). Based on the above results, we used 8.89 $\mu$g/$\mu$L dsRNAs for each injection for further RNAi experiments.

### *Role of CSP4, CSP5 and CSP6 genes in the search for host plants*

In the olfactometer assay, there was a strong response to host plant odors in *R. padi* (Fig. 6A). *R. padi* could clearly discriminate wheat ($F = 224.45$; df = 2, 6; $P < 0.001$), barley ($F = 154.76$; df = 2, 6; $P < 0.001$), triticale ($F = 159.40$; df = 2, 6; $P < 0.001$), maize ($F = 116.54$; df = 2, 6; $P < 0.001$), and sorghum ($F = 253.84$; df = 2, 6; $P < 0.001$) from the air in the respective searching test.

Silencing of *CSP4*, *CSP5* and *CSP6* resulted in behavioral changes in *R. padi* in response to the host plant. RNAi of *CSP4*, *CSP5* and *CSP6* genes decreased the ability of *R. padi* to search for the host plant wheat (Fig. 6B). Among *CSP5*-silenced aphids, 26% moved away from wheat, 50% were attracted by the host plant, and the remaining 24% showed no response. After knockdown of the *CSP5* gene, the percentage of the aphids choosing

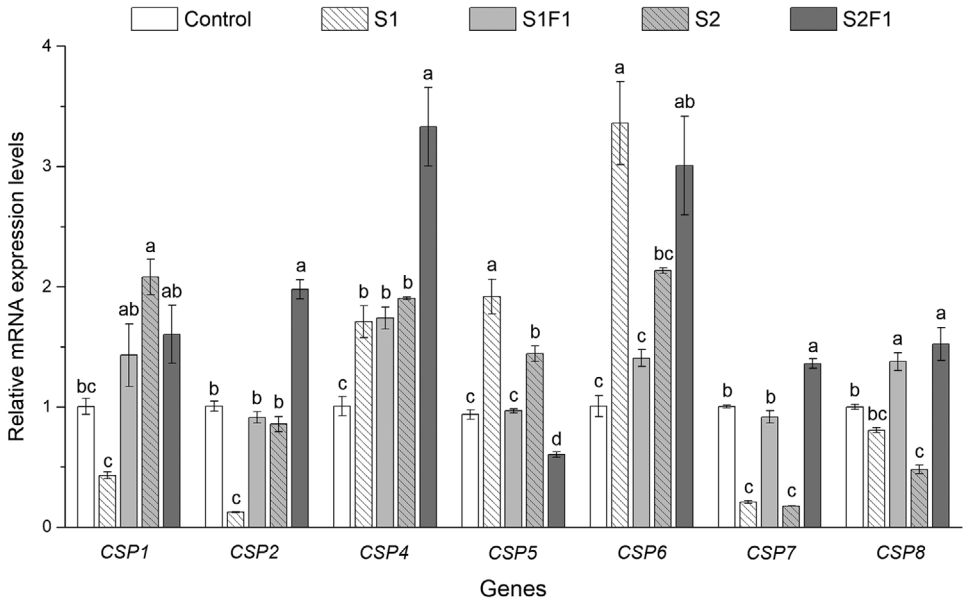


**Fig. 4** Expression profiles of the seven *CSP* genes in *Rhopalosiphum padi* adults after starvation conditions and refeeding after starvation. S1, starvation for 24 h; S1F1, starvation for 24 h and feeding for 24 h; S2, starvation for 48 h; S2F1, starvation for 48 h and feeding for 24 h. Values of each gene are normalized to the average expression of that gene. Different letters on the bars indicate significant differences ($P < 0.05$, Tukey's honestly significant difference [HSD] test).

wheat was significantly lower than that of those who received the ds*GFP* injection ($P < 0.05$), and more aphids moved away from the wheat or showed no choice compared with those who received the ds*GFP* injection ($P < 0.05$). A similar result was observed after knockdown of the *CSP4* gene.

RNAi of *CSP4*, *CSP5* and *CSP6* genes also affected the behavior of *R. padi* when searching for barley (Fig. 6C), triticale (Fig. 6D), maize (Fig. 6E), and sorghum (Fig. 6F). More aphids in the treatment groups (injection of *dsCSP4*, *dsCSP5* and *dsCSP6*) moved away from the host plant or showed no choice compared with those that received the ds*GFP* injection. Only 46% and 50.67% of *CSP5*-silenced and *CSP6*-silenced aphids, respectively, were able to choose barley, which was significantly lower than the percentage of control aphids that received the ds*GFP* injection ($P < 0.05$). Only 51.33% of *CSP4*-silenced aphids were able to move toward barley. The olfactometer assay showed that *CSP5* silencing in *R. padi* significantly decreased the aphid's strong preference for triticale and sorghum ($P < 0.05$) compared to control aphids. *CSP6* silencing in *R. padi* dramatically decreased the aphid's strong preference for triticale and maize, and *CSP4* silencing in *R. padi* significantly decreased the aphid's strong preference for triticale, maize and sorghum ($P < 0.05$) compared to control aphids.

### *Effects of CSP genes on the response of R. padi to volatiles*

*R. padi* stayed significantly longer in the arm containing octanal ($P < 0.001$), linalool ($P = 0.03$) or (*E*)-2-hexenol ($P = 0.01$) than in the corresponding controls (Fig. 7). No significantly differences were found in the staying time of *R. padi* between the treatment arm, that is (*E*)-2-hexenyl acetate, (*E*)-$\beta$-ocimene, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, benzaldehyde, $\beta$-myrcene, decanal, heptanal, linalool oxide, methyl salicylate or nonanal, and the corresponding control arm.

We selected the compound octanal in which the aphid showed strongest response with most significant differences between the treatment and control for further molecular mechanism analyses of *R. padi* host searching. The results showed that RNAi of *CSP4* ($P < 0.05$) or *CSP5* ($P < 0.05$) genes could significantly affect the staying time of *R. padi* in octanal arms (Fig. 8).

### *Molecular docking study on the interaction of CSPs and ligand*

The sequence similarities of the CSP protein and corresponding homologous protein template were higher than 30% (CSP4: 44.90% of the protein template 2gvs.1.A;

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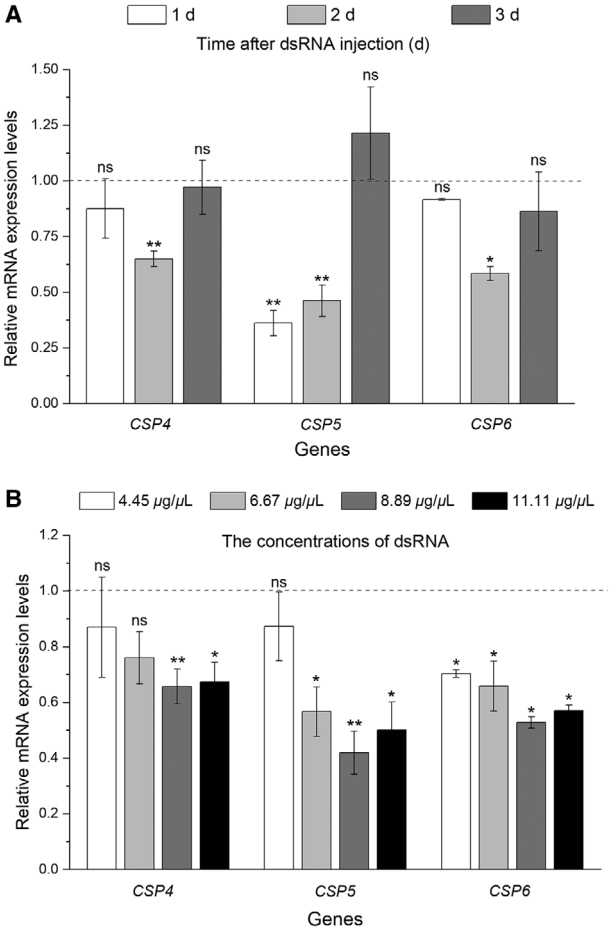


**Fig. 5** Relative expression levels of *CSP4*, *CSP5* and *CSP6* from *Rhopalosiphum padi* at different times after injection of double-stranded (ds)*CSP4*, ds*CSP5* and ds*CSP6*, respectively (A). Relative expression levels of *CSP4*, *CSP5* and *CSP6* from *R. padi* after injected with four different concentrations of ds*CSP4*, ds*CSP5* and ds*CSP6*, respectively (B).

CSP5: 36.17% of 2gvs.1.A; CSP6: 47.22% of 1k19.1.A). The lowest negative binding energy values being considered as the optimal binding position of the CSP proteins (CSP4, CSP5 and CSP6) and volatile compounds octanal, (*E*)-2-hexenol and linalool are shown in Table 1. As shown in Table 2, a hydrogen bond was found between Gln86 of CSP4 and octanal, and the distance of the hydrogen bond was 2.0 Å. In 3D conformer structure, hydrogen bonds could be found between Gln74 of CSP5 or Glu74 of CSP6 and octanal; the distances of the two hydrogen bonds were 2.7 and 3.1 Å, respectively. There was a hydrogen bond between Asp118 of CSP4 and (*E*)-2-hexenol with a 1.9 Å distance. The hydrogen bond was found between Lys41 of CSP5 (3.1 Å) or Val45 of CSP6 (1.8 Å) and (*E*)-2-hexenol. In 3D conformer structure of the CSP4-linalool complex, there was a hydrogen bond between Lys123 and linalool with a 1.9 Å distance. No hydrogen bond was found in the CSP5-linalool complex. There was a hydrogen bond between Asp31 of CSP6 and linalool with a 2.6 Å distance (Fig. S1).

## Discussion

The host-location behavior of small insects is largely based on the semiochemicals emitted by plants. Aphids rely on sensitive and powerful olfactory systems to detect relevant volatile compounds and increase their chance of finding host sources (Webster, 2012; Pickett *et al.*, 2013). Quiroz and Niemeyer (1998) assessed the responses of *R. padi* to volatiles of wheat and oats by olfactometer and found that winged and wingless individuals of *R. padi* could be attracted by volatiles extracted from wheat and oat seedlings. Another study showed that this aphid is also attracted by volatiles emitted from maize cultivars (Schröder *et al.*, 2015). To simulate the natural conditions, we used the commonly used Y-tube olfactometer bioassay to test the olfactory response of the aphid to the intact plant in the current study (Ruther & Thiemann, 1997; Cao *et al.*, 2018). We tried to collect the volatiles from the five host plants (wheat, sorghum, maize, triticale and barley) by head-space solid phase microextraction coupled with gas chromatography mass spectroscopy, and found there are many compounds including aldehydes (hexanal, octanal, decanal, heptanal, nonanal, benzaldehyde), alcohols (1-pentanol, 1-hexanol, [*E*]-2-hexenol, [*Z*]-3-hexenol, linalool), esters ([*E*]-2-hexenyl acetate, [*Z*]-3-hexenylacetate), alkanes (3-methyl pentadecane, 3-methyl tetradecane), terpenoids ([*E*]-$\beta$-ocimene, $\beta$-myrcene, linalool, linalool oxide), aromatic (methyl salicylate), hydrocarbons (nonane, hexadecane), ketones, and so on. We studied the response of *R. padi* to common volatiles in the five host plants. Unfortunately, the attractiveness of a single volatile was not very strong for *R. padi* (Peng & Chen, unpublished data). We then speculated that *R. padi* does not rely on a single chemical compound to search for host plants, but may rely on combinations of compounds as shown in other aphids (Bruce & Pickett, 2011). Further studies are needed to identify which plant compounds, alone or in combination and at a given dose or ratio, induce an olfactory response in *R. padi*.

CSPs are widespread and play pivotal roles in the host-location behavior of arthropods by capturing outside volatiles and transporting them to the ORs (Pelosi *et al.*, 2014; Waris *et al.*, 2018). In this study, we identified seven CSPs from *R. padi*, a number in the range

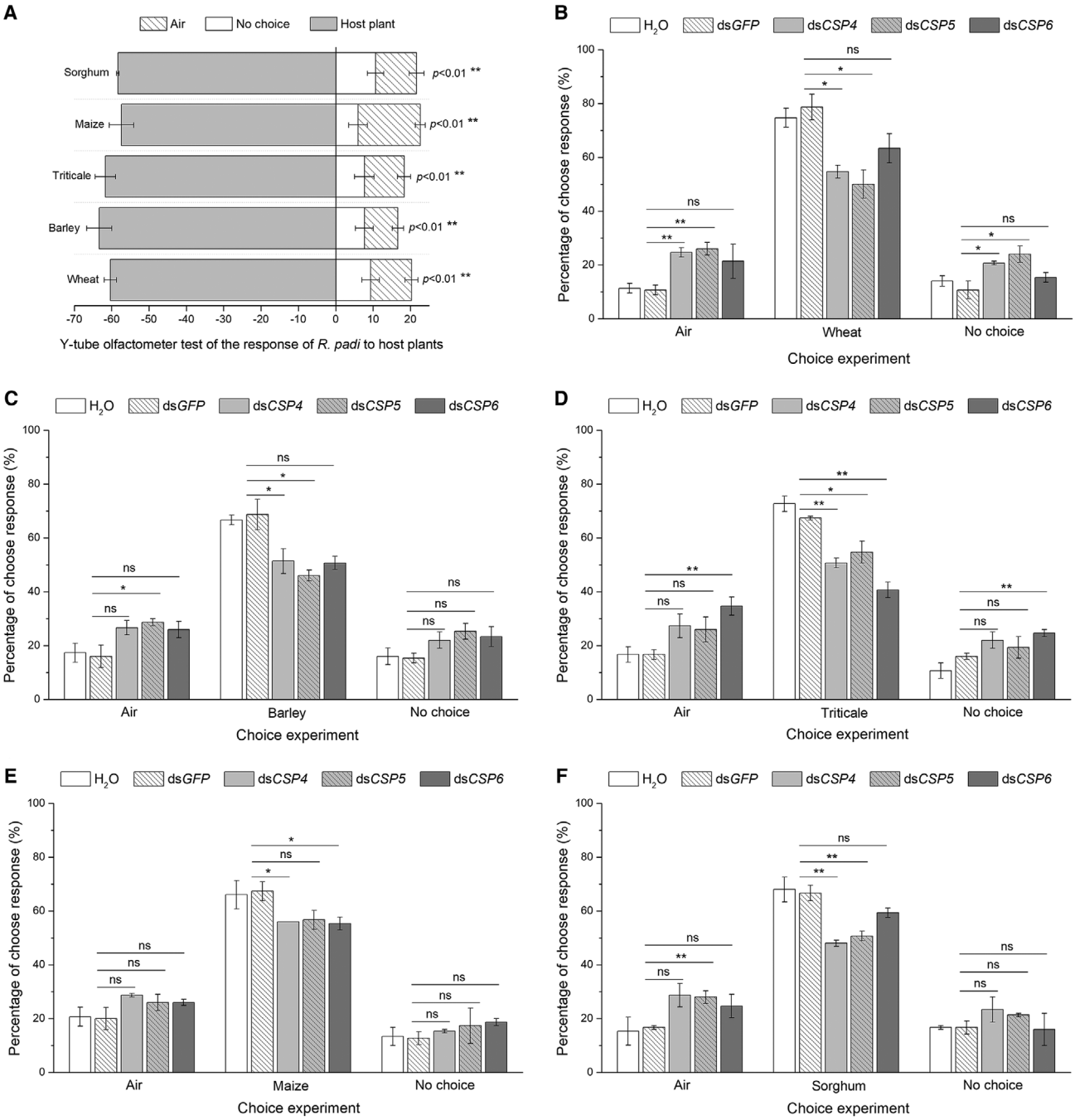


**Fig. 6** Response of *Rhopalosiphum padi* to a single host plant using the Y-tube olfactometer test (A), and effect of RNA interference targeting the *CSP5*, *CSP6* and *CSP4* genes on *R. padi* searching for wheat (B), barley (C), triticale (D), maize (E) and sorghum (F). Asterisks on the top of the bars specify that the values were significantly different (*$P < 0.05$; **$P < 0.01$; ns, no significant difference; Tukey's honestly significant difference [HSD] test).

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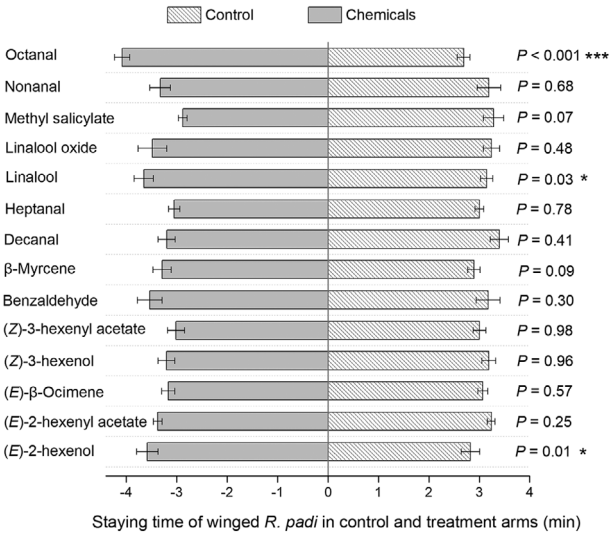


**Fig. 7** Time spent by winged *Rhopalosiphum padi* in each arm when exposed to control and treatments. The concentration of each compound was 0.05 $\mu$g/$\mu$L. Significant differences between two groups were assayed by *t*-test using a threshold *P*-value < 0.05.

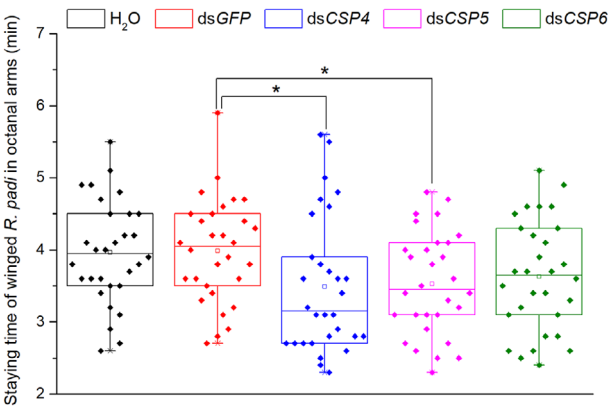


**Fig. 8** Effect of RNA interference targeting the *CSP4*, *CSP5* or *CSP6* genes on the response of *Rhopalosiphum padi* to volatiles. Significant differences between two groups were assayed by *t*-test using a threshold *P*-value < 0.05.

of what has been found in aphids (between 5–13). The seven CSPs had four conserved cysteine motifs, which are typical of CSPs and are conserved in many other insect species (Zhou *et al.*, 2010; Gu *et al.*, 2013; Xue *et al.*, 2016; Mathers *et al.*, 2017; Wenger *et al.*, 2017; Zhao *et al.*, 2017; Kang *et al.*, 2018). Although a common domain (Insect pheromone-binding domain) exists in the different CSPs, low sequence similarity was found in the conserved domains of the seven *CSP* genes. This may be due to the fact that aphids require CSP proteins with varied structures to bind volatiles with different structures under natural conditions (Pelosi *et al.*, 2014, 2017).

**Table 1** The lowest predicted negative binding energy value for the optimal binding position of the chemosensory proteins (CSPs) and volatile compounds.

| Volatiles | Lowest negative binding energy value | | |
|---|---|---|---|
| | CSP4 | CSP5 | CSP6 |
| Octanal | −3.87 | −3.65 | −3.44 |
| (*E*)-2-hexenol | −2.79 | −3.16 | −3.53 |
| Linalool | −3.44 | −3.26 | −4.48 |

The analysis of the relative expression level of the seven *CSP* genes in wingless and winged aphids showed that four *CSP* genes (*CSP1*, *CSP4*, *CSP5* and *CSP6*) are highly expressed in winged individuals, indicating these genes might have a role in the host-location behavior of *R. padi*. Similar results were found in the cotton aphid *Aphis gossypii*, for which two *CSP* genes (*AgosCSP4* and *AgosCSP6*) were significantly upregulated in the winged morph compared to the expression in wingless morphs (Gu *et al.*, 2013).

Antennae, the main sensory organs of insects, can sense a large number of volatiles and play an important role in chemodetection (Younas *et al.*, 2018a; Younas *et al.*, 2018b). Previous works showed that some *CSP* genes are highly expressed in moth and beetle antennae (Gong *et al.*, 2007; Li *et al.*, 2018). In this study, the analysis of different tissues revealed highest expression levels of *CSP1*, *CSP4*, *CSP5* and *CSP6* in head with antennae, suggesting the four genes may be involved in chemoreception. To further identify genes that may be involved in host location, we analyzed the expression patterns of *CSP* genes following starvation and refeeding after starvation. Our results showed that the expression of four *CSP* genes (*CSP1*, *CSP4*, *CSP5* and *CSP6*) tended to be upregulated following starvation, and refeeding after starvation reduced the expression of *CSP5* and *CSP6* in *R. padi*. Starvation and refeeding after starvation could affect the expression levels of four *CSP* genes in *R. padi*. Our unpublished data showed that starvation could affect the host searching sensitivity of the aphid, and the starved *R. padi* could find host plants more easily than the control aphids without starvation treatment. Based on these findings, we speculated that the four *CSP* genes might play important roles in locating host plants under unfavorable environmental conditions, and high expression levels of *CSP* genes might be essential for maintaining the sensitivity to odor in the insect's olfactory system (Pelosi et al.,

**Table 2** Number of hydrogen bonds and distance between amino acid residues of *Rhopalosiphum padi* chemosensory proteins (CSPs) and volatile compounds.

| Volatiles | Number of hydrogen bonds | | | Distance between amino acid residues and volatiles | | |
|---|---|---|---|---|---|---|
| | CSP4 | CSP5 | CSP6 | CSP4 | CSP5 | CSP6 |
| Octanal | 1 | 1 | 1 | Gln86 (2.0 Å) | Gln74 (2.7 Å) | Glu74 (3.1 Å) |
| (*E*)-2-hexenol | 1 | 1 | 1 | Asp118 (1.9 Å) | Lys41 (3.1 Å) | Val45 (1.8 Å) |
| Linalool | 1 | 0 | 1 | Lys123 (1.9 Å) | – | Asp31 (2.6 Å) |

2006). On the other hand, the expression levels of *CSPs* in *R. padi* were increased under food shortage conditions, suggesting that *CSP* genes might be helpful for insects to resist starvation stress. Previous reports showed that the physiological regulation of insects under starvation stress might be related to carbohydrates, lipids, proteins, nerve signals and hormones (i.e., insulin signaling pathway, dopaminergic signaling pathway and juvenile hormone titer) (Zhang *et al.*, 2019a). CSPs might be involved in the physiological mechanisms of aphid starvation resistance; however, further analyses are needed to investigate the role of CSPs in the physiological processes.

In order to assess the importance of the three *CSP* genes in host-location, RNAi experiments were conducted. The sensitivity to host plant odors in *R. padi* decreased after injection of ds*CSP4*, ds*CSP5* and ds*CSP6*. The expression levels of *CSP* genes might be important for maintaining the sensitivity to odor in the insect's olfactory system (Pelosi *et al.*, 2014). We could confirm that RNAi reduced the expression levels of these three genes, which could result in insufficient CSP proteins for *R. padi* to bind plant volatiles, thus affecting the aphids' sensitivity to odors and decreasing the ability of *R. padi* to search for the host plants. We asserted that *CSP4*, *CSP5* and *CSP6* are pivotal recognition proteins for searching for host plants in *R. padi*. Silencing of *CSP* genes has been shown to affect odor preferences or weaken olfactory performance in other insects. *DarmCSP2* plays a critical role in the olfactory perception of *D. armandi* (Li *et al.*, 2018). Similar functions of *MsepCSP5*, *MsepCSP8*, *GmolCSP8*, *NlugCSP8* and *NlugCSP10* genes on olfactory response were respectively found in *M. separata*, *G. molesta* and *N. lugens* (Waris *et al.*, 2018, 2020; Younas *et al.*, 2018a; Younas *et al.*, 2018b; Li *et al.*, 2019). The interference of three *CSP* genes (*CmedCSP1*, *CmedCSP2* and *CmedCSP3*) in *C. medinalis* remarkably decreased the electroantennogram responses to host-related volatiles and sex pheromones, suggesting an involvement of the three *CmedCSPs* in the reception of semiochemicals (Zeng *et al.*, 2018). RNAi of *BdorCSP2* in *B. dorsalis* weakens the effect of oviposition deterrence and antifeedant activity of Rhodojaponin-III (Yi *et al.*, 2013). Different CSP proteins could have different roles in host plant searching of *R. padi*. On the other hand, the five plants tested in this study could have some similarities and differences in the types and contents of volatiles, which would result in varied response of the aphids to host plants when different dsRNA are injected. For example, RNAi of the *CSP5* gene in our study decreased the response of *R. padi* to wheat, barley, triticale and sorghum but not to maize. Similarly, ds*CSP6*-treated *R. padi* had a reduced response to barley, triticale and maize but not to wheat and sorghum; and RNAi of *CSP4* decreased the response of *R. padi* to wheat, triticale, maize and sorghum but not to barley. Further studies are needed to explore in more details the role of CSPs in the recognition of specific odors or volatile blends by *R. padi*.

The results showed that winged *R. padi* responded to three compounds octanal, linalool and (*E*)-2-hexenol, and could be attracted by these compounds. A similar phenomenon was also found by Quiroz and Niemeyer (1998). The behavioral responses of *R. padi* to some synthetic compounds were different in different studies, for example, Quiroz and Niemeyer (1998) found that *R. padi* was significantly attracted to (*E*)-2-hexenyl acetate, (*Z*)-3-hexenol and methyl salicylate. However, Schröder *et al.* (2015) found that (*E*)-2-hexenyl acetate, (*Z*)-3-hexenol and methyl salicylate did not attract or repel *R. padi*. The difference of behavioral responses to compounds in *R. padi* might be due to aphid lineages and the concentration of compounds (Quiroz & Niemeyer, 1998; Padmaja *et al.*, 2010; Dong *et al.*, 2015; Schröder *et al.*, 2015). To further analyze how the three CSP proteins affect the host search ability of *R. padi*, we predicted the binding of CSP protein and compounds using molecular docking. The results proved that some CSP proteins could bind with octanal, linalool or (*E*)-2-hexenol. RNAi of *CSP4* or *CSP5* genes could significantly decrease staying time of *R. padi* in octanal arms, suggesting *CSP4* and *CSP5* genes might play an important role in the host search process by combining octanal.

Intensive use of pesticides could result in environmental hazards and negative impacts on human health; new eco-friendly pest control strategies are thus needed as an alternative to chemical spray (Carvalho *et al.*, 2006). Our study shows that three *CSP* genes are involved in searching for host plants. These results shed light into the function of CSPs in host-location behavior in *R. padi*. The olfactory recognition system plays a vital role in the interactions of insects with congeners, host plants and natural enemies. Understanding how aphids locate and select their host plants is an important step for the development of new pest control strategies.

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## Disclosure

The authors declare they have no conflicts of interest.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Molecular docking analysis for the binding of CSP4 (A, D, G), CSP5 (B, E, H) and CSP6 (C, F, I) from *Rhopalosiphum padi* with octanal (A, B, C), (*E*)-2-hexenol (D, E, F) and linalool (G, H, I) using 3D binding mode.

**Table S1** Primers used to amplify the seven chemosensory proteins in *Rhopalosiphum padi* and primer sequences for real-time quantitative reverse transcription polymerase chain reaction assays and RNA interference of target and reference genes.