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Identification and expression profiles of putative chemosensory protein genes in *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae)

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ABSTRACT

Insect chemosensory proteins (CSPs) are small, water-soluble proteins which can bind and transport hydrophobic odorants through sensillum lymph to activate odorant receptors, thus play important roles in the olfactory recognition. In the present study, by searching the transcriptome data sets, a total of 22 candidate CSP genes were identified from *Cnaphalocrocis medinalis*, a serious lepidopteran rice pest in Asia. CSP proteins encoded by these genes showed typical characteristics: an N-terminal signal peptide, four conserved cysteine residues and the pattern of cysteine spacing. The expression profiles of these putative CSP genes were investigated using real-time quantitative PCR. The results showed that five genes (*CmedCSP4*, *CmedCSP8*, *CmedCSP11*, *CmedCSP18* and *CmedCSP21*) were expressed primarily in antennae, suggesting their involvement in olfactory processes; whilst other genes are mainly expressed in non-olfactory tissues, such as abdomen and legs, indicating a broader physiological function for these CSPs. The findings will lead to a better understanding of the molecular mechanisms of chemoreception in *C. medinalis*.

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Introduction

The detection of odorants is of vital importance for insect survival and reproduction. Although insects sense their environment mainly rely on specific olfactory receptors (ORs) and ionotropic receptors (IRs) located in specialized sensilla in antennae (Touhara and Vosshall, 2009), small soluble proteins, including odorant-binding proteins (OBPs) and chemosensory proteins (CSPs), also play important roles in transporting hydrophobic odorants through sensillum lymph to reach and activate ORs (Pelosi et al., 2006; Leal, 2013). The CSPs are small, extracellular proteins with a length of 100–120 amino acid residues (Pelosi et al., 2006). Unlike OBPs, CSPs contain four cysteines with a conserved spacing pattern, and forming two disulfide bridges (Pelosi et al., 2006; Zhou, 2010). Some CSPs are enriched in antennal sensillar lymph and are capable of binding host volatiles and pheromone constituents, suggesting that they are involved in olfactory recognition (Dani et al., 2011; Gu et al., 2012; Iovinella et al., 2013; Zhang et al., 2014); however, other CSPs are highly expressed in non-olfactory tissues such as pheromonal gland, legs and wings, indicating that they may participate in other physiological processes (Jacquin-Joly et al., 2001; Dani et al., 2011; Pelletier and Leal, 2011). To date, a variety of biochemical and structural data have been accumulated on CSPs in a wide range of insect species, and the role CSPs played in chemoreception was more and more clear (Lartigue et al., 2002; Gu et al., 2012; Liu et al., 2012a,b; Iovinella et al., 2013; Yi et al., 2014; Zhang et al., 2014). However, the precise functions of CSPs in other physiological processes remain uncertain (Maleszka et al., 2007; Liu et al., 2014; Pelosi et al., 2014).

The rice leaffolder, *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae), is a serious rice insect pest in Asia and caused great economic losses (Gurr et al., 2012). In many countries, pheromone traps have been made to attract male *C. medinalis* (Kawazu et al., 2002; Kawazu et al., 2005), but sometimes it was not effective. Recently, semiochemical-based reverse chemical ecology approach has been applied for screening better attractants to effectively manage insect pests (Leal et al., 2008). Therefore, identification of olfactory-related genes may pave the way for the development of novel attractants for *C. medinalis*. Previously, 15 OBP genes and 3 CSP genes have been isolated in the moth species (Li et al., 2012; Zeng et al., 2013). In the present study, 22 members of the CSP family, including 19 novel genes, were identified and characterized in *C. medinalis*. Their distribution in different tissue was also determined by real-time quantitative PCR analysis. Some of the *C. medinalis* CSP genes were predominantly expressed in antennae, suggesting that they could be involved in odorant detection.

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Materials and methods

Insects

The *C. medinalis* adults used in the whole study was collected from an experimental field of Anhui Agricultural University, Hefei, Anhui Province, China. Moths were brought back to the laboratory, anesthetized on ice and dissected into different tissues (antennae, abdomen, and legs of both females and males). All the samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Identification of CSP genes

Previously, a transcriptome data set was obtained from pooled RNA samples of *C. medinalis* eggs, first- to fifth-instar larvae, pupae, and adults (Li et al., 2012). A second *C. medinalis* transcriptome data set was constructed in the authors' laboratory (Based on Illumina paired-end sequencing technology, unpublished), using a mixture of RNA derived from several different tissues of adults (antennae, heads, thoraces, abdomens, legs and wings from females and males) at equal ratios. Putative CSP genes were identified by searching the two transcriptome data sets with keywords (chemosensory protein and CSP), and by using the basic local alignment search tool (BLAST) (Altschul et al., 1997). The annotated CSPs from other lepidopteran species, such as *Bombyx mori* (Gong et al., 2007), *Papilio xuthus* (Ozaki et al., 2008) and *Helicoverpa armigera* (Liu et al., 2012b), were used as references. The candidates of *C. medinalis* CSP genes were confirmed by searching against the NCBI non-redundant (nr) protein database using BLASTX (cut-off E-value of 10^{-5}).

Bioinformatic analysis

The open reading frames (ORFs) of putative *C. medinalis* CSP genes were predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Putative signal peptides were predicted with SignalP (http://www.cbs.dtu.dk/services/SignalP) and removed from the data set. The matured CSP protein sequences from *C. medinalis* and other moth species were aligned by using ClustalW (http://www.ebi.ac.uk/tools/msa/clustalw2) to generate a percent identity matrix, and a phylogenetic tree was constructed in MEGA5.05 software (Tamura et al., 2011) using neighbor-joining method with 1000-fold bootstrap resampling. The Genbank accession numbers of amino acid sequences of CSPs used were listed in Supplementary data Table S1.

Real-time quantitative PCR

Total RNA from various tissues of *C. medinalis*, including antennae, abdomen and legs of adult females and males, was extracted using Trizol reagent (Life Technologies, USA). 1 μg total RNA from each sample was reverse-transcribed using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan).

Real-time quantitative PCR (RT-qPCR) were performed using SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara, Japan). Each reaction (20 μl) contained a SYBR Premix Ex Taq II (10 μl), a cDNA template (20 ng), a sense primer and an anti-sense primer (0.2 μM each), and nuclease-free water. Primers were listed in Supplementary data Table S2, the *β-actin* (Genbank no. JN029806) of *C. medinalis* was used as a housekeeping gene to correct for sample-to-sample variation.

RT-qPCR was run on a StepOne Plus Real-time PCR system (Applied Biosystems, USA) for one cycle of 95 °C for 2 min, 40 cycles of 95 °C for 5 s and 60 °C for 25 s. At the end of each thermal cycle, the PCR products were analyzed using a heat-dissociation protocol to confirm that only one single gene was detected by fluorescence dye. A no-template control (NTC) was also run to detect possible contamination. Three biological repeats were done, relative expression levels of CSP genes across the samples were measured by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Data were converted to logarithmic form, a one-way analysis of variance (ANOVA) with the least significant difference (LSD) test were applied to compare means, using the Data Processing System (DPS) software v9.5 (Tang and Zhang, 2013).

Results

Identification of CSP genes in C. medinalis

By searching the two transcriptome data sets of *C. medinalis*, a total of 22 putative CSP gene fragments were identified (Table 1, Supplementary data Figs. S1 and S2). Of these, 20 sequences had complete ORFs, while two sequences, *CmedCSP9* and *CmedCSP11*, are incomplete cDNAs with partial ORFs (Table 1). Among the 22 *CmedCSP* sequences, three showed 100% identities in nucleic acids with previously reported *C. medinalis CSP1*, *CSP2* and *CSP3* (Genbank No. AGI37361, AGI37363 and AGI37365) (Zeng et al., 2013), respectively. Therefore, we named these three sequences *CmedCSP1*, *CmedCSP2* and *CmedCSP3*, in accordance with their original names (Table 1). The remaining 19 sequences were successively named from *CmedCSP4* to *CmedCSP22* (Genbank acc. nos: KM365188–KM365206, Table 1). It is worth to mention that, of all 22 CSP candidates identified in Li et al.'s data set (Li et al., 2012), only 12 candidates were identified in the authors' data set (Table 1).

The signal peptides were predicted from the deduced proteins of 20 full-length *CmedCSP* genes and a partial fragment *CmedCSP9* (Fig. 1, Table 1). The prediction was not performed for *CmedCSP11* due to its incomplete N-terminus. Besides the presence of a signal peptide, all the deduced *CmedCSP* proteins have the characteristic hallmarks of the CSP family: the highly conversed four cysteine residues and the spacing pattern $C_1–X_6–C_2–X_{17–19}–C_3–X_2–C_4$ (Fig. 1).

The information about BLASTX best hit of all the 22 *CmedCSP*s was listed in Table 1. Almost all the *CmedCSP*s had high identities to known lepidopteran CSPs, except for *CmedCSP8* and *CmedCSP19*, both showed only 30% identities to their respective orthologs. In addition, *CmedCSP22* showed a high identity (67% identity) with a CSP of parasitoid wasp *Microplitis mediator* (Hymenoptera: Braconidae) (Table 1). The percent identity matrix was constructed for the 22 *CmedCSP* proteins; the result showed that these proteins share low amino acid identity with each other (Supplementary data Table S3).

Phylogenetic analysis

The phylogenetic analysis was performed to better understand the relationships of the *CmedCSP* proteins with CSPs in other lepidopteran species, including *B. mori* (Gong et al., 2007), *H. armigera* (Liu et al., 2012b), *P. xuthus* (Ozaki et al., 2008), *Sesamia inferens* (Zhang et al., 2013), *Plutella xylostella* (Liu et al., 2010), *Agrotis ipsilon* (Gu et al., 2013) and *Chilo suppressalis* (Genbank data, unpublished). In the neighbor-joining tree (Fig. 2), it could be seen that the 22 *CmedCSP*s were spread out on various branches, and that almost all the *CmedCSP*s were clustered with at least one lepidopteran ortholog. The exceptions were *CmedCSP5*, which fall into a branch independent of other CSPs; and *CmedCSP1* and *CmedCSP8*, which were clustered together in a single branch separated from other members (Fig. 2).

Tissue-specific expression of C. medinalis CSPs

Relative expression levels of *CmedCSP* genes in different tissues of *C. medinalis* were investigated by RT-qPCR (Fig. 3). *CmedCSP1*, *CmedCSP2* and *CmedCSP3* were excluded from the analysis since their expression profiles were determined previously (Zeng et al., 2013).

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