Identification and functional characterization of odorant-binding proteins 69a and 76a of Drosophila suzukii

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ARTICLE INFO
Keywords:
Drosophila suzukii
Odorant-binding proteins
Competitive binding
cis-vaccenyl acetate (cVA)
(2)-7-tricosene
β-ionone
Berberine

ABSTRACT
The fruit fly Drosophila suzukii is a fruit crop pest that causes a severe economic threat to soft summer fruit worldwide. The male sex pheromone, cis-vaccenyl acetate (cVA) has multiple functions in intra-species communication in Drosophila melanogaster, which is required in male to suppress male-male courtship. D. suzukii males do not produce cVA; however, the odorant receptor for cVA (Or67d) is still functional. The lack of cVA in D. suzukii casts the question of whether this pheromone might have been replaced by another compound similar to cVA that disrupts mating in D. suzukii. In order to address this question, we cloned two D. suzukii adult antenna-specific odorant-binding proteins (OBPs) DsOBP69a and DsOBP76a and aligned with their D. melanogaster orthologues. Moreover, we examined the binding properties of the newly identified recombinant proteins against 26 potential ligands including cVA, using the fluorescence-based ligand binding assay. The alignment showed that DsOBP69a and DsOBP76a, have six conserved cysteines and belong to the classic OBP family. Furthermore, our results revealed that cVA did not bind to DsOBP69a or DsOBP76a proteins. Interestingly, the floral odorant β-ionone and the bitter substance berberine chloride and coumarin displayed high binding ability. It is also worth noting that DsOBP69a and DsOBP76a have different affinities to (2)-7-Tricosene that may reflect different functional roles. These findings suggest that DsOBP69a and DsOBP76a are potentially involved in olfaction and gustation of D. suzukii.

1. Introduction

The fruit fly Drosophila suzukii (also known as Spotted Wing Drosophila) is an important polyphagous insect pest infesting many fruit crops, causing damage to soft-skinned fruits such as strawberries, cherries, grapes and others. D. suzukii prefers to lay eggs in fresh and ripening fruits, while most other Drosophila species, such as Drosophila melanogaster, only deposit eggs on decaying, fermented fruits (Mitsui et al., 2006). The fly was first observed in Mainland (Honshu) Japan in 1916, and then it broke out in Europe and the United States in 2008 (Rota-Stabelli et al., 2013). Currently, it is widely distributed in Asia, Europe, and America, and may spread to Africa and Australia (dos Santos et al., 2017; Asplen et al., 2015). If proper control measures are not taken, the annual loss to the American fruit industry from D. suzukii could reach $500 million (Goodhue et al., 2011; Walsh et al., 2011). At present, the main method for reducing the economic losses caused by D. suzukii is the frequent use of chemical pesticides (Farnsworth et al., 2017). However, because the eggs and larvae are inside the fruit, the effectiveness of chemical control is very limited, and it is not conducive to environmental protection and food safety. Therefore, developing of species-specific baits that may provide a successful management method for the control of D. suzukii, is still required. According to the scent of fresh and fermented apple juice, a mixture of five compounds is more effective and more specifically targeted for attracting D. suzukii (Feng et al., 2018).

Pheromones are important targets for pest monitoring. There are two pheromonal systems known in D. melanogaster: cuticular hydrocarbons (CHCs) produced by oenocytes, which are located directly under the cuticle of the abdomen, and cis-vaccenyl acetate (cVA) produced by the...
male ejaculatory bulb (Brieger and Butterworth, 1970). cVA is the most thoroughly studied pheromone in fruit flies. The cVA promotes mating of the female and it is transferred to the female during the mating process. After mating, the cVA in the female will inhibit further male mating (Kurtovic et al., 2007; Jallon, 1984; Biller et al., 2009; Ruta et al., 2010). cVA also promotes aggression between the males of D. melanogaster (Wang and Anderson, 2010). Adult males of D. Suzuki do not produce cVA due to atrophy of the ejaculation tube, and artificial application of cVA to males will inhibit their mating (Dekker et al., 2015; Snellings et al., 2018).

Drosophila senses pheromones signals through the olfactory and taste sensory neurons in the sensilla. And the neurons are bathed in an aqueous phase called sensory lymph. The odorant molecules bind to the odorant binding proteins (OBPs) in the sensory lymph, and they are transported to the sensory neuron dendrites where they bind to the receptors, stimulating the neuron to transmit sensory signals to the central nervous system (Leal, 2013; Kohl et al., 2015; Joseph and Carlson, 2015). Some D. suzukii OBPs, like D. melanogaster OBPs (Hekmat-Scafe et al., 2002; Larter et al. 2016; Rihani et al., 2019), are expressed in the olfactory sensilla on the antenna and taste sensilla of the labellum and leg (Ahn et al., 2020; Crava et al., 2019; Ramsammy, 2016). D. melanogaster perception of cVA requires OBP76a (also known as Lush) to promote cVA transport for binding through OR67d and SNMP proteins (Laughlin et al., 2008; Gomez-Diaz et al., 2013). Recently, OBPs has been also be reported to be involved in the response of D. melanogaster to cVA (Bentzur et al., 2018). However, whether cVA or some other substances bind directly to OBPs69a remains unclear.

The olfactory system of D. suzukii has changed to adapt to its unique ecological niche. There are 53 OBPs in D. suzukii (Chiu et al., 2013; Ramsammy et al., 2016) while there are 52 OBPs in D. melanogaster (Hekmat-Scafe et al., 2002; Larter et al. 2016), and four OBPs show signs of different selective properties and positive selection, including DsOBP69a in D. suzukii (Ramasamy et al., 2016). There is no functional research on OBPs reported for D. suzukii, except for our earlier report on DsOBP56h (Li et al., 2019). In this study, we identified DsOBP69a and DsOBP76a as orthologs to D. melanogaster OBP69a and OBP76a. We also evaluated the binding properties of DsOBP69a and DsOBP76a proteins to a panel of potential ligands using the fluorescence competition assay. cVA did not bind directly with them and the floral odor compound β-ionone and the bitter tasting berberine chloride and coumarin showed good affinity.

2. Materials and methods

2.1. Insect rearing

The D. suzukii colonies were initially collected from a cherry orchard in Shiyuan, Hubei, China and reared on artificial diets based on banana under laboratory conditions of the constant temperature of 24 ± 1 °C, the relative humidity (HR) of 65 ± 5% and the photoperiod of 16 h light: 8 h dark (Dalton et al., 2011).

2.2. Cloning and sequence analysis of DsOBP69a and DsOBP76a

Ten male and female adults of D. suzukii were frozen in liquid nitrogen followed by total RNA extraction using the RNeasy Plus reagent (TaKaRa, Beijing, China). PrimerScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Beijing, China) was employed to synthesize the first-strand cDNA, following the manufacturer’s protocol. Since the D. suzukii genome has been published (Chiu et al., 2013; Ometto et al., 2013), the coding sequences (CDS) of DsOBP69a and DsOBP76a (accession number: XM_017093981.1 and XM_017075568.1) were amplified. Signal peptides were predicted using SignalP-5.0 server (Armenteros et al., 2019). The sequences encoding mature DsOBP69a and DsOBP76a were amplified using the following primers: GGAATTCATATG CAGGACATTAACCCCATGATT (DsOBP69a forward, Ndel underlined); CCGCTAGCTCGAGGATCCATCATGTT (DsOBP76a reverse, Xhol underlined); GGAATTCATATGACGTACGAGCTGACGACGCA (DsOBP76a forward, Ndel underlined) and CCGCTAGCTCGAGGATCCATCATGTT (DsOBP76a reverse, Xhol underlined).

The PCR amplifications were performed using PrimeSTAR HS DNA polymerase (TaKaRa, Beijing, China). The PCR program included an initial denaturation at 94 °C for 3 min; followed by 30 cycles of 98 °C for 10 s, 60 °C for 5 s, and 72 °C for 1 min; then 72 °C for 10 min. PCR products were detected on a 1% agarose gel and sequenced by Tsingke Biological Technology (Beijing, China).

The OBP putative orthologs from other insects were obtained from the National Center for Biotechnology Information (NCBI) (Table s1). Sequence alignment and phylogenetic tree generation were performed using the ClustalW and maximum likelihood methods in the software MEGA 5 (Tamura et al., 2011).

2.3. Bacterial expression and protein purification of DsOBP69a and DsOBP76a

Using Ndel and Xhol as restriction sites, DsOBP69a and DsOBP76a were ligated into vector pET-30a (+) and transferred to Escherichia coli BL21 (DE3) cells. The cells were cultured in LB/kanamycin (50 μg/mL) for 4–6 h (OD600 = 0.4–0.6) at 37 °C and 180 rpm. Recombinant protein expression was induced with adding IPTG (1 mmol L⁻¹) at 28 °C and 180 rpm for 8 h. All bacterial cultures were harvested by centrifugation at 12000 x g for 1 min. Each cell pellet was resuspended using bacterial lysate solution (5000 mL/L Tris-HCl, 2 mmol/L ethylenediaminetetraacetic acid, 100 mol/L NaCl, 0.5% Triton X-100 and 1 mg/mL lysozyme). Then cells were disrupted by ultrasonication (active for 3 s, stopped for 5 s, 30 min in total). DsOBP69a and DsOBP76a were mainly detected in the inclusion bodies, then dissolved with 8 M urea, and added into high affinity Ni-NTA resin (Genscript, China). Bound proteins were eluted with 50 mM Tris containing 20 mM, 100 mM, 250 mM and 500 mM imidazole, and detected by SDS-PAGE. Eluted proteins were refolded by the method of cystine/cysteine (Prestwich, 1993). Dialysis was carried out for 24 h with 50 mM Tris-HCl (pH 7.4) at 4 °C, during which time the dialysate was changed 3 times. Protein concentrations were measured using the bicinchoninic acid (BCA) Protein Assay Kit (ThermoFisher Scientific-Life Technologies, Carlsbad, CA, USA), following the manufacturer’s protocol.

2.4. Ligand binding assays of DsOBP69a and DsOBP76a

All ligands were diluted to 1 mmol L⁻¹ with chromatographic grade methanol. The protein was diluted with 50 mM Tris-HCl (pH 7.4) to a final concentration of 2 μmol L⁻¹. An aliquot of 2 mL of the diluted protein was placed into the cuvette and titrated with bis-ANS. Protein was detected and affinity was probed by a fluorescence spectrophotometer. The excitation wavelength was set to 295 nm and the scanning range was 300–550 nm. The binding curve was linearized according to the Scatchard plot, assuming that the protein was 100% active, and its stoichiometric ratio with the ligand was 1:1; and the binding dissociation constant Ki was calculated as: Ki = [I]₀/[I₀]₁ × [I]₀, where [I]₀ is the concentration of the ligand when the fluorescence intensity is reduced by half and [I₀]₁ is the free bis-ANS concentration.

2.5. Homology modeling

The protein sequences of DsOBP69a and DsOBP76a, from which signal peptides were removed, were used for matching suitable templates and predicting the 3D structures of the proteins in SWISS-MODEL (Biasini;
et al., 2014). The rationality of protein structure was evaluated using a Ramachandran plot generated by ProCheck (Laskowski et al., 1993). The secondary structure of the protein was predicted using the ESPript 3.0 program (Robert and Gouet, 2014).

3. Results

3.1. Gene cloning and sequence analysis

The CDS of DsOBP69a and DsOBP76a were successfully cloned, consistent with the sequences in NCBI (XM_017079398.1 and XM_017075568.1). According to SignalP-5.0 server (Armenteros et al., 2019), the N-terminus of the DsOBP69a protein contains a signal peptide consisting of 24 residues. The N-terminus of the DsOBP76a protein contains a signal peptide consisting of 29 residues. Both DsOBP69a and DsOBP76a, having six conserved cysteines, belong to the classic OBP family (Figure 1). The phylogenetic tree analysis indicated that Dipteran OBP69a and OBP76a were each clustered into one branch, with branch support value of 0.9. DsOBP69a and DmOBP69a have the closest relationship, with branch support value of 0.76. Similarly, DsOBP76a and DbLush have the closest relationship, with branch support value of 0.97 (Figure 2).

3.2. Binding characteristics of DsOBP69a and DsOBP76a

The DsOBP69a and DsOBP76a sequences lacking the signal peptides were successfully ligated into pET30a and transferred to E. coli BL21 (DE3) cells. Both DsOBP69a and DsOBP76a proteins were expressed in inclusion bodies, and after renaturation, the pure proteins of interest were obtained (Figure 3 and s1).

1-NPN is a commonly used fluorescent probe, but it can react with cVA to produce fluorescence in the absence of protein addition (Figure s2), thus affecting subsequent experiments. Therefore, we chose bis-ANS as the fluorescent probe for this experiment (Figure 4). The dissociation constant of DsOBP69a and bis-ANS is $0.8292 \mu M$, and the linear equation of the Scatchard plot is $y = -4.357x + 8.183$ ($R^2 = 0.9867$). The dissociation constant of DsOBP76a and bis-ANS is $0.8116 \mu M$, and the linear equation of the Scatchard plot is $y = -4.509x + 8.497$.

Figure 1. Sequence alignment of the OBPs. Cysteines are highlighted in black.

Figure 2. Phylogenetic tree of DsOBP69a, DsOBP76a and their putative orthologues from other insects. DsOBP69 and DsOBP76a is marked with an asterisk. Ds, Drosophila suzukii; Ot, Oranthophagus taurus; Rf, Rhynchophorus ferrugineus; Gd, Galeruca daurica; Nv, Nesa r viridula; Pp, Papilio polytes; Cs, Carposina sasakii; Px, Papilio xuthus; Db, Drosophila biarmipes; Dm, Drosophila melanogaster; Md, Musca domestica; Bd, Bactrocera dorsalis; Aa, Aedes aegypti; Tn, Trichoplusia ni; Eo, Ectropis obliqua; At, Amyelois transstella; Hc, Hyyleus cichori. The number on the branch indicates branch support value.
Bis-ANS binds well to both DsOBP69a and DsOBP76a, so we chose bis-ANS for subsequent fluorescence competition binding experiments. The ligand was gradually added to the 2 μM protein and bis-ANS mixture, and the change in fluorescence intensity was recorded to calculate the IC50 and the dissociation constant Kd (Table s3). Of the 26 potential ligands selected, only five were able to bind to DsOBP69a (Figure 5A). β-Ionone has the strongest binding ability with DsOBP69a, and the Kd is 4.03 μM. Two kinds of bitter substances, berberine chloride and coumarin, can also bind to DsOBP69a with Kd values of 7.64 and 7.76 μM, respectively. The Kd of naringenin and DsOBP69a is 24.10 μM. The cVA and other selected ligand compounds associated with OBP69a and OBP76a, which are more thoroughly studied in Drosophila, do not bind to either DsOBP69a or DsOBP76a.

3.3. Homology modeling

According to SWISS-MODEL (Biasini et al., 2014), DsOBP69a has the highest degree of matching with Ceratitis capitata OBP22 (ID: 6hhe.1.A), with a Global Model Quality Estimation (GMQE) value of 0.71, and a sequence similarity of 36.44%. The similarity between CcapObp22 and D. melanogaster OBP69a was 37% [40]. DsOBP76a has the highest degree of matching with D. melanogaster OBP76a (ID: 3b86.1.A), with a GMQE value of 0.96 and sequence similarity of 83.87%. The evaluation of the DsOBP69a model showed that 92.9% of the amino acid residues fell in the optimal region and 7.1% of them fell in the allowable region (Figure s3 A). The evaluation of the DsOBP76a model showed that 92.8% of the amino acid residues fell in the optimal region and 7.2% of them fell in the allowable region (Figure s3 B). The 3D structural model predicted for DsOBP69a consists of seven alpha helices (Figure 6A). The 3D structural model predicted for DsOBP76a consists of six alpha helices: Met3-Ser15 (α1), Ile25-Arg32 (α2), Thr79-Cys92 (α5), Thr79-Cys92 (α5), and Ser102-Glu115 (α6) (Figure 6B).

4. Discussion

Insects need to find a suitable host to complete a normal life history and breeding population. Its host selection relies on smell and taste (Knolhoff and Heckel, 2014). OBPs play an important role in the host and coumarin, can also bind to DsOBP69a with Kd values of 7.21 and 20.37 μM, respectively. Drosophila pheromone (Z)-7-tricosene can also bind to DsOBP69a with a Kd of 19.34 μM. Naringenin can also be combined with DsOBP69a with a Kd of 23.76. The binding ability of DsOBP76a to the ligands is similar to that of DsOBP69a, with the exception of (Z)-7-tricosene (Figure 5B). β-Ionone has the strongest binding to DsOBP76a, and its Kd is 3.37 μM. Two kinds of bitter substances, berberine chloride and coumarin, can also bind to DsOBP76a with Kd values of 7.64 and 7.76 μM, respectively. The Kd of naringenin and DsOBP76a is 24.10 μM. The cVA and other selected ligand compounds associated with OBP69a and OBP76a, which are more thoroughly studied in Drosophila, do not bind to either DsOBP69a or DsOBP76a.
selection of Drosophila. For example, OBP57d and OBP57e of D. sechellia affect the recognition of the plant-derived poisons caproic acid and caprylic acid produced by host Morinda citrifolia, which in turn affects host selection behavior (Matsuo et al., 2007). In this study, we identified OBP69a and OBP76a in D. suzukii, whose function may have changed compared to D. melanogaster.

RNA-seq analysis detected expression of Obp69a and Obp76a genes in D. suzukii third antennal segments (Crava et al., 2019). In D. melanogaster, the expression of OBP69a was significantly increased after females were exposed to males; whereas for isolated males, the expression of OBP69a were significantly higher than that in males when females were also in the population. In contrast, no significant changes were observed for OBP76a in the expression level. In addition, cVA treatment did not impact the expression levels of OBP69a in male and female (Bentzur et al., 2018). However, the atrophied ejaculation bulb of the D. suzukii male does not produce cVA (Dekker et al., 2015). Some Drosophila species do not produce but still detect cVA (Khallaf et al., 2020). Electrophysiological recording revealed that the D. suzukii females both can able to detect cVA by olfactory neurons in antennal trichoid sensilla (Khallaf et al., 2020). The amino acid sequence of OBP69a and OBP76a between D. melanogaster and D. suzukii are highly consistent. They are theoretically orthologous genes and have similar functions. However, in this study, fluorescence competition binding assays showed that cVA had not affinity with the DsOBP69a and DsOBP76a. Their difference may be related to the protein structure, which is worthy of further study. Moreover, we speculate that there are other OBPs to promote cVA transport in the D. suzukii, which will be our future work.

(Z)-7-Tricosene among eight tested Drosophila pheromones was the only one detected by the DsOBP69a, although electrophysiological recording revealed that the D. suzukii females do not detect (Z)-7-Tricosene via single sensillum recordings (Khallaf et al., 2020). On the contrary, all tested pheromones were not affinity with the DsOBP76a. This suggests that there may be other unknown sex pheromones or other substances that regulate the expression of the DsOBP76a. So far, many D. melanogaster pheromones are still unknown. (Z)-7-Tricosene, the male-specific sex pheromone, was detected by the D. melanogaster (Khallaf et al., 2020). The currently known Drosophila sex pheromones are cVA produced by males and CHCs produced by oenocytes, but males still show strong courtship for females without oenocytes (Billeter and Levine, 2015), indicating that females produce sex pheromones other than CHCs, and further research is clearly needed.
β-ionone is a floral odor substance that attracts D. melanogaster and has a strong affinity with OBP28a (Gonzalez et al., 2019). Ab4 and pb2 sensilla are the most sensitive to β-ionone, and OBP28a mutants cause the responses of ab4 and pb2 sensors to β-ionone to be reduced, but they did not completely disappear, indicating that ab4 and pb2 sensors can also detect β-ionone by means other than OBP28a (Gonzalez et al., 2019). Similarly, in our experiments, β-ionone bind to DsOBP69a and DsOBP76a and have the highest affinity compared to all the ligands tested. Strawberry leaves have an attractive effect on D. suzukii, and strawberry leaves also volatilize β-ionone, but the effect of β-ionone on D. suzukii is still unclear (Keese, et al., 2015). Therefore, we guessed that D. suzukii detect β-ionone emitted by strawberry leaves through DsOBP69a and DsOBP76a, and find their host strawberries. However, behavioral research of DsOBP69a and DsOBP76a is needed for further verification, which is very important.

The DsOBP69a and DsOBP76a proteins not only play a crucial role in olfactory, but also play an important role in taste. This is consistent with the researches that OBP have an important role in non-olfactory, such as taste (Jeong et al., 2013; Rihani et al., 2019). In this study, berberine chloride and coumarin have a good affinity with the DsOBP69a and DsOBP76a proteins. These two bitter substances also have a high affinity with the DsOBP59b proteins (Li et al., 2019). However, other bitter substances, including denatonium benzoate and N-phenylthiourea, were not affinity with the DsOBP69a, DsOBP76a and DsOBP76a proteins (Li et al., 2019). In insects, identifying bitter substances can help them avoid plant toxins and unfavorable spawning points. OBP49a can suppress the appetite for sweet-tasting compounds through the perception of bitter stimuli. The deletion of OBP49a reduced the inhibition of sucrose-induced action potential by bitter chemicals (Jeong et al., 2013). The reduction of expression of individual OBP genes induced either an increase or a decrease of sucrose intake in the presence of bitter compounds (Swarup et al., 2013). The roles of DsOBP69a and DsOBP76a in the taste of D. suzukii remain to be further studied.

The D. melanogaster OBP19b protein, a taste-expressed OBP, not only is detected some bitter compounds, such as berberine, but also plays a crucial role in the detection of indispensable nutrients, such as L-phenylalanine (Rihani et al., 2019).

DsOBP69a has the highest matching degree with C. capitata OBP22 (ID: 6hhe.1.a), while CcapOBp22 has a closer genetic relationship with D. melanogaster OBP69a, with a sequence similarity of 37%. CcapOBp22 can interact with male pheromone (E,E)-α-farnesene. Using CcapOBp22 (ID: 6hhe.1.a) as a template, we constructed a 3D structural model of the DsOBP69a protein, which is composed of seven alpha helices like CcapObp22. After cVA binds to OBP76a in D. melanogaster, the conformation of F121 changes. The change of the F121 residue will affect the binding activity of OBP76a to cVA. In addition, the binding of OBP76a to cVA will lead to the fracture of DsOBP76a and DsOBP76a and have the highest affinity compared to all the ligands tested, which is very important.

This work was supported by the National Key R & D Program of China (Grant no.: 2019YFD1002100) and the China Agriculture Research System (Grant no.: CARS-24-05B).
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