Expression Profiles and Binding Properties of the Chemosensory Protein PxylCSP11 from the Diamondback Moth, *Plutella xylostella* (Lepidoptera: Plutellidae)

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Abstract

The diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae) is one of the most destructive pests to cruciferous plants worldwide. The oligophagous moth primarily utilizes its host volatiles for foraging and oviposition. Chemosensory proteins (CSPs) are soluble carrier proteins with low molecular weight, which recognize and transport various semiochemicals in insect chemoreception. At present, there is limited information on the recognition of host volatiles by CSPs of *P. xylostella*. Here, we investigated expression patterns and binding characteristics of *PxylCSP11* in *P. xylostella*. The open reading frame of *PxylCSP11* was 369-bp encoding 122 amino acids. *PxylCSP11* possessed four conserved cysteines, which was consistent with the typical characteristic of CSPs. *PxylCSP11* was highly expressed in antennae, and the expression level of *PxylCSP11* in male antennae was higher than that in female antennae. Fluorescence competitive binding assays showed that *PxylCSP11* had strong binding abilities to several ligands, including volatiles of cruciferous plants, and (Z)-11-hexadecenyl acetate (Z11-16:Ac), a major sex pheromone of *P. xylostella*. Our results suggest that *PxylCSP11* may play an important role in host recognition and spouse location in *P. xylostella*.

Key words: chemosensory protein, tissue expression, fluorescence competitive binding, host volatiles

Insects mainly rely on their chemosensory system, especially olfactory repertoires, to distinguish the specific chemical information in the external environment to mediate their behaviors, such as foraging, mating, oviposition, and avoiding natural enemies (Field et al. 2000, Arimura et al. 2009, Hansson and Stensmyr 2011, Leal 2013). The entire olfactory system of insects has become a complex and precise chemical information processing network in the long-term evolution. As one of the most important olfactory organs in insects, antennae are used to perceive chemical cues from the environment (Yan et al. 2014, Li et al. 2018). There is an abundance of olfactory sensilla on antennae. Sensilla basiconica mainly sense host volatiles, and sensilla trichodea detect sex pheromones (Ammagarahalli and Gemeno 2014, Yan et al. 2017, Fauchex et al. 2019). Furthermore, the high sensitivity and specificity of the olfactory system are closely related to olfactory proteins located in antennae, such as odorant binding proteins (OBPs), chemosensory proteins (CSPs), olfactory receptors (ORs), ionotropic receptors (IRs), sensory neuron membrane proteins (SNMPs), and odorant-degrading enzymes (ODEs) (Pelosi et al. 2006, Vogt et al. 2009, Abuin et al. 2011, Leal 2013, Pelosi et al. 2018, Fleischer et al. 2018). The first step in olfactory recognition is the entrance of chemosensory proteins into the hemolymph through microprobes in olfactory sensilla (Kaissling 2001, Pelosi et al. 2006, Kaissling 2009, Leal 2013, Sun et al. 2016). Due to the hydrophillicity of the lymph fluid, hydrophobic odors are transported by carrier proteins to reach the dendritic membranes of sensory neurons (Pelosi et al. 2018). CSPs, as a type of carrier proteins, play a significant role in the process of binding and delivering odors.

CSPs, also known as olfactory specific-D (OS-D) (Mckenna et al. 1994) and sensory appendage proteins (Robertson et al. 1999), are soluble proteins with low molecular weight and are generally composed of 100–120 amino acid residues. Almost all CSPs contain four highly conserved cysteines, which is a typical characteristic of these proteins (Wänner et al. 2004, Zeng et al. 2018b). The first CSP member of insects was obtained from the antennae of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) through subtractive cDNA hybridization (Mckenna et al. 1994). Subsequently, CSPs have also been identified from the antennae of different insect species, including *Bombbyx mori* L. (Lepidoptera: Bombycidae) (Gong et al. 2007), *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) (Zhang and Lei 2015), *Sitobion avenae* Fabricius (Hemiptera: Aphididae) (Xue et al. 2016), and *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) (Ali et al. 2019). Many studies have demonstrated that antennae-enriched CSPs bind and transport a variety of host plant volatiles and/or sex pheromones (Zhang et al. 2013, He et al. 2017, Chen et al. 2018). Three CSPs of *Adelphocoris lineolatus* Goeze (Hemiptera: Miridae) highly
expressed in antennae are related to host recognition (Gu et al. 2012). When *Mythimna separate* Walker (Lepidoptera: Noctuidae) *MsepCSP5* specifically expressed in the antennae is silenced by RNAi, the behavioral responses of adults to volatile attractants decrease (Younas et al. 2018).

CSPs are also broadly distributed in other insect tissues, such as head, maxillary palps, labial palps, legs, thorax, wings, proboscis, pheromone glands, and ejaculatory ducts, indicating that CSPs have multiple physical functions (Maleszka and Stange 1997, Marchese et al. 2000, Jin et al. 2005, Zhou et al. 2010, Gu et al. 2012, Zhu et al. 2016). Interestingly, a CSP has been demonstrated to participate in the morphological and behavioral transformation of *Locusta migratoria* Meyen (Orthoptera: Acrididae) from the solitary phase to the gregarious phase (Hassanali et al. 2005). Other special functions of CSPs have also been reported, such as CO$_2$ detection in *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) (Maleszka and Stange 1997), leg regeneration of *Periplaneta Americana* L. (Blattaria: Blattidae) (Kitabayashi et al. 1998), embryonic development of *Apis mellifera* L. (Hymenoptera: Apidae) (Forêt et al. 2007, Maleszka et al. 2007), and insecticide resistance of *Spodoptera littura* Fabricius (Lepidoptera: Noctuidae) (M. Li et al. 2018). In addition, the function of CSPs is related to the age, sex, and mating status in insects (Zhou et al. 2013, Xue et al. 2016).

The diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), is a destructive pest that damages the quality and quantity of cruciferous vegetables worldwide. *P. xylostella* mainly relies on volatile compounds released by host plants to find food and oviposition sites (Pivnick et al. 1994). Olfactory recognition in insects requires the cooperation of various olfactory proteins. Silencing olfactory genes responsible for identifying signals affects the feeding and mating behaviors of insects, thereby efficiently controlling the pest population (Pitino et al. 2011). Thus, identification and functional characterization of olfactory genes are of significant importance. In *P. xylostella*, a total of 118 olfactory genes, including 54 ORs, 16 IRs, 7 gustatory receptors (GRs), 15 CSPs, 24 OBPs, and 2 SNMPs, have been identified from antennal transcriptome (Yang et al. 2017). *PxyCSP1* and *PxyCSP2* have a high expression in the antennae of both male and female adults, while *PxyCSP3* and *PxyCSP4* are distributed in various tissues (Liu et al. 2010b). At present, only the binding characteristic of *PxyCSP1* has been deeply studied. The protein has a binding specificity to Rhodojaponin-II (R-III), a nonvolatile oviposition inhibitor (Liu et al. 2010b). However, limited information has been focused on functions of other CSPs in diamondback moth.

We have detected the tissue expression profiles of some CSPs in *P. xylostella* (unpublished data), and the results show that *PxyCSP11* is highly expressed in the antennae of the male and female moth, indicating its potential olfactory function. In order to better understand the function of *PxyCSP11* in the olfactory communication system, we analyzed characteristics of *PxyCSP11* sequence, constructed the recombinant expression vector of *PxyCSP11* in a prokaryotic expression system, and tested the binding affinities of *PxyCSP11* to volatile molecules through the fluorescence competitive binding assays. Our study enriches the understanding in functions of CSPs from *P. xylostella* and complex olfactory mechanisms in insects.

**Materials and Methods**

**Insects Rearing and Tissue Collection**

*P. xylostella* was reared in the Insect Neuroethology and Sensory Biology Laboratory, Shanxi Agricultural University and was reared in the Insect Neuroethology and Sensory Biology Laboratory, Shanxi Agricultural University and was maintained under constant conditions of 25 ± 1°C, 75% relative humidity, and a 14:10 (L:D) h photoperiod. For collecting various tissues, we dissected 300, 50, 30, 30, 100, and 100 3-day-old adults for antennae, heads without antennae, thoraxes, abdomens, legs, and wings, respectively. The adults were classified into two groups by sex. There were 12 tissue samples with three biological replicates. All samples were immediately frozen in liquid nitrogen and stored at −80°C.

**RNA Extraction and cDNA Synthesis**

Total RNA of different tissue samples was isolated using TRizol reagent (Sangon, Shanghai, China) according to the manufacturer’s instructions. The integrity of the extracted RNA was detected by 1% agarose gel electrophoresis, and the purity was examined by the Ultra low volume spectrometer (BioDrop, United Kingdom). The first strand of cDNA was synthesized from total RNA (1 μg) using the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Dalian, China), and was used for PCR and qRT-PCR.

**cDNA Cloning and Sequence Analysis**

We obtained the sequence of *PxyCSP11* (Accession no. XM_011564896.1) from the transcriptional data of *P. xylostella* submitted by NCBI ([https://www.ncbi.nlm.nih.gov/genome/?term=txid51655[orgn]], and validated the nucleotide sequence of the gene by PCR. PCR assays were performed in a mixture of 25 μl containing 2 μl of antennae cDNA (100 ng), 12.5 μl of 2×Taq PCR Mastermix (Tiangen, Beijing, China), 1 μl of each primer (10 μM), and 8.5 μl of sterilized ddH$_2$O. The PCR amplification procedure was as follows: pre-denaturation at 95°C for 1 min; 30 cycles of 95°C for 30 s, 48°C for 30 s, 72°C for 1 min; and final extension at 72°C for 10 min. The PCR product was visualized by 1% agarose gel electrophoresis. The target gene was recovered, and ligated into T-Vector pMD™ 19 (Simple) (Takara). The recombinant T-Vector was transformed into *E. coli* (DH5α) competent cells (Takara), and sequenced with standard M13 primers. The obtained sequence was used to analyze basic characteristics of *PxyCSP11*. The open reading frame (ORF) of *PxyCSP11* was found by utilizing the ORF Finder ([http://www.ncbi.nlm.nih.gov/orffinder.html])(Min et al. 2005). The signal peptide of *PxyCSP11* was predicted by SignalP 5.0 Server ([http://www.cbs.dtu.dk/services/SignalP/])(Almagro Armenteros et al. 2019). Molecular weight (MW) and isoelectric point (pI) were computed through the ExPaSy ProtParam tool ([http://web.expasy.org/protparam/]). Multiple sequence alignment was produced with DNAMAN (Lynnon Biosoft, USA). A phylogenetic tree was constructed using MEGA7.0 based on the neighbor-joining method with 1,000 bootstrap replicates ([Hall 2013]).

**qRT-PCR Analysis**

The qRT-PCR assay was performed with the Bio-Rad CFX Connect Real-Time Detection System (USA). Ribosomal protein S4 (RP54, GenBank XM_011553732) was used as an endogenous gene. The specificity of each primer pair was validated by melting curve analysis, and the efficiency was calculated by analyzing standard curves with a fivefold cDNA dilution series. In the experiment, the amplification efficiencies of all primers were 90–110%. The qRT-PCR assay was conducted in a 20 μl mixture containing 10 μl of 2× SG Fast qPCR Master Mix (Sangon), 0.4 μl of each primer (10 μM), 2 μl of sample cDNA, and 7.2 μl of sterilized ddH$_2$O. The procedure of qRT-PCR reaction was as follows: denaturation at 95°C for 3 min; 40 cycles of 95°C for 10 s, 58°C for 10 s, and 72°C for 30 s; and melting curve analysis from 60 to 95°C. Three biological replicates
and three technical replicates were performed for each tested sample. The expression level in different tissues was calculated by the comparative 2−ΔΔCt method (Livak and Schmittgen 2001). All data were analyzed through a one-way analysis of variance (ANOVA) followed by Tukey’s honest significant difference (HSD) test (P < 0.05) in SPSS Statistics 17.0 software (SPSS Inc., Chicago, IL).

Expression Vector Construction

PxylCSP11 sequence without signal peptide for prokaryotic expression vector construction was amplified by PCR with a forward primer containing a BamHI I-restriction site and a reverse primer containing a Xho I-restriction site (listed in Supp Table 1 [online only]). The recovered target gene was ligated into the T-Vector pMD™ 19 (Simple), and the product was transformed into E. coli (DH5α) competent cells. After sequencing, the correct reconstructed T-Vector was digested with BamHI and Xho I at 30°C for 4 h. The purified fragment was ligated into the expression vector pET28a (+) (Novagen, Madison, WI) linearized with the same restriction enzymes, and transformed into E. coli (DH5α) competent cells. Positive clones were sequenced to verify whether the sequence was consistent with PxylCSP11. The confirmed recombinant plasmid was then transformed into chemically competent E. coli BL21 (DE3) cells, and the strains were sequenced. Correct strains were induced to express recombinant proteins.

Induction and Purification of Recombinant PxylCSP11

The PxylCSP11 recombinant protein was induced at 30°C for 4 h with a final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Bacteria cells were collected by centrifugation at 8,000 g for 10 min, suspended in 20 mM Tris-HCl (pH 7.4), sonicated in ice, and centrifuged at 12,000 × g for 10 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) showed that recombinant PxylCSP11 was expressed mainly in the supernatant. Protein purification was accomplished by Ni-NTA Resin (TransGen Biotech, Beijing, China). Purified recombinant proteins were analyzed by 15% SDS–PAGE and diazylated overnight with 20 mM Tris–HCl (pH 7.4).

Fluorescence Competitive Binding Assays

Fluorescence binding assays were performed with a 1 cm light path quartz cuvette on an RF-5301 fluorescence spectrophotometer (Shimadzu, Japan). The slit widths of excitation and emission were set to 5 nm. The emission spectra of the fluorescent probe, 4,4′-diaminobenzene-1,1′-binaphthyl-3,3′-sulfonic acid (Bis-ANS, Sigma–Aldrich, St. Louis, MO), were recorded from 400 to 580 nm with an excitation wavelength of 365 nm (Zhang et al. 2011, Hua et al. 2013). To measure the affinity of Bis-ANS to PxylCSP11, 1 µM protein in 20 mM Tris–HCl (pH 7.4) was titrated with 1 mM Bis-ANS to final concentrations ranging from 1 to 10 µM. The recorded fluorescence intensity at the maximum emission wavelength of 485 nm was linearized using the Scatchard equation (Sideris et al. 1992), and the dissociation constants for Bis-ANS were then calculated.

Twenty-five ligands, including host volatiles and two main sex pheromone components, (Z)-11-hexadecenyl acetate (Z11-16:Ac) and (Z)-11-hexadecenal (Z11-16:Ald), were used to detect the affinities of PxylCSP11. Ligands with concentrations ranging from 1 to 32 µM were added into the mixed solution of protein (1 µM) and probe (1 µM). All values were obtained from three independent measurements. The data were analyzed based on the assumption that the protein was 100% active with a stoichiometry of protein and ligand at a 1:1 ratio. Dissociation constants (Kd) of the competitor were calculated using the following equation: Kd = [IC50]/(1 + [Bis-ANS]/Kd Bis-ANS), where [IC50] is the ligand’s concentration, [Bis-ANS] is the free concentration of Bis-ANS, and Kd Bis-ANS is the dissociation constant of the complex of PxylCSP11 and Bis-ANS (Campanacci et al. 2001).

Results

Sequence Analysis of PxylCSP11

The cDNA sequence of PxylCSP11 contained a 369-bp ORF encoding 122 amino acid residues. At the N-terminus of the polypeptide chain, PxylCSP11 possessed a predicted 16-residue signal peptide. As the signal peptide was cleaved off, molecular weight of the mature protein PxylCSP11 was 12.34 kDa with an isoelectric point of 8.89. The results of multiple alignments showed that PxylCSP11 presented four conserved cysteines (Fig. 1), which formed two pairs of disulfide bonds. The sequence identity of PxylCSP11 with Spodoptera exigua Hubner (Lepidoptera: Noctuidae) CSP11 was 87.10%. A phylogenetic tree was constructed based on the amino acid sequences of P. xyllostella and different insect orders, including Lepidoptera, Coleoptera, Orthoptera, Hemiptera, and Hymenoptera (Fig. 2). The phylogenetic analysis showed that the CSPs of the same order were clustered in the same group and that PxylCSP11 was clearly located in the Lepidoptera group. PxylCSP11 and Lobesia botrana (Denis and Schiffermüller) (Lepidoptera: Tortricidae) LborCSP16 shared the highest bootstrap support value of 100%, suggesting that these two proteins may be evolutionarily homologous.

Tissue Expression Patterns of PxylCSP11

To better understand the function of PxylCSP11, we examined its expression pattern in different tissues by qRT-PCR. PxylCSP11 had a broad expression profile in all tissues of male and female moths, but it was predominantly expressed in antennae (Fig. 3). The expression level of PxylCSP11 in male antennae was higher than that in female antennae. Additionally, its expression was evident in the male moth abdomen. The expression of PxylCSP11 in other tissues was low, and there was no significant difference.

Purification of the Recombinant Protein

After the PxylCSP11 cDNA was cloned into the pET28a (+) prokaryotic expression vector, the PxylCSP11 recombinant protein was induced to express in E. coli BL21 (DE3) strains. The induced recombinant PxylCSP11 was confirmed by 15% SDS–PAGE (Fig. 4A). The target protein mainly existed in the supernatant after bacterial lysis by ultrasonication (Fig. 4A). Proteins were subsequently purified using ProteinLiso Ni-NTA Resin. Figure 4B shows the SDS–PAGE analysis indicated a single protein band of approximately 17 kDa, which is consistent with the predicted recombinant protein molecular weight. The purified recombinant proteins were used for fluorescent competitive binding assays.

Binding Properties of Recombinant PxylCSP11

For analysis of the PxylCSP11 binding affinity, Bis-ANS was used as a competitive fluorescent reporter. When Bis-ANS was added dropwise to the protein solution, the maximum emission peak at 418 nm was shifted to approximately 485 nm. The Kd of Bis-ANS to PxylCSP11 was 1.98 ± 0.09 µM as calculated using the Scatchard equation (Fig. 5). The competitive binding curves of PxylCSP11 to various ligands are shown in Fig. 6. The IC10 values and Kd of...
PxylCSP11 to different ligands were then calculated (Table 1). In all tested volatiles, PxylCSP11 showed strong binding abilities to two special volatiles of cruciferous vegetables, namely, phenethyl isothiocyanate (19.53 µM) and allyl isothiocyanate (19.79 µM). PxylCSP11 also exhibited strong binding affinities to several other volatiles, including α-terpineol (10.69 µM), 2,4-dimethylheptane (16.34 µM), α-terpinene (20.19 µM), phenethyl alcohol (21.00 µM). In addition, PxylCSP11 had a high binding affinity for Z11-16:Ac (13.80 µM), which is one of the major sex pheromone components of P. xylostella.

Discussion

In this study, we cloned and sequenced the PxylCSP11 cDNA from P. xylostella and analyzed the properties of PxylCSP11 protein. The PxylCSP11 from P. xylostella possesses four conserved cysteines, which are common in a typical CSP. The low molecular, N-terminal signal peptide and two disulfide bridges of PxylCSP11 also represent the common characteristics of insect CSPs (Angeli et al. 1999, Zhang and Lei 2015, Ali et al. 2019). Generally, insect CSPs are highly conserved in evolution, and their sequence identities are between 40 and 50% even among different insect orders (Wanner et al. 2004, Pelosi et al. 2006, Zhu et al. 2016). Our results showed that the sequence identity of PxylCSP11 and other Lepidoptera CSPs was as high as 80% and that PxylCSP11 was clustered with lepidopterous CSPs in a group according to the phylogenetic tree, indicating the conservation of CSP evolution in insects. As the bootstrap support value of PxylCSP11 and L. botrana LbotCSP16 reached 100%, two proteins might come from a close ancestor. We speculated that PxylCSP11 and LbotCSP16 have similar functions.

LbotCSP16 was expressed in antennae of male and female moths (Rojas et al. 2018). However, there are no reports on the function of LbotCSP16.

The expression of CSPs in various tissues is closely related to their functions (Gu et al. 2012, Yang et al. 2014). Increasing studies show that CSPs highly expressed in antennae participate in olfactory recognition (Khuhro et al. 2018, Wu et al. 2019, Zhang et al. 2020). In our study, the result that PxylCSP11 was highly expressed in antenna of male and female adults suggested that PxylCSP11 may be involved in olfactory perception. It has been shown that CSPs with high expression in the antennae can recognize host volatiles (Zhang et al. 2013, He et al. 2017, Chen et al. 2018). M. alternatus MaltCSP5 is mainly expressed in male and female antennae, and the protein has a high binding affinity to most pine volatiles (Ali et al. 2019). An antenna-enriched CmedCSP33 protein in Cnaphalocrocis medinalis Guenée (Lepidoptera: Pyralidae) exhibits strong binding abilities to seven compounds of rice volatiles (Duan et al. 2019).
Based on the above-mentioned study, the antennae-predominant PxylCSP11 may be responsible for binding host volatiles in olfactory recognition.

Interestingly, the expression of PxylCSP11 in antennae of males was higher than that in antennae of females, which was similar to the expression profiles of other insect CSPs, including SinfCSP19 of Sesamia inferens Walker (Lepidoptera: Noctuidae) (Zhang et al. 2014), SexiCSP3 of S. exigua (Zhu et al. 2015) and MaltCSP5 of M. alternatus (Ali et al. 2019). The male-biased SinfCSP19 has strong binding abilities to three sex pheromones released from females (Zhang et al. 2014). Unlike female insects, which need to seek host plants for surviving and oviposition, males are less dependent on host plants. The main task of male adults is to locate sexual partners for mating in their life (Pivnick et al. 1990, Bonduriansky 2001, Reddy and Guerrero 2004). These complex physiological activities are also reflected in the difference of perception of host volatiles and sex pheromones of males and females. It is common that electroantennogram (EAG) responses to sex pheromone components

Fig. 2. Phylogenetic analysis of PxylCSP11 with other CSPs from various insect orders. Different orders were marked as follows: Lepidoptera (red), Orthoptera (purple), Coleoptera (green), Hemiptera (yellow), Hymenoptera (blue). Lepidoptera insects included L. botrana (Lbot), C. medinalis (Cmed), Helicoverpa armigera (Hübner (Lepidoptera: Noctuidae) (Harm), O. furnacalis (Ofur), and Ectropis oblique Warren (Lepidoptera: Geometridae) (Eobl); Orthoptera insects included Oedaleus asiaticus (Bey-Bienko) (Orthoptera: Acrididae) (Oasi); Coleoptera insects included Tribolium castaneum Herbst (Coleoptera: Tenebrionidae) (Tcas) and Tenebrio molitor L. (Coleoptera: Tenebrionidae); Hemiptera insects included Adelphocoris lineolatus (Alin) and Myzus persicae Sulzer (Homoptera: Aphididae) (Mper); Hymenoptera insects included Meteorus pulchricornis Wesmael (Hymenoptera: Braconidae) (Mpul), Microplitis mediator Haliday (Hymenoptera: Braconidae) (Mmed), A. mellifera (Amell), and Apis cerana cerana (Acer) Fabricius (Hymenoptera: Apidae). Accession no. of CSPs for constructing phylogenetic tree are in Supp. Table 2 (online only).
are stronger in males than in females but weaker to host volatiles. In *P. xylostella*, the response of males to sex pheromone constituents is higher than to host plant odorants, but females have a higher EAG response to plant odorants than to sex pheromones (Palaniswamy et al. 1986, Pivnick et al. 1994, Wu et al. 2020). Therefore, it is reasonable to speculate that *Pxyl*CSP11 may be sensitive to the sex pheromone besides plant volatiles in the olfactory communication.

In addition, *Pxyl*CSP11 was also highly expressed in male abdomen. Although the function of CSPs in the abdomen is not completely clear, it has been confirmed that CSPs are expressed in this tissue in other insects. For example, *B. mori* BmorCSP6, BmorCSP9, BmorCSP11, and BmorCSP14 are expressed in the pheromone gland, and BmorCSP11 and BmorCSP13 are strictly limited in the spermary (Gong et al. 2007). More importantly, *L. migratoria* LmigCSP91, which is abundant in the reproductive organs of male locusts, participates in the transmission of pheromones during mating (Zhou et al. 2013). Additionally, the low expression of *Pxyl*CSP11 in other tissues does not exclude other potential unknown functions of the protein.

The premise that CSPs possess olfactory recognition function is that the proteins recognize and bind semiochemicals released from host plants and intraspecific or interspecific insects. Therefore, we investigated if *Pxyl*CSP11 binds to chemical cues by fluorescence competitive binding assays. The results showed that *Pxyl*CSP11 had strong binding affinities to several chemicals, including Z11-16:Ac, α-terpineol, phenethyl isothiocyanate, allyl isothiocyanate, α-terpinene, phenethyl alcohol, and 2,4-dimethylheptane. Among these chemicals, Z11-16:Ac is one of the major sex pheromone components of *P. xylostella*, α-terpineol, phenethyl isothiocyanate, allyl isothiocyanate, α-terpinene, and phenethyl alcohol are cruciferous plant volatiles, and 2,4-dimethylheptane is released from non-host plants. The binding of *Pxyl*CSP11 to Z11-16:Ac supported the above hypothesis that *Pxyl*CSP11 was sensitive to the sex pheromone. Over decades, the management strategies based on sex pheromones of *P. xylostella* in the agronomy practices can lure the male moths and reduce the mating rate of the adults, thereby effectively controlling the damage of *P. xylostella* (Reddy and Guerrero 2000b, Li et al. 2012, Wu et al. 2012). Although PBPs (pheromone binding proteins) are as the transporters of sex pheromones, there are some reports on binding properties of CSPs to sex pheromones in various insect species recently. In *C. medinalis*, CmedCSP3 has a binding affinity to Z11-16:Ac, one of the major sex pheromones of *C. medinalis*, and the EAG response of the insect to Z11-16:Ac is decreased when the CmedCSP3 gene is silenced by RNAi (Zeng et al. 2018a). *Pxyl*CSP11 showed a binding specificity to the sex pheromone, suggesting the protein may function in courtship and mating.

The preference of herbivorous insects for plants is determined by host volatiles. Therefore, the volatiles of cruciferous plants play an inevitable role in the life activities of *P. xylostella*. Allyl isothiocyanate and phenethyl isothiocyanate are special volatiles of cruciferous vegetables, which are from the glucosinolates by hydrolysis (Reddy and Guerrero 2000a, Bones and Rossiter 2006, Tian et al. 2018). Allyl isothiocyanate is an attractant to *P. xylostella*, and reduces the mating rate of the adults, thereby effectively controlling the damage of *P. xylostella* (Griffiths et al. 2001, Dai et al. 2008). One of the main sex pheromone components, Z11-16:Ac, is released from cruciferous plants. The binding of *Pxyl*CSP11 to Z11-16:Ac supported the above hypothesis that *Pxyl*CSP11 was sensitive to the sex pheromone. In addition, the premise that CSPs possess olfactory recognition function is that the proteins recognize and bind semiochemicals released from host plants and intraspecific or interspecific insects. Therefore, we investigated if *Pxyl*CSP11 binds to chemical cues by fluorescence competitive binding assays. The results showed that *Pxyl*CSP11 had strong binding affinities to several chemicals, including Z11-16:Ac, α-terpineol, phenethyl isothiocyanate, allyl isothiocyanate, α-terpinene, phenethyl alcohol, and 2,4-dimethylheptane. Among these chemicals, Z11-16:Ac is one of the major sex pheromone components of *P. xylostella*, α-terpineol, phenethyl isothiocyanate, allyl isothiocyanate, α-terpinene, and phenethyl alcohol are cruciferous plant volatiles, and 2,4-dimethylheptane is released from non-host plants. The binding of *Pxyl*CSP11 to Z11-16:Ac supported the above hypothesis that *Pxyl*CSP11 was sensitive to the sex pheromone. Over decades, the management strategies based on sex pheromones of *P. xylostella* in the agronomy practices can lure the male moths and reduce the mating rate of the adults, thereby effectively controlling the damage of *P. xylostella* (Reddy and Guerrero 2000b, Li et al. 2012, Wu et al. 2012). Although PBPs (pheromone binding proteins) are as the transporters of sex pheromones, there are some reports on binding properties of CSPs to sex pheromones in various insect species recently. In *C. medinalis*, CmedCSP3 has a binding affinity to Z11-16:Ac, one of the major sex pheromones of *C. medinalis*, and the EAG response of the insect to Z11-16:Ac is decreased when the CmedCSP3 gene is silenced by RNAi (Zeng et al. 2018a). *Pxyl*CSP11 showed a binding specificity to the sex pheromone, suggesting the protein may function in courtship and mating.

The preference of herbivorous insects for plants is determined by host volatiles. Therefore, the volatiles of cruciferous plants play an inevitable role in the life activities of *P. xylostella*. Allyl isothiocyanate and phenethyl isothiocyanate are special volatiles of cruciferous vegetables, which are from the glucosinolates by hydrolysis (Reddy and Guerrero 2000a, Bones and Rossiter 2006, Tian et al. 2018). Allyl isothiocyanate is an attractant to *P. xylostella*, which lures the moth to foraging and oviposition (Griffiths et al. 2001, Dai et al. 2008, Pivnick et al. 1994, Wu et al. 2020). Therefore, it is reasonable to speculate that *Pxyl*CSP11 may be sensitive to the sex pheromone besides plant volatiles in the olfactory communication.

In addition, *Pxyl*CSP11 was also highly expressed in male abdomen. Although the function of CSPs in the abdomen is not completely clear, it has been confirmed that CSPs are expressed in this tissue in other insects. For example, *B. mori* BmorCSP6, BmorCSP9, BmorCSP11, and BmorCSP14 are expressed in the pheromone gland, and BmorCSP11 and BmorCSP13 are strictly limited in the spermary (Gong et al. 2007). More importantly, *L. migratoria* LmigCSP91, which is abundant in the reproductive organs of male locusts, participates in the transmission of pheromones during mating (Zhou et al. 2013). Additionally, the low expression of *Pxyl*CSP11 in other tissues does not exclude other potential unknown functions of the protein.
to be performed by different types of proteins to ensure the high efficiency of olfactory recognition. As an example, PBPs usually are the carrier proteins of sex pheromones, while GOBPs (general odorant binding proteins) and CSPs also have abilities to bind sex pheromones (Zhou et al. 2009, Zhang et al. 2014). Alpha-terpinene released from cruciferae plants (Geervliet et al. 1997) has an effect on feeding and oviposition of *P. xylostella* (Zhang et al. 2004).

Our results showed that *Pxyl*CSP11 has a strong binding affinity to \(\alpha\)-terpinene. Meanwhile, *Pxyl*CSP11 showed a binding activity towards \(\alpha\)-terpineol. Alpha-terpineol is similar to \(\alpha\)-terpinene in structure, both of which have a six-carbon ring with a double bond. It has been reported that the binding of CSPs to ligands depends on the interaction between the functional atoms of ligands and the amino acids of CSPs (Tan et al. 2018, Younas et al. 2018). Therefore, the binding of *Pxyl*CSP11 to these two chemicals may be accomplished through similar binding sites in its active binding cavity. Phenethyl alcohol, a binding ligand of *Pxyl*CSP11, is an aromatic compound, which is commonly present in various plants such as Brassicaceae species (Ômura et al. 1999). The chemical can cause EAG responses and attraction behaviors of some lepidopterous insects (Honda et al. 1998). In addition, phenylethanol is also found in the hairpencils of male moths, such as *Mamestra brassicae* L. (Lepidoptera: Noctuidae) (Jacquin et al. 1991) and *Mamestra configurata* Walker (Lepidoptera: Noctuidae) (Clearwater 1975). When the male moth mates with the female of the same species, the hairpencils of the male will release some volatiles (e.g., phenethyl alcohol). The chemicals may inhibit the release of sex pheromones by the female, and prevent other males from interfering with mating process (Huang et al. 2015).

*Pxyl*CSP11 showed similar levels of sensitivities to the sex pheromone component and plant volatiles, suggesting that *Pxyl*CSP11 plays important roles not only for mating but also for host plant finding in the olfactory communication system.

2,4-dimethylheptane is another binding ligand of *Pxyl*CSP11. To date, the compound has been reported to exist in Sims (Parietales: Passifloraceae) (Liu et al. 2010a) and *Ambrosia trifida* Fig. 6. Competitive binding curves of *Pxyl*CSP11 with different volatile ligands.
L. (Campanulales: Asteraceae) (Wang and Zhu 1996), non-host plants of *P. xylostella*. In our previous work, 2,4-dimethylheptane was identified from *Mentha haplocalyx* Briq. (Lamiales: Labiatae), a repellent plant against *P. xylostella*, by GC-EAD with electrophysiological activity to *P. xylostella*, and the compound was a repellent of the moth investigated by the Y-tube olfactometer (unpublished data).

**PxylCSP11** has a binding affinity to 2,4-dimethylheptane, suggesting that **PxylCSP11** may mediate *P. xylostella* to distinguish the non-hosts and host plants from the complex environment, and tune the corresponding physiological or behavioral responses of *P. xylostella*.

In conclusion, our results showed that **PxylCSP11** mainly expressed in the antennae of male and female adults and that **PxylCSP11** mainly had an affinity to host volatiles and **Z11-16:Ac**. Therefore, **PxylCSP11** may play a dual role in the perception of host volatiles and the sex pheromone in the olfactory recognition of *P. xylostella*. The characterization and function of **PxylCSP11** from *P. xylostella* contribute to understanding the underlying mechanisms of the complex olfactory communication in insects.

**Supplementary Data**

Supplementary data are available at *Journal of Insect Science* online.

**Table 1. Different affinity of PxylCSP11 with different volatile ligands**

| Ligands                  | CSA No. | Company          | Purity% | IC₅₀ (μM) | Kd (μM) |
|--------------------------|---------|------------------|---------|-----------|---------|
| **Plant volatiles**      |         |                  |         |           |         |
| Monoterpenes             |         |                  |         |           |         |
| Myrcene                  | 123-35-3| Sigma–Aldrich    | 90      | >32       | —       |
| α-Terpinene              | 99-86-5 | Sigma–Aldrich    | 95      | 28.79     | 20.19   |
| (S)-(−)-Limonene         | 5989-54-8| Sigma–Aldrich | 95      | >32       | —       |
| Linalool                 | 78-70-6 | Sigma–Aldrich    | 97      | >32       | —       |
| **Esters**               |         |                  |         |           |         |
| Allyl isothiocyanate     | 57-06-7 | Sigma–Aldrich    | 95      | 27.99     | 19.79   |
| Phenetidyl Isothiocyanate| 2257-09-2| Sigma–Aldrich | 99      | 27.57     | 19.53   |
| (Z)-3-Hexenol acetate   | 3681-71-8| Sigma–Aldrich | 98      | >32       | —       |
| Methyl salicylate        | 119-36-8| Sigma–Aldrich    | 99      | >32       | —       |
| **Aldehydes**            |         |                  |         |           |         |
| Phenylacetaldehyde       | 122-78-1| Sigma–Aldrich    | 95      | >32       | —       |
| 1-Nonanal                | 124-19-6| Sigma–Aldrich    | 95      | >32       | —       |
| Heptaldehyde             | 111-71-7| Sigma–Aldrich    | 95      | >32       | —       |
| Benzaldehyde             | 100-52-7| Sigma–Aldrich    | 98      | >32       | —       |
| (E)-2-Hexenal            | 6728-26-3| Sigma–Aldrich | 98      | >32       | —       |
| **Esters**               |         |                  |         |           |         |
| (Z)-2-Penten-1-ol        | 1576-95-0| Sigma–Aldrich | 95      | >32       | —       |
| (Z)-3-Hexen-1-ol         | 928-96-1| Sigma–Aldrich    | 98      | >32       | —       |
| Phenethyl alcohol        | 60-12-8 | Sigma–Aldrich    | 99      | 29.84     | 21.00   |
| (E)-2-Hexenol            | 928-95-0| Sigma–Aldrich    | 95      | >32       | —       |
| α-Terpineol              | 98-55-5 | Sigma–Aldrich    | 96      | 15.14     | 10.69   |
| **Ketones**              |         |                  |         |           |         |
| β-Ionone                 | 79-77-6 | Sigma–Aldrich    | 96      | >32       | —       |
| (R)-(-)-Carvone          | 6485-40-1| Sigma–Aldrich | 99      | >32       | —       |
| Methyl heptenone         | 110-93-0| Sigma–Aldrich    | 98      | >32       | —       |
| **Alkanes**              |         |                  |         |           |         |
| 2,4-Dimethylheptane      | 2213-23-2| Aladdin         | 98      | 23.12     | 16.34   |
| 2-Methylnonane           | 871-83-0| Sigma–Aldrich    | 98.5    | >32       | —       |
| **Sex pheromone components from Plutella xylostella** | | | | | |
| **Z11-16:Ac**            | 34010-21-4| Shin-Etsu     | >95%    | 19.63     | 13.80   |
| **Z11-16:Ald**           | 33939-28-9| Shin-Etsu     | >95%    | >32       | —       |

For IC₅₀ values more than 32 μM, the corresponding Kd values are not calculated and was represent by ‘—’.

2,4-Dimethylheptane was from *Mentha haplocalyx* (a non-host plant of *Plutella xylostella*) in our previous work (unpublished data). The other plant volatiles were detected in cruciferous vegetables, including *Brassica oleracea*, *Brassica campestris*, and *Brassica rapa* (Blaakmeer et al. 1994, Geervliet et al. 1997, Ômura et al. 1999, Reddy et al. 2002, Bukovinszky et al. 2005).

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