

BIOTYPE EXPRESSION AND INSECTICIDE RESPONSE OF *Bemisia tabaci* CHEMOSENSORY PROTEIN-1

Guo Xia Liu, Ning Xuan, Dong Chu, Hong Yan Xie, Zhong Xue Fan, Yu Ping Bi, and Jean-François Picimbon

Biotechnology Research Center, Shandong Academy of Agricultural Sciences, Shandong Provincial Key Laboratory for Genetic Improvement Cultivation and Physiology of Crops, Jinan, China

Yu Chuan Qin and Su Ting Zhong

Department of Entomology, China Agricultural University, Beijing, China

Yao Fa Li, Zhan Lin Gao, and Wen Liang Pan

Plant Protection Institute of Hebei Academy of Agricultural and Forestry Sciences, IPM Centre of Hebei Province, Baoding, China

Guo Ying Wang

Institute of Crop Science, Chinese Academy of Agricultural Sciences-CAAS, Beijing, China

Balaji Rajashekar

Institute of Computer Science, University of Tartu, Tartu, Estonia

Chemosensory proteins (CSPs) are a group of small soluble proteins found so far exclusively in arthropod species. These proteins act in chemical communication and perception. In this study, a gene encoding the Type 1 CSP (BtabCSP1) from the agricultural pest Bemisia tabaci (whitefly) was analyzed to understand sequence variation and expression specificity in different biotypes. Sequence analysis of BtabCSP1 showed significant

Contract grant sponsor: Natural Sciences Foundation of Shandong Province; contract grant number: ZR2011CM046; contract grant sponsor: Overseas Talent, Taishan Scholar (Jean-François Picimbon); contract grant number: NO.tshw20091015.

Correspondence to: Jean-François Picimbon, Functional Genomics and Proteomics of Chemical Ecology, Biotechnology Research Center, Shandong Academy of Agricultural Sciences, No 202 Industry North Road, Jinan 250100, China. E-mail: jfpicimbon@gmail.com

differences between the two genetically characterized biotypes, B and Q. The B-biotype had a larger number of *BtabCSP1* mutations than the Q-biotype. Similar to most other CSPs, *BtabCSP1* was more expressed in the head than in the rest of the body. One-step RT-PCR and qPCR analysis on total messenger RNA showed that biotype-Q had higher *BtabCSP1* expression levels than biotype-B. Females from a mixed field-population had high levels of *BtabCSP1* expression. The interaction of *BtabCSP1* with the insecticide thiamethoxam was investigated by analyzing the *BtabCSP1* expression levels following exposure to the neonicotinoid, thiamethoxam, in a time/dose-response study. Insecticide exposure increased *BtabCSP1* expression (up to tenfold) at 4 and 24 h following 50 or 100 $\mu\text{g/ml}$ treatments. © 2014 Wiley Periodicals, Inc.

Keywords: whitefly; B-biotype; Q-biotype; chemosensory protein; neonicotinoid thiamethoxam insecticide

INTRODUCTION

The tobacco whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), also known as the cotton, sweet potato, or silverleaf whitefly, is a pest of several plants including ornamental plants and agricultural crops grown around the world (Brown and Bird, 1995; De Barro et al., 2011). Whiteflies damage crops through feeding and transmission of various plant pathogenic viruses (Jones, 2003). *B. tabaci* comprises multiple genetically characterized biotypes (or biological forms) that spread through the world due to host-plant adaptation and insecticide resistance (Brown et al., 1995; Nauen et al., 2002). About twenty *B. tabaci* biotypes have been identified and biotypes B and Q are the most common (Perring, 2001). These two types are invasive and transmit plant pathogenic viruses (Leshkowitz et al., 2006; Liu et al., 2007). Both biotypes feed on cotton, eggplant, or cabbage but occasionally they display host-plant preferences, suggesting that the biotypes B and Q have developed specific olfactory systems (Bernays, 1999).

In insects, the odor receptor mechanism involves seven-transmembrane domain receptors located in olfactory neuronal dendrites and small soluble-binding proteins that mediate transport of odors to olfactory receptors. Two families of small soluble proteins, odorant-binding proteins (OBPs), and chemosensory proteins (CSPs), have been described in the insect antennae (Picimbon, 2003). Chemosensory proteins (CSPs, also called p10, A10, OS-Ds, and SAPs) have been detected in cockroaches, flies, locusts, and moths before other insect species, such as the pine shoot beetle (Picimbon, 2003; Zhu et al., 2012).

In *B. tabaci*, extensive use of insecticides such as imidacloprid has resulted in biotype replacements due to differing insecticide resistance. Resistance to imidacloprid occurs in some B-biotype populations (Byrne et al., 2003). However, the Q-biotype populations are extremely resistant to imidacloprid and they become the predominant whitefly bio-form (Nauen and Denholm, 2005; Horowitz et al., 2005; Ghanim and Kontsedalov, 2007). Cytochrome P450 enzymes, in particular *CYP6CM1*, are linked to metabolism of xenobiotics and insecticide resistance in biotype-Q whiteflies (Feyereisen, 2006; Karunker et al., 2008). The existence of specific insecticide resistance mechanisms occurring in *B. tabaci* biotypes B and Q has been suggested (Wang et al., 2010).

We recently reported a whitefly CSP (BtabCSP1; #ADG56568; Li et al., 2012), which opened the question of whether the B and Q biotypes express BtabCSP1 in a similar manner. Here, we describe the cloning and sequencing of BtabCSP1 from both biotypes. We report on the tissue- and sex-expression profile of BtabCSP1, the differences in gene expression between the two biotypes and expression of the gene following treatment with an insecticide, thiamethoxam.

MATERIALS AND METHODS

Sampling of B. tabaci Biotypes

Whiteflies were originally collected from eggplant fields nearby Shouguang and Jinan cities (Shandong province, China). The biotypes B and Q were identified from these field-populations using Cleaved Amplified Polymorphic Sequences (CAPS) of mitochondrial cytochrome oxidase I (about 620 bps) amplified with primers (C1-J-2195/R-BQ-2819) as described by Chu et al. (2011). The mtCOI fragment was cleaved by the restriction endonucleases *VspI*. All of the mtCOIs that could be cut by *VspI* were *B. tabaci* Q. All others were *B. tabaci* B (Chu et al., 2011).

The two biotypes were maintained in our laboratory on mature Dalong eggplant before transfer to cotton in a climatic incubator under constant conditions for optimal rearing (temperature: 26°C, relative humidity: 60% and photoperiodic cycle L/D: 16/8 h). Mixed field populations were collected from cabbage plants from the greenhouse of Plant Protection Institute at SAAS in Licheng district of Jinan. Males were distinguished microscopically from females based on size and shape of the abdominal tip. Before dissection, the insects were anesthetized on ice for 10 min. Heads were removed using a razor blade under dissecting microscope. Whole bodies as well as heads and decapitated bodies were quickly frozen in liquid nitrogen then freeze-dried before storage at -70°C.

Cloning of BtabCSP1-RNA Variants

Total RNA from pools of 20 male and 20 female adults (both biotypes) was extracted using the Trizol method (Invitrogen, San Diego, California, USA) and treated with DNase I (Fermentas, Vilnius, Lithuania). Messenger RNA quality was assessed by electrophoresis of total RNA. RNA concentration was determined by measuring the absorbance at 260 nm (Eppendorf Biophotometer, Hamburg, Germany). Complementary DNAs were obtained using M-MuLV transcriptase from RevertAid™ First Strand cDNA Synthesis Kit, as described by the manufacturer (Fermentas, Vilnius, Lithuania). BtabCSP1-specific sense 5'-ATGCAGGTTTTGACTTTAGTT-3' and anti-sense 5'-TTACAGGGTTTGTCCGAAG-3' primers were used to clone BtabCSP1s from biotype- and sex-specific cDNA samples. 30 ng of cDNAs from whole body served as a template in the PCR experiments. The PCR reaction used EasyTaq DNA polymerase (Beijing TransGen Biotech Company, Beijing, China) and was performed in a TaKaRa PCR Thermal Cycler Dice (Takara, Dalian, China), programmed as follows: 94°C for 5 min; 35 cycles of 94°C for 30s, 52°C for 30s, and 72°C for 30 s; final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis on 1% agarose and visualized under UV-light after staining with ethidium bromide. The PCR products of expected size (380 bps) were purified using the EasyPure Quick Gel Extraction Kit (TransGen Biotech) and cloned into pEasy T1 vector (TransGen Biotech). Positive recombinant vectors were identified

by ampicillin resistance and BtabCSP1 target gene PCR amplification. For each sample (B-male, B-female, Q-male, and Q-female), nine to twelve positive recombinant vectors were sequenced in the laboratory on an ABI3700 sequencer instrument using the RR Dye Deoxy terminator cycle sequencing kit (Perkin-Elmer, Weiterstadt, Germany) with Sp6/T7 sequencing primer.

Sequence Alignment and Phylogenetic Analysis

Homologs to BtabCSP1 (ADG56568) were identified by searching the NCBI protein database using BLASTP tool (<http://blast.ncbi.nlm.nih.gov>). Using sequence similarity, BtabCSP1 showed clear homology to the Olfactory-Specific protein D family (Pfam, PF3392). The alignment profile of OS-D family was downloaded from the Pfam database and used as a template to generate the alignment of BtabCSP1 and orthologous sequences using ClustalX2.1 software. The consensus sequence was generated using Jalview software. Amino acid sequence alignment and consensus sequence were calculated using the most closely related sequences (more than 53% identity) to identify the amino acid motifs characteristic to this group of CSPs.

For phylogenetic analysis, a total of 28 resulting sequences having a score above 120, covering more than 90%, *e*-value below $1e-33$ and identity of at least 46% were selected as significant matches to BtabCSP1. A paralog of BtabCSP1 (AEY84055) was included in the analysis. We included also 25 CSP consensus sequences from different groups of insect species (ants, aphids, bees, beetles, flies, lice, locusts, mosquitoes, moths, and wasps). Species-specific consensus CSP sequences were from Ban et al. (2003), Wanner et al. (2004), Forêt et al. (2007), Gong et al. (2007), and Zhou et al. (2010). *Aedes*, *Culex*, *Drosophila*, *Harpegnatos*, *Nasonia*, *Pediculus*, and *Tribolium*-specific consensus CSP sequences were from insect genome analysis (Picimbon et al., in preparation). Consensus sequences (C*) were built using block-maker (<http://bioinformatics.weizmann.ac.il/blocks>). Optimal alignment was achieved by aligning all the CSP sequences in ClustalX 2.1 software as described before. The phylogenetic tree was inferred using maximum likelihood (PROTMIX model, Dayhoff, PROTCAT model, 100 bootstraps) in RAxML 7.0.4 program (Stamatakis et al., 2005). The crustacean consensus generated from species *Daphnia pulex* (ABH88167, ABH88166) and *Artemia franciscana* (ABY62738) was used as outgroup to infer phylogenetic relationships between BtabCSP1 and CSPs from other insect species.

One-Step Reverse Transcriptase PCR

The expression profiles of BtabCSP1 in the different biotypes, sexes, and various tissues were estimated first using semiquantitative one-step RT-PCR. Total RNA (100 ng) was extracted following the TRIzol® Reagent method (Invitrogen, San Diego, California, USA) and used as template for sequential reverse transcription and PCR amplification in a single-tube/two-enzymes reaction (PrimeScript™ One Step RT-PCR Kit, TAKARA). The RT-PCR experiments were carried out in a TAKARA PCR thermal Cycler Dice (TAKARA Bio Inc., Dalian, China) using the reaction cycles at 50°C for 30 min, 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 72°C for 7 min. The primers used for the amplification of BtabCSP1 are listed just above. Sense and antisense primers for the actin gene are 5'-CTTGGGTATGGAATCTTGC-3' and 5'-AAGGTGGACAGGGAAGC-3', respectively. The final RT-PCR products were separated by electrophoresis in a 1% (1 g/100 ml) agarose gel, and the intensity of the RNA band visualized after staining with ethidium bromide.

qPCR

For qRT-PCR analysis, cDNA templates (20 ng/ μ l) were synthesized from 250 ng of whole body RNA using M-MuLV transcriptase as described before (Fermentas, Vilnius, Lithuania). In one of the qRT-PCR experiments, head (including antennae) was analyzed separately from the rest of the body. Primers were specifically designed to amplify 150–300 bps long qRT-PCR products for both BtabCSP1 (BtabCSP18F: 5'-AGTTGTCTTGGTCGGCTG-3', BtabCSP325R: 5'-GGTCGTATTTCTGCTGGAG-3') and actin (sense: 5'-TCTTCCAGCCATCCTTCTTG-3' and antisense: 5'-CGGTGATTT CCTTCTGCATT-3'). *Actin* was used as endogenous control for gene expression analysis. Reactions were run with initial denaturing step consisting of 2 min at 50°C and 5 min at 95°C, followed by 40 cycles of 5 s at 94°C, 20 s at 60°C, and 31 s at 72°C, and terminated by a dissociation curve program from 60 to 95°C with a heating rate of 0.1°C and a continuous fluorescence acquisition. The SYBR Green I fluorescence (Takara) was monitored in Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Foster City, California). All samples were tested in three technical replicates on 96-well plates with optical sealing tape. The experiment was repeated three times ($n = 9$). We used the 2(-Delta Delta C(T)) method to analyze the relative changes in gene expression following Livak and Schmittgen (2001). Data were statistically analyzed by one-way ANOVA in SPSS.

Insecticide Treatment

Mature adult forms of a mixed whitefly population were collected from cabbage fields in the northern section of Jinan and exposed in the laboratory conditions to 0 (control), 25, 50, 100 μ g/ml of thiamethoxam using a cotton leaf-dip method (Rauch and Nauen, 2003). Cotton plant leaf discs (2.5 cm diameter) were dipped for 10 sec in formulated thiamethoxam (25% WG, Syngenta, Shanghai, China) diluted with distilled water. After drying, the plant discs were individually placed on agar base in 20 ml glass vials. Twenty-eight whiteflies were aspirated into each vial. Vials were then capped with an absorbent cotton stopper, inverted and held at room temperature. Insects were collected following 4, 24, and 72 h insecticide or water (control) exposure, and directly placed in -70°C until RNA extraction (see qRT-PCR).

RESULTS

Sequence and Phylogenetic Analysis of BtabCSP1-RNA Sequences

Gene specific primers were used to amplify cDNA sequences from males and females of the two biotypes B and Q. The amplification resulted in a 380 bp long cDNA product in all the clones. Forty-six clones were sequenced; twenty-three haplotypes were identified (B28, B29, B31, B33, B34, B40, B41, B42, B45, B46, B47, B48, G40, G41, G44, G45, G46, G47, G49, G51, Y46, Y49, and Y50; NCBI accessions JQ678974–JQ678996). Pairwise comparison of the twenty-three haplotype sequences to genomic DNA showed a total of 16 point mutation sites corresponding to missense (nonsynonymous), silent (synonymous), and nonsense nucleotide substitutions.

Between the two biotypes, the biotype-B showed significantly a higher number of mutations compared to biotype-Q (Fig. 1, Table 1). The nucleotide switches from T to C

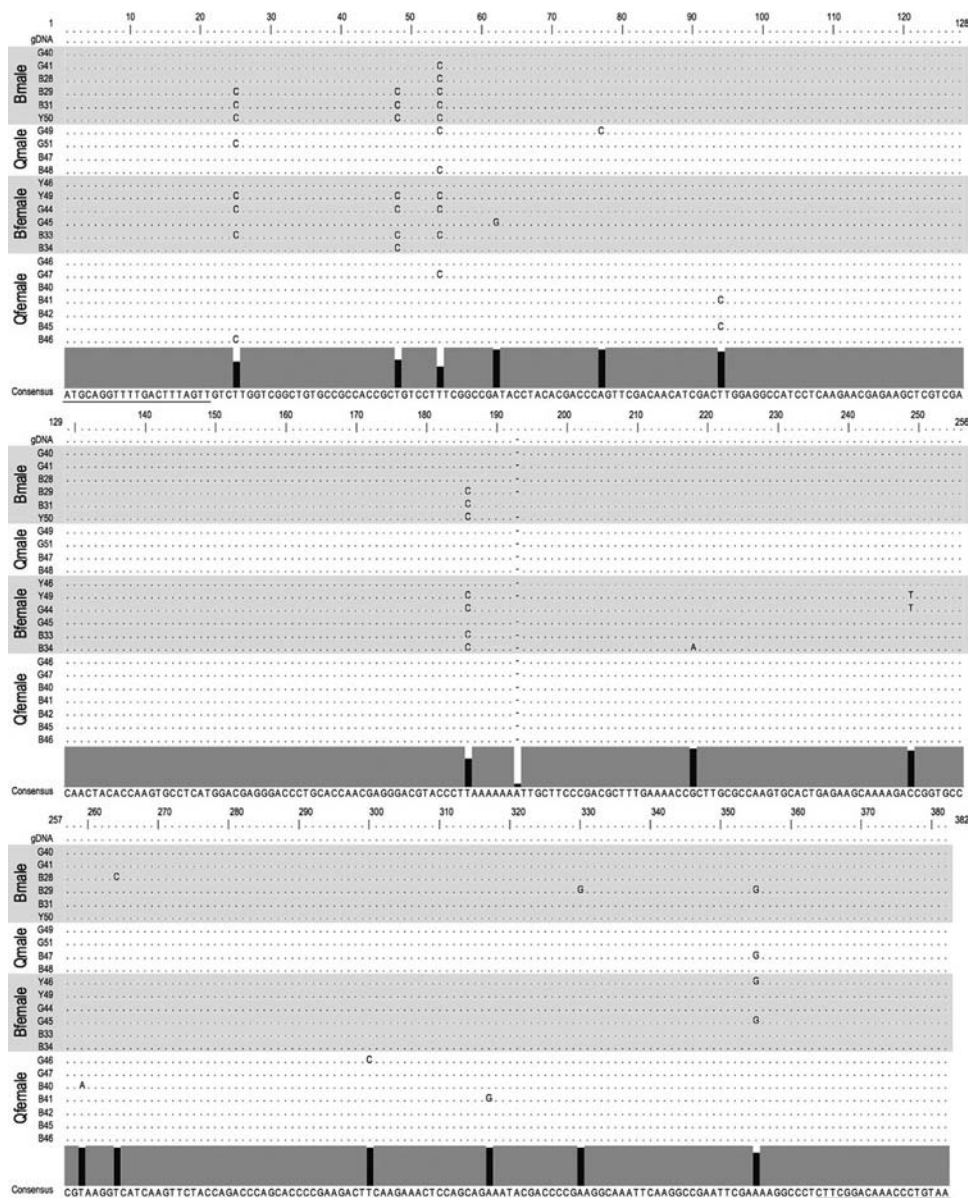


Figure 1. Identifying substitution sites in BtabCSP1 from the biotypes B- and Q of *B. tabaci*. The alignment shows BtabCSP1 nucleotide sequences from gDNA and cDNA clones in B-males, Q-males, B-females, and B-males of *B. tabaci* (Acc. Num. JQ678974–JQ678996). Horizontal lines indicate the positions of primers used to amplify BtabCSP1s. Nucleotide motifs identical to the BtabCSP1 consensus sequence are represented as dots. Presence of a nucleotide indicates a substitution at this site. The symbol “-” indicates a gap in the alignment (insertion of A at position 193 in two B-clones, JQ678978 and JQ678982). Numbering starts at the initial codon ATG. The graph below the alignment indicates nucleotide conservation (in gray) or substitution (in black). Nucleotide sequences from biotype B are shaded in gray.

Table 1. Silent Nucleotide Substitutions on BtabCSP1 RNA from B and Q Biotypes

Biotype	Site	Nucleotide change	Frequency	Conserved amino acid (Y/N)	Accession no.
Q	25	T→C	2/11	N	JQ678992, 94
	54	T→C	3/11	N	JQ678987, 93, 96
	77	A→C	1/11	Y	JQ678993
	94	T→C	2/11	N	JQ678989, 91
	259	T→A	1/11	N	JQ678988
	300	T→C	1/11	Y	JQ678986
	317	A→G	1/11	Y	JQ678989
	355	A→G	1/11	N	JQ678995
B	25	T→C	6/12	N	JQ678977-9, 81-2, 84
	48	T→C	7/12	N	JQ678977-9, 81-2, 84-5
	54	T→C	8/12	N	JQ678975-9, 81-2, 84
	62	A→G	1/12	Y	JQ678983
	186	T→C	7/12	N	JQ678977-9, 81-2, 84-5
	193	A-plus	2/12	Y ^a	JQ678978, 82
	218	G→A	1/12	Y	JQ678985
	249	C→T	2/12	Y	JQ678981-2
	264	T→C	1/12	Y	JQ678976
	330	A→G	1/12	Y	JQ678977
	355	A→G	3/12	N	JQ678977, 80, 83

^aInsertion in the sequence resulting in frame-shift and early-stop codon.

at bases 25, 48, 54, and 186 were found to occur at a rate of 50–66% in the biotype-B, while switches occurred in biotype-Q only in bases 25 and 54 at a rate of 16–25%. In addition, an insertion of nucleotide A in position 193 was identified only in biotype-B (B31-JQ678978; G44-JQ678982) but not in biotype-Q (Table 1).

Silent and missense mutations were observed in males and females of the two biotypes. Females showed six amino acid changes while males showed three missense mutations. These missense nucleotide transitions (A-G:62 [read as A to G at base 62], A-C:77, G-A:218, C-T:249, T-C:264, and 300, A-G:317, and 330) lead to amino acid changes at specific positions (Asp-Gly:21 [read as Asp to Gly at position 21], Gln-Pro:26, Ala-Thr:73, Thr-Ile:83, Val-Ala:88, Phe-Ser:100, Lys-Glu:106, and Glu-Gly:110; Table 2).

Except for one mutation (A-T:193) that inserts a premature stop codon and leads to a C-terminus truncated protein, the BtabCSP1-mRNA sequence variants all encode a protein with 126 amino acid residues, a calculated molecular weight of 12.3 Kda (a signal peptide of 19 residues), an isoelectric point of 8.3, a high number of lysines (17) and a characteristic motif of four conserved cysteines (Fig. 2). Comparing the amino acid sequence of BtabCSP1 variants with CSPs from other insect species, BtabCSP1 showed particular relatedness (more than 53% identity) with *Aedes Aegypti* AAEL001963, *Anopheles darlingi* AND_23774, *A. gambiae* AGAP008059, and CAG26923, CSP1 from *Delia antiqua* and CSP3 from *Glossina morsitans morsitans* (Acc. Num. CAG26923.1, CBA11329.1, BAI82449.1, EFR29504.1, XP_001660749.1, and XP_317400.2; Fig. 2). The protein BtabCSP1 and most closely related proteins from flies and mosquitoes are characterized by 37 strictly conserved amino acid residues (scattered all along the molecule but mainly at 27-FDNI-30, 44-NYXKCL-49, 66-LPDAL-70, and 106-KYDPEG-111) and about 25 well-conserved amino acid residues (mainly in the amino acid motifs 33–43, 96–106, and 116–119). In addition to the signal peptide, most variable regions are found in the N-terminus (amino acid motif 34–43), the central core (amino acid motif 82–102), and the C-terminus (amino

Table 2. Missense Nucleotide Substitutions on BtabCSP1-RNA from Biotypes B and Q

Clone no.	Nucleotide motif	Amino acid motif	Location ^a	Accession no.
Bmale-B28	AAGGTCATC	KVI	88	JQ678976
	AAGGCCATC	KAI		
Bmale-B29	CCCGAAGGC	PEG	110	JQ678977
	CCCGGAGGC	PGG		
Bfemale-G45	GCCGATACC	ADT	21	JQ678983
	GCCGGTACC	AGT		
Bfemale-B34	ACCGCTTGC	TAC	73	JQ678985
	ACCACTTGC	TTC		
Bfemale-Y49	AAGACCGGT	KTG	83	JQ678981
	AAGATCGGT	KIG		
Qmale-G49	ACCCAGTTC	TQF	26	JQ678993
	ACCCCGTTC	TPF		
Qfemale-G46	GACTTCAAG	DFK	100	JQ678986
	GACTCCAAG	DSK		
Qfemale-B41	CAGAAATAC	QKY	106	JQ678989
	CAGGAATAC	QEY		

^aThe amino acid motifs are highlighted on BtabCSP1 in Figure 2.

acid motif 112–126). The C-terminus of the mosquito CSPs is found to be two amino acid residues shorter than BtabCSP1 (Fig. 2).

The rooted maximum likelihood analysis of the BtabCSP1 amino acid sequences together with its main orthologs and consensus amino acid CSP sequences obtained from various insect species indicates that BtabCSP1 is phylogenetically related to bee and wasp CSPs (*A. mellifera* CAJ01448 sequence and wasp *N. vitripennis* consensus sequence form a group together with *B. tabaci* ADG56568 and the consensus sequence of BtabCSP1 variants). The BtabCSP1 paralog (AEY84055) falls more closely to the consensus sequence of CSPs from the moth species, *Bombyx mori*, and the crustacean consensus sequence (Fig. 3).

Biotype, Sex, and Tissue-Specific Expression of BtabCSP1

We investigated variations in expression levels of BtabCSP1 in the B and Q biotypes, sexes, and head/body tissues (Fig. 4). In pilot PCR using a mixed whitefly field-population, BtabCSP1-specific primers amplified products of about 380 bps in the head and decapitated body samples, but expression levels of BtabCSP1 in the head were significantly higher than the rest of the body (Fig. 4A). BtabCSP1 expression levels in the female whole-body were higher than those in the male whole-body (Fig. 4A). Expression levels of BtabCSP1 in males and females from the biotype-Q were higher than those from the biotype-B (Fig. 4B).

The expression of BtabCSP1 in the two biotypes was analyzed in more detail by qPCR and pure laboratory colonies of insects. Transcription levels of BtabCSP1 were 2.5 to 4 times higher in the biotype-Q than in the biotype B (Bf BtabCSP1-Actin value normalized to 1; Fig. 4C). No differences in transcription levels were observed between males and females in the biotype-B (Fig. 4C). However, BtabCSP1 was expressed in higher levels in biotype Q (Fig. 4C). The qPCR measurements on mixed field-population samples showed BtabCSP1 expression is low in the head and decapitated body tissues from a pool of individuals collected on the same leaf (Fig. 4D, left), but BtabCSP1 expression in whole

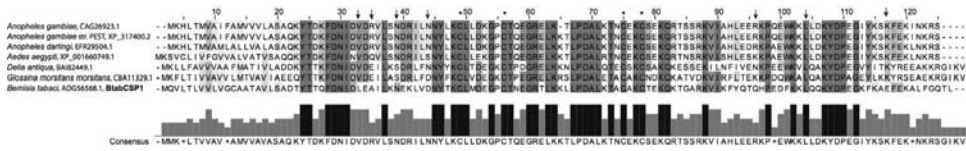


Figure 2. Sequence alignment of BtabCSP1 with insect orthologs. Sequences are from ADG56568, CAG26923.1, CBA11329.1, BAI82449.1, EFR29504.1, XP_001660749.1, and XP_317400.2 (NCBI). Residues showing >50% identity in the alignment are shaded in gradient of light to dark gray. The consensus sequence is shown below the alignment. Numbers at the top indicate amino acid positions in the BtabCSP1 protein. The asterisk indicates the position of the conserved Cysteine residue. The arrow indicates missense mutation site (amino acid changes).

body samples from a pool of insects collected on another leaf was very high in females (relative gene expression over 140). About fourteen times lower expression levels were recorded in males (DBody BtabCSP1-Actin value normalized to 1; Fig. 4D, right).

Effect of Insecticide Treatment on BtabCSP1 Expression

In this experiment, whiteflies from a mixed field-population were treated with thiamethoxam (at 0, 25, 50, and 100 $\mu\text{g}/\text{ml}$). Whiteflies were then assayed for changes in expression of BtabCSP1 at 4, 24, or 72 h following insecticide treatment (control BtabCSP1-Actin value normalized to 1; Fig. 5).

Induction of BtabCSP1 expression was observed in treated (50 $\mu\text{g}/\text{ml}$) whiteflies. After 4 h of treatment, the same or slightly lower expression of BtabCSP1 was registered in whiteflies treated with 25 or 100 $\mu\text{g}/\text{ml}$ doses insecticide. However, significantly higher BtabCSP1 expression was induced following exposure to the 50 $\mu\text{g}/\text{ml}$ dose of thiamethoxam (Fig. 5). The low dose of insecticide had no effects, the responses to moderate doses started to decrease, but a tenfold response to high doses of insecticide was seen at 24 h (Fig. 5).

After 72 h, the stimulatory effects of thiamethoxam had completely disappeared (Fig. 5).

DISCUSSION

Our study answers our question of whether the B and Q biotypes of *B. tabaci* express BtabCSP1 in a similar manner.

The sequence analysis suggests to us a micro-diversity of protein variants possibly originating from pinpoint mutations at the level of RNA. Most of the RNA mutations are silent but some result in specific amino acid changes. More data on the functional structure of BtabCSP1 are required to assess how these mutations affect the functioning of the protein. Similar phenomena (mutations on RNA sequences) have been recorded in CSPs from various tissues (the antennae and pheromone gland) from other insect species such as moths (Vogel et al., 2010; Grosse-Wilde et al., 2011). In whiteflies, the mutations on CSP-encoding RNA may be fixed in a biotype-specific manner. Alternatively, there are variations in the genomic sequence of the BtabCSP1 gene between biotypes. Some of the RNA mutations in B-biotype are not detected in the Q-biotype and vice-versa. Correlatively, there are behavioral differences among the biotypes in response to plant chemical cues (Inbar and Gerling, 2008; Bleeker et al., 2009). In addition to plant chemicals that can attract or repulse biotypes B and Q, there may be some specific

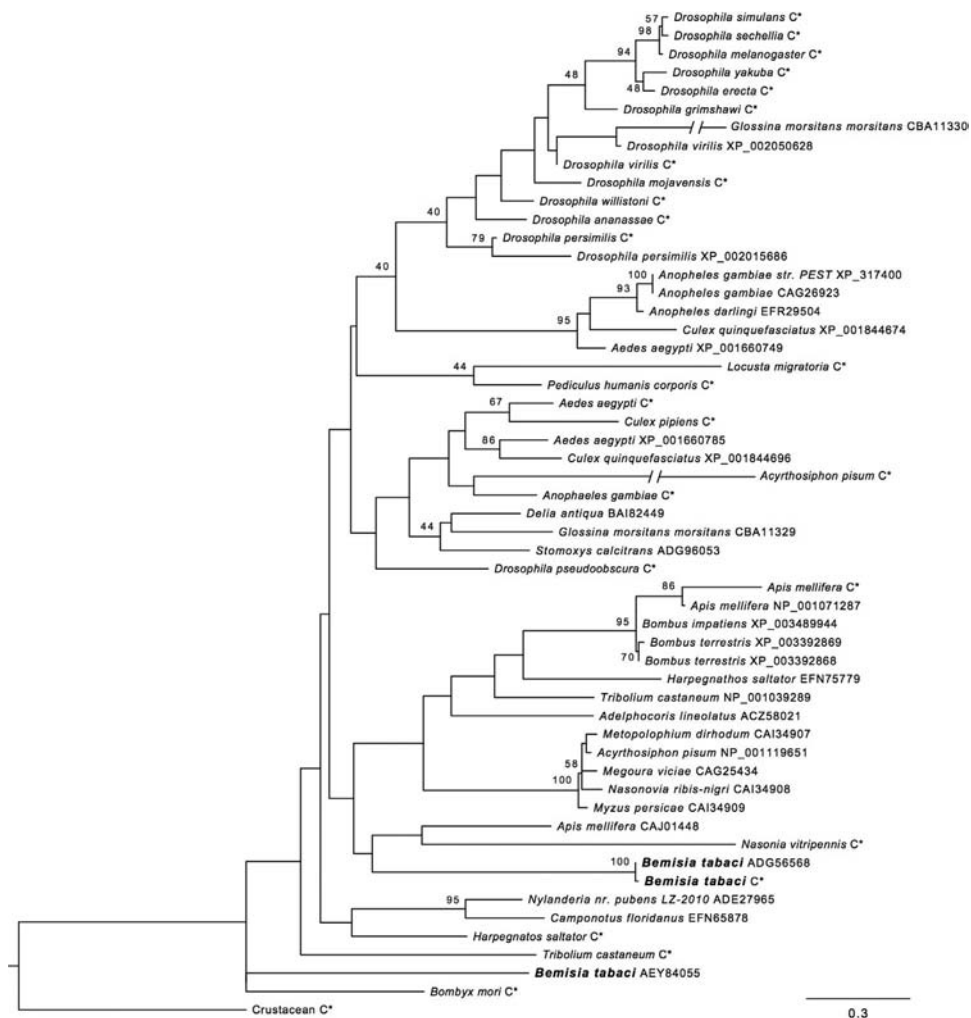


Figure 3. Phylogenetic tree of BtabCSP1 and consensus CSPs. Sequences are from BtabCSP1 (ADG56568), *B. tabaci* CSP AEY84055, and various CSPs from other insect species (*Acyrthosiphon pisum*, *Adelphocoris lineolatus*, *Anopheles darlingi*, *A. gambiae*, *Aedes aegypti*, *Apis mellifera*, *Bombus impatiens*, *B. terrestris*, *Camponotus floridanus*, *Culex pipiens*, *C. quinquefasciatus*, *Delia antiqua*, *Drosophila persimilis/pseudoobscura*, *D. virilis*, *G. morsitans morsitans*, *Harpegnathos saltator*, *Megoura viciae*, *Metopolophium dirhodum*, *Myzus persicae*, *Nasonovia ribis-nigri*, *Nylanderia pubens*, *Stomoxys calcitrans*, and *Tribolium castaneum*; XP_317400, XP_003489944, XP_003392868, CBA11329, XP_001660749, CAG26923, EFR29504, XP_001844674, ADE27965, BAI82449, NP_001071287, ADG96053, XP_003392869, EFN65878, NP_001039289, XP_001660785, CAI34909, XP_001844696, EFN75779, XP_002015686, ACZ58021, CAG25434, CAI34907, NP_001119651, CAJ01401448, CAI34908, CBA11330, and XP_002050628). Numbers at the nodes indicate the bootstrap support values (>40,100 bootstraps). The consensus CSP sequences from crustacean species *Daphnia pulex* and *Artemia franciscana* are used as outgroup (ABH88167, ABH88166, and ABY62738). BtabCSP1 (in bold) groups with the honeybee ASP3c (CAJ01401448).

chemicals (sex pheromones) that are exchanged between males and females of the same biotype. The B and Q biotypes do not cross-mate (Perring and Symmes, 2006).

Comparing amino acid sequences using alignment and phylogenetic tools, it can be seen that BtabCSP1 is closely related to fly and mosquito CSPs as well as to the bee ASP3c (#CAJ01448.1, AAN59784.1, ABH88171.1), which is unexpected considering

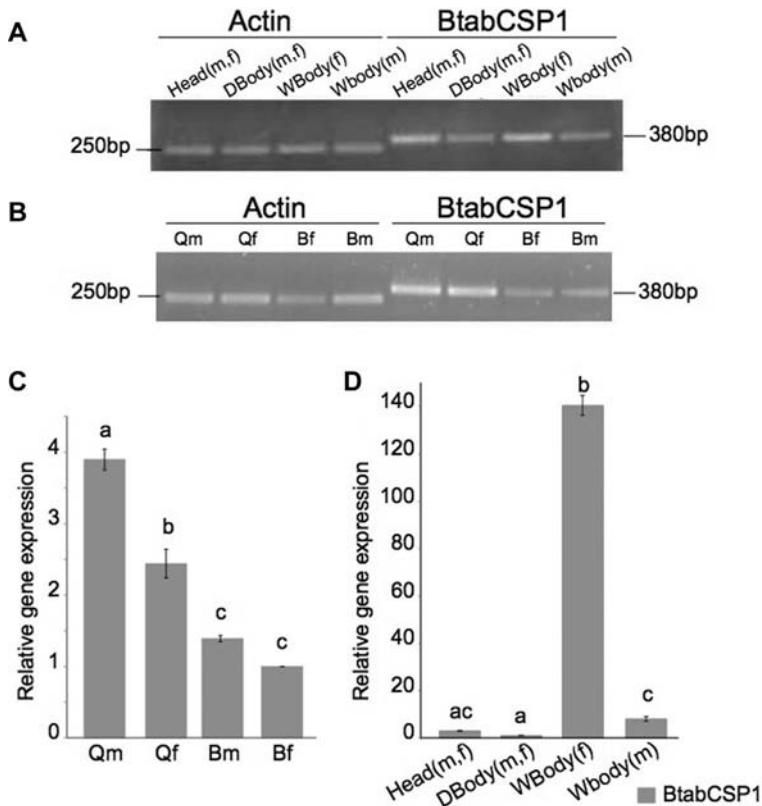


Figure 4. Expression of BtabCSP1 in different tissues, sexes, and biotypes. (A and B) RT-PCR calibration experiments using Actin primer show the same amplicon (250 bps) in similar amounts in all samples. Test experiments using BtabCSP1 primers show an amplicon (380 bps) with preferential expression in head (left; A), female whole-body (right; A), and biotype Q (B). (C) Relative quantification of BtabCSP1 gene expression in Q-males (Qm), Q-females (Qf), B-females (Bf), and B-males (Bm). The relative expression levels observed in B-females (Bf) were used as reference (Bf BtabCSP1-Actin value normalized to 1). (D) Relative quantification of BtabCSP1 gene expression in field-collected insects (DBody BtabCSP1-Actin value normalized to 1). Data are means $\pm$ standard deviation. Different letters indicate significant differences at $\alpha = 0.05$ by one-way ANOVA.

the phylogenetic distances between these insect groups. The protein honeybee ASP3c is a brood pheromone-binding protein (Briand et al., 2002). However, Western blot experiments show that ASP3c is detected in different parts of the body from all castes (Calvello et al., 2005). qPCR of ASP3c transcripts showed that ASP3c is highly expressed in wings and legs (Li et al., 2007). We find that BtabCSP1 is more expressed in the head than in the rest of the body.

Our gene expression data show that BtabCSP1 is more highly expressed in the Q biotype than in the B.

The biotypes B and Q have high resistance to various insecticides such as abamectin, neonicotinoids, pyrethroids, and pyriproxyfen. However, biotype-Q is more resistant than B (Ma et al., 2007; Luo et al., 2010). A function of BtabCSP1 in relation to pesticide is supported by the high BtabCSP1 expression levels in field-collected *B. tabaci* females (see Fig. 4D). Similarly, high expression of chemosensory proteins has been reported in females from the tsetse fly *G. morsitans* (Liu et al., 2012). In *B. tabaci*, the females are more resistant to insecticides than males (Crowder et al., 2008).

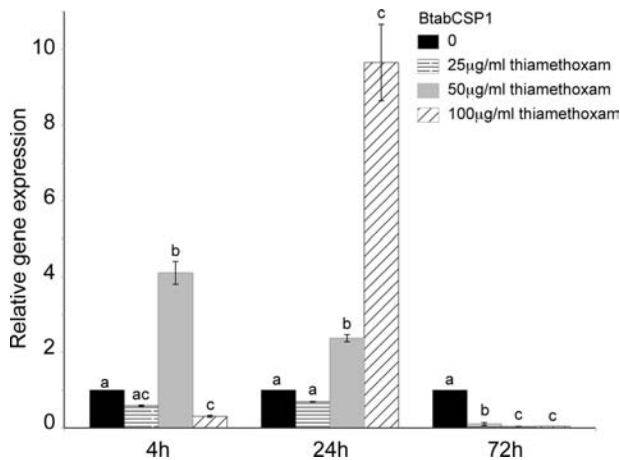


Figure 5. Effects of different doses of insecticide neonicotinoid thiamethoxam on BtabCSP1 expression. Expression of BtabCSP1 was measured after 4, 24, and 72 h of thiamethoxam treatment. Actin was used as internal control (control BtabCSP1-Actin value normalized to 1). Data are means $\pm$ standard deviation. Different letters indicate significant differences at $\alpha = 0.05$ by one-way ANOVA.

A role of BtabCSP1 in relation with insecticide is further supported by the effects of different doses of neonicotinoid thiamethoxam on BtabCSP1 expression after 4, 24, and 72 h of treatment. Our experiments show that whiteflies exposed to thiamethoxam express dose-dependent increases (four to ten folds more) in BtabCSP1 mRNA. Different doses of insecticide are known to differentially affect the insect physiology (Haynes, 1988; Desneux et al., 2004; Tricoire-Leignel et al., 2012). There are also probably individual variables such as age, gender, genetic, and physiological status (mainly in detoxification mechanisms) that could influence the responses to insecticide in whiteflies (Karalliede et al., 2003).

The reason behind the differential kinetics observed with different thiamethoxam doses is unknown. The main result of our study is support for the idea that some insecticides interact with CSP proteins, although we do not yet understand the mechanism. We propose continued research on how CSPs (and OBPs) interact with environmental chemicals. If these proteins are inducible and their behavior triggered by insecticide chemicals, this surely makes them interesting targets for insect population control.

ACKNOWLEDGMENTS

We thank Ma Hong-Mei (SAAS) for helping in whitefly rearing. We thank the Estonian Center of Excellence in Computer Science (EXCS) and Estonian Research Mobility Scheme (ERMOS) for support of Balaji Rajashekar.

The authors declare that no conflict of interest exists.

LITERATURE CITED

Ban LP, Scaloni A, Brandazza A, Angeli S, Zhang L, Yan Y, Pelosi P. 2003. Chemosensory proteins of *Locusta migratoria*. *Insect Mol Biol* 12:125–134.

- Bernays EA. 1999. When choice is a problem for a generalist herbivore: experiments with the whitefly *Bemisia tabaci*. *Ecol Entomol* 24:260–267.
- Bleeker PM, Diergaarde PJ, Ament K, Guerra J, Weidner M, Schütz S, de Both MTJ, Haring MA, Schuurink RC. 2009. The role of specific tomato volatiles in tomato-whitefly interaction. *Plant Physiol* 151:925–935.
- Briand L, Swasdipan N, Nespoulos C, Bézirad V, Blon F, Huet JC, Ebert P, Pernollet JC. 2002. Characterization of a chemosensory protein (ASP3c) from honeybee (*Apis mellifera* L.) as a brood pheromone carrier. *Eur J Biochem* 269:4586–4596.
- Brown JK, Bird J. 1995. Variability within the *Bemisia tabaci* species complex and its relation to new epidemics caused by Geminivirus. *CEIBA* 36:73–80.
- Brown JK, Frohlich DR, Rosell RC. 1995. The sweet potato or silverleaf whiteflies: biotype of *Bemisia tabaci* or a species complex. *Annu Rev Entomol* 40:511–534.
- Byrne FJ, Castle S, Prabhaker N, Toscano NC. 2003. Biochemical study of resistance to imidacloprid in B biotype *Bemisia tabaci* from Guatemala. *Pest Manag Sci* 59:347–352.
- Calvello M, Brandazza A, Navarrini A, Dani FR, Turillazzi S, Felicioli A, Pelosi P. 2005. Expression of odorant-binding proteins and chemosensory proteins in some hymenoptera. *Insect Biochem Mol Biol* 35:297–307.
- Chu D, Gao CS, De Barro P, Zhang YJ, Wan FH. 2011. Investigation of the genetic diversity of an invasive whitefly in China using both mitochondrial and nuclear DNA markers. *Bull Entomol Res* 101:467–475.
- Crowder DW, Ellers-Kirk C, Yafuso CM, Dennehy TJ, Degain BA, Harpold VS, Tashnik BE, Carrière Y. 2008. Inheritance of resistance to pyriproxyfen in *Bemisia tabaci* (Hemiptera: Aleyrodidae) males and females (B biotype). *J Econ Entomol* 101:927–932.
- De Barro PJ, Liu SS, Boykin LM, Dinsdale AB. 2011. *Bemisia tabaci*: a statement of species status. *Annu Rev Entomol* 56:1–19.
- Desneux N, Rafalimanana H, Kaiser L. 2004. Dose-response relationship in lethal and behavioral effects of different insecticides on the parasitic wasp *Aphidius ervi*. *Chemosph* 54: 619–627.
- Feyereisen R. 2006. Evolution of insect P450. *Biochem Soc Transactions* 34:1252–1255.
- Forêt S, Wanner KW, Maleszka R. 2007. Chemosensory proteins in the honeybee: Insights from the annotated genome, comparative analysis and expression profiling. *Insect Biochem Mol Biol* 37:19–28.
- Ghanim M, Kontsedalov S. 2007. Gene expression in pyriproxyfen-resistant *Bemisia tabaci* Q biotype. *Pest Manag Sci* 63:776–783.
- Gong DP, Zhang H, Zhao P, Lin Y, Xia QY, Xiang ZH. 2007. Identification and expression pattern of the chemosensory protein gene family in the silkworm, *Bombyx mori*. *Insect Biochem Mol Biol* 37:266–277.
- Grosse-Wilde E, Kuebler LS, Bucks S, Vogel H, Wicker D, Hansson BS. 2011. Antennal transcriptome of *Manduca sexta*. *Proc Natl Acad Sci USA* 108:7449–7454.
- Haynes KF. 1988. Sublethal effects of neurotoxic insecticides on insect behavior. *Ann Rev Entomol* 33:149–168.
- Horowitz AR, Kontsedalov S, Khasdan V, Ishaaya I. 2005. Biotypes B and Q of *Bemisia tabaci* and their relevance to neonicotinoid and pyriproxyfen resistance. *Arch Insect Biochem Physiol* 58:216–225.
- Inbar M, Gerling D. 2008. Plant-mediated interactions between whiteflies, herbivores, and natural enemies. *Annu Rev Entomol* 53:431–448.
- Jones DR. 2003. Plant viruses transmitted by whiteflies. *Eur J Plant Pathol* 109:195–219.
- Karalliedde LD, Edwards P, Marrs TC. 2003. Variables influencing the toxic response to organophosphates in humans. *Food Chem Toxicol* 41:1–13.

- Karunker I, Benting J, Lueke B, Ponge T, Nauen R, Roditakis E, Vontas J, Gorman K, Denholm I, Morin S. 2008. Over-expression of cytochrome P450 CYP6CM1 is associated with high resistance to imidacloprid in the B and Q biotypes of *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Insect Biochem Mol Biol* 38:634–644.
- Leshkowitz D, Gazit S, Reuveni E, Ghanim M, Czosnek H, McKenzie C, Shatters RL Jr, Brown JK. 2006. Whitefly (*Bemisia tabaci*) genome project: analysis of sequenced clones from egg, instar and adult (viruliferous and non-viruliferous) cDNA libraries. *BMC Genomics* 7:79–98.
- Li HL, Lou BG, Chang JA, Gao QK. 2007. The chemosensory protein of Chinese honeybee, *Apis cerana cerana*: molecular cloning of cDNA, immunocytochemical localization and expression. *Chinese Sci Bull* 52:1355–1364.
- Li YF, Qin YC, Gao ZL, Dang ZH, Pan WL, Xu GM. 2012. Cloning, expression and characterization of a novel gene encoding a chemosensory protein from *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). *Afr J Biotech* 11:758–770.
- Liu R, He X, Lehane S, Lehane M, Hertz-Fowler C, Berriman M, Field LM, Zhou JJ. 2012. Expression of chemosensory proteins in the tsetse fly *Glossina morsitans morsitans* is related to female host-seeking behaviour. *Insect Mol Biol* 21:41–48.
- Liu SS, DeBarro PJ, Xu J, Luan JB, Zang LS, Ruan YM, Wan FH. 2007. Assymetric mating interactions drive widespread invasion and displacement in a whitefly. *Science* 318:1769–1772.
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25:402–408.
- Luo C, Jones CM, Devine G, Zhang F, Denholm I, Gorman K. 2010. Insecticide resistance in *Bemisia tabaci* biotype Q (Hemiptera: Aleyrodidae) from China. *Crop Protection* 29:429–434.
- Ma DY, Gorman K, Devine G, Luo W, Denholm I. 2007. The biotype and insecticide-resistance status of whiteflies, *Bemisia tabaci* (Hemiptera: Aleyrodidae), invading cropping systems in Xinjiang Uygur Autonomous Region, northwestern China. *Crop Protection* 26:612–617.
- Nauen R, Denholm I. 2005. Resistance of insect pests to neonicotinoid insecticides: Current status and future prospects. *Arch Insect Biochem Physiol* 58:200–215.
- Nauen R, Stumpf N, Elbert A. 2002. Toxicological and mechanistic studies on neonicotinoid cross resistance in Q-type *Bemisia tabaci* (Hemipteran: Aleyrodidae). *Pest Manag Sci* 58: 868–875.
- Perring TM. 2001. The *Bemisia tabaci* species complex. *Crop Protection* 20:725–737.
- Perring TM, Symmes EJ. 2006. Courtship behavior of *Bemisia argentifolii* (Hemiptera: Aleyrodidae) and whitefly mate recognition. *Ann Entomol Soc Am* 99:598–606.
- Picimbon JF. 2003. Biochemistry and evolution of CSP and OBP proteins. In: Blomquist, GJ, Vogt RG, editors. *Insect pheromone biochemistry and molecular biology, the biosynthesis and detection of pheromones and plant volatiles*. London: London Academic Press, p. 539–566.
- Rauch N, Nauen R. 2003. Identification of biochemical markers linked to neonicotinoid cross resistance in *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Arch Insect Biochem Physiol* 54:165–176.
- Stamatakis A, Ludwig T, Meier H. 2005. RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. *Bioinformatics* 4:456–463.
- Tricoire-Leignel H, Thany SH, Gadenne C, Anton S. 2012. Pest insect olfaction in an insecticide-contaminated environment: info-disruption or hormesis effect. *Front Physiol* 3:58.
- Vogel H, Heidel AJ, Heckel DG, Groot AT. 2010. Transcriptome analysis of the sex pheromone gland of the noctuid moth *Heliothis virescens*. *BMC Genomics* 11:29.
- Wang XW, Luan JB, Li JM, Bao YY, Zhang CX, Liu SS. 2010. De novo characterization of a whitefly transcriptome and analysis of its gene expression during development. *BMC Genomics* 11:400.
- Wanner KW, Willis LG, Theilmann DA, Isman MB, Feng Q, Plettner E. 2004. Analysis of the insect os-d-like gene family. *J Chem Ecol* 30:889–911.

- Zhou JJ, Vieira FG, He XL, Smadja C, Liu R, Rozas J, Field LM. 2010. Genome annotation and comparative analyses of the odorant-binding proteins and chemosensory proteins in the pea aphid *Acyrtosiphon pisum*. *Insect Mol Biol* 19:113–122.
- Zhu JY, Zhao N, Yang B. 2012. Global transcriptional analysis of olfactory genes in the head of pine shoot beetle, *Tomicus yunnanensis*. *Comp Funct Genomics* 2012:491748.