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Identification and expression profile analysis of putative odorant-binding proteins in *Sitodiplosis mosellana* (Gehin) (Diptera: Cecidomyiidae)

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ABSTRACT

Odorant binding proteins (OBPs) contribute to the remarkable sensitivity of the insect's olfactory system and play important roles in the olfactory recognition. The orange blossom midge, *Sitodiplosis mosellana* is a cereal specialist, and utilizes pheromone and host odorant as a cue for its mating and oviposition. However, OBP genes have not been largely identified in *S. mosellana*. Based on the sequenced transcriptome database, twenty-six OBP genes were identified in *S. mosellana* for the first time. Phylogenetic analysis revealed that *S. mosellana* OBP genes are more closely related to *Mayetiola destructor* OBP genes than to *Aedes aegypti* OBP genes. Most OBP genes seemed to be antenna-specific, but differentially expressed in male and female antennae. Three OBP genes (OBP9, OBP19 and OBP23) are leg-specific. And also, most OBP genes have higher expression levels in adults. Only one OBP gene (OBP10) has higher expression levels in larval stages. These findings serve as an important basis for understanding the molecular mechanisms of chemosensory perception.

1. Introduction

The orange wheat blossom midge (OWBM), *Sitodiplosis mosellana* (Gehin) (Diptera: Cecidomyiidae), is a periodic pest of wheat crops in the Northern Hemisphere and occasionally inflicts severe damage, particularly where a sequence of seasons favoring the midges triggers an outbreak [1]. *S. mosellana* is a cereal specialist, for which wheat is the most attractive crop for oviposition and in the absence of a wheat crop at a suitable growth stage, midges will fly to crops of rye, triticale, or barley, or may complete their life cycle in weed grasses [2]. The percentage of infested spike was above 50% in Roegneria ciliaris [3]. Female *S. mosellana* are attracted by volatile compounds from preanthesis wheat spikes [2]. Stimulatory volatiles may be the cues that females use to find potential hosts and initiate oviposition. But sometimes volatile compounds released by wheat spikes contribute to the reduced oviposition on some wheat genotypes and growth stages [4]. On the other hand, a monitoring system for male *S. mosellana* for use in pheromone traps in Canada and UK has been made [5]. But in China, it was not effective (data not shown). Therefore it is very important to study the recognition mechanism of *S. mosellana*.

A better knowledge of the molecular mechanism of *S. mosellana* olfaction will contribute to the development of new tools for the control of this species since inhibition/artificial activation of the proteins implicated in the olfactory process would lead to the disruption of its chemical communication [6]. Secreted proteins including odorant-binding proteins (OBPs) play important roles in the olfactory recognition. Experimental evidence has demonstrated that OBPs could selectively bind odorants, pheromones or oviposition deterrents [7–15].

Despite the economical importance of *S. mosellana*, no data described the olfactory proteins. Here, we took advantage of next generation sequencing technologies (NGS) to enrich the study of the olfactory in this pest. We identified genes encoding OBPs. The tissue and the development stages specificity of the transcripts of these genes were analysed. These finding would serve as an important basis for identification of the *S. mosellana* OBPs that is required perception of the host compound or pheromone.

2. Materials and methods

2.1. Insects

The cocoons of *S. mosellana* were originally collected in Wuzhi, Henan province, China, during March 2011. The field studies did

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not involve endangered or protected species. No specific permits were required for the described field collections, and the location is not protected in any way. *S. mosellana* is common agricultural pest and is not included in the "List of Protected Animals in China". The third instar larvae, pupae and male and female adults were collected, respectively. Meanwhile, the emerged adults were collected within 24 h and temporarily stored at −80 °C. Each part of *S. mosellana* (male and female antennae, male and female legs) was dissected on ice and collected in an Eppendorf tube. Finally, these samples were frozen at −80 °C until use.

2.2. Identification of odorant binding protein genes from S. mosellana transcriptome

The available odorant binding protein gene sequences from other insect species including (*Anopheles gambiae*, *Aedes aegypti* and *Culex quinquefasciatus*) were used as references to screen the *S. mosellana* transcriptomic database [16]. OBP genes were identified by searching the sequences in the transcriptome database for keywords (OBP, odorant binding protein) or by using the basic local alignment search tool (BLAST) algorithm to search for other

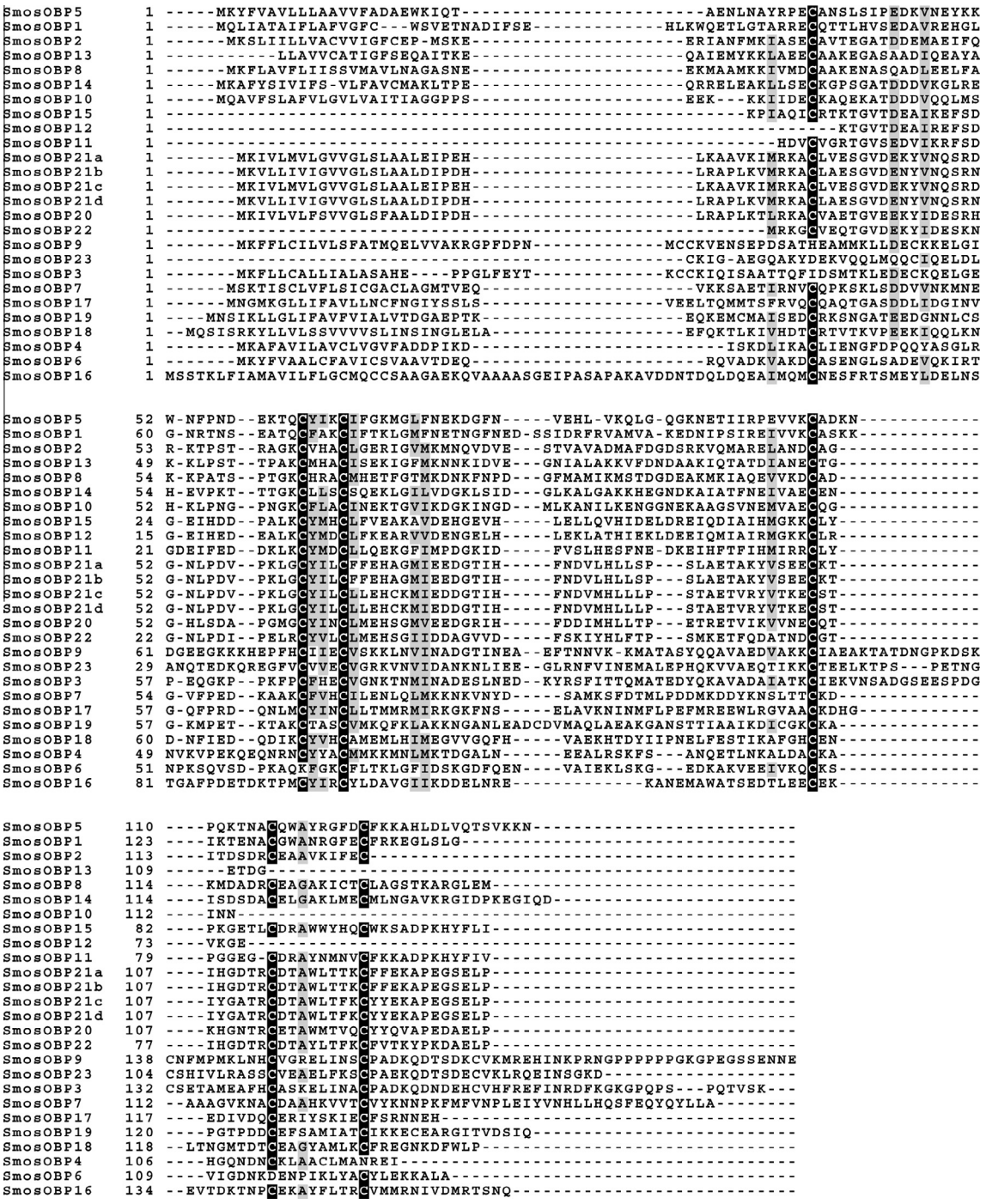

Fig. 1. Alignment of deduced amino acid sequences of *S. mosellana* OBP family members. The alignment was performed using Clustal X 1.83 and their homologous areas were marked by the Boxshade 3.21 program. GenBank accession numbers are available in Table S2.

known insect OBP genes. The candidates of odorant binding protein genes were confirmed by searching the BLASTX algorithm against the non-redundant (nr) NCBI nucleotide database using a cut-off *E*-value of 10^{-5}. OBPs were searched for the presence of a signal peptide using SignalP 4.0 [17].

2.3. Phylogenetic analyses

The amino acid sequence alignment of the candidate OBPs was performed using CLUSTALX (1.83) [18]. Signal peptide sequences were removed from the data set. The 26 OBPs along with OBPs from other insect species, were used to construct phylogenetic trees based on the amino sequences. The OBPs data set contained OBPs from *A. aegypti* [19], *Mayetiola destructor* (ftp://ftp.bioinformatics.ksu.edu/pub/HessianBase/). All alignments were curated manually to remove highly divergent regions. Neighbor-joining trees were produced using MEGA5 [20] with *p*-distance model, and 1000 bootstrap replicates were performed.

2.4. Nucleic extraction and cDNA synthesis

Total RNA was isolated from different tissues (male antennae, MA; female antennae, FA; female legs, FL; male legs, ML) and different development stages (larvae, LA; pupae, PU; female adult, FeA; male adult, MaA) of *S. mosellana* with Trizol (Invitrogen, CA, USA), according to the manufacturer's protocol. 1 μg total RNA from each sample was reverse-transcribed in a 20 μl reaction to generate the first-strand cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Tokyo, Japan).

2.5. Tissue and development stage specificity of SmosOBPs

For tissue expression profiling, FA, MA, FL and ML of adults were dissected. Meanwhile, RNA of different development stages including LA, PU, FeA, MaA were also conducted. Real-time PCR was performed in the StepOne Plus Real-time PCR system (Applied Biosystems, Forster City, CA, USA), using the SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa) as DNA-binding fluorescence dye in the fluorescence PCR (TaKaRa, Tokyo, Japan). Primer pairs were designed from the nucleotide sequences using the primer3 (v. 0.4.0) software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). All primer sequences are listed in Table S1. 18s rRNA was used as an endogenous control to normalize the results of a variable target gene and to correct for sample-to-sample variation.

Each amplification reaction was performed using a 20 μl reaction mixture, under the following conditions: denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and at 60 °C for 30 s. The fluorescent signals yielded by the PCR products were detected by subjecting the products to a heat-dissociation protocol during the last step of each cycle. Following amplification, melting

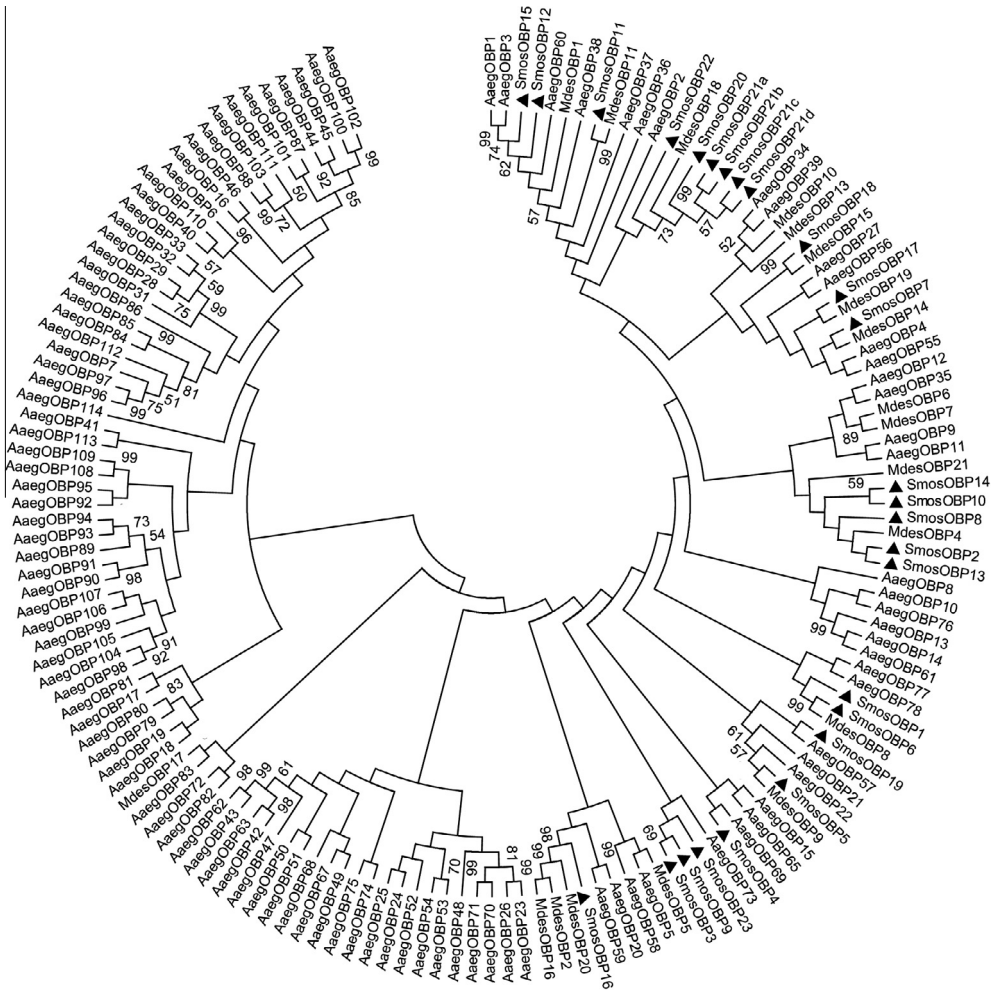

Fig. 2. Phylogenetic relationship between Diptera OBPs. The tree was constructed by neighbor-joining method using the program MEGA 5.1 (1000 bootstrap replications). Bootstrap values >50% are shown. Signal peptides and extended C-terminal were removed before alignment in Clustal X. The amino acid sequences of OBPs used in this analysis were listed in Table S4.

curves were constructed, and data analysis was performed by using the StepOne software 2.1. The reaction was carried out in triplicate, and the average threshold cycle value was used to quantify the relative copy number. A no-template control (NTC) sample was run to detect contamination and to determine the degree of dimer formation. The $2-\Delta\Delta Ct$ method was used to measure relative expression levels across the samples [21].

3. Results

3.1. Annotation of putative odorant-binding protein genes

As genome information is not available for *S. mosellana*, we decided to perform a transcriptome analysis of the genes. We focused on putative members of olfactory gene families, starting with OBPs-coding genes. OBPs are a class of small, water-soluble, extracellular proteins that are thought to aid in the capture and transport of odorant and pheromone to olfactory receptors (ORs). In total, 26 OBP-related sequences were obtained from our transcriptome analysis. Of these, 20 SmosOBP sequences seemingly had full-length open reading frames (ORF). And only one (SmosOBP5) were the same as sequences deposited in the GenBank: EW780463.

Sequence analysis identified 20 genes with a full length ORF with a predicted signal peptide sequence. Signal peptide sequence was not detected in the remainder of putative OBPs due to incomplete N-termini. The information including unigene, length, and BLASTx best fit hit and so on of all the 26 OBPs was listed in Table S2. The nucleotide sequences of all the 26 OBPs were listed in Table S3.

Almost all the deduced proteins have the characteristic hallmarks of the OBP protein families: the presence of a signal peptide, and the highly conversed six cysteine profiles (Fig. 1, Table S2). Some of the SmosOBPs clustered in the 'plus-C' and 'minus-C' OBP sub-families, in correlation with the cysteine number.

3.2. Phylogenetic analysis

We performed a BLAST search on the assembled Official Gene Set (OGS) proteins to identify OBPs and sequences of the *A. aegypti* and *A. gambiae* as templates. This search yielded 22 sequences encoding OBPs (named MdesOBP1-OBP22, Table S4). The phylogenetic relationship of the predicated OBPs in *S. mosellana* and other insect species was shown in Fig. 2. From this phylogenetic tree, it could be seen that SmosOBP have the closest relationship with MdesOBPs. The SmosOBPs were spread out on various branches

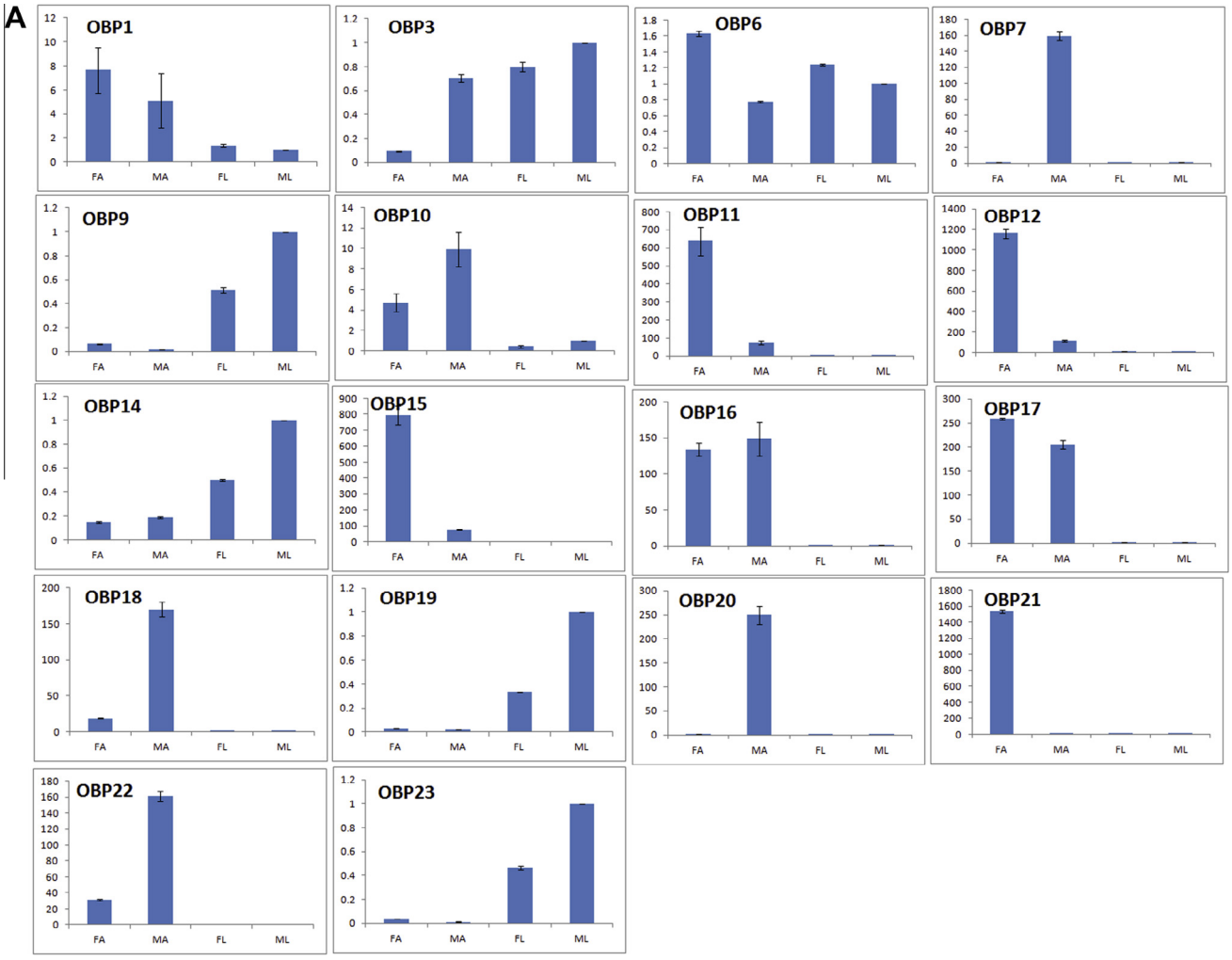

Fig. 3. Expression profiles of *S. mosellana OBPs* in different tissue (A) and in different development stages (B). Total RNA was extracted from dissected male antennae (MA), female antennae (FA), male legs (ML), female legs (FL), larvae (LA), pupae (PU), female adult (FeA) and male adult (MaA). Data were normalized according to the expression level of 18S rRNA determined. The expression levels were quantified relative to the value obtained from leg samples. The bars indicates Mean ± SE of relative mRNA copy numbers ($n = 3$).

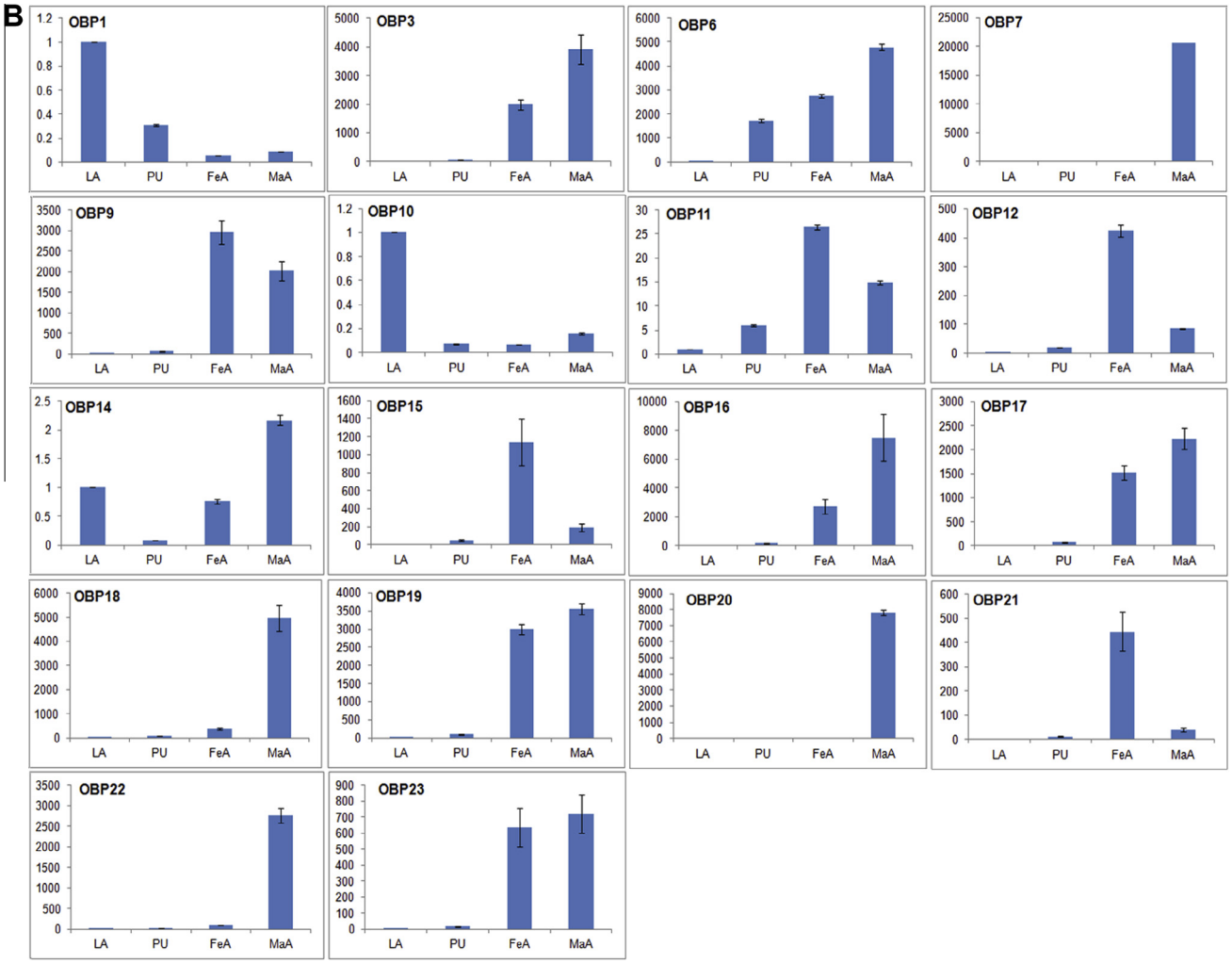

Fig. 3 (*continued*)

where they generally formed small subgroups together with OBPs mostly from *M. destructor*.

3.3. Expression patterns of S. mosellana OBP genes

We conducted quantitative RT-PCR analyses in different tissues (FA, MA, FL and ML) and in different development stages (LA, PU, FeA, MaA) to assess the expression of *S. mosellana* OBPs. Of the tested eighteen OBP genes, the transcript levels of thirteen were much higher in both male and female antennae than legs (Fig. 3A). The expression of genes for OBP11, OBP12, OBP15 and OBP21 in the antennae of female OWBM was much higher than in the antennae of male OWBM, while OBP10 and OBP16 genes were expressed at a slightly lower level in females than in males. Meanwhile, OBP3, OBP6 and OBP14 genes ubiquitously expressed, barely antenna-specific, and highly antenna-specific. Moreover, OBP17 gene was expressed at slightly higher levels in the antennae of female OWBM than in those of males. The expression of genes for OBP7, OBP18, OBP20 and OBP22 in the antennae of male OWBM was much higher than in the antennae of female OWBM. They were likely to be male antenna-specific. OBP9, OBP19 and OBP23 were highly expressed only in the legs and the transcript level is higher in male legs than female legs.

In order to study the expression patterns of different development stages, we conducted the real-time PCR. Most of OBPs genes (13 of 18 genes) highly expressed in the male and female adults (Fig. 3B). The expression of genes for OBP12, OBP15 and OBP21 in the female adults was much higher than in the male adults. In contrast, the expression of genes for OBP7, OBP18, OBP20 and OBP22 in the male adults was much higher than in the female adults. The expression patterns in male and female adults are consistent with that the expression pattern in the different tissues. For OBP1, the expression levels are decreased from larvae to adults. Noticeably, OBP10 only has higher expression levels in larval stages. OBP3 and OBP16 have higher expression in the male than female adults. The higher expression level of OBP9, OBP19 and OBP23 in adults may be because its higher expression level in legs.

4. Discussion

Using a de novo transcriptome of *S. mosellana*, we focused the Illumina sequencing on OBP genes, which involved in host plant sensing. A total of 26 candidate OBPs could be deduced from the analysis of the data. They are further referred to as SmosOBP1-26. And the further studies using this transcriptome data could provide insights into insect physiology and pest control strategy [22].

The number of SmosOBPs is low compared to what has been identified in other species via genome or transcriptome analyses, especially in Diptera insects including *Drosophila*, *A. gambiae*, *A. aegypti* and *C. quinquefasciatus* [19]. For OBPs, we can assume that most numbers of this family present in the *S. mosellana* have been

identified, based on the fact that only 22 OBPs were identified from *M. destructor*, both belong to Cecidomyiidae.

To better understand the OBPs, we constructed a phylogenetic tree based on the amino acid sequences of OBPs. The candidate SmosOBPs showed higher homology to MdesOBPs. Almost each candidate SmosOBP was clustered together with at last one orthologue gene in *M. destructor*. But the SmosOBPs and MdesOBPs were not spread out on various branches. They only clustered into the higher homology AaegOBPs groups. The presence of new genes subfamilies in *A. aegypti* maybe related to their roles in adaptation to severe environment.

The expression patterns of OBPs in *S. mosellana* may help us to characterize the function of these proteins in future research. The results of real time PCR showed that the expression levels of most OBPs in *S. mosellana* were mainly expressed in antennae during the adult stage and mainly expressed in adults among larvae, pupae and adults. *S. mosellana* only has one pheromone [23]. So OBPs which are male biased in expression may play the same roles as PBPs. Several OBPs have already been reported to bind the pheromone component [13,14,24,25]. Three genes (OBP9, OBP19, and OBP23) are expressed at high level in legs and the transcript level is higher in male legs than female legs. And OBPs higher expressed in legs have been reported in other insects, such as *Adelphocoris lineolatus* [26], *Bactrocera dorsalis* [27] and *C. quinquefasciatus* [28]. In *C. quinquefasciatus*, 21 OBPs are more expressed in legs than in antennae. It is unlikely that proteins predominantly expressed in non olfactory tissues play a role in odorant reception in antennae [29]. These proteins highly expressed in non olfactory tissues may be carriers of ligands other than odorants [30].

In *S. mosellana*, of the eighteen studied OBP genes, thirteen of them were olfactory specific, suggesting an olfactory role of these OBPs. Predominantly, some SmosOBP transcripts appeared to be female specific (OBP11, OBP12, OBP15 and OBP21) or male specific (OBP7 and OBP22). These genes could play a role in odorant perception of certain plant volatiles or sex pheromone in the specific host-searching and oviposition behavior [31,32]. In *Helicoverpa armigera* and *Helicoverpa assulta*, OBP 10 is expressed in antennae and could be a carrier for oviposition deterrents [7]. In the mosquito *A. aegypti*, OBP22 is expressed in antennae and in male reproductive organs, and is transferred to females during mating [33,34]. And In *C. quinquefasciatus*, OBP1 is a carrier for an oviposition pheromone [35,36].

OBP1 and OBP10 have high expression level in larvae stages. Since OBPs are proposed to participate in odor discrimination by binding a defined group of molecular structures [37], the OBP specifically expressed in larvae (SmosOBP1 and OBP10) may define larvae-specific olfactory capacities.

The results of real time PCR showed that the expression levels of all OBPs in *S. mosellana* were very low in pupal stage. This phenomenon is also observed in *Cnaphalocrocis medinalis* [38]. The OBP genes may have no role in odorant recognition in *S. mosellana*. But in *Apis mellifera*, OBP13 is highly expressed in the old larvae and throughout the pupal stage [39]. And in *Bombyx mori*, expression of OBP31 gradually rises and reaches its highest level in late pupae. So some members of the OBPs may play general physiological roles as carriers or may mediate responses to ligands that are important for metamorphosis and development [40].

Overall, the expression study displaying the presence of SmosOBPs in different tissues and different development stages makes an important contribution to our understanding of specific odorant reception.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.036.

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