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Identification of odorant-binding protein genes in *Galeruca daurica* (Coleoptera: Chrysomelidae) and analysis of their expression profiles

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Abstract

Odorant-binding proteins (OBPs) play a fundamental role in insect olfaction. In recent years, *Galeruca daurica* (Joannis) (Coleoptera: Chrysomelidae) has become one of the most important insect pests in the Inner Mongolian grasslands of China. This pest only feeds on the species of *Allium* plants, implying the central role of olfaction in its search for specific host plants. However, the olfaction-related proteins have not been investigated in this beetle. In this study, we identified 29 putative OBP genes, namely GdauOBP1–29, from the transcriptome database of *G. daurica* assembled in our laboratory by using RNA-Seq. All 29 genes had the full-length open reading frames except GdauOBP29, encoding proteins in length from 119 to 202 amino acids with their predicted molecular weights from 12 to 22 kDa with isoelectric points from 3.88 to 8.84. Predicted signal peptides consisting of 15–22 amino acid residues were found in all except GdauOBP6, GdauOBP13 and GdauOBP29. The amino acid sequence identity between the 29 OBPs ranged 8.33–71.83%. GdauOBP1–12 belongs to the Classic OBPs, while the others belong with the Minus-C OBPs. Phylogenetic analysis indicated that GdauOBPs are the closest to CbowOBPs from *Colaphellus bowringi*. RT-PCR and qRT-PCR analyses showed that all GdauOBPs were expressed in adult antennae, 11 of which with significant differences in their expression levels between males and females. Most GdauOBPs were also expressed in adult heads (without antennae), thoraxes, abdomens, legs and wings. Moreover, the expression levels of the GdauOBPs varied during the different development stages of *G. daurica* with most GdauOBPs expressed highly in the adult antennae but scarcely in eggs and pupae. These results provide insights for further research on the molecular mechanisms of chemical communications in *G. daurica*.

Keywords: expression profile, *Galeruca daurica*, odorant binding protein, phylogenetic analysis

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Introduction

Galeruca daurica (Joannis) (Coleoptera: Chrysomelidae) has become one of the most important insect pests in the Inner Mongolian grasslands of China since its abrupt outbreak in 2009 (Yang *et al.*, 2010). It is mainly distributed in Mongolia, Russia (Siberia), Korea and China including Inner Mongolia, Xinjiang and Gansu province. This leaf beetle forages only on the species of *Allium* plants, including *Allium mongolium*, *Allium polyrhizum* and *Allium ramosum* (Hao *et al.*, 2014, 2015). Extensive outbreaks of this pest since 2009 have caused great losses to pasture in the Inner Mongolian grasslands and the damage continues to increase (Li *et al.*, 2014). Thus far, molecular studies on this pest is limited with much focus on

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the occurrence (Ma *et al.*, 2012), host plant selection (Hao *et al.*, 2014), life history (Hao *et al.*, 2015), cold hardiness (Li *et al.*, 2014, 2015*a*; Gao *et al.*, 2015), insecticide screening (Chang *et al.*, 2015), genetic diversity (Zhang *et al.*, 2015), diapause (Zhou *et al.*, 2016*a*), thermal requirement (Zhou *et al.*, 2016*b*) and mitochondrial genome (Zhou *et al.*, 2016*c*).

Odorant binding proteins (OBPs) are small amphipathic proteins involved in insect olfaction (Vogt, 2003). They have six cysteines in general with a conserved spacing pattern leading to the formation of three disulfide bridges (Vogt, 2005). In insects, OBPs may be the first specific biochemical step in odor reception (Vogt *et al.*, 1999). They are concentrated in the sensilla lymph of the antennae and are thought to play an important role in transporting odors to the odorant receptors, thus triggering a behavioral response (Zwiebel, 2003). Gene transcripts encoding OBPs are mainly found in chemosensory tissues, and can bind pheromones and other odorants (Vogt, 2005). Functionally, OBPs have roles in the behavioral responses of insects to pheromones (Laughlin *et al.*, 2008) and taste perception (Matsuo *et al.*, 2007). Therefore, studying insect OBPs is useful for developing novel pest management strategies to interfere with pest insect behaviors such as host location and mating. Moreover, the studies of OBPs reveal the molecular mechanisms of insect olfaction.

In the order Insecta, Coleoptera has the most number of species and diversity with many species classified as important pests of agricultural crops, forestry as well as humans. Current knowledge of coleopteran olfaction stems from studies of olfactory genes in coleopterans such as *Ips typographus* and *Dendroctonus ponderosae* (Andersson *et al.*, 2013), *Holotrichia parallela* (Ju *et al.*, 2014), *Monochamus alternatus and Dastarcus helophoroides* (Wang *et al.*, 2014), *Colaphellus bowringi* (Li *et al.*, 2015*b*), *Dendroctonus valens* (Gu *et al.*, 2015), *Rhynchophorus ferrugineus* (Antony *et al.*, 2016), and *Ambrostoma quadriimpressum* (Wang *et al.*, 2016). However, the olfactory genes of *G. daurica* are unknown.

In the present study, using the transcriptome data of *G. daurica* adults assembled in our laboratory (unpublished), 29 putative OBP genes were identified and analyzed by using bioinformatics. Moreover, the tissue-specific and developmental stage-specific expression profiles of the OBP genes were analyzed using semi-quantitative reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR). The findings of this study provide insights for future functional research on the olfactory reception in *G. daurica* and will help design pest management strategies to control this insect pest.

Materials and methods

Insects and sample collection

The larvae of *G. daurica* were collected from Xilinhot, Inner Mongolia, China (43°54′53″N, 115°39′13″E) in 2015, and reared with *A. mongolicum* in incubators at 26 ± 1°C, 16 h light: 8 h dark cycle and 60–80% relative humidity. Antennae, heads (without antennae), thoraxes, abdomen, legs and wings were obtained from both 3-days-old male and female adults, which fed on *A. mongolicum* without mating, transferred to Eppendorf tubes, frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Identification and analysis of OBP transcripts

We identified putative OBP genes by searching the transcriptome database of *G. daurica* adults assembled in our laboratory (unpublished). Putative OBP genes were searched using 'OBP' and 'odorant-binding protein' as the key words to screen the annotated sequences in the transcriptome database (Zhang *et al.*, 2015). Moreover, tBlastn was used to screen the transcriptome database and identify putative OBP genes using known OBP sequences of Chrysomelidae as 'query'. All putative OBP genes were manually confirmed using the Blastx program against the NR nucleotide database at NCBI with a cut-off E-value 10^{-5}. The open reading frames (ORFs) of the putative OBP genes were predicted using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The signal peptides of the amino acid sequences were predicted using Signal IP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The molecular weight and isoelectric point (pI) of the amino acid sequences were predicted using DNAMAN V6 and the amino acid identity between the putative OBP genes was calculated using Clustal Omega (www.ebi.ac.uk/tools/msa/clustalo/).

Phylogenetic analysis of OBPs from G. daurica *and other insects*

Phylogenetic analysis was performed based on the putative amino acid sequences of 29 OBPs from *G. daurica* and 181 OBPs from seven other insect species from six different orders obtained from the NR nucleotide database in GenBank. Amino acid sequences were downloaded in FASTA format. Putative N-terminal signal peptide sequences predicted using Signal IP (http://www.cbs.dtu.dk/services/SignalP/) were removed before alignment. The tree was constructed using the neighbor-joining method with Poisson correction for distances as implemented in MEGA6 (Tamura *et al.*, 2013). Branch support was assessed with 1000 bootstrap replicates.

Expression analysis of G. daurica *OBP genes by semi-quantitative RT-PCR*

Total RNA was extracted with TaKaRa Mini BEST Universal RNA Extraction Kit (Takara, Dalian, China) from the dissected antennae, heads (without antennae), thoraxes, abdomens, legs, and wings of male and female adults. The cDNA was synthesized using PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. The primers were designed using Primer Premier 5.0. The succinate dehydrogenase complex (*SDHA*) gene was used as a reference gene (Tan *et al.*, 2016). Reactions were conducted on a BIO-RAD T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) using the following conditions: denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, primer-specific temperature (50–55°C) for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min.

Expression analysis of G. daurica *OBP genes by qRT-PCR*

Total RNA was extracted from eggs, first- to third-instar larvae, pupae, and antennae of male and female adults. Gene-specific primers of 28 OBPs (one OBP did not have a complete ORF) were designed using Primer3 Input (http://primer3.ut.ee/). Experiments were performed using the FTC-3000P Real-Time Quantitative Thermal Cycler (Funglyn Biotech, Canada) with BRYT Green®dye as the fluorescence reporter for each elongation cycle (GoTaq®qPCR Master Mix, Promega, USA). The *SDHA* gene was used as a reference

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Table 1. List of OBP genes in *Galeruca daurica* transcriptome. '—'means not detected.

Gene name	Accession number	ORF length (aa)	MW (kDa)	PI	SP	BLAST annotation	Score	Query cover (%)	*E*-value	Identity (%)	BlastP accession
GdauOBP1	KX900453	131	15.1998	4.60	19	OBP 3 [*Batocera horsfieldi*]	59.3	87	8e-09	29	AHA33381.1
GdauOBP2	KX900454	147	16.4622	4.50	16	OBP 3 [*Phyllotreta striolata*]	169	99	2e-51	51	ANQ46502.1
GdauOBP3	KX900455	135	15.4943	4.85	15	OBP 17 [*Colaphellus bowringi*]	186	94	2e-58	61	ALR72505.1
GdauOBP4	KX900456	119	13.5361	3.88	22	OBP 14 [*Colaphellus bowringi*]	104	90	1e-26	45	ALR72502.1
GdauOBP5	KX900457	136	14.9087	4.23	21	OBP 2 [*Phyllotreta striolata*]	162	93	4e-49	56	ANQ46501.1
GdauOBP6	KX900458	119	12.9294	7.77	–	OBP 4 [*Monochamus alternatus*]	161	99	7e-49	59	AHA39269.1
GdauOBP7	KX900459	124	13.5234	4.90	19	OBP, partial [*Lissorhoptrus oryzophilus*]	57	75	6e-08	32	SHE13795.1
GdauOBP8	KX900460	126	14.1288	4.62	20	OBP 8 [*Colaphellus bowinqi]*	61.2	54	1e-09	35	ALR72496.1
GdauOBP9	KX900461	146	16.8615	4.43	20	OBP 11[*Colaphellus bowinqi*]	62.4	99	1e-09	27	ALR72499.1
GdauOBP10	KX900462	146	16.6495	7.72	22	OBP 1[*Phyllotreta striolata*]	199	97	2e-63	65	ANQ46500.1
GdauOBP11	KX900463	178	20.0584	4.89	18	OBP 18 [*Colaphellus bowinqi*]	245	86	2e-80	72	ALR72506.1
GdauOBP12	KX900464	202	22.6722	4.32	18	OBP 12 [*Colaphellus bowinqi*]	157	99	1e-44	39	ALR72500.1
GdauOBP13	KX900465	152	17.1199	7.87	–	OBP 21[*Dastarcas helophoroides*]	132	86	6e-37	47	AIX97067.1
GdauOBP14	KX900466	140	15.6669	4.13	19	OBP 15[*Colaphellus bowinqi*]	75.5	73	9e-15	43	ALR72503.1
GdauOBP15	KX900467	143	16.3362	5.03	17	OBP 11[*Colaphellus bowinqi*]	94.4	97	4e-22	38	ALR72499.1
GdauOBP16	KX900468	143	16.6513	6.91	19	OBP 16 [*Colaphellus bowinqi*]	73.2	96	7e-14	31	ALR72504.1
GdauOBP17	KX900469	129	14.4022	5.92	16	OBP 13[*Dendroctonus armandi*]	62.0	99	1e-09	34	ALM64971.1
GdauOBP18	KX900470	141	15.8448	6.52	18	OBP 16 [*Tenebrio molitor*]	86.7	83	3e-19	35	AJM71490.1
GdauOBP19	KX900471	135	15.0176	5.32	18	OBP 29 [*Dendroctonus ponderosae*]	97.4	86	2e-23	38	AGI05182.1
GdauOBP20	KX900472	139	15.4643	7.39	16	OBP [*Chilo suppressalis*]	77.8	95	9e-16	30	AGM38609.1
GdauOBP21	KX900473	137	15.345	8.38	16	OBP [*Colaphellus bowinqi*]	146	99	1e-42	50	ALR72494.1
GdauOBP22	KX900474	136	15.2027	8.34	18	OBP 15 [*Colaphellus bowinqi*]	84.3	75	3e-18	39	ALR72503.1
GdauOBP23	KX900475	144	16.388	4.85	17	OBP 11 [*Colaphellus bowinqi*]	106	97	9e-27	42	ALR72499.1
GdauOBP24	KX900476	140	16.1109	7.34	20	OBP [*Rhynchophorus ferruqineus*]	76.3	87	8e-15	32	AMK48596.1
GdauOBP25	KX900477	137	15.5374	8.84	16	OBP [*Colaphellus bowinqi*]	150	99	3e-44	51	ALR72494.1
GdauOBP26	KX900478	142	15.7641	7.01	17	OBP 15 [*Colaphellus bowinqi*]	88.6	72	7e-20	42	ALR72503.1
GdauOBP27	KX900479	145	17.0476	5.21	19	OBP 83b [*Drosophila ananassae*]	55.1	94	6e-07	27	XP 001955184.1
GdauOBP28	KX900480	130	14.8516	5.81	17	OBP 14 [*Tenebrio molitor*]	55.8	94	2e-07	27	AJM71488.1
GdauOBP29	KX900481	5′missing	12.9264	5.73	–	OBP 21[*Dastarcus helophoroides*]	137	97	8e-40	57	AIX97067.1

Table 2. The consensus (%) of 29 GdauOBP amino acid sequences alignment.

	OBP1	OBP2	OBP3	OBP4	OBP5	OBP6	OBP7	OBP8	OBP9	OBP10	OBP11	OBP12	OBP13	OBP14
OBP2	21.85													
OBP3	19.83	21.26												
OBP4	18.27	15.74	15.18											
OBP5	18.80	17.46	26.52	22.94										
OBP6	19.00	17.80	18.42	21.51	25.66									
OBP7	24.79	17.24	20.00	14.15	20.54	21.00								
OBP8	27.12	17.95	22.61	19.39	20.35	15.38	31.03							
OBP9	12.20	17.78	18.52	11.30	18.66	16.67	12.50	13.22						
OBP10	17.74	18.25	19.26	19.66	21.97	54.62	16.26	13.82	15.83					
OBP11	12.10	17.61	13.64	15.04	11.45	12.71	9.92	14.75	11.89	14.08				
OBP12	15.52	17.27	11.11	22.43	17.60	16.24	13.27	17.54	14.39	15.79	21.15			
OBP13	14.16	17.74	17.50	18.81	21.14	15.74	16.67	14.81	20.47	17.46	17.29	13.60		
OBP14	19.47	17.69	16.10	15.00	21.19	10.38	14.81	18.35	10.85	9.45	21.01	16.79	17.32	
OBP15	15.00	11.19	19.70	18.58	18.32	17.54	14.53	15.38	23.94	20.44	15.60	17.69	25.81	18.25
OBP16	12.40	15.27	20.30	14.91	20.00	15.18	24.37	15.00	20.57	18.12	9.35	12.50	19.84	13.39
OBP17	13.89	21.85	12.61	13.00	19.82	19.00	14.29	16.33	15.25	16.95	18.90	16.13	19.33	36.72
OBP18	14.02	17.56	13.33	16.00	22.03	14.81	15.09	16.82	19.05	16.67	17.16	13.95	28.79	20.31
OBP19	20.18	21.14	19.33	14.00	23.33	13.08	18.69	18.69	19.05	13.60	18.05	13.60	52.24	19.84
OBP20	17.09	12.21	19.38	19.09	24.81	17.70	17.54	15.65	15.33	22.39	17.39	16.80	22.31	18.33
OBP21	18.18	20.47	19.66	15.31	16.24	12.38	11.43	10.48	14.29	13.60	19.26	15.50	16.67	24.26
OBP22	13.89	12.90	16.67	9.90	18.33	12.04	14.29	15.24	17.60	12.80	15.67	15.50	23.66	30.00
OBP23	13.33	9.63	21.21	17.86	19.85	15.65	15.38	14.53	25.35	16.79	16.78	14.62	23.39	17.46
OBP24	11.61	14.29	11.11	12.00	14.53	14.00	15.60	16.36	12.50	11.90	18.25	12.70	21.26	24.24
OBP25	14.55	16.54	21.37	17.35	18.80	16.19	13.33	10.48	14.29	16.00	19.26	17.83	23.02	23.53
OBP26	14.68	17.83	19.83	12.75	21.49	12.04	16.04	14.95	15.75	14.29	16.18	16.42	22.73	27.48
OBP27	12.20	12.03	22.22	15.52	20.45	12.28	19.01	13.93	18.88	14.29	11.35	12.31	18.25	16.54
OBP28	18.52	18.64	15.79	12.12	14.78	8.74	19.05	21.15	10.74	11.67	17.19	12.82	17.60	22.13
OBP29	15.38	20.18	22.33	18.60	25.24	13.46	16.48	20.65	18.87	14.95	15.04	10.81	41.59	24.55

	OBP15	OBP16	OBP17	OBP18	OBP19	OBP20	OBP21	OBP22	OBP23	OBP24	OBP25	OBP26	OBP27	OBP28
OBP16	25.36													
OBP17	17.24	16.24												
OBP18	24.80	15.87	23.73											
OBP19	25.20	16.80	18.64	28.91										
OBP20	29.41	20.30	12.73	21.49	22.50									
OBP21	15.32	14.40	24.41	25.40	20.00	16.10								
OBP22	22.95	14.52	28.23	24.22	16.92	21.85	21.71							
OBP23	71.83	23.91	16.38	24.80	26.02	27.54	14.52	20.49						
OBP24	14.52	11.90	26.67	21.26	12.70	13.22	21.88	23.44	12.90					
OBP25	13.71	16.00	29.92	26.98	20.80	16.95	70.07	24.03	12.90	27.34				
OBP26	21.14	12.80	25.00	23.13	18.32	18.33	26.36	55.15	21.14	22.14	27.91			
OBP27	22.86	63.64	12.82	15.08	16.80	20.74	11.20	12.90	20.00	15.87	13.60	12.80		
OBP28	17.80	8.33	20.34	25.20	19.35	14.66	19.83	17.74	14.41	23.77	19.83	24.00	10.00	
OBP29	17.92	17.92	24.30	33.63	33.04	18.45	21.10	17.70	16.98	26.42	21.10	23.89	17.92	18.02

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gene (Tan *et al.*, 2016). qRT-PCR was performed in a 10 µl reaction mixture and repeated three times for each sample. All reactions used the following conditions: denaturation at 95°C for 10 min followed by 45 cycles at 95°C for 15 s, 60°C for 1 min and a dissociation at the end. Each reaction was performed with three biological replicates and three technical replicates. The relative expression levels of each OBP gene was estimated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Results

Identification of putative OBP genes in G. daurica

From the transcriptome database, a total of 29 putative OBP genes were identified, which were named as GdauOBP1–29 (GenBank ID: KX900453–KX900481), and all except GdauOBP29 had the full-length ORFs. Summary statistics was compiled for each GdauOBP gene discovered (table 1). The ORFs for these genes ranged in length from 119 to 202 amino acids and their calculated molecular weights ranged 12–22 kDa. Their isoelectric points ranged from 3.88 to 8.84. Signal peptides consisting of 15–22 amino acid residues were present in all except OBP6, OBP13, and OBP29, which had no predicted signal peptides. The amino acid identities between the 29 OBPs ranged 8.33–71.83%, which showed high divergence (table 2).

Alignment of the amino acid sequences of all 29 GdauOBPs is shown in fig. 1. Based on the number and location of the conserved cysteines, the 29 GdauOBPs could be divided into two subfamilies: GdauOBP1–12 had six conserved cysteines and belonged with the Classic OBPs while the others belonged with the Minus-C OBPs and had four conserved cysteines with C2 and C5 missing.

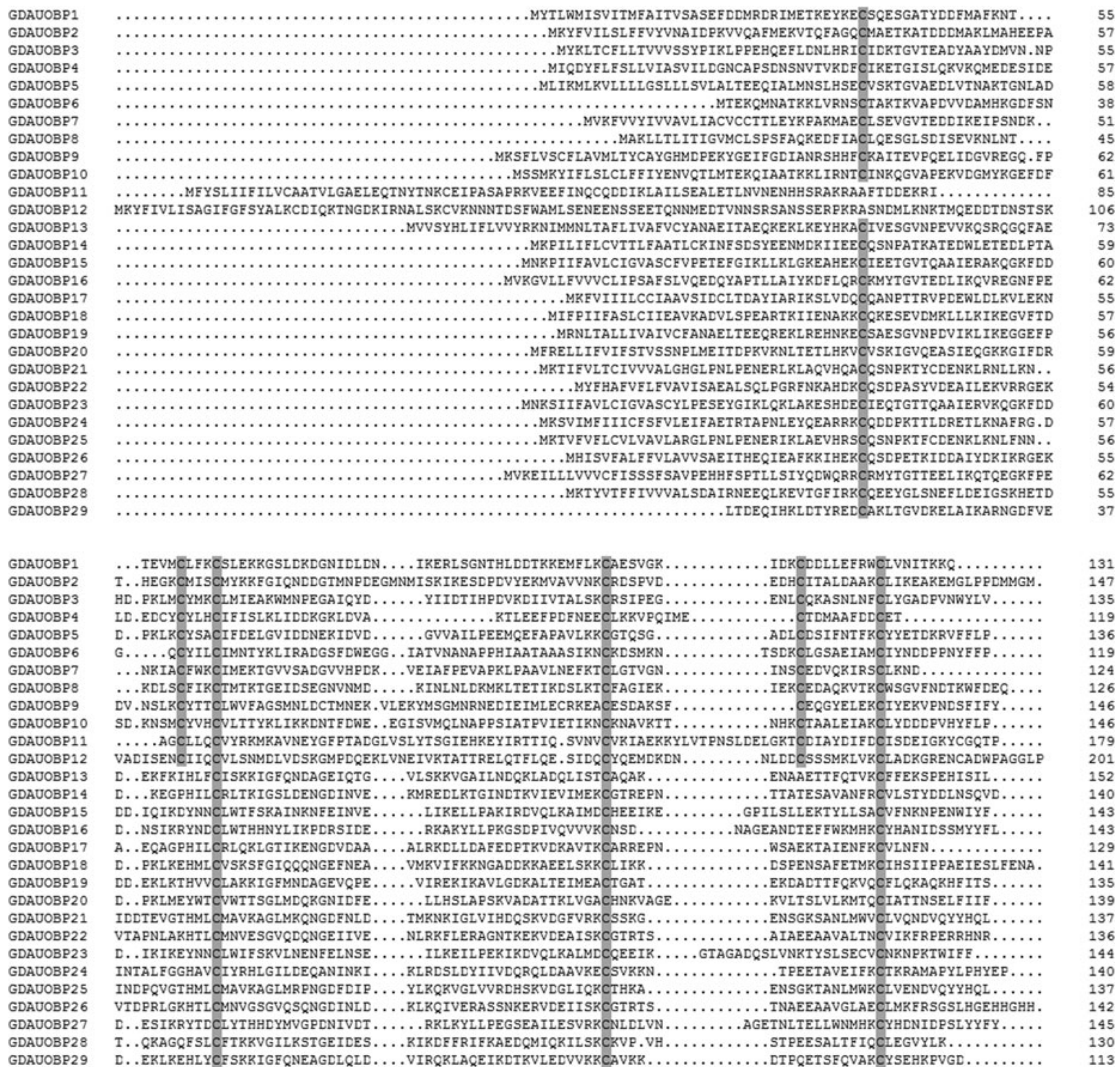

Fig. 1. Alignment of 29 putative OBPs in *G. daurica*. Red boxes show conserved cysteines.

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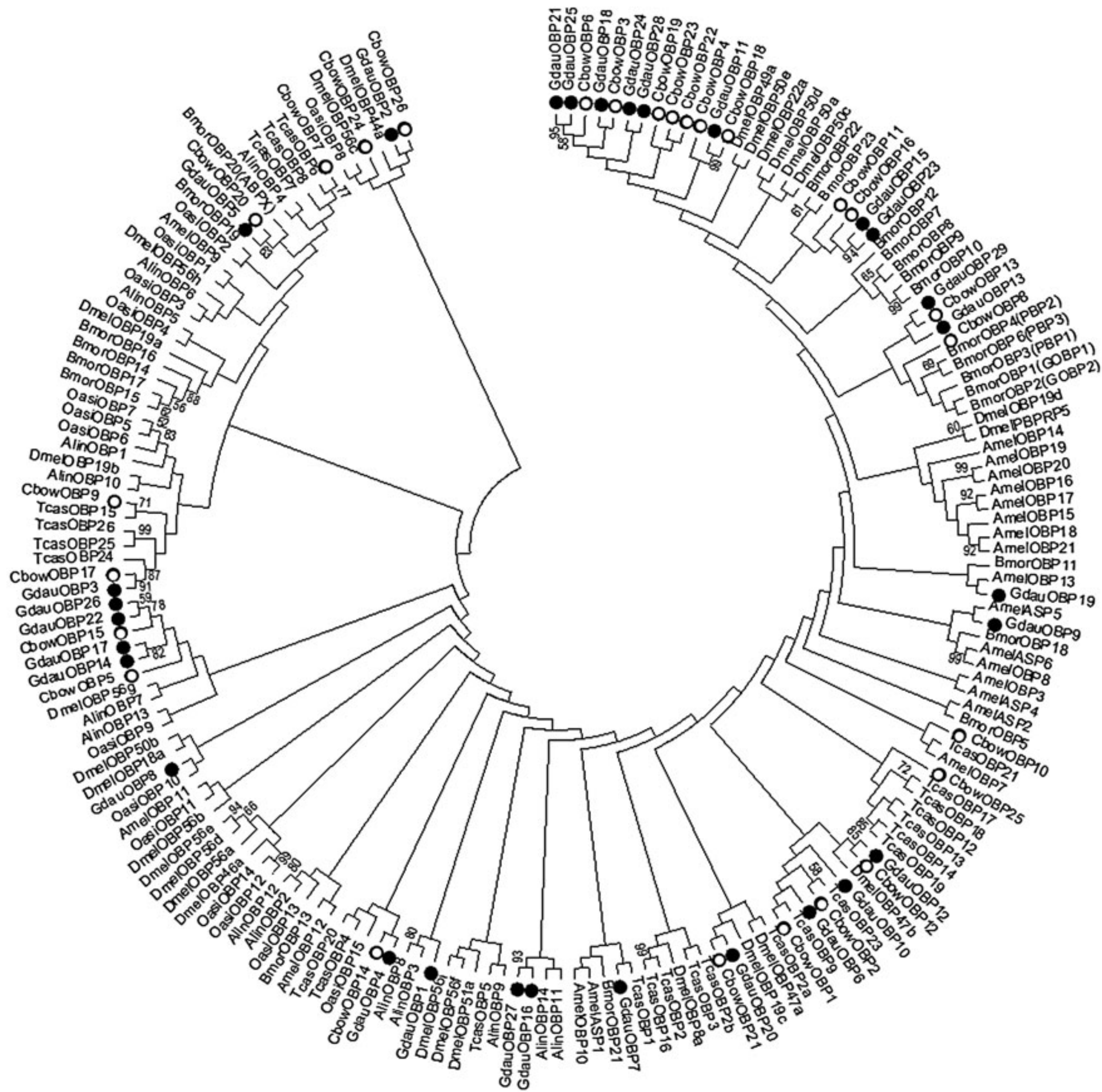

Fig. 2. Phylogenetic tree was constructed by neighbor-joining method using the program MEGA 6.0 with 1000 bootstrap replications. Bootstrap values >50% are shown. Red dots indicate *G. daurica* OBPs. Blue dots indicate *C. bowringi* OBPs. Amel: *Apis mellifera*; Dmel: *Drosophila melanogaster*; Bmor: *Bombyx mori*; Tcas: *Tribolium castaneum*; Alin: *Adelphocoris lineolatus*; Oasi: *Oedaleus asiaticus*.

Phylogenetic analysis of OBPs in G. daurica *and other insects*

A phylogenetic tree was generated to infer the relationships between the 29 OBPs of *G. daurica* and 181 OBPs of seven other insect species from six orders (fig. 2). The GdauOBPs did not form a single clade, although six pairs of clusters were observed (GdauOBP14/GdauOBP17, GdauOBP15/GdauOBP23, GdauOBP16/GdauOBP27, GdauOBP21/GdauOBP25, GdauOBP22/GdauOBP26, and GdauOBP24/GdauOBP28) with bootstrap support ranging from 59 to 96%. At the same time, the phylogenetic tree showed that ten pairs of GdauOBPs/CbowOBPs were clustered into the same clade (Gdau2/Cbow26, Gdau3/Cbow17, Gdau4/Cbow14, Gdau5/Cbow20, Gdau6/Cbow2, Gdau11/Cbow18, Gdau12/Cbow12, Gdau18/Cbow3, Gdau20/Cbow21, Gdau29/Cbow13). However, GdauOBP1, GdauOBP7–GdauOBP9, and GdauOBP19 did not cluster into the same clade.

Tissue-specific expression profiling of OBP genes by semi-quantitative RT-PCR

Tissue-specific expression profiling by RT-PCR was performed with cDNA prepared from total RNA extracted from antennae, heads (without antennae), thoraxes, abdomens, legs and wings of male and female adults. Figure 3 shows that GdauOBP2, 4, 8, 11, 13–15, 17, 19, 21–26, and 28 were ubiquitously expressed in all tested adult tissues, whereas GdauOBP15, 17 and 22 were expressed at lower levels in the antennae. Moreover, GdauOBP17 was expressed in female thoraxes but it was undetectable in male thoraxes; GdauOBP24 was expressed in male wings whereas it was not detectable in female wings while GdauOBP7 was expressed in female wings but it was undetectable in male wings. Both GdauOBP7 and GdauOBP12 were ubiquitously expressed in the antennae, heads, thoraxes, legs, and wings.

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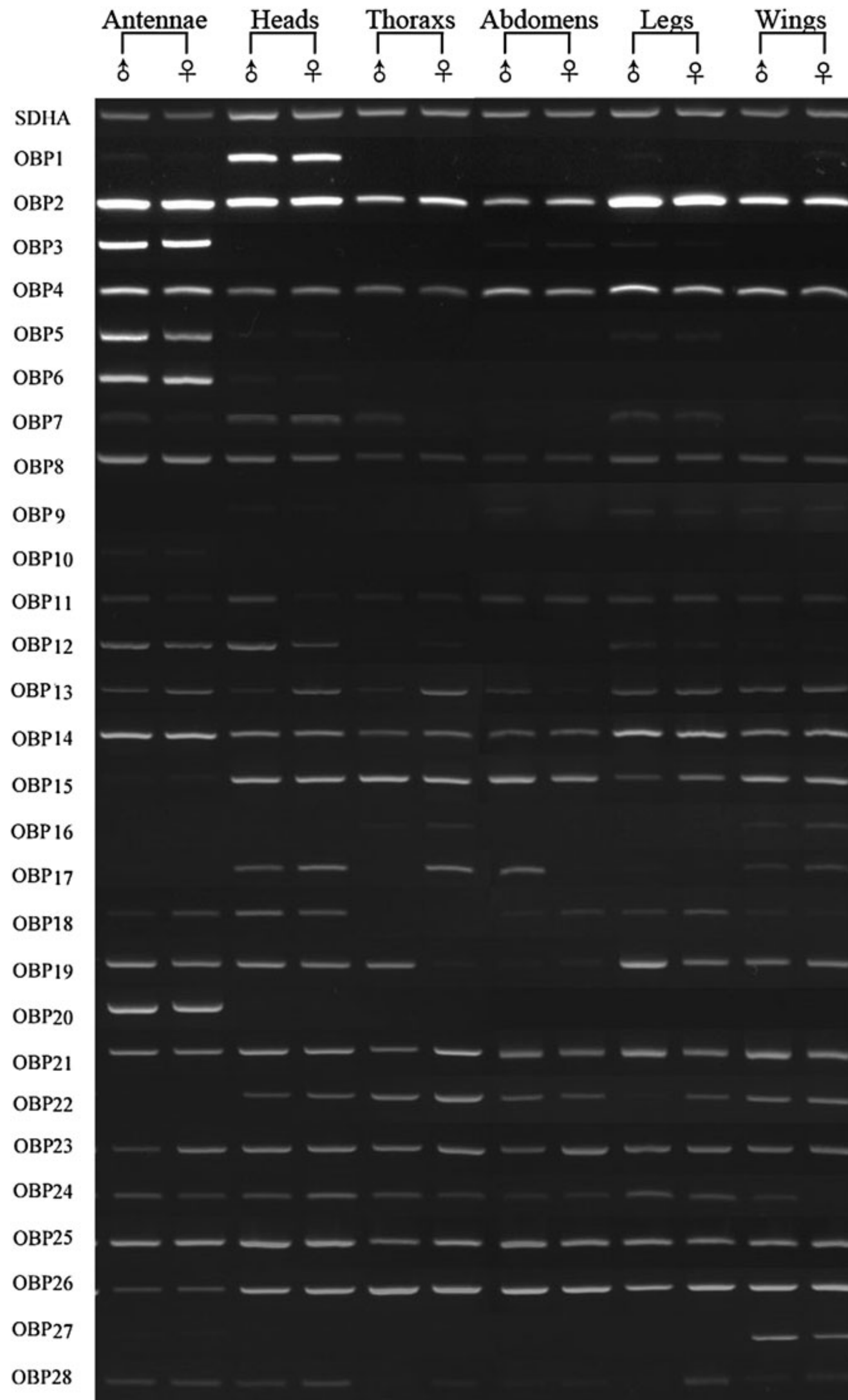

Fig. 3. Tissue expression of 28 OBP genes in *G. daurica.* SDHA: the succinate dehydrogenase complex (SDHA) gene of *G. daurica.*

GdauOBP9 and GdauOBP18 were expressed in the antennae, heads, abdomens, legs, and wings. The expression of GdauOBP10 and GdauOBP20 were limited to the antennae in both genders, and GdauOBP16 was primarily expressed in thoraxes and wings. GdauOBP27 was uniquely expressed in the antennae and wings, and the PCR amplification showed robust expression in the wings than in the antennae. GdauOBP3, 5 and 6 were primarily expressed in the antennae, and the intensity of the PCR bands of these OBPs in other tissues were very weak, such as GdauOBP3 in abdomens and legs, GdauOBP5 in heads and legs, and GdauOBP6 in heads.

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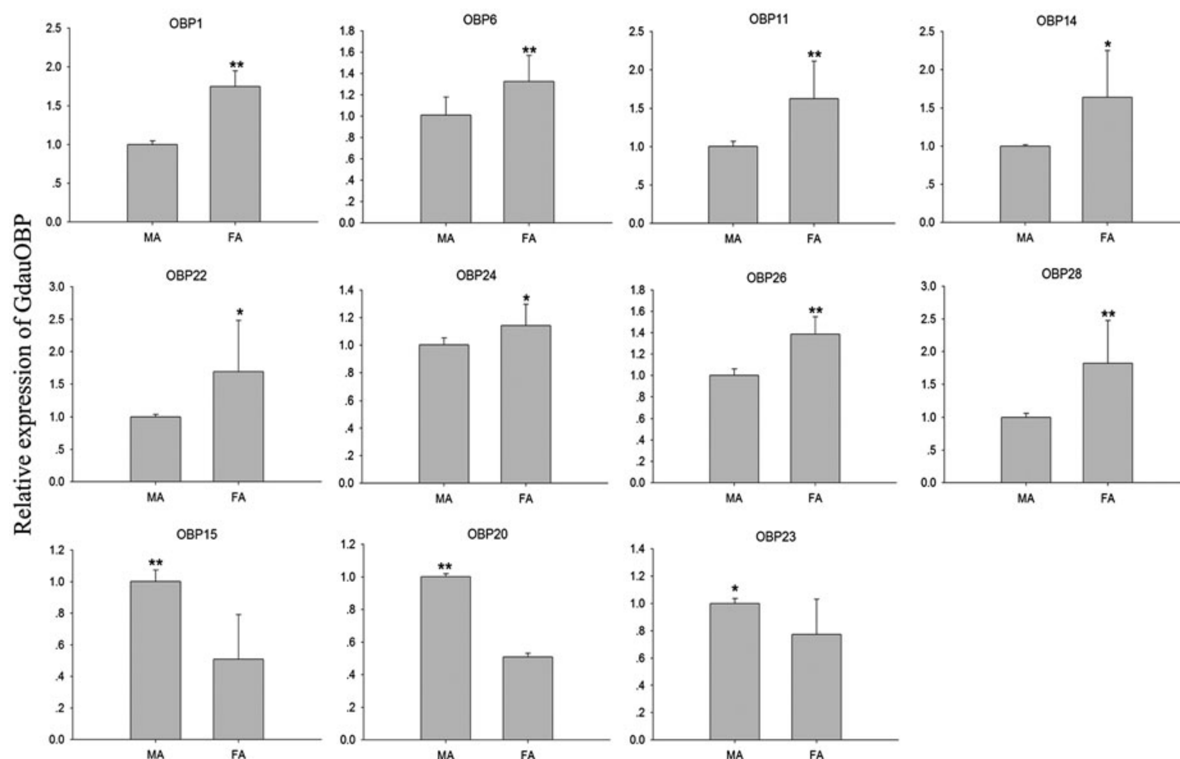

Fig. 4. Sex-biased expression of some OBP genes in *G. daurica* antennae measured by qRT-PCR (*t*-test; *, $P < 0.05$; **, $P < 0.01$). MA: male antennae; FA: female antennae. Columns indicate the mean ± standard error of three independent experiments.

Sex-biased expression of OBP genes in antennae by qRT-PCR

Sex-biased expression of GdauOBP1–28 in antennae was analyzed by qRT-PCR and relative expression levels of each GdauOBP gene was estimated in the antennae of males and females with the antennae of male selected as the reference. The results showed that the expression levels of GdauOBP15, 20 and 23 in the male antennae were significantly higher than in the female antennae. In contrast, GdauOBP1, 6, 11, 14, 22, 24, 26, and 28 had significantly higher expression levels in females than in males (fig. 4). The remaining 17 GdauOBPs did not show significant differences in gene expression levels between males and females; these include GdauOBP2–6, 7–10, 12, 13, 16–19, 21, 25, and 27.

Expression profiling of OBPs genes in different developmental stages by qRT-PCR

We further conducted qRT-PCR to assess the expression of GdauOBP genes during the various developmental stages of *G. daurica*. The expression level of each GdauOBP gene was estimated in eggs, first- to third- instar larvae, pupae, and adult antennae; the expression level in pupae was selected as the reference (fig. 5). Among the 28 OBP genes tested, the expression levels of 15 OBPs including GdauOBP2–6, GdauOBP8, GdauOBP10, GdauOBP12-14, GdauOBP18-20, GdauOBP24, and GdauOBP27, were significantly higher in the adult antennae than in other stages. Notably, GdauOBP28 was found to be expressed mainly in the eggs with the expression levels approximately 12–2800-fold higher than in other stages. Furthermore, five OBPs (GdauOBP15–17, GdauOBP23 and GdauOBP25) had significantly higher expression levels in the pupae than in the other stages. GdauOBP9 was highly expressed in larvae but the expression levels decreased from the first-instar larvae to third-instar larvae. The expression levels of GdauOBP21 were about 2500-fold higher in the third-instar larvae and adult antennae than in other stages. The expression levels of GdauOBP7 were approximately 160 to 300-fold higher in the first- and second-instar larvae and adult antennae than in other stages.

Discussion

In this study, we identified 29 OBP genes from the *G. daurica* transcriptome, all of which are reported here for the first time. This number is close to the number of OBP genes identified in the antennal transcriptomes of *C. bowringi* (26) (Li *et al.*, 2015*a*, *b*) and *D. ponderosae* (31) (Andersson *et al.*, 2013) while more than that reported in *D. valens* (21) (Gu *et al.*, 2015), but much less than in *Tribolium castaneum* (49) (Dippel *et al.*, 2014) and in *Rhynchophorus ferrugineus* (38) (Antony *et al.*, 2016). The likely factors that may have influenced the numbers of these OBP genes may include the evolution of divergent behaviors of different insects during their adaptation to various environmental factors, such as diets, mating, and oviposition (Lavagnino *et al.*, 2012; Zhou *et al.*, 2012; Goldman-Huertas *et al.*, 2015). The phylogenetic analysis showed that GdauOBPs are closely related to CbowOBPs from *C. bowringi*, which is also a member of Chrysomelidae (Coleoptera) like *G. daurica*. Thus, it is reasonable that they have homologous chemosensory systems. However, there are also many

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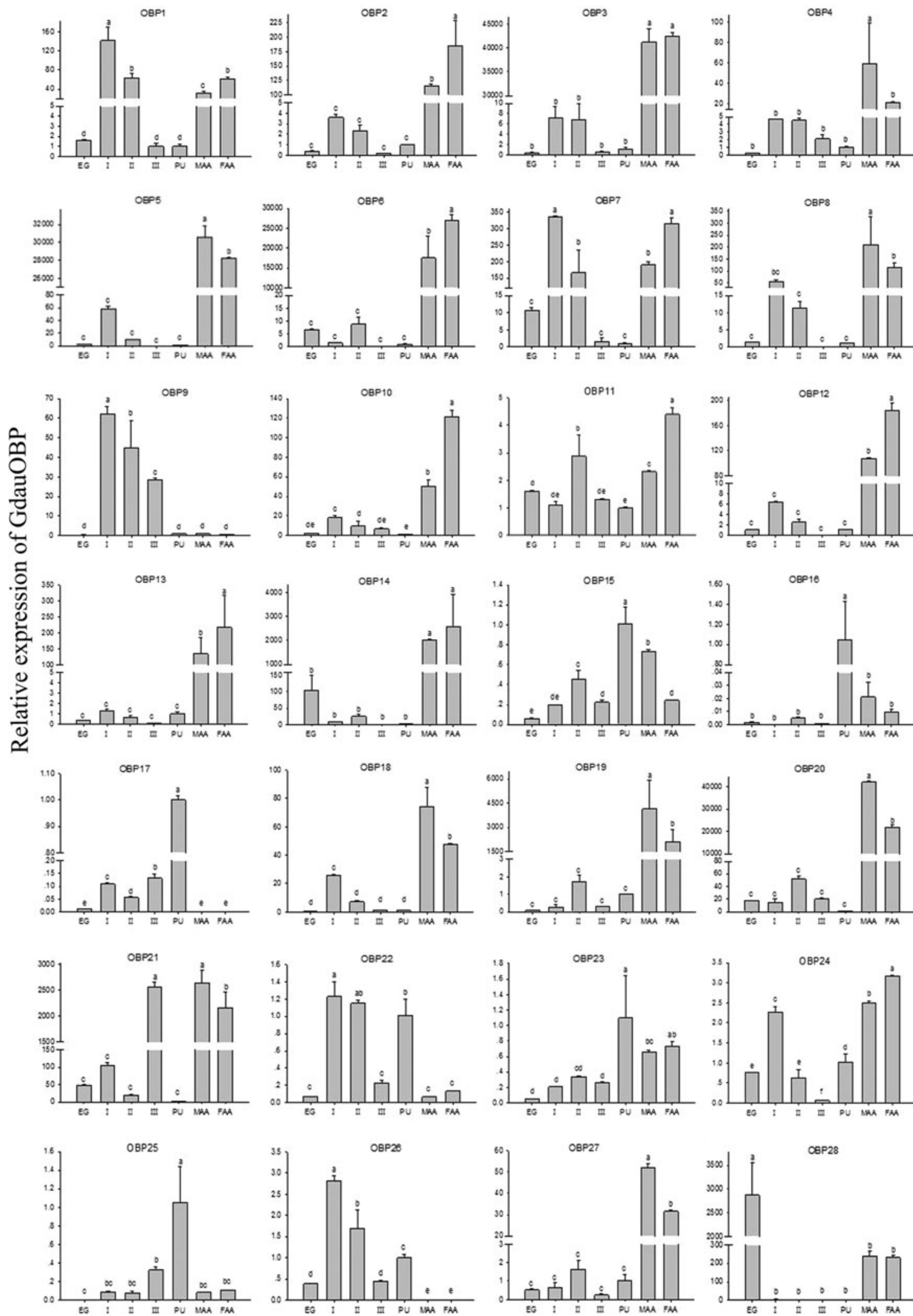

Fig. 5. Expression profiles of *G. daurica* OBPs in different development stages. EG, egg; I, first instar larvae; II, second instar larvae; III, third instar larvae; PU, pupae; MA, male antennae; FA, female antennae. Columns indicate mean ± standard error of three independent experiments. Different letters above each column denote significant differences ($P < 0.05$).

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differences between the GdauOBPs and CbowOBPs, and the likely reasons for the differences may be the evolution of unique chemosensory systems to adapt to different environments.

Some studies have suggested that the OBPs that have high expression in non-antennal tissues may be associated with taste perception and could participate in other physiological functions (Shanbhag *et al*., 2001; Jeong *et al*., 2013). In this study, the cumulative results of RT-PCR and qRT-PCR showed that not only all GdauOBPs were expressed in the antennae of both male and female adults, but also that most GdauOBPs were expressed in non-antennal tissues such as heads, thoraxes, abdomens, legs, and wings of adults, suggesting that these genes might also participate in taste or general functions. Our results are consistent with previous reports that OBPs are expressed in different tissues (Zhang *et al*., 2013; Zhu *et al*., 2013; Dippel *et al*., 2014; Sparks *et al*., 2014). Moreover, in our study, there were significant differences in expression levels between males and females; three OBPs (GdauOBP15, 20, and 23) were male-biased, indicating that these OBPs may detect pheromones released by females, like in moths (Gong *et al*., 2014). In contrast, eight OBPs (GdauOBP1, 6, 11, 14, 22, 24, 26, and 28) were female-biased and may be involved in female-specific chemosensory processes, such as egg laying (Zheng *et al*., 2013).

Surprisingly, chemosensory genes have been seldom investigated in different developmental stages of insects. Several studies have shown that OBPs are expressed not only in adults but also in larvae of insects, such as *Spodoptera littoralis* (Poivet *et al*., 2013) and *Cryptolaemus montrouzieri* (Pan *et al*., 2016). In our study, the developmental stage-specific expression showed difference in the expression levels of these OBPs in the eggs, larvae, pupae, and adult antennae of *G. daurica*. Moreover, among the 28 OBPs of *G. daurica*, half (14) were significantly up-regulated in adult antennae than in other developmental stages, suggesting an olfactory role for these genes with antennae being the major olfactory organ. However, one OBP (GdauOBP28) in eggs, two OBPs (GdauOBP9 and 26) in larvae and three OBPs (GdauOBP15, 17, and 25) in pupae were significantly up-regulated than in adult antennae, and most OBPs (21) were expressed at the lowest levels in eggs and pupae. Gong *et al*. (2014) reported that all 18 OBPs in *Sitodiplosis mosellana* pupae were scarcely expressed or not expressed at all. Qin *et al*. (2016) indicated that one OBP (*PxylOBP31*) from *Plutella xylostella* was expressed highly in adult antennae but weakly in eggs, larvae and pupae. The qRT-PCR results of Jia *et al*. (2015) showed that *CpunPBP1* from *Conogethes punctiferalis* was dominantly expressed in adult antennae, whereas scarcely expressed in egg stage and not expressed in larval and pupal stages. We presume that the low or no expression of OBPs in eggs and pupae indicate an evolutionary significance because insect eggs and pupae live in immobile status when an advanced olfactory system is not necessary. Interestingly, one OBP (GdauOBP28) was abundantly expressed exclusively in eggs, suggesting its involvement in egg development. This is the first report of an OBP that has abundant expression in insect eggs. Taken together, our results show the extremely complex expression profile of OBPs both in tissues and developmental stages, which are likely due to their different roles in *G. daurica* behaviors. These findings provide insights for further research on the molecular mechanisms of chemical communication in *G. daurica*.

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