A Chemosensory Protein BtabCSP11 Mediates Reproduction in Bemisia tabaci

Yang Zeng1, Austin Merchant2, Qingjun Wu1, Shaoli Wang1, Lan Kong3, Xuguo Zhou2, Wen Xie1* and Youjun Zhang1*

1 Department of Plant Protection, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China, 2 Department of Entomology, University of Kentucky, Lexington, KY, United States, 3 Department of Computer Science, Eastern Kentucky University, Richmond, KY, United States

The olfactory system serves a vital role in the evolution and survival of insects, being involved in behaviors such as host seeking, foraging, mating, and oviposition. Odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) are involved in the olfactory recognition process. In this study, BtabCSP11, a CSP11 gene from the whitefly Bemisia tabaci, was cloned and characterized. The open reading frame of BtabCSP11 encodes 136 amino acids, with four highly conserved cysteine residues. The temporal and spatial expression profiles showed that BtabCSP11 was highly expressed in the abdomens of B. tabaci females. Dietary RNA interference (RNAi)-based functional analysis showed substantially reduced fecundity in parthenogenetically reproduced females, suggesting a potential role of BtabCSP11 in B. tabaci reproduction. These combined results expand the function of CSPs beyond chemosensation.

Keywords: chemosensory proteins, Bemisia tabaci, RNA interference, expression profiles, reproduction

INTRODUCTION

The insect olfactory system is used extensively in a variety of contexts, such as during host seeking, foraging, mating, and oviposition behaviors. Odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) are involved in the olfactory recognition process (Pelosi et al., 2014, 2018). Both OBPs and CSPs are globular water-soluble acidic proteins with low isoelectric points found at high concentrations surrounding the chemosensory neurons (Pelosi et al., 2006). However, CSPs share minimal sequence similarity with OBPs. Generally, CSPs appear to be more conserved and are usually smaller (10–15 kDa) than OBPs (15–17 kDa). All CSPs possess four conserved cysteines (Pelosi et al., 2018). Previous studies have shown that CSPs are widely distributed in chemosensory organs, excluding the antennae (Maleszka and Stange, 1997; Nagnan-Le Meillour et al., 2000; Jin et al., 2005). In addition, CSPs are broadly expressed in non-chemosensory tissue, such as the subcuticular layer and wings (Marchese et al., 2000; Ban et al., 2003; Lu et al., 2007). The ubiquitous expression of CSPs suggests that, in addition to their known role in chemosensation, CSPs are involved in other physiological functions. In the honeybee, Apis mellifera, and the diamondback moth, Plutella xylostella, CSPs play a role in embryonic development (Maleszka et al., 2007; Gong et al., 2012). Pheromone production, sexual behavior, mating, ovulation and oviposition are the main
reproductive events. These events play roles in regulation of reproduction in insects (Raabe, 1987). Several studies have reported that insect CSPs can bind sex pheromone analogs and involved in reproduction (Dani et al., 2011; Iovinella et al., 2013).

The whitely species Bemisia tabaci, one of the world’s most invasive agricultural pests, causes substantial crop losses worldwide by feeding on phloem and transmitting plant viruses. Bemisia Middle East-Asia Minor1 (MEAM1 or “B”) and Mediterranean (MED or “Q”) are the two most invasive biotypes, and have invaded nearly 60 countries in the past two decades (De Barro et al., 2011; Gilbertson et al., 2015; Wan and Yang, 2016). Because of its short life cycle and high fecundity, whitefly management has been extremely challenging. Whitefly has developed resistance to most commercially available insecticides, particularly the neonicotinoids (Elbert and Nauen, 2000; Horowitz et al., 2004; Wang et al., 2010; Zheng et al., 2019). Functional analyses, however, are limited. Based on previous research, we hypothesized that BtabCSP11 is associated with reproductive behavior in B. tabaci. To test this hypothesis, we carried out the following experiments: (1) we cloned and analyzed the structure of BtabCSP11, and constructed a phylogenetic tree to analyze its evolutionary relationship with other insect CSPs; (2) we measured the temporospatial distribution of BtabCSP11 throughout different developmental stages and across different tissue types; and finally (3) we investigated the function of BtabCSP11 using dietary RNAi.

### MATERIALS AND METHODS

**Bemisia tabaci MED populations** were maintained on cotton plants at 27 ± 1°C under a L:D 16:8 photoperiod and 70 ± 10% relative humidity (RH). The identities of these strains were monitored every three generations using a mitochondrial marker, cytochrome oxidase I (mtCO I) (Chu et al., 2010). Different developmental stages, including eggs, the four nymphal stages, and adult females and males; and adult tissue types, including head, abdomen and a mixture of thorax, legs and wings, were collected separately from three B. tabaci MED populations. A total of 500 B. tabaci were collected per replicate for the egg and four nymphal stages, while 100 individuals were collected per replicate for adult females and males. Three independent biological replicates were used for each sample. Samples were rapidly frozen in liquid nitrogen and stored at -80°C for the subsequent RT-qPCR analyses.

**RNA Extraction, cDNA Synthesis, and Molecular Cloning of BtabCSP11**

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. RNA was quantified using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, United States), and integrity was checked with 1% Tris/borate/EDTA (TBE) agarose gel electrophoresis. For gene cloning and RT-qPCR analysis, first-strand cDNA was either used immediately or stored at −80°C for later use. We obtained the full-length sequence of BtabCSP11 from the B. tabaci MED genome and transcriptome (Xie et al., 2017).

**Sequence Analysis**

The program SignalPV5.0 was used to predict the putative N-terminal signal peptides and cleavage sites of BtabCSP11. The

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**TABLE 1** Primsers used for this study.

| Primer Name | Anneal Temp (°C) | Primer Sequence (5’−3’) |
|-------------|------------------|------------------------|
| RACE PCR    |                  |                        |
| BtabCSP11-F | 58               | CGTTTGCGCGTCTGGATG     |
| BtabCSP11-R | 58               | GCAACTCAAGACGGGGGAC    |
| RT-qPCR     |                  |                        |
| BtabCSP11-F-RT | 60          | GTCCTTGACAACTGAGGAGG   |
| BtabCSP11-R-RT | 60          | AACTGTGCGACATCTCCTCC   |
| EF-1a-F     | 60               | TGGTGCCAACAGATTAGGTGC   |
| EF-1a-R     | 60               | GCGACTGATTTCTTCTGTCC   |
| Actn-F      | 60               | TGCTTGGAAGACAGGATGGC   |
| Actn-R      | 60               | CCGTGATTTCTTCTGTCCAT   |
| SDHA-F      | 60               | GGGCTGACGAGGCTCTGCG    |
| SDHA-R      | 60               | TGTTGCGCAACAGATTAGGTGC |

**Dietaary RNAi**

| Primer Name | Anneal Temp (°C) | Primer Sequence (5’−3’) |
|-------------|------------------|------------------------|
| dsBtabCSP11-F | 58             | TAATACGACTCTATAGAGGC   |
| dsBtabCSP11-R | 58             | GATCCGCCATTAGTGGATGATGA |
| dsEGFP-F    | 58               | GATCCGCCATTAGTGGATGATGA |
| dsEGFP-R    | 58               | GATCCGCCATTAGTGGATGATGA |

**Collection**

**B. tabaci** MED populations were maintained on cotton plants at 27 ± 1°C under a L:D 16:8 photoperiod and 70 ± 10% relative humidity (RH). The identities of these strains were monitored every three generations using a mitochondrial marker, cytochrome oxidase I (mtCO I) (Chu et al., 2010). Different developmental stages, including eggs, the four nymphal stages, and adult females and males; and adult tissue types, including head, abdomen and a mixture of thorax, legs and wings, were collected separately from three B. tabaci MED populations. A total of 500 B. tabaci were collected per replicate for the egg and four nymphal stages, while 100 individuals were collected per replicate for adult females and males. Three independent biological replicates were used for each sample. Samples were rapidly frozen in liquid nitrogen and stored at −80°C for the subsequent RT-qPCR analyses.

**RT-qPCR analysis**

The program SignalPV5.0 was used to predict the putative N-terminal signal peptides and cleavage sites of BtabCSP11. The

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1. http://www.cbs.dtu.dk/services/SignalP/
occurrence of α-helices and molecular weight were predicted using ExPaSy. The CSPs of other insect species discussed in this study were retrieved from the NCBI database. ClustalW was used to align the sequences with default gap penalty parameters of gap opening = 10 and extension = 0.2. An alignment graph was generated using WebLogo.

Phylogenetic Analysis
A neighbor-joining tree was constructed using MEGA 6.0 with a p-distance model and pairwise deletion of gaps (Tamura et al., 2013). The bootstrap support of tree branches was assessed by resampling amino acid positions 1,000 times. Sequences used in the phylogenetic analysis were open reading frames (Supplementary Table S1). Phylogenetic trees were then presented in circular shape and colored taxonomically using online tools provided by Evolview (He et al., 2016).

Expression Profiles of BtabCSP11
Expression profiles of BtabCSP11 throughout different developmental stages of B. tabaci MED were obtained using transcriptome data (SRP064690). In addition, we validated transcriptomic profiles using RT-qPCR analysis (Zeng et al., 2019). Five two-fold serial dilutions of whitefly cDNA template were used to determine RT-qPCR primer amplification efficiencies through dissociation curve analysis. Only primers with 90–110% amplification efficiencies were used for subsequent analysis. Relative quantification was calculated using the $2^{-\Delta \Delta Ct}$ method, and mRNA expression values were normalized to the recommended reference genes EF1-α, SDHA and Actin (Li et al., 2013). Three biological and four technical replicates were used for each sample. Significant differences among samples were determined using one-way ANOVA with Tukey's HSD test. SPSS20.0 was used to analyze correlations between RT-qPCR and RNA-seq data.

Dietary RNAi
dsRNA primers of BtabCSP11 and EGFP (GenBank: KC896843) with a T7 promoter sequence were designed using Primer Premier 5.0 (Table 1). dsRNAs of BtabCSP11 and EGFP were synthesized using the T7 Ribomax™ Express RNAi System (Promega, Madison, WI, United States). The quality of dsRNA was evaluated by gel electrophoresis, and dsRNA concentration was quantified using a NanoDrop spectrophotometer.

Knockdown of BtabCSP11 was performed by orally feeding dsRNAs to B. tabaci MED adult females in a feeding chamber. The feeding chambers contained 200 µL of diet solution, which consisted of 30% sucrose, 5% yeast extract (weight/volume), ddH2O and 0.5 µg/µL dsBtabCSP11. Approximately 45 newly emerged (<2 days old) B. tabaci MED adult females were released into the feeding chambers, and were kept at 25°C, 80% RH, and a L:D 16:8 photoperiod. The effectiveness of RNAi was evaluated by RT-qPCR 2 days post-feeding. Each RNAi treatment was repeated six times.

Oviposition Bioassay
After feeding, fecundity (Berger et al., 2008), i.e., the number of eggs laid per adult female, was counted at day 1, 3, 7, and 10. Fresh cotton leaves were provided every day. Bemisia tabaci reproduction was recorded as the mean number of eggs per surviving whitely laid. A total of six independent biological replicates were used for each sample. Data were analyzed with SPSS20.0. Differences among treatments were evaluated using one-way ANOVA with Tukey's HSD test. Figures were generated using SigmaPlot 12.5.

RESULTS
Identification and Sequence Analysis of BtabCSP11
The full-length cDNA of BtabCSP11 contains a 408 bp open reading frame (ORF) encoding 136 amino acids (GenBank number: XP_018916537.1) with a 16.2 kDa molecular weight. There is a signal peptide with 18 residues at the N-terminus of BtabCSP11. Consistent with a classical model Cys-X6-8-Cys-X16-21-Cys-X2-4-Cys, BtabCSP11 has four conserved cysteine residues and six α-helices (Figure 1 and Supplementary Figure S1).

The CSP11 genes of other insect species were chosen for multiple sequence alignment with BtabCSP11 (Supplementary Table S1). A total of seven CSPs with >40% sequence similarity with BtabCSP11 (Pelosi et al., 2018), including DhouCSP, DvitCSP8, DkikCSP, CmedCSP3, PrapCSP10, and CbowCSP7, were included in the WebLogo alignment (Figure 1). The
results of the alignment showed four highly conserved cysteine residues at positions 67, 74, 94, and 97; other positions such as 35 (Y), 50 (D), 57 (N), 64 (Y), 66 (K), 72 (G), 73 (P), 75 (T), 77 (E), 82 (K), 87 (P), 108 (V), 126 (K), 128 (D), and 133 (Y) were also highly conserved. The complete sequence alignment including all insect CSP11s is displayed in Supplementary Figure S1. Phylogenetic analysis indicated that the hemipteran CSPs formed four large branches, of which \textit{BtbCSP11}, \textit{MperCSP4}, \textit{DvitCSP8}, and \textit{AgosCSP8} were clustered into one group (Figure 2).
Temporospatial Expression Profiles of BtabCSP11

The transcriptomic profiles of BtabCSP11 were confirmed by RT-qPCR analysis ($P < 0.05$, $r = 0.908$, Figure 3A). Among different developmental stages, BtabCSP11 expression was significantly higher in adult females ($F = 76.988$, $P < 0.0001$), and there were no significant differences among the remaining developmental stages (Figure 3A). Among different tissue types, BtabCSP11 expression was significantly higher in abdomen tissue than in head and thorax tissue (Figure 3B).

Functional Analysis of BtabCSP11

To explore the function of BtabCSP11 in B. tabaci, we silenced this gene using dietary RNAi. In comparison to control groups, BtabCSP11 expression in dsBtabCSP11-treated groups was suppressed significantly at 48 h post-feeding (Figure 4A). The total number of eggs laid by dsBtabCSP11 females at days 1, 3, 7, and 10 post-feeding was significantly lower than that in controls ($P < 0.05$; Figure 4B). There was no significant difference in hatching rate between the treatment (dsBtabCSP11) and control (dsEGFP) groups (Figure 4C).

DISCUSSION

In this study, we analyzed the genetic sequence of BtabCSP11 in B. tabaci and found that BtabCSP11 has four conserved cysteines and six helices connected by $\alpha$-$\alpha$ loops (Wanner et al., 2004). BtabCSP11 displayed about 40% sequence similarity to the CSPs of phylogenetically distant species such as the cabbage beetle Colaphellus bowringi (Pelosi et al., 2005, 2018).

Kulmuni and Havukainen (2013) showed that 5-helical CSPs are the only highly conserved CSPs in Arthropoda and are likely involved in functions other than chemosensation, whereas the vastly divergent 6-helical CSPs carry out solely chemosensory functions. Evidence strongly suggests that CSPs might be involved in chemodetection, as are OBPs (Waris et al., 2018, 2020b). In the honeybee, CSP3 specifically binds some components of brood pheromone (Briand et al., 2002). In the paper wasp Polistes dominulus (Calvello et al., 2003), the tsetse fly Glossina morsitans morsitans (Liu et al., 2012), and several species of ants, some CSPs are specifically expressed in antennae (McKenzie et al., 2014) and have been proposed to be associated with host-seeking behavior. In the plant bug Adelphocoris lineolatus, three CSPs have high binding affinity with host-related chemicals (Gu et al., 2012). Similarly, SinfCSP19 plays a role in the reception of host plant volatiles by the stem borer Sesamia inferens (Zhang et al., 2014). In the planthopper Nilaparvata lugens, NlugCSP10 may detect volatiles emitted from host plants (Waris et al., 2020a). In addition to host plant volatiles, study has reported that insect CSPs can bind $\beta$-carotene (Zhu et al., 2016).

CSPs are widely expressed in the head, thorax, abdomen, legs, wings, testes and ovaries (Gong et al., 2007; Lu et al., 2007). This expression pattern indicates that CSPs may be involved in a variety of physiological processes. Previous reports show that CSPs have many functions outside of simply transporting and binding odor molecules. For example, studies have shown that CSPs are involved in the growth and development of honeybees, the molting process of ant larvae, the reproduction of the beet armyworm S. exigua, and insecticide resistance in the mosquito A. gambiae (Malessza et al., 2007; Gong et al., 2012; Pelosi et al., 2018; Ingham et al., 2019). Inhibition of CSP9 expression by RNAi affects fatty acid biosynthesis and prevents cuticle development in the fire ant Solenopsis invicta (Cheng et al., 2015). In addition, some CSPs act as carriers of visual pigments, as in the cotton bollworm, Helicoverpa armigera (Zhu et al., 2016). CSPs

**FIGURE 3** Temporospatial expression profiles of BtabCSP11. (A) Expression analysis of the BtabCSP11 gene by RT-qPCR (black bars) and RNA-seq (black lines). E = egg, N = nymph stages 1–4 as indicated, F = adult female, and M = adult male. (B) B. tabaci BtabCSP11 gene transcript levels in different tissue types. Standard error for each sample is represented by error bar and different letters (a, b, c) above each bar denote significant differences ($P < 0.05$).
have also been found to play roles in the reproductive processes of insects, such that a decrease in CSP gene expression negatively affects reproduction (Gong et al., 2012; Ma et al., 2019).

Given that phylogenetic analysis clustered BtabCSP11 with MperCSP4, DvitCSP8, and AgosCSP8, additional functional studies are warranted to resolve the evolutionary relationship within this group. This kind of evolutionary relationship also occurs among other insects (Vieira and Rozas, 2011; Eyun et al., 2017). Additional functional studies should be carried out to understand the evolutionary history of CSPs in Hemiptera.

Our temporospatial distribution study demonstrated that BtabCSP11 was highly expressed in the abdomens of adult females, suggesting a potential role in reproduction. Our dietary RNAi-based functional study confirmed that silencing of BtabCSP11 significantly reduced oviposition in B. tabaci (Figure 4). Similarly, Gong et al. (2012) reported that silencing of CSP3 in S. exigua led to a 71.4% reduction in oviposition in comparison to uninjected controls. In addition, silencing of CSP12 in the leaf beetle Ophraella communa resulted in a 28% reduction in the number of eggs laid (Ma et al., 2019). Potential causes for this change include altered reproductive behavior (e.g., mating and oviposition). In this study, however, males were not included and females were not provided a choice of oviposition site. Therefore, we hypothesize that BtabCSP11 may affect the oviposition behavior of B. tabaci.

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article/Supplementary Material.
AUTHOR CONTRIBUTIONS

The research was designed by WX and YJZ. The experiments were conceived by YZ. Data was analyzed by YZ, LK, and XZ. Manuscript was drafted by YZ. Manuscript was revised and finalized by WX, XZ, AM, SW, QW, LK, and YJZ. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00709/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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