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Binding properties of four antennae-expressed chemosensory proteins (CSPs) with insecticides indicates the adaption of *Spodoptera litura* to environment

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A B S T R A C T

Insects receive a variety of chemical signals from the environment. Chemosensory protein (CSP) is one of the olfactory proteins that can accommodate a variety of small molecules and have the ability to bind to lipophilic compounds, transmitting nonvolatile odor molecules and chemical stimuli to target cells. To understand the correlation between the insect olfactory system and environment, we identified four antennae-expressed *SlituCSP* genes and investigated their expression profiles after treatment with different temperatures, starvation and three commonly used pesticides: chlorpyrifos, emamectin benzoate and fipronil. The transcriptions of four *SlituCSP* genes are affected by pesticide treatment and less affected by starvation and different temperatures. To further understand the molecular function of CSPs and their correlation with pesticides, we expressed and purified four SlituCSPs and assayed their binding ability with pesticides. The binding of four SlituCSPs with three pesticides were determined using a fluorescence competitive binding assay. We found direct binding between CSPs and pesticides, especially between SlituCSP18 and chlorpyrifos/fipronil and between SlituCSP6 and all three pesticides. The high binding affinity with pesticides and the significant down-regulation of *SlituCSP18* by chlorpyrifos suggests that SlituCSP18 is more sensitive to pesticide treatment and may play an important role in mediating the interaction of the olfactory system and the pesticide. This study can help us understand the role of CSP proteins in the adaption of *S. litura* to the environment.

1. Introduction

Insects receive a variety of chemical signals from the environment. Favorable chemicals such as some of the volatile chemicals from the host plant help insects to find food and reproductive sites and to avoid natural enemies, while harmful chemicals such as insecticides help insects to produce a corresponding avoidance behavior or self-activation of the corresponding degradation mechanism to maintain homeostasis [1]. Chemosensory protein (CSP) is an protein that is characterized by two small disulfide bonds with small molecular weight, high solubility and acidity [2,3]. The secondary structure of CSP is mainly composed of α-helices; its protein cavity is hydrophobic and can accommodate a variety of small molecules [2–7]. Studies showed that CSPs have the ability to bind to lipophilic compounds and transmit nonvolatile odor molecules and chemical stimuli to target cells and that the expression of CSPs were found in multiple organs such as antennae, head, thorax, abdomen, leg, wings, proboscis and gonads [8,9].

Determination of in vitro expressed proteins and their ligand-binding ability is now common in measuring the association of olfactory-related proteins and their chemical ligands. The ability of CSP proteins to bind to chemicals has been reported in a wide range of species, such as the binding of cotton bollworm CSP4 to terpenoids and related chemicals [10] and response of *Anopheles gambiae* to aldehydes and ketones [11]. In silkworm *Bombyx mori*, CSP1 and CSP2 have high binding abilities with retinal, 4-hydroxy-4′-isopropylazobenzene and octyl benzoate [12]. A previous study described the binding of SlituCSP6 to lutein III and predicted the three-dimensional structure of the protein [13]. Interestingly, some CSPs are able to cooperate with each other, CSP5 and CSP6 of the alfalfa plant bug *Adelphocoris lineolatus* (Goeze) have higher affinity with the three compounds (trans-β-farnesene, α-humulene and β-caryophyllene) when CSP5 and CSP6 were presented together [14]. Most of the compounds binding to CSP are plant volatiles, and some CSPs, such as CSP2, CSP3 and CSP4 of *Apolygus lucorum*, have a higher binding capacity for cotton secondary metabolites [15]. CSP2, CSP3 and CSP4 can also respond to insecticides containing rapeseed [15]. The use of pesticides has a greater impact on the insect olfactory system [16]. Different doses of thiamethoxam against *Bemisia tabaci* affected the expression of the *CSP1* gene to

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different degrees [17]. Expression levels of CSP1, CSP2, CSP3 of B-type (the Middle East-Asia Minor I species) and Q-type (the Mediterranean species) in *Bemisia tabaci* were also changed to varying degrees in 25 different doses of thiamethoxam [18], suggesting that CSP is involved in the physiological effects of pesticides. CSP is an indispensable factor for the insect sensory system. Therefore, studying the effects of pesticides on CSPs of *S. litura* and further exploring the intrinsic relationship between them will help us to understand the molecular mechanism of pesticide resistance and the molecular function of CSPs.

S. litura is an important intermittent pest, its larvae can feed on a variety of crops, with strong resistance to pesticides [19–22]. The degree of resistance of *S. litura* to pesticides has gradually increased, as has the generation of cross-resistance [23,24]. Here, we investigated the expression profiles of four *SlituCSP* genes in antennae by qRT-PCR under varying environmental factors (temperature, starvation) and chemical factors (three pesticides). Four *SlituCSP* genes were then cloned and SlituCSP proteins were obtained by expression in *E. coli* and subsequent purification. The binding ability of four SlituCSPs to three pesticides was determined using a fluorescence competitive binding assay. This study provides a basis for studying the association of olfactory systems with pesticides and the role of CSP proteins in the adaption of *S. litura* to the environment.

2. Materials and methods

2.1. Insects

Male *S. litura* adults were collected from Hangzhou (30°19'34.19"N, 120°22'50.77"E), Zhejiang, China. The moths were kept in the laboratory at 25 ± 1 °C at a relative humidity of 75% ± 10%, with light conditions of L16:D8. Male *S. litura* adults were collected by traps (PT-NT, Ningbo Newcomb Biotechnology Company, China), using the PVC pheromone SL02-SP (Ningbo Newcomb Biotechnology Company, China). The main components were Z9, Z11-14: OAc and Z9, Z12-14: OAc (Ningbo Newcomb Biotechnology Company). The adults were fed with 10% sucrose water.

2.2. Treatment

Male *S. litura* adults were divided into groups (20 per group) and treated with pesticides. Each of the three different pesticides, chlorpyrifos, emamectin benzoate or fipronil (Aladdin Bio-Chem Technology Co., LTD, Shanghai, China) was added to water with 10% sugar at a concentration of 1 ppm. No pesticide was added to the control. For temperature treatment, twenty moths were placed at 5 °C, 15 °C, 25 °C, 35 °C at a relative humidity of 75% ± 10%, with light conditions of L16:D8. Twenty *S. litura* male adults were used in each of the starvation treatment and the control group: no 10% sucrose water was provided for the starvation group, while the control group was supplied with 10% sucrose water; other conditions remained unchanged. All the treatments lasted 24 h after which antennae were cut and ground in Trizol, stored at −80 °C. Three biological repeats were made for each group.

2.3. RNA extraction and first strand cDNA synthesis

Total RNA of antennae was extracted using RNAiso Plus kit (TAKARA, Dalian, China). RNA was dissolved in DEPC treated water, and concentration and purity were measured on an ultramicro spectrophotometer (NanoDrop, ThermoFisher Scientific, USA). First strand cDNA was synthesized with the PrimeScript RT-PCR kit (TAKARA, Dalian, China) and RNase H (TAKARA, Dalian, China) was used to degrade RNA. The newly synthesized cDNA was stored at −20 °C.

Table 1
Primers for qRT-PCR.

Gene	5′	3′
SlituCSP6	GTGTTCCTCGTGTGTGTGT	CTTCCTTAATGTGCGACTTC
SlituCSP8	AACTGTCTGATGGATCTTGG	TTCCATCCGATTTGTATTTC
SlituCSP9	CAATTTTCACGAACAAGAGAC	CAGGATATTCGTTCTTCAGC
SlituCSP18	AAGCTATCGTTTCTGATGACA	CTCTTGTGGCCTTTTCTTC
SlituRPL10	GACTTGGGTAAGAAGAAG	GATGACATGGAATGGATG

2.4. Quantitative real-time PCR (qRT-PCR)

The expression of *SlituCSP6*, *SlituCSP8*, *SlituCSP9* and *SlituCSP18* were detected by qRT-PCR after the pesticide, starvation and different temperature treatments. The primers were designed using the NCBI Primer tool online software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) according to the previous report [25]. *RpL10* was selected as a reference gene [26]. Primer sequences are shown in Table 1. The KAPA SYBR FAST qPCR Kit Master Mix Universal was used, with a total volume of 20 μL. An ABI StepOnePlus was used by a two-step method: 1. pre-denaturation at 95 °C for 3 min, 2. PCR amplification reaction at 95 °C for 3 s, 55 °C for 20 s, 40 cycles. All qRT-PCR data were analyzed using the $2^{-\triangle\triangle Ct}$ method [27].

2.5. Sequence analysis, cloning and expression of SlituCSP genes

A phylogenic tree was constructed using the Neighbor-Joining method [28]: homologous sequences were downloaded from NCBI, sequences were aligned with ClustalW2 [46], and the comparison graph was generated from WebLogo (http://weblogo.berkeley.edu/logo.cgi).

Cloning primers were designed according to the previous report [25], and primers with restriction enzyme sites were used for cloning into pCold-TF (TAKARA, Dalian, China) for expression in *E. coli*. Primers for cloning *SlituCSP*s are shown in Table 2. The *CSP* gene was amplified by PCR and then was purified ligated with PMD18-T vector (TAKARA, Dalian, China), the ligation was then transformed into the *E. coli* competent cell and the clones were screened by PCR. Positive clones were sequenced and confirmed (Shanghai Sunny Biotech, China). The pCold-TF expression vector was digested with the corresponding endonucleases: *SlituCSP6* (BamHI and XbaI), *SlituCSP8* (KpnI and EcoRI), *SlituCSP9* (KpnI and BamHI), *SlituCSP18* (KpnI and EcoRI). All enzymes were bought from NEB, Beijing. The same restriction enzymes were used to cut each *CSP* gene from the previously mentioned PMD18-T vector. The *CSP* gene was ligated with pCold-TF expression vector and transformed into *E. coli* competent cell, then the clones were screened by PCR. All of the clones were confirmed by sequencing (Shanghai Sunny Biotech, China). Then, 2 μL of *E. coli* (BL21(DE3)) culture transformed with pCold-TF-CSP was added to 2 mL of LB liquid medium at 37 °C and shaking at 200 rpm for 3 h. Then, 2 mL of the bacterial solution was transferred to 200 mL of LB liquid medium and incubated at 37 °C, shaking at 200 rpm to an OD600 = 0.4 to 0.5 (10 mol/L) at 15 °C for 24 h. SDS-PAGE was used to detect protein expression. The images were analyzed using a Tanon 4100 automatic digital gel image analysis system (Tanon, Shanghai, China) and Adobe Photoshop CS6.

2.6. Purification of recombinant SlituCSP protein

Two hundred milliliters of LB medium was used for expression of recombinant proteins. The optimal induction conditions of four SlituCSPs were as follows: 1 mmol/L IPTG, 15 °C, induced at 20 r/min for 24 h. Cells were centrifuged at 9560 rcf at 4 °C for 10 min after induction by IPTG. The supernatant was discarded, and the cells were resuspended with 10 mmol/L Phosphate-buffered saline (PBS) (pH = 7.4). The cells were sonicated for 15 min on ice (ultrasound 3 s,