Comparative Genomics Provide Insights Into Function and Evolution of Odorant Binding Proteins in *Cydia pomonella*

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Insect olfaction is vital for foraging, mating, host-seeking, and avoidance of predators/pathogens. In insects, odorant binding proteins (OBPs) are involved in transporting hydrophobic odor molecules from the external environment to receptor neurons. The codling moth, *Cydia pomonella*, one of the most destructive insect fruit pests, causes enormous economic losses. However, little is known about the number, variety, gains and losses, and evolution of OBP genes in *C. pomonella*. Here we report the identification of 40 OBP genes in *C. pomonella*, most (75%) of which are classic OBPs, using genomic and transcriptomic analyses. Two OBP genes were lost in *C. pomonella* relative to possible distant ancestor in Lepidoptera lineage based on an analysis of gene gains and losses. The phylogenetic tree and chromosome location showed that the expansion of OBP genes mainly resulted from tandem duplications, as the *CpomGOBP2* gene was duplicated twice along with loss of *CpomPBPB*. Two positive selection sites of the *CpomGOBP1* gene were identified while other OBP genes evolved under purifying selection. Our results provide fundamental knowledge of OBP genes allowing further study of their function in *C. pomonella*.

Keywords: odorant binding proteins, codling moth, *Cydia pomonella*, positive selection, comparative genomics, gene gains and losses

**INTRODUCTION**

Insects rely on their olfactory system to sense environmental odors related to behaviors such as foraging, host-seeking, mating, and oviposition, as well as avoiding predators and pathogens (Andersson et al., 2015). Odorant binding proteins (OBPs) are small water-soluble globular proteins with molecular masses of 10–30 kDa (Sun et al., 2018). OBPs are highly expressed in the hydrophilic
lymph of insect olfactory sensilla (Pelosi et al., 2014). When lipophilic semiochemicals from the environment enter the lymph through micropores on the surface of olfactory sensilla, the OBPs bind, solubilize, and deliver the semiochemicals to the receptor proteins, e.g., odorant receptors (ORs) or ionotropic receptors (IRs), which are located on the membranes of olfactory sensory neurons. This delivery activates a series of downstream olfactory signal transductions accompanied by corresponding behavioral movements in insects (Zhang et al., 2020). OBPs are clearly essential in communications between insects and environmental semiochemicals including both pheromones and host volatiles.

OBPs are involved in the initial step of recognizing host volatiles or sex pheromones, suggesting that the functional divergence of OBPs is associated with speciation or host diversity. Considering the low sequence identities between orthologous/paralogous OBP genes, OBP genes have likely been evolving at a rapid rate through gene gains or losses (McKenzie et al., 2014) and positive selection (Campanini and de Brito, 2016). Most OBP genes are tandemly arranged in chromosomes, indicating that the occurrence of these genes arose from tandem duplication (Hekmat-Scafe et al., 2002; Gong D. P. et al., 2009; Manoharan et al., 2013; Dippel et al., 2014). The duplicate genes then gradually diverge in function through mutation or pseudogenization (Nei and Rooney, 2005; Vieira et al., 2007).

Studies on the origin, evolution, and structural variation of OBP genes provide insight into the functional differentiation of OBPs and host preference in insects. However, there is little knowledge of the numbers, structures, and evolution of the OBP gene family in important insect crop pests such as the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). *C. pomonella* is an economically threatening pest worldwide (Witzgall et al., 2008; Kumar et al., 2015; Zhu et al., 2017). It mainly destroys apples and pears as well as other seed and stone fruits. Some studies focused on the structures and functions of pheromone binding proteins (PBPs) (Liu et al., 2016; Tian et al., 2016a,b; Tian and Zhang, 2016) and identification of general odorant binding proteins (GOBPs) (Garczynski et al., 2013). In contrast, studies on OBPs are lacking in *C. pomonella*, with little information of their roles in recognizing hosts or locating mates.

To understand the evolution and function of OBPs in *C. pomonella*, we identified and annotated its OBP genes by combining transcriptome data with the high quality genome we released previously (Wan et al., 2019). The gene gains and losses of OBP genes were estimated by CAFE 3.0 (Han et al., 2013) for seven moth species. Subsequently, a phylogenetic tree of OBP genes from three lepidopteran insects (*C. pomonella*, *Bombyx mori*, and *Manduca sexta*) was constructed to explore their evolutionary relationships. The collinearity and chromosome locations were used to compare the divergence of OBP genes between *C. pomonella* and *B. mori*. Finally, the positive selection of genes and structural homology model were analyzed to predict the functional divergence of selected OBPs. Our results provide insights into the evolution of OBP genes in *C. pomonella*, which will facilitate future functional studies.

### MATERIALS AND METHODS

#### Identification of OBP Genes in the C. pomonella Genome

The protein sequences of seven lepidopteran insect OBPs were collected from deposited data of published articles, which have been identified from their genomes, and these species included *B. mori* (Gong D. P. et al., 2009), *M. sexta* (Vogt et al., 2015), *Plutella xylostella* (Cai et al., 2020), *Spodoptera littoralis* (Cheng et al., 2017), *Spodoptera frugiperda* (Gouin et al., 2017), *Helicoverpa armigera* (Pearce et al., 2017), and *Danaus plexippus* (Zhan et al., 2011). These protein sequences were then used as queries in iterative BLASTP searches with parameter “-e-value 1e–5” against the *C. pomonella* genome (Wan et al., 2019) to find candidate OBP genes. A local command line HMMER (version 3.1b2) search was conducted for these candidate OBP genes against the Pfam-A database (El-Gebai et al., 2019) to find the PBP_GOBP (PF01395) HMM profile. The identified OBP genes were subsequently used as queries to align the *C. pomonella* genome using tblASTn search with parameter “-e-value 1e–5” to identify the missing OBP genes during gene prediction for the genome. We used an in-house Perl script to extract DNA sequences of novel genes from the genome, followed by predicting the CDS using the online website FGENESH (Solovyev et al., 2006). Gene prediction was verified by comparing with the transcriptome data that we used in the *C. pomonella* genome paper to confirm the complete gene structure (Wan et al., 2019). Finally, we used GMAP (Wu and Watanabe, 2005) to rebuild gene structures of all OBP genes. For *B. mori*, we used the 44 OBPs of *B. mori* which were identified by Gong (Gong D. P. et al., 2009), to perform tblASTn search against the newest version of the *B. mori* genome (Kawamoto et al., 2019) and rebuilt their gene structures by GMAP (Wu and Watanabe, 2005).

To check the conserved cysteine pattern, which is the predominant feature of OBP genes, we first performed multiple sequence alignment of OBP sequences using MAFFT v7 (Katoh et al., 2002) with default parameters. Then, the aligned sequences were trimmed by trimAl v1.2 (Capella-Gutierrez et al., 2009) to remove gaps and low-quality regions with the parameter “--automated1.” The trimmed sequences were subsequently submitted to ESPript 3.0 Server1 for visualization.

#### Estimation of Gene Gains and Losses

To explore gene gains and losses of OBPs in moths, seven moth species with available genomes and past investigations of the OBP gene family were selected, including *S. littura*, *S. frugiperda*, *H. armigera*, *B. mori*, *M. sexta*, *C. pomonella*, and *P. xylostella*. Orthologous and paralogous groups of these species were inferred by OrthoFinder v2.3.1 (Emms and Kelly, 2015) with default parameters. Orthologous groups including only single copy genes for each species were selected to construct the species tree. Protein sequences of each orthologous group were independently aligned using MAFFT v7 (Katoh et al., 2002), trimmed by trimAl v1.2 (Capella-Gutierrez et al., 2009), and

1[http://escript.ibcp.fr/EScript/cgi-bin/EScript.cgi](http://escript.ibcp.fr/EScript/cgi-bin/EScript.cgi)
then concatenated into one super-sequence. The phylogenetic tree was inferred using maximum likelihood (ML) in RAxML with the best-fit model (JTT + I + F) estimated by ProtTest3 v3.4.2 (Darriba et al., 2011). The Bayesian Relaxed Molecular Clock (BRMC) approach was adopted to estimate the neutral evolutionary rate and species divergence time using the program MCMCTree, implemented in PAML v4.9b package (Yang, 2007). The tree was calibrated with the following time frames adopted from TimeTree (Kumar et al., 2017) to constrain the age of the species. Orthologous gene pairs or blocks were linked by lines. The tree was used to test which genes or sites might have evolved under positive selection. We performed a test of heterogeneity across sites by comparing the M0 and M3 models with $K = 3$ categories. Another test of positive selection on sites involved fitting a beta distribution of $\omega$ values across sites by comparing M7 and M8 models. Considering the evolutionary specificity of GOBP and PBP genes in lepidopteran insects (Yasukochi et al., 2018), we used the branch-site model to test the genes as well as their amino acid sites that evolved under positive selection in nine lepidopteran insects including B. mori, C. pomonella, D. plexippus, Helicinius melpomene, M. sexta, Operopherta brumata, Papilio xuthus, P. xylostella, and S. litura. In the phylogenetic tree of each gene (GOBP1, GOBP2, PBPA, PBPC, and PPBD), we labeled the branch composed of genes from C. pomonella as the foreground branch and the remaining branches as background branches to test positive selection in C. pomonella GOBP and PBP genes. We compared model A (the alternative model), in which some sites on the foreground branch were allowed to change to a value of $\omega > 1$, with the null model of neutral evolution. 

The likelihood ratio tests (LRTs) statistic ($2\Delta L$), which approximates a $\chi^2$ distribution, was used for comparisons between models, and significant results were determined using $\chi^2$-tests. If the LRT was significant, Bayes empirical Bayes (BEB) was used to identify sites of positive selection. The sites with posterior probabilities (PPs) of $\geq 0.95$ were considered positively selected, thus they were defined as positively selected sites (PSS).
Expression Profiling of 40 CpomOBPs

We calculated the expression levels of 40 CpomOBPs in several sensory tissues based on transcriptome data. The sensory tissues were collected from female and male adults, including the antennae, head, leg, labial palp, each sample with three biological replicates. The paired-end clean reads were mapped to the C. pomonella genome using HISAT2 (Kim et al., 2015). The FPKM (Fragments Per Kilobase of transcript per Million mapped reads) was calculated by StringTie software (Pertea et al., 2016). The heatmap.2 function of R package “gplots” to draw the heatmap of expression profiling based on the FPKM values.

Results

Identification of OBP Genes in C. pomonella

A total of 40 OBP genes were identified in the C. pomonella genome. Complete CDS were determined by cross-checking with transcriptome assemblies. All gene information including gene names, CDS, amino acid sequences, chromosomes, and gene lengths and classification are provided in Supplementary Table 1. We compared the 40 CpomOBPs with those previous reported (Garczynski et al., 2013) and renamed them, and those that were not well matched were renamed by the sequence length. The amino acid sequences range from 133 to 339 amino acids (Supplementary Table 1). Multiple sequence alignments show that most have six typical conserved cysteine residues (Figure 1 and Supplementary Table 1). Based on the number and location of the conserved cysteine, the 40 CpomOBPs were classified into four subfamilies, including classic, minus-C, plus-C, and atypical. In total, 30 CpomOBPs belong to the classic subfamily, which had six typical conserved cysteine residues. Four CpomOBPs contained more cysteines than the classic OBPs, and were classified into the plus-C subfamily. Four CpomOBPs belong to the minus-C subfamily, which had fewer than six cysteines. The remaining two CpomOBPs, which exhibited none of the above characteristics, were classified into the atypical subfamily.

Estimation of Gene Gains and Losses

A statistical gene birth and death analysis for OBP genes from seven moth species (S. litura, S. frugiperda, H. armigera, B. mori, M. sexta, C. pomonella, and P. xylostella) was performed by CAFÉ (Figure 2). Forty-two OBP genes were inferred in the common ancestor node of moth species considered in this study at 162 Mya. The gene gains and losses range from -1 (lost one gene) to +1 (gained one gene) between the adjacent ancestor nodes. However, different species have various gene gains or losses ranging from 1 to 7 compared with their adjacent ancestors. For example, gene gains occurred in S. frugiperda (+7) and M. sexta (+6), while gene losses occurred in S. litura (-7), H. armigera (-2), B. mori (-1), C. pomonella (-2), and P. xylostella (-3) compared with their adjacent ancestors. Our results suggested that the gains and losses of OBP genes may be associated with functional divergence which results from adaptation.

Phylogenetic Analysis of OBP Genes

The phylogenetic tree was inferred using a total of 133 amino acid sequences of OBP genes, including 40 OBPs from C. pomonella,
43 OBPs from *B. mori*, and 50 OBPs from *M. sexta* (Figure 3). We classified these OBP genes into 12 groups (Groups 1–12) according to the clusters in the phylogenetic tree. Eleven of them were orthologous groups shared among these three species with nearly 1:1 orthologous genes in each group from each species, except that Group 2 has a lineage-specific expansion in *M. sexta*. The GOBP/PBP subfamily is a specific cluster in lepidopteran species (Vogt et al., 2015; Yasukochi et al., 2018), consisting of six typical tandem genes including GOBP1, GOBP2, and PBPA-PBPD. In our study, Group 1 was the conserved GOBP/PBP cluster, including six genes from *B. mori* (GOBP1, GOBP2, PBPA-1, PBPA-2, PBPC, and PBPD), six genes from *M. sexta* (GOBP1, GOBP2, PBPA, PBPC, and PBPD), and seven genes from *C. pomonella* (GOBP1, GOBP2a, GOBP2b, GOBP2c, PBPA, PBPC, and PBPD). The PBPD gene was lost in *C. pomonella*, while the GOBP2 gene is duplicated twice, which suggested that GOBP2 may be under positive selection. In general, the OBP gene family is evolutionarily conserved in Lepidoptera insects.

**Collinearity and Chromosomal Distribution of OBP Genes**

All 40 OBP genes were located on 11 *C. pomonella* chromosomes (Figure 4). These genes are organized into two major clusters on chromosomes 18 and 8, while the other chromosomes contain scattered and few OBP genes. The largest cluster contains 11 tandem OBP genes on chromosome 18, accounting for 27.5% of the total number of OBP genes. These genes have a collinearity block in chromosome 18 of *B. mori* that contains 12 tandem OBP genes. Furthermore, this collinearity block of OBP genes was clustered into Groups 5, 7, and 11 in the phylogenetic tree, which belong to antennal binding protein I (ABPI) and antennal binding protein II (ABPIII) (Figure 3) (Gong D. P. et al., 2009). Another big cluster on chromosome 8 contains seven OBP genes, which account for 17.5% of the total number of OBP genes. Six of them were in a tandem GOBP/PBP gene cluster, including GOBP1, GOBP2a, PBPA, PBPC, and PBPD. There was also a tandem GOBP/PBP gene cluster on chromosome 19 in *B. mori*. There was a collinearity block of the GOBP/PBP gene cluster between *C. pomonella* and *B. mori*, and they were clustered into Group 1 in the phylogenetic tree (Figure 3). Seven OBP genes in *C. pomonella* have no collinearity compared with *B. mori*.

**Tests of Selective Pressures on Lepidopteran OBP Genes**

We selected eight clades (groups) from the phylogeny to test whether some orthologous/paralogous OBP genes of three moths (including *B. mori*, *C. pomonella*, and *M. sexta*) evolved under positive selection. Selected groups included Groups 2–6, 9, 11, and 12 (Table 1). Groups 7–8 and 10 were excluded since they had too few genes. Group 1 was later tested independently using the branch-site model.

According to tests of the one-ratio model (M0), which assumes a single $\omega$ for all amino acid sites, the $\omega$ values of eight clades ranged from 0.00547 to 0.15846 (Table 1), suggesting the existence of strong purifying selection. However, the comparison between models M0 and M3 (discrete) provided strong evidence of variation in selective pressures at different amino acid sites in Groups 2–3, 5, 9, and 11 ($P < 0.01$, Table 1), indicating that purifying selection has been relaxed at some amino acid sites. We further compared models M7 and M8 for clades showing $0.5 < d_{S} < 1$ to investigate whether some amino acid
sites actually evolved under positive selection. Only Group 2 presented evidence of positive selection ($P = 0.0008$) with one positively selected site (PSS). However, the Bayes empirical Bayes (BEB) analysis showed that the PSS only had a 93.4% posterior probabilities (PPs), which is not statistically significant.

We used the branch-site model to test the positive selection in each codon for different gene clades of GOBPs and PBPs from nine species (*B. mori*, *C. pomonella*, *D. plexippus*, *Heliconius melpomene*, *M. sexta*, *Operophtera brumata*, *Papilio xuthus*, *P. xylostella*, and *S. litura*) (Figure 5). Only GOBP1 was identified as being under positive selection for *C. pomonella* after the likelihood ratio test ($P = 0.0318$). We further used the BEB approach to detect the positive sites in GOBP1, which showed that sites 41S and 43G were significant signs of positive selection with PPs of 0.994 and 0.972, respectively (Table 2).

**Structural Links to Protein Function**

To get additional insight into the functional significance of PSSs, we mapped the PSSs to the multiple sequence alignments of GOBP1 protein sequences from nine species, and labeled them on the structural homology model of *C. pomonella* GOBP1 (Figure 6A). Compared to the other eight species, the 41st amino acid Glutamic acid (E) was substituted by Serine (S), while the 43rd amino acid Glutamine (Q) was substituted by Glycine (G) (Figure 6B). The structural homology model of *C. pomonella* GOBP1 showed that both the 41st and 43rd amino acids were located in the loop near the first helix (Figure 6A).

The binding energies that *CpomGOBP1* bind with 48 odorant molecules were assessed by AutoDock Vina (Trott and Olson, 2010). Among them, β-bourbonene had the lowest binding energy (-9.3 KJ/mol) with *CpomGOBP1*. 

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![Figure 3](https://example.com/figure3.png)

**Figure 3** The phylogenetic tree of the OBP gene family in lepidopteran insects. *C. pomonella* OBPs are classified as Classic, Minus-C, Plus-C, and Atypical, which are represented by blue, red, orange, and green stars, respectively. The GOBP/PBP genes are shown in orange.
Chromosomal distribution of OBP genes in *C. pomonella* (red) and *B. mori* (blue). Cyan solid lines represent the correspondence between *C. pomonella* and *B. mori* OBP genes.

TABLE 1 | Tests of positive selection on the orthologous/paralogous OBP genes of moths by site model.

| Clade  | n   | $d_N/d_S$ | $2\Delta l$ M0 vs. M3 | 2$\Delta l$ M7 vs. M8 |
|--------|-----|-----------|------------------------|------------------------|
| Group2 | 14  | 0.15846   | 55.305518 $^*$(P = 0)  | 14.265552 $^*$ (P = 0.0008) |
| Group3 | 16  | 0.11555   | 52.788572 $^*$(P = 0)  | 7.520000 $^*$ (P = 0.9996) |
| Group4 | 8   | 0.04517   | 0 (P = 1)             | 0.001418 $^*$ (P = 0.9994) |
| Group5 | 15  | 0.00687   | 135.091616 $^*$(P = 0) | 0.001886 $^*$ (P = 0.9991) |
| Group6 | 9   | 0.01691   | 0 (P = 1)             | 0.001218 $^*$ (P = 0.9994) |
| Group9 | 7   | 0.00547   | 16.037206 $^*$(P = 0.0030) | 3.500000 $^*$ (P = 0.9998) |
| Group11| 17  | 0.02222   | 13.897966 $^*$(P = 0.0076) | 7.020000 $^*$ (P = 0.9996) |
| Group12| 17  | 0.01384   | 0 (P = 1)             | 0.001418 $^*$ (P = 0.9993) |

**Clade Parameter estimated under M8 model**

| Clade  | $p_0 = 0.98223, p = 3.14309, q = 15.84241, p_1 = 0.01777, \omega = 3.42058$ |

*Number of genes tested; $d_N/d_S$, Estimated under M0; $2\Delta l$, Likelihood ratio test.
$^*$Significant within the 1% interval after Bonferroni correction.*

(Supplementary Table 2). β-bourbonene was located in the binding cavity composed of 11 hydrophobic amino acid residues, including Phe12, Phe33, Phe36, Phe76, Phe118, Ile52, Ile94, Val8, Trp37, Met5, and Leu61 (Figures 7A,B).

**Expression Profiling of 40 CpomOBPs**

The expression profiling of all 40 CpomOBPs were assessed using FPKM values based on transcriptome data (Figure 8). The result showed that 31 CpomOBPs expressed (FPKM ≥ 10) in the
antennae, head, leg, wing, and labial palp, except CpomOBP23, CpomOBP2, CpomOBP18, CpomOBP28a, CpomOBP28b, CpomOBP19, CpomOBP21, CpomOBP27, and CpomOBP17. There were 25 and 26 CpomOBPs that are expressed in the antennae of female and male adults, respectively, 22 of them were classic OBPs. The other enriched expression tissue is the labial palp, in which there are 27 and 25 CpomOBPs expressed in female and male adults. Three CpomPBPs, CpomGOBP1, CpomGOBP2a, and CpomGOBP2b, were mainly expressed in the antennae and labial palp. It is interesting to note that CpomGOBP2c was specifically expressed in wing.

**DISCUSSION**

In this study, we identified 40 OBP genes in *C. pomonella*, which is similar to several lepidopteran moth species e.g., 43 OBPs in *B. mori* (Gong D. P. et al., 2009), 39 OBPs in *P. xylostella* (Cai et al., 2020), 40 OBPs in *H. armigera* (Pearce et al., 2017), and 36 OBPs in *S. litura* (Cheng et al., 2017). However, *C. pomonella* has fewer OBPs than *M. sexta* and *S. frugiperda*, both of which have 50 OBP genes (Gouin et al., 2017). Variation in the numbers of OBP genes among moth species suggests that the evolution of OBP genes occurred during the speciation adaptation process.
and functional requirements for each species. A study in *B. mori* found that classic OBPs were dominant in the OBP gene family: *B. mori* has 29 classic OBPs, five plus-C OBPs, and eight minus-C OBPs (Gong D. P. et al., 2009). Similarly, in our study the 40 OBP genes of *C. pomonella* were classified as 30 classic OBPs, four plus-C OBPs, four minus-C OBPs, and two atypical OBPs. A previous study suggested that most of the classic OBPs and all ABPIIs are likely involved in chemoreception, since they show increased chemosensory tissue expression (Dippel et al., 2014). The fact that 75% of OBPs in *C. pomonella* are classic OBPs indicated that these genes are essential in recognizing host plants or pheromones such as sex pheromones. The result of expression profiling indicates that 22 classic OBPs were expressed in the antennae, which is similar to the finding in *Tribolium castaneum* (Dippel et al., 2014).

We used the CAFÉ software to estimate gene gains and losses, rather than directly comparing the number of OBP genes, because it considers a birth-and-death model in the evolutionary process (Han et al., 2013). In the most recent common ancestor of moths considered in this study, approximately 162 Mya inferred by two time frames adopted from TimeTree (see section “Materials and Methods”), 42 OBP genes were shared. There were no more than two expanded and contracted genes in each ancestor node, which indicated that speciation may not be driven by the evolution of OBP genes. The gene gains or losses of each species compared to their distant ancestors range from 1 to 7. According to this result, we suggest that the functional divergence of OBP genes occurred mainly after speciation, as a result of adapting to a new diversity of environments such as new host plants or pheromones. As a result, the OBP genes may be under positive selection. However, the variation of OBPs in moths is smaller than the odorant receptors (ORs) or gustatory receptors (GRs): the expanded or contracted genes of these two gene families is as high as 54 (Engsontia et al., 2014). In general, we found that *C. pomonella* lost two OBP genes compared to its closest ancestor.

### TABLE 2 | Tests of positive selection on Lepidopteran GOBP and PBP genes by branch-site model (Branch labels referring to Figure 5).

| Branch-site model | H0 lnL Vs. H1 lnL | df | 2△l and P-value | Parameter Estimated under H1 | Positively Selected Sites (PSSs) |
|-------------------|------------------|----|-----------------|-----------------------------|---------------------------------|
| GOBP1             | -3159.24         | 1  | 4.61            | $p_0 = 0.809, p_1 = 0.140$   | 41S 43G                        |
|                   | -3156.94         |    | $P = 0.0318^*$  | $p_2 = 0.043, p_3 = 0.007$  |                                 |
|                   |                  |    |                 | $\omega_1 = 1.000, \omega_2 = 533.665$ |                     |
| GOBP2             | -3897.96         | 1  | 2.89            | $p_0 = 0.861, p_1 = 0.107$   | N/A                            |
|                   | -3896.51         |    | $P = 0.0890$    | $p_2 = 0.028, p_3 = 0.004$  |                                 |
|                   |                  |    |                 | $\omega_1 = 1.000, \omega_2 = 999.000$ |                     |
| PBP3              | -3313.48         | 1  | 0.18            | $p_0 = 0.639, p_1 = 0.104$   | 26Q 46E 112D 115T 148K 149G 150M 153S |
|                   | -3313.39         |    | $P = 0.6755$    | $p_2 = 0.021, p_3 = 0.036$  |                                 |
|                   |                  |    |                 | $\omega_1 = 1.000, \omega_2 = 1.269$ |                     |
| PBP2              | -3414.50         | 1  | 0               | $p_0 = 0.759, p_1 = 0.191$   | N/A                            |
|                   | -3414.50         |    | $P = 1.0000$    | $p_2 = 0.040, p_3 = 0.010$  |                                 |
|                   |                  |    |                 | $\omega_1 = 1.000, \omega_2 = 1.000$ |                     |
| PBP1              | -3254.95         | 1  | 0.41            | $p_0 = 0.769, p_1 = 0.197$   | N/A                            |
|                   | -3254.75         |    | $P = 0.5212$    | $p_2 = 0.027, p_3 = 0.007$  |                                 |
|                   |                  |    |                 | $\omega_1 = 1.000, \omega_2 = 2.733$ |                     |

*Significant within the 5% interval after Bonferroni correction.

**Significant within the 1% interval after Bonferroni correction.
PSSs in bold show 99% posterior probability confidence.

N/A: No positively selected sites were detected.

### FIGURE 6 | Structural homology model and positively selected sites of *C. pomonella* GOBP1. (A) Structural homology model of *C. pomonella* GOBP1, positively selected sites are marked in red. (B) Multiple sequence alignments of GOBP1 genes from ten moth species.
FIGURE 7 | Molecular docking of CpomGOBP1 with β-bourbonene. (A) β-bourbonene located in the binding cavity of CpomGOBP1. (B) Key amino acid residues that interact with β-bourbonene.

FIGURE 8 | Expression profiling of 40 CpomOBPs in different tissues. F, female; M, male; AT, antenna; HD, head (antennae removed); WG, wing; LG, leg; LP, labial palp.
To further explore which OBP genes have expanded or contracted in C. pomonella, we built a phylogenetic tree and performed collinearity analysis in chromosome location by comparing with related moth species. The results showed that OBP genes were conserved except the genes in Group 2, which contains many expanded genes in M. sexta. We also noticed some gene gains and losses in the conserved clade Group 1, composed of GOBP and PBP genes. The GOBPs and PBPAs were a specific conserved subfamily in butterflies and moths, including GOBP1-2 and PBPA-D, which are in a tandem array with a fixed order in the same chromosome. These genes were thought to be involved in the recognition of volatile organic compounds (VOC) and sex pheromones of insects (Liu et al., 2020). However, recent studies showed some variations in this subfamily, including gene gains, losses, inversions, and translocations (Yasukochi et al., 2018). Although GOBP1 and GOBP2 were regarded as conserved in lepidopteran species (Vogt et al., 2015), some studies found that gene gains occurred in the GOBP1 genes, such as duplication events of GOBP1 in P. xylostella (Yasukochi et al., 2018) and Operophtera brumata (Yasukochi et al., 2018). In our study, we found a duplication event of a GOBP2 gene that generated three GOBP2 (GOBP2a, GOBP2b, and GOBP2c), two of which have been reported by Garghynski (Gargasneyk et al., 2013). PBP gene gains and losses occurred more commonly; most Lepidoptera have lost the PBPB gene (Yasukochi et al., 2018), while PBPA was expanded in B. mori (Gong D. P. et al., 2009). Similarly, we found that the PBPB gene was also lost in C. pomonella, which suggests that this gene may be undergoing a gene fusion event (Yasukochi et al., 2018).

Most OBP genes result from tandem duplications in insects, such as Drosophila melanogaster (Hekmat-Scafe et al., 2002) and Anopheles gambiae (Xu et al., 2003) in Diptera; Tribolium castaneum (Dippel et al., 2014) in Coleoptera; and B. mori (Gong D. P. et al., 2009) and C. pomonella (this study) in Lepidoptera. However, in earlier diverging ancestor orders including Hemiptera and Hymenoptera, there are fewer OBP genes without large tandem duplications (Vieira and Rozas, 2011), as in Acyrthosiphon pisum (Zhou et al., 2010) and Bemisia tabaci (Zeng et al., 2019) in Hemiptera; and Apis mellifera (Foret and Maleszka, 2006) and Solenopsis invicta (Pracana et al., 2017) in Hymenoptera. We also found a very consistent collinearity between B. mori and C. pomonella. These findings strongly suggest that the expansion of most OBP genes is caused by tandem duplications, and the tandem duplications of OBP genes in Lepidoptera occurred before speciation, indicating the existence of mainly purifying selection in moth OBP genes. In addition, the duplicated CpomGOBP2c gene is located in chromosome 21, instead of the GOBP/PBP gene cluster in chromosome 8, which indicates functional differentiation.

Some studies showed that single-point mutation of an amino acid could cause functional differentiation (Leary et al., 2012; Yang et al., 2017). Therefore, we further tested whether there are some positive sites in the OBP genes in C. pomonella. The results of evolutionary analysis by site model showed that most OBP genes evolved under purifying selection with ω ranging from 0.00547 to 0.15846 estimated by the M0 model. Similarly, most OBP genes in B. mori also evolved under purifying selection (Gong D. P. et al., 2009). The purifying selection of OBP genes is potentially due to functional constraints (Gong D. P. et al., 2009). However, among the OBP genes of lepidopteran species, the major function of PBPs is mainly to sense pheromones (Gong Y. et al., 2009), while GOBPs mainly sense the volatiles of host plants (Vogt et al., 2002). We assumed that GOBP/PBP genes may evolve under positive selection due to the vast diversity of sex pheromones and host volatiles. The results of the branch-site model on GOBP/PBP genes suggested that the GOBP1 gene in C. pomonella evolved under positive selection. We detected two positively selected sites (41 S and 43 G) in CpomGOBP1, both located in the loop, close to the first disulfide bridge on helix 1. The mutations of these two amino acid residues may influence the fold shape of the binding cavity by modifying the disulfide bridge, which will cause functional differentiation (Sanchez-Gracia and Rozas, 2008). The docking result suggests that CpomGOBP1 may have the ability to bind with β-bourbonene, however this must be functionally validated.
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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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