Mol Genet Genomics (2016) 291:2159–2171
DOI 10.1007/s00438-016-1245-5

ORIGINAL ARTICLE

Genome-wide identification and analysis of JHBP-domain family members in the silkworm *Bombyx mori*

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Received: 9 June 2016 / Accepted: 29 August 2016 / Published online: 8 September 2016

Abstract Juvenile hormone (JH) regulates the insect growth and development. JH appears in the hemolymph bound by a specific glycoprotein, juvenile hormone-binding protein (JHBP), which serves as a carrier to release the hormone to target tissues and cells. However, *JHBP* family candidates, expression patterns, and functional implications are still unclear. In this study, we identified 41 genes-containing conserved JHBP domains distributed across eight chromosomes of the silkworm *Bombyx mori*. A phylogenetic tree showed that the silkworm *JHBP* (*BmJHBP*) genes could be classified into two major branches and four subfamilies. Microarray data revealed that *BmJHBP* genes exhibit various expression patterns and are expressed in different tissues, periods, and sexes. The expression of *BmJHBP* genes was generally higher in the head, integument, midgut, fat body, testis, and ovary than in the anterior of the silk gland (ASG), median of the silk gland (MSG), posterior of the silk gland (PSG), hemocyte, and Malpighian tubule. BmJHBPd2, in particular, was investigated by Western Blotting, and immunofluorescent assay and was found to be highly expressed in the PSG cytoplasm on day 3 of the fifth instar, coinciding with silk production. Taken together, our findings will be useful in improving understanding the complexity of the *JHBP* family, and will lay the foundation of explaining functional characterization for further research.

Keywords JHBP · Gene family · Identification · Expression pattern · PSG

Introduction

Juvenile hormone (JH) controls the growth and development of insects (Truman and Riddiford 1999; Gilbert et al. 2000). During the larval stages, JH regulates metamorphosis to the adult stage via up-regulation and down-regulation of specific genes (Riddiford 1986). In the hemolymph, JH may bind three types of juvenile hormone-binding proteins (JHBPs), including lipophorins, hexameric proteins, and low molecular weight proteins of approximately 30 kDa (Kramer et al. 1976; Dekort and Koopmanschap 1986, 1987; Zalewska et al. 2011). In the hemolymph of *Lepidoptera*, almost 99 % of JH molecules exist in a complex with a specific low weight 30 kDa JHBP, which serves a supporting role, transporting the hormone from the site of its synthesis (corpora allata) to target tissues and cells (Kramer et al. 1974; Trowell 1992; Touhara et al. 1993; Hidayat and Goodman 1994). The previous studies suggested that the JHBP complex or JH-JHBP complex was bound by a membrane receptor, with JHBP acting as the first crucial member in the long JH signal transmission chain (Wiśniewski et al. 1987; Trowell 1992; Kochman and Wieczorek 1995; Sok et al. 2008). It has also been demonstrated that JHBP protects JH against degradation by non-specific esterases (Debski et al. 2004). In these ways, JHBP is crucial to JH signal transport. However, the knowledge about the

Communicated by S. Hohmann.

Electronic supplementary material The online version of this article (doi:10.1007/s00438-016-1245-5) contains supplementary material, which is available to authorized users.

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precise role, the protein plays in cellular compartments and in tissues is rather limited and obscure. The identification, expression patterns, and evolutionary relationship of *JHBP* family candidates are still incomplete.

Early studies described the sequencing, structure, and expression of *JHBP* in *Manduca sexta* (Orth et al. 2003). The *MsJHBP* gene is present as a single copy and is composed of five exons spanning 6.7 kb. The first exon encodes the upstream non-coding sequence (5′ UTR), signal peptide, and the first two amino acids of the open reading frame, while the other exons encode the rest of the mature protein and the downstream non-coding sequence (3′ UTR). The structure of the *Galleria mellonella JHBP* gene is similar, consisting of five exons and four introns (Sok et al. 2005).

Analysis of the JHBP structure has aided understanding of this protein's function. Using ^{1}H, ^{13}C, and ^{15}N NMR chemical shift signal assignments, the solution secondary structure of the JH III-bound mature JHBP from the silkworm *Bombyx mori* was estimated, contributing to the determination of the three-dimensional structure of JHBP complexed with JH (Suzuki et al. 2009). Based upon the crystal and solution structures of apo- and JH-bound JHBP from the silkworm, related JH signaling revealed a gate-latch mechanism of JH delivery in the hemolymph by JHBP (Suzuki et al. 2011). Furthermore, the structure of JHBP in complex with two 2-methyl-2, 4-pentanediol (MPD) molecules revealed that one molecule (MPD1) was found in the same hydrophobic cage as the epoxide of the JHBP-bound JH in the JH-binding pocket, while the other (MPD2) was bound in a second cavity, generating a significant change in conformation (Fujimoto et al. 2013).

At present, there are few reports that have characterized and analyzed the *JHBP* gene family in insects. By searching the silkworm EST database with the *Drosophila takeout* gene, nine *BmJHBP* genes were identified (Saito et al. 2006). The cDNAs of these genes were cloned, and the sequences were used to construct a phylogenetic tree of takeout/JHBPs revealed that *JHBP* genes could be divided into two major clades. Functional analysis also found that the development and feeding conditions of the silkworm larvae regulated the expression of *BmJHBP* genes in various tissues. Studies implied that the *BmJHBP* genes may be involved in the regulation of metabolism, growth, or development. Another study indicated that *Omphisa fuscidentalis* JHBPs share moderate homology with the JHBPs of the lepidopteran *Heliothis virescens* (52 % amino-acid identity) but less homology with BmJHBPs (45 %) and MsJHBPs (44 %). *OfJHBP* genes are expressed at a moderately high level in the fat body during the third, fourth, and fifth instars, after which expression rapidly rises, peaking during the early diapause (Ritdachyeng et al. 2012).

The silkworm, a typical representative of lepidopteran insects, has extensively been used as a model organism in agricultural studies and has been considered to be a putative experimental animal in biology and human disease research (Xia et al. 2004; Goldsmith et al. 2005; Tabunoki et al. 2016). However, the previous studies investigating *JHBP* genes have not been conducted at a genome-wide or evolutionary scale. Moreover, the expression patterns of *BmJHBP* genes have not been analyzed comprehensively in various tissues or developmental periods. The functional distinctions between different types of JHBPs have not yet been elucidated. Identifying *JHBP* genes in a genome-wide screen of the silkworm will contribute to greater functional understanding of this gene family.

In this study, we used BLAST to query the genome sequence of *B. mori* and identified 41 *BmJHBP* candidates. We analyzed these *BmJHBP* candidates through the multiple sequence alignment and construction of a phylogenetic tree. Using RT-PCR, the expression patterns of these genes were analyzed in multiple silkworm tissues on day 3 of the fifth instar larvae. In addition, we further analyzed one of the genes, *BmJHBPd2* (Accession Number BGIBMGA011460), which was specifically expressed in the posterior of the silk gland (PSG), by Western Blotting, and immunofluorescent assay.

Materials and methods

Materials

The Chinese silkworm strain Dazao (usually yield silk strain) was obtained from the Gene Resource Library of Domesticated Silkworm, Southwest University, China. Fresh mulberry leaves were maintained at 70–80 % relative humidity, and silkworm larvae were fed in an environment maintained at 25 °C under a photoperiod of 12 h light/12 h dark.

Identification of *JHBP* gene family members

The hidden Markov model (HMM) in the Pfam database (Finn et al. 2016) was used to search Pfam for the JHBP superfamily. The JHBP Pfam sequence (PF06585) was then used to search for *BmJHBP* genes in the silkworm database (SilkDB) using BLAST (E value $\leq$1e − 10) (Xia et al. 2004; Wang et al. 2005; Duan et al. 2010). To conduct the most comprehensive search for *BmJHBP* genes, the PF06585 sequence was also used to search the publically available Geneset-A table file in the KAIKObase (Mita et al. 2004; Suetsugu et al. 2013). In the case of *BmJHBP* genes that were identical, similar, or overlapping between SilkDB and KAIKObase, we selected the longer of the two.

To determine whether the genes contained JHBP domains, protein domains were predicted with hmmscan (Finn et al. 2015) and SMART (Schultz et al. 1998; Letunic et al. 2015). The same procedures were employed to search for *JHBP* family candidates in the protein databases of the following insects: *Drosophila melanogaster* (Attrill et al. 2016), *Danaus plexippus* (Zhan and Reppert 2013), *Heliconius Melpomene* (ftp://ftp.ensemblgenomes.org/pub/), *Apis mellifera* (http://www.ncbi.nlm.nih.gov/), and *Plutella xylostella* (http://www.ncbi.nlm.nih.gov/).

Chromosomal distribution of *BmJHBP* genes

The silkworm genome sequence and single-nucleotide polymorphism (SNP) marker linkage map (Duan et al. 2010) were used (E value $\leq 1e-6$) to determine the chromosomal positions of *BmJHBP* genes.

Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment of BmJHBP was conducted using ClustalX (Larkin et al. 2007). Alignment output was visualized using ENDscript 2.0 (Robert and Gouet 2014). A neighbor-joining (NJ) tree was constructed using MEGA 6.06 with a Poisson model, pairwise deletion of gaps, and 1000 bootstrap replicates. Branch lengths were based on phylogenetic distances. The phylogenetic tree was visualized using MEGA 6.06 (Tamura et al. 2013).

Expression patterns based on microarray database

To determine the expression patterns of *BmJHBP* genes, we downloaded the microarray data for the *BmJHBP* genes from the SilkMDB (Xia et al. 2007), revealing the genome-wide expression levels across multiple tissues and developmental periods. Normalized and filtered expression data were used to generate a heat map for hierarchical clustering with the program Multi-Experiment Viewer (MeV) (Yeung et al. 2001).

RNA extraction

Larval tissues from day 3 of the fifth instar were isolated and ground, including the following regions: ASG, MSG, PSG, hemocyte, brain, integument, midgut, fat body, Malpighian tubule, testis, and ovary. In addition, the PSG was isolated and ground from larvae across the development stages, including day 3 of the fourth instar (IV-D3), 1 h of fourth molting (IV-M-1 h), 24 h of fourth molting (IV-M-24 h), day 0 of the fifth instar (V-D0), day 1 of the fifth instar (V-D1), day 3 of the fifth instar (V-D3), day 5 of the fifth instar (V-D5), day 7 of the fifth instar (V-D7), day 1 of wandering (W-D1), and day 2 of wandering (W-D2). Total RNAs were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA concentrations were quantified by spectrophotometer at 260 nm. Single-stranded cDNAs were synthesized from total RNA (1 μg) of the various tissues using a cDNA synthesis kit (Takara Bio, Japan). Synthesized cDNA products were assessed using 1 % agarose gel electrophoresis. The *B. mori* ribosomal protein L3 (*RPL3*, forward primer: 5′ TCGTCATCGTGG TAAGGTCAA 3′; reverse primer: 5′ TTTGTATCCTTTG CCCTTGGT 3′) was used as an internal control.

Expression pattern validation with RT-PCR

To verify the expression patterns of the microarray data, we used Primer Premier 5.0 to design primers for cloning the *BmJHBP* genes. RT-PCR amplification was then performed on the cDNAs from various silkworm tissues to examine their expression profiles. Each 10 μL PCR reaction was conducted under the following conditions: the initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 15 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 7 min before storing at 4 °C. RT-PCR products were analyzed on 1.2 % agarose gels.

BmJHBPd2 expression pattern, tissue distribution, and localization

BmJHBPd2 peptides (N → C: CQNLKFDMDKKIIAA) were synthesized and used to prepare a polypeptide antibody against BmJHBPd2. Total proteins were extracted with the Total Protein Extraction Kit (Beyotime Biotechnology, China) from the various tissues of larvae on day 3 of the fifth instar, including the ASG, MSG, PSG, brain, integument, midgut, fat body, Malpighian tubule, and genital gland (testis and ovary). Western Blotting assays were conducted with JHBP antibody, with tubulin used as an internal control. Fresh silk glands were removed from larvae on day 3 of the fifth instar and were divided into ASG, MSG, and PSG sections (Maekawa and Suzuki 1980). These were immediately fixed for 2 h with 4 % paraformaldehyde solution and were then stored in 70 % alcohol. Tissues were dehydrated by an ethanol gradient and invaded with paraffin, chopped into 5 μm sections (Leica, Germany), and heated at 60 °C for 1 h. The silk gland sections were then incubated for 20 min for antigen retrieval using 10 mM citrate buffer (pH 6.0) at 95 °C. The samples were treated with 10 % (w/v) bovine serum in 10 mM phosphate-buffered saline (PBS, pH 7.5) at 37 °C for 30 min, incubated with prepared antibody (1:200) in 10 mM PBS and secondary antibodies (1:500; Beyotime) in 10 mM PBS at 37 °C for 1.5 h, and then treated with 1 % DAPI solution in 10 mM PBS. The antibody and DAPI solutions were

washed with PBS three times after incubation (Nie et al. 2015). Fluorescent images were captured with a fluorescence microscope (Carl Zeiss, Germany).

Results

Identification, chromosomal distribution, and structure analysis of *JHBP* gene family members

A total of 41 *BmJHBP* gene family candidates were identified in the silkworm genome sequence, and the information, including number of exons, open reading frame (ORF) length, amino-acid length, JHBP-domain length, chromosomal distribution, number of signal peptides, and gene names was collected for each (Table 1). The 41 identified *BmJHBP* genes are located on eight chromosomes (Fig. 1), though the majority are distributed on just two chromosomes: chromosome 15 (15 genes) and chromosome 23 (20 genes). The remaining six chromosomes contain only one *BmJHBP* gene each: *BmJHBPc14* on chromosome 5, *BmJHBPd7* on chromosome 7, *BmJHBPa7* on chromosome 11, *BmJHBa8* on chromosome 12, *BmJHBPc11* on chromosome 13, and *BmJHBPc9* on chromosome 16.

To analyze *BmJHBP* gene structures, ORF sequences were aligned to the genome, revealing a high degree of structural complexity. All *BmJHBP* genes contain introns, with exon numbers ranging from two to ten, though four to six is typical (Fig. 2). Five genes (*BmJHBPc6*, *BmJHBPc8*, *BmJHBPd4*, *BmJHBPd5*, and *BmJHBPd12*) exceed 14 kb in length. *BmJHBPd12* contains the longest introns and is also the longest gene, while *BmJHBPd7* is the shortest gene and contains only two exons. *BmJHBP* genes exhibit a relative diversity in intron–exon structure, and parallel intron–exon structure of *BmJHBP* has not been found.

All of the BmJHBPs contain only one JHBP-domain each, except for BmJHBPd4 and BmJHBPd13, which contain two and three JHBP domains, respectively. The protein BmJHBPa1, in addition to containing a JHBP domain in the N-terminal region, also contains a Grp7_allergen domain near the C-terminal region (Supplementary Fig. 1).

Multiple sequence alignment and evolutionary analysis of BmJHBPs

To assess the sequence conservation of BmJHBPs, we constructed a multiple sequence alignment (Supplementary Figs. 2, 3, and 4). The alignment revealed that the conserved JHBP domains were consistently situated near the C-terminal and N-terminal regions, while the remainder of the protein was not highly conserved. To analyze evolutionary relationships among *BmJHBP* genes, a phylogenetic tree was constructed with JHBP sequences from *B. mori* and five other insect species, including 40 *D. plexippus*, 27 *H. melpomene*, and 43 *P. xylostella* (*Lepidoptera*) sequences, 29 *D. melanogaster* (*Diptera*) sequences, and 17 *A. mellifera* (*Hymenoptera*) sequences (Fig. 3). The phylogenetic tree showed that *BmJHBP* genes can be divided into two major clades and four subfamilies: JHBP-A, JHBP-B, JHBP-C, and JHBP-D. Clade I includes the subfamilies, JHBP-A and JHBP-B, and contains nine *BmJHBP* genes, while clade II consists of the JHBP-C and JHBP-D subfamilies and the remaining 32 *BmJHBP* genes. Of the nine *BmJHBP* genes in clade I, five genes contain six or more exons, while in clade II, 25 of the 32 *BmJHBP* genes contain five or fewer exons (Figs. 2, 3). Among the four subfamilies, JHBP-A exhibited the highest conservation. A subset of the JHBP-A genes, including three *BmJHBP* genes, four *DmJHBP* genes, four *AmJHBP* genes, four *PxJHBP* genes, and six *DpJHBP* genes, clustered together. In subfamily JHBP-B, only one JHBPs from each of the three insects exhibited close, orthologous relationships.

Expression pattern of *BmJHBP* genes

The microarray data for 35 *BmJHBP* genes were downloaded from the SilkMDB database, detailing expression patterns in various tissues and developmental periods, as well as both sexes of silkworm (Fig. 4). Data for six genes were not founded in the database. *BmJHBP* genes generally exhibited higher expression levels in the testis, ovary, head, fat body, integument, and Malpighian tubule, and exhibited relatively lower expression in the hemocyte, ASG, MSG, and PSG. The expression levels of *BmJHBPa8*, *BmJHBPc4*, *BmJHBPc8*, *BmJHBPc9*, *BmJHBPc10*, *BmJHBPd6*, and *BmJHBPd10* were lower overall than those of other genes, regardless of the tissue, developmental period, or sex, while the expression levels of *BmJHBPa2*, *BmJHBPc12*, and *BmJHBPd5* were higher overall.

To validate the microarray data that have been detected and to supplement the incomplete data, tissue expression patterns of BmJHBP gene family members in larvae on day 3 of the fifth instar were analyzed by RT-PCR (Fig. 5). Primers are listed in Table 2. The tissue expression levels based on RT-PCR are consistent with the microarray results, revealing generally higher expression in the head, integument, midgut, fat body, testis, and ovary and relatively lower expression in the ASG, MSG, PSG, hemocyte, and Malpighian tubule. The highest expression overall was found in the head tissue, particularly from *BmJHBPc12* and *BmJHBPc14*. *BmJHBPa4*, *BmJHBPa5*, *BmJHBPa6*, *BmJHBPa7*, *BmJHBPc6*, and *BmJHBPd6* were highly expressed in the midgut, while *BmJHBPd3* was highly expressed in the

Table 1 Characteristics of genes-encoding juvenile hormone-binding proteins in the silkworm *B. mori*

Gene name	Number of exons	cDNA length (ORF)	Protein length (amino acids)	Number of JHBP domains (positions)	Location (Chr.)	Number of signal peptides	Accession numbers
BmJHBPa1	3	1371	456	1 (4–228)	15	1	BGIBMGA003344
BmJHBPa2	7	711	236	1 (26–233)	15	1	BMgn003322
BmJHBPa3	7	684	227	1 (29–224)	15	1	BGIBMGA003323
BmJHBPa4	8	681	226	1 (6–226)	15	1	BMgn003325
BmJHBPa5	7	765	254	1 (54–254)	15	1	BGIBMGA014298
BmJHBPa6	7	675	224	1 (3–224)	15	1	BGIBMGA003326
BmJHBPa7	7	816	271	1 (47–265)	11	–	BMgn001762
BmJHBPa8	6	783	260	1 (21–248)	12	1	BMgn010410
BmJHBPb1	6	774	257	1 (3–257)	15	–	BGIBMGA003408
BmJHBPc1	7	774	257	1 (18–240)	15	1	BMgn016212
BmJHBPc2	4	720	239	1 (18–239)	23	1	BMgn011552
BmJHBPc3	5	735	244	1 (18–241)	23	1	BMgn011076
BmJHBPc4	5	789	262	1 (35–262)	15	1	BMgn003407
BmJHBPc5	5	726	241	1 (18–240)	23	1	BMgn016913
BmJHBPc6	6	795	264	1 (42–264)	15	–	BGIBMGA003345
BmJHBPc7	5	870	289	1 (1463–287)	15	–	BGIBMGA003404
BmJHBPc8	5	735	244	1 (18–244)	15	1	BMgn003405
BmJHBPc9	6	741	246	1 (18–245)	16	1	BMgn012768
BmJHBPc10	5	750	249	1 (17-–244)	15	1	BMgn003406
BmJHBPc11	5	750	249	1 (21–246)	13	1	BMgn001164
BmJHBPc12	6	717	238	1 (18–237)	23	1	BMgn011078
BmJHBPc13	5	693	230	1 (13–230)	23	1	BMgn016915
BmJHBPc14	6	750	249	1 (18–247)	5	1	BMgn003736
BmJHBPd1	5	714	237	1 (16–237)	23	1	BMgn001325
BmJHBPd2	5	732	243	1 (18–243)	23	1	BGIBMGA011460
BmJHBPd3	5	711	236	1 (18–236)	23	1	BGIBMGA001324
BmJHBPd4	10	1431	476	3 (1–78; 68–192; 256–476)	23	–	BGIBMGA011077
BmJHBPd5	6	711	236	1 (18–236)	23	1	BMgn001308
BmJHBPd6	5	720	239	1 (21–239)	23	1	BMgn011575
BmJHBPd7	2	465	154	1 (32–143)	7	–	BGIBMGA010179
BmJHBPd8	5	780	259	1 (20–251)	23	1	BGIBMGA011459
BmJHBPd9	5	774	257	1 (17–249)	23	1	BGIBMGA011458
BmJHBPd10	5	753	250	1 (24–245)	23	1	BGIBMGA011555
BmJHBPd11	5	726	241	1 (22–241)	23	1	BGIBMGA011457
BmJHBPd12	5	753	250	1 (25–246)	23	1	BMgn011556
BmJHBPd13	4	579	192	2 (1–74; 66–191)	15	–	BGIBMGA003346
BmJHBPd14	5	720	239	1 (16–238)	23	1	BGIBMGA011075
BmJHBPd15	5	687	228	1 (1–228)	15	–	BGIBMGA003342
BmJHBPd16	4	687	228	1 (7–228)	23	1	BMgn011553
BmJHBPd17	3	711	236	1 (18–236)	23	1	BMgn016916
BmJHBPd18	5	732	243	1 (18–241)	23	1	BGIBMGA011549

"–" indicates that no signal peptide was predicted

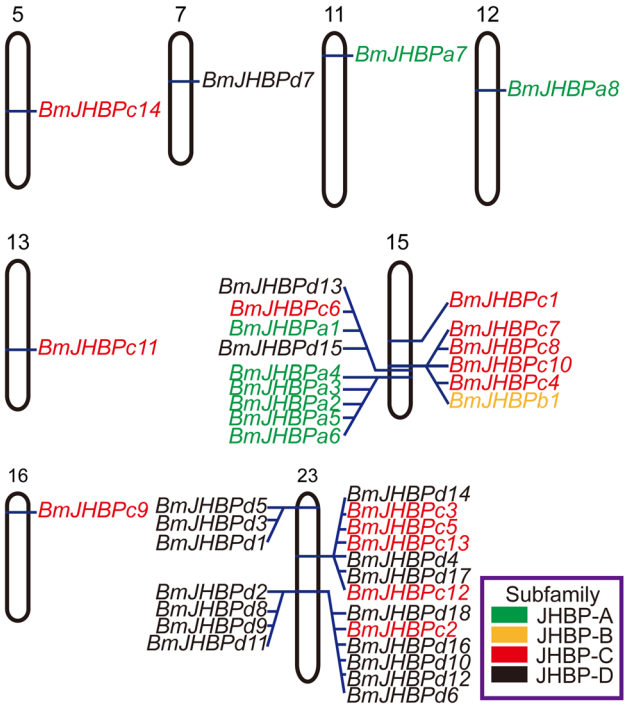


Fig. 1 Chromosomal location of the juvenile hormone-binding protein family genes in silkworm. The eight chromosomes of the silkworm that contain *BmJHBP* genes are shown, indicating by the *blue lines* (remaining 20 chromosomes are not displayed). *Different font colors* are used to represent the four subfamilies of *BmJHBP* genes (colour figure online)

MSG. *BmJHBPb1* was highly expressed in the Malpighian tubule, and *BmJHBPa2* was highly expressed in multiple tissues. Notably, *BmJHBPd2* was specifically expressed in the PSG at a high level, and was the only gene to demonstrate this particular expression pattern.

Expression pattern, tissue distribution, and localization of BmJHBPd2

The expression of the BmJHBPd2 protein over various developmental periods, as well as the tissue distribution and localization, were further assessed. *BmJHBPd2* mRNA level was found to be present at high levels in IV-D3 larvae but at low levels at the fourth molting. Expression was then high again in V-D1, V-D3, and V-D5 larvae, after which point expression decreased in V-D7, with no expression in W-D1 or W-D2 larvae (Fig. 6a). Based on Western Blotting, total protein isolated from day 3 of the fifth instar also showed that BmJHBPd2 was most highly expressed in the PSG, followed by the MSG (Fig. 6b). To determine the location of BmJHBPd2 expression in silk gland sections, immunofluorescence assays were performed with a prepared BmJHBPd2 antibody. Results indicated that the BmJHBPd2 protein is mainly distributed in the cytoplasm in PSG (Fig. 7).

Discussion

BmJHBP genes with different structures and expression patterns may be related to various functions

JH is known to control and regulate a variety of physiological processes in insects (Riddiford 1994; Wyatt and Davey 1996). As the JH carrier and first protein in the JH signal transmission chain, JHBP plays a vital role in proper JH functioning (Sok et al. 2008). In this study, we identified 41 JHBP-domain-containing proteins in silkworm (Table 1).

Among the eight subfamily JHBP-A genes, six are located on chromosome 15 (Fig. 1). *BmJHBPa7* and *BmJHBPa8*, however, are located on other chromosomes, and their different locations in the phylogenetic tree suggest that they may play distinct biological roles. Some *BmJHBP* genes from different subfamilies are nonetheless located on the same chromosomes, suggesting they have undergone duplication or splicing events since divergence from a common ancestor. In addition, as described in Fig. 3, proteins from subfamily JHBP-A exhibited higher conservation and homology compared with those of subfamilies JHBP-B, JHBP-C, and JHBP-D, which may reflect structural or functional differences.

Gene structures of the 41 *BmJHBP* genes revealed a high level of complexity (Fig. 2), with exon numbers ranging from two to ten per gene. In clade I, genes overall exhibited more exons than clade II genes (Fig. 3), which may indicate that intron–exon structure has played a role in the evolution of the JHBP gene family (Long et al. 1995). The first 20 amino acids of the proteins generally consist of a signal peptide (Table 1), which may be related to the secretory characteristics of BmJHBP proteins. BmJHBPd4 and BmJHBPd13 contain two and three JHBP domains, respectively, which may affect their JH-binding ability. Interestingly, BmJHBPa1 also encodes a Grp7_allergen domain (Supplementary Fig. 1), and related genes were detected in *D. melanogaster* (accession number FBPP0077261), *A. mellifera* (gil328791671), *D. plexippus* (DPOGS208697), and *P. xylostella* (Px005557.1). A previous study involving a DALI search for structures homologous to Der f 7 showed that, aside from Der p 7, JHBP is the protein most structurally related to Der f 7 (Tan et al. 2012). Further studies of *JHBP* genes-containing Grp7_allergen domains may elucidate the specific functions and structures of these genes.

Using silkworm JHBP sequences, we identified JHBP family members in five other insect species (Fig. 3). The number of *JHBP* genes in *B. mori* is most similar to that in *D. plexippus* and *P. xylostella*, both lepidopteran insects, while the number in *H. melpomene* (*Lepidoptera*), *D. melanogaster* (*Diptera*), and *A. mellifera* (*Hymenoptera*) differed considerably. This may indicate that the *JHBP* gene family has undergone gene expansion or reduction during

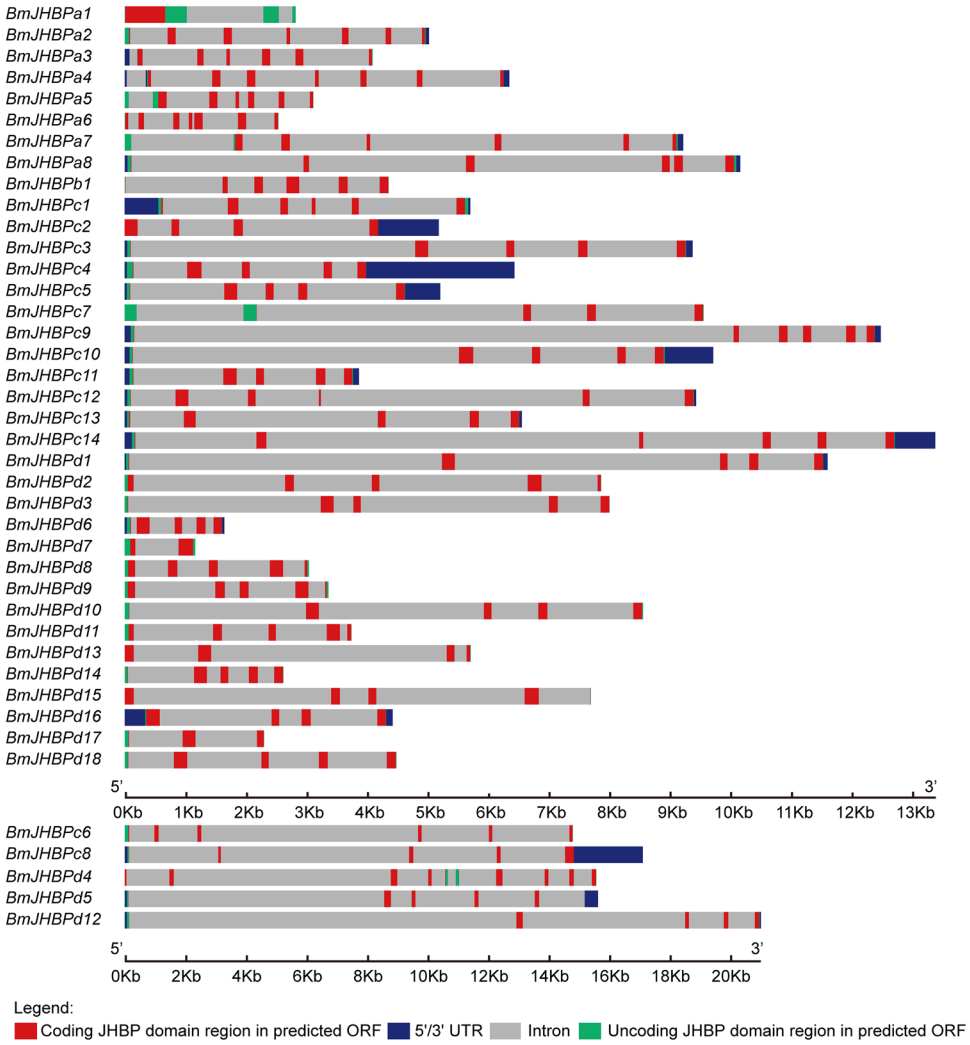


Fig. 2 Structural analysis of the *BmJHBP* genes. Different plotting scales are used, because of distinction of gene lengths. Various gene structures are represented by different *line colors*. *Red box* as coding JHBP domain in predicted ORF; *blue box* as 5′ and 3′ UTR region of mRNA; *grey box* as intron; and *green box* as uncoding JHBP domain in predicted ORF (colour figure online)

the course of evolution among lepidopteran, dipteran, and hymenopteran insects. Furthermore, lepidopteran, dipteran, and hymenopteran insects may exist different JH regulating mechanism, because of *JHBP* amount differences.

BmJHBP family members exhibit different expression levels in different tissues. The previous studies showed that *JHBP* genes were mainly expressed in the fat body, which is an important tissue in silkworm, as it stores nutrients and is responsible for metabolism (Ritdachyeng et al. 2012). In our study, *BmJHBP* genes revealed high levels of expression in the head, integument, midgut, testis, and ovary (Fig. 5). Different expression levels of *BmJHBP* among different tissues may be related to functional differences in the ASG, MSG, PSG, hemocyte, brain, integument, midgut, fat body, Malpighian tubule, testis, and ovary. In this case, JHBP may regulate insect tissue growth and development in different ways or at different levels in each tissue.

BmJHBPd2 may be related to silk protein synthesis

Silk, composed of proteins generated by the silk glands and stored in the lumen of the glands before being secreted, is a product with high economic value (Xia et al. 2014). Two major silk proteins, fibroin and sericin, are produced in the PSG and MSG, respectively. In our study, BmJHBPd2 was found to be unique in that it was highly expressed in the PSG cytoplasm in larvae on day 3 of the fifth instar (Fig. 7). In studying the expression pattern of *BmJHBPd2* in the PSG from the fourth instar to wandering, we found that it was highly expressed from day 1 to 5 of the fifth instar (Fig. 6a), coinciding with the production of silk proteins, particularly the fibroin heavy chain (*fib-H*) (Zhao et al. 2015).

BmJHBP, as the JH carrier and the first element of the JH signal transmission chain, is present in tissues and cells

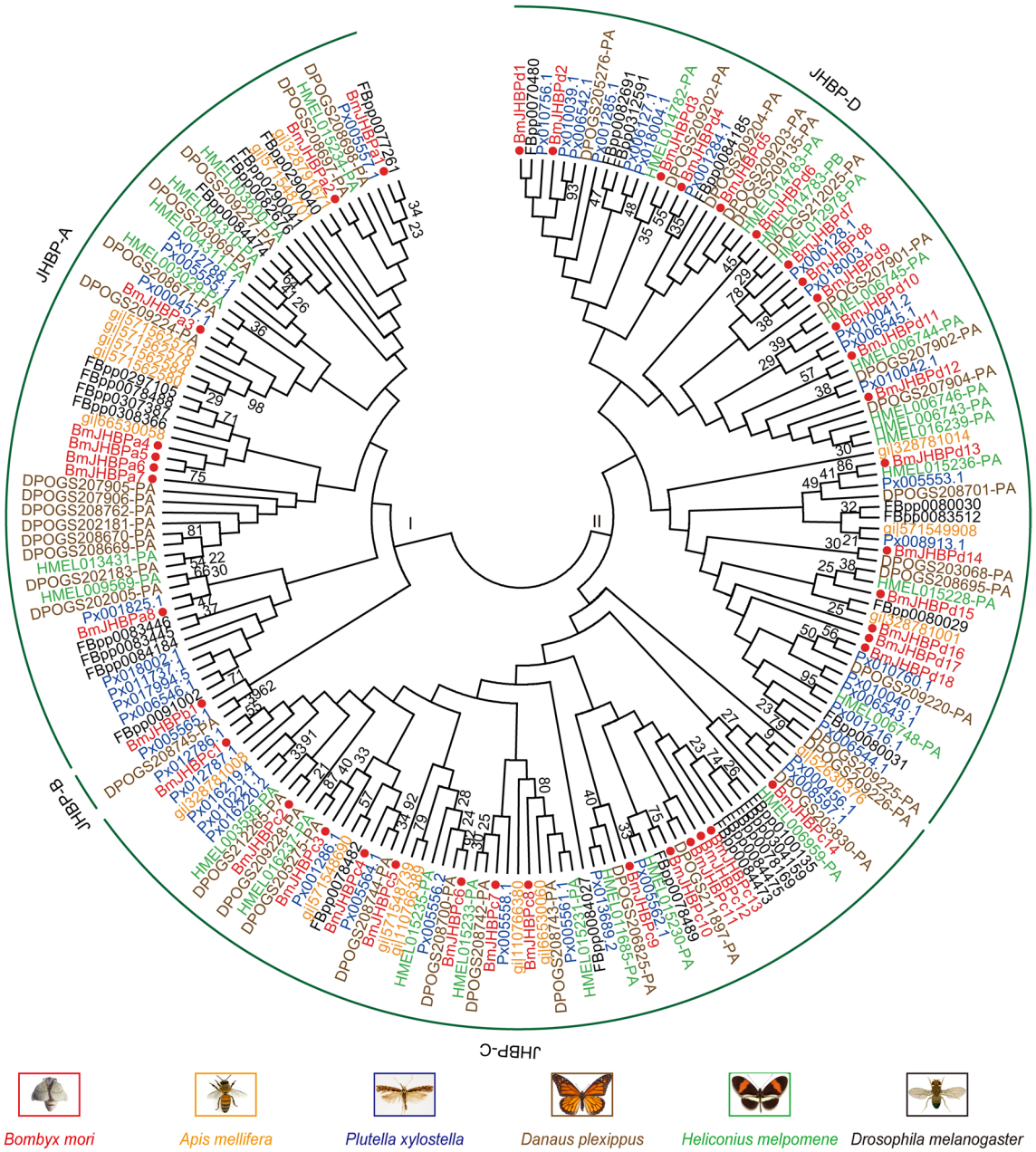
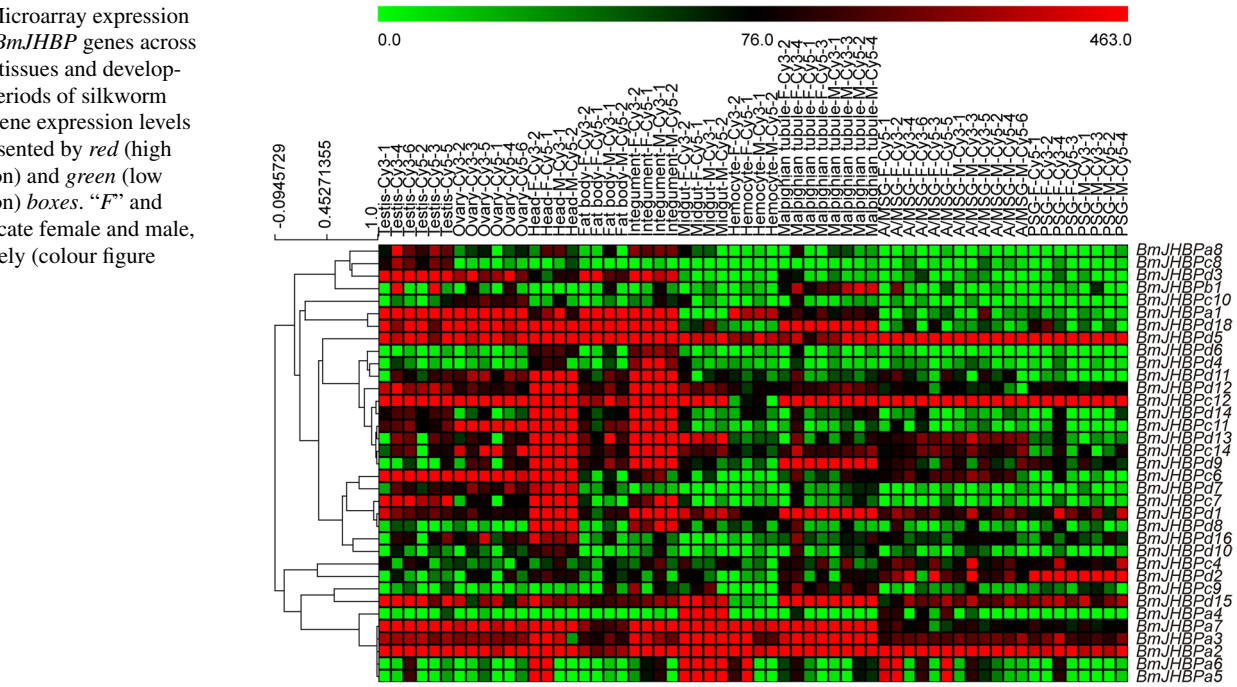


Fig. 3 Evolutionary analysis of the JHBP superfamily. A phylogenetic tree was constructed with the neighbor-joining method in MEGA6. Bootstrap values (%) for 1000 replicates are shown next to the nodes (those ≤20 % are omitted). JHBP-A, JHBP-B, JHBP-C, and JHBP-D subfamilies are shown. The roman numerals *I* and *II* indicate two major clades. Accession numbers for 41 BmJHBP genes are shown in Table 1. Font colors represent genes from different insects: *Bombyx mori* (*red*), *Apis mellifera* (*orange*), *Plutella xylostella* (*blue*), *Danaus plexippus* (*brown*), *Heliconius melpomene* (*green*), and *Drosophila melanogaster* (*black*). The species photos are cited from http://www.arkive.org/coco-de-mer/lodoicea-maldivica/image-G4793.html (*B. mori*), http://bugguide.net/node/view/603265 (*Apis mellifera*), http://www.vilkenart.se/Art.aspx?Namn=Plutella_xylostella (*Plutella xylostella*), https://commons.wikimedia.org/wiki/Danaus_plexippus (*Danaus plexippus*), http://butterfliesofamerica.com/heliconius_melpomene_rosina_specimens.htm (*Heliconius melpomene*), and http://www.uniprot.org/taxonomy/7227 (*Drosophila melanogaster*) (colour figure online)

along with JH (Trowell 1992; Kochman and Wieczorek 1995). The JH titer sharply decreases on day 1 of the fifth instar in the silkworm hemolymph and reaches its lowest level before day 6 of the fifth instar (Furuta et al. 2013). The JH titer of the silk gland itself, however, has not been examined. Therefore, there are three possibilities for the function of BmJHBPd2. One is that BmJHBPd2 binds JH which from the hemolymph of the larvae at the beginning of the fifth instar, and releases into silk gland cell continuously, which maintain the young status of the silk gland, so



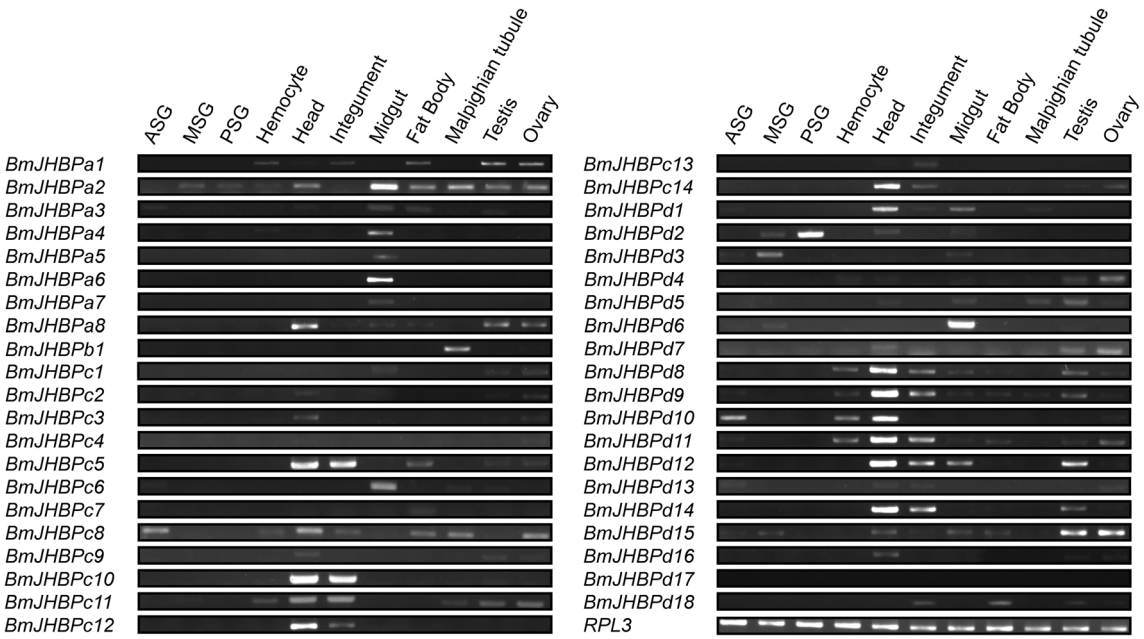


Fig. 4 Microarray expression data for *BmJHBP* genes across multiple tissues and developmental periods of silkworm larvae. Gene expression levels are represented by *red* (high expression) and *green* (low expression) *boxes*. "*F*" and "*M*" indicate female and male, respectively (colour figure online)

Fig. 5 Expression patterns of *BmJHBP* genes in larvae on day 3 of the fifth instar. RT-PCR was performed using gene-specific primers, and the *RPL3* gene was used as an internal control

that the silk protein synthesis can be continued. The other is that BmJHBPd2 involves in the expression regulation of the *fib-H* indirectly. In our previous reports, we found that *Bmdimm* directly regulated the expression of *fib-H* through JH-Met-Kr-h1 signaling pathway (Zhao et al. 2015). The third is that there is no evidence that JH can be synthesized in silk gland independently, but also no data showed that silk gland cannot synthesis JH, whether it is possible that BmJHBPd2 binds JH which is synthesized in silk gland and protected it, just play the role of other JHBP in hemolymph. However, which one is closed to the truth, it needs further researches.

In summary, 41 *B. mori JHBP* genes, 40 *D. plexippus JHBP* genes, 27 *H. Melpomene JHBP* genes, and 43 *P.*

Table 2 Primer sequences used in this study

Gene	F (5′–3′)	R (5′–3′)
BmJHBPa1	ACTGAGGGTGACAGCGAACG	GCTTTTACCGATGATGCTTGAC
BmJHBPa2	GGAGATAAACGCACGTCAAGAG	CAATAGTGGTTGGCACGGTCT
BmJHBPa3	ATCTTTGCCCTTGCTGTT	GACGTTCCTGACGGTGTAT
BmJHBPa4	AAGGAGTTCCGAGCTTGCAC	TCAGACTCGACATCACCAACG
BmJHBPa5	AAGCAGGTGGAACATGATTTGA	TGACTGTGGAGACCAGACGAAG
BmJHBPa6	GCCTTCGCATTTGTTCTTGTT	CGAGGAGTTCGGGAAGTCTGT
BmJHBPa7	TCAAACGATACCGCTTCC	ACCGCCTAAATCCAAGTC
BmJHBPa8	CGTGCTTGAAAAGTGACCCC	TCCTCCAAAGATTGGATGCC
BmJHBPb1	AAATTAGCCACAGGACTGCC	GCTTCCTCCATCGGCACT
BmJHBPc1	TGGAAATCGCAACATTCTCATC	AGCCAGGTAATGCCAGGTGT
BmJHBPc2	ATTCGGAGCCTTACCTGATGTC	TCAACGCTTTCCAATTTTGATT
BmJHBPc3	TCTCCCAACGCCTGAACTATAC	TGACGCTTTCGATGAACTTTTC
BmJHBPc4	ACGACATCACCCGATTTACCA	TGCGTCCCTTATCACCATTCT
BmJHBPc5	TTCGCAAGCCCTTTATGATTT	GCCCTGGAGTTCTTTCGCTAC
BmJHBPc6	CATATTCGGAGGGAAATGTTCA	TGGAGTACGGCGTTTGAGTTAT
BmJHBPc7	TGTTGGAGGTGATGTTGAGTC	CCCTTGTTCTGATTGATTGC
BmJHBPc8	ATTGCGTTGGCTAGAGGTCC	TTTCGCCGTTATTTTGTTCG
BmJHBPc9	AACGCGATATTATTGGGTGC	CTCCGATGAGGTCGTGCTT
BmJHBPc10	ACGCCCGACTTCTTTCCTC	CGGCAGCCAGTGGTCTTT
BmJHBPc11	TACGAAGGGTATTCCTGAACTGG	ATTTGCTTGATAGCAGGAGGTG
BmJHBPc12	GTCAACGCCGATAATGGAGAA	AAGCTGGCAGCTCGATTTCT
BmJHBPc13	TTTTTCAGTGCTTCGGGTG	TGGTATTTCCTGCCCGTT
BmJHBPc14	GCGACAGGCATCGAGGAG	AGCTCGCGGTACAGCACTCT
BmJHBPd1	TTGGAGATTCTGCCTGTTTTGT	GATGGGCGGGGCTACTTC
BmJHBPd2	ATGTGGACCGGTCTGTTTTTAGT	TTATTCGGGCATTAATTCGTCA
BmJHBPd3	GCGTCCGATGCGACAGAT	CACGGTTGCCGTTAAACAAAT
BmJHBPd4	CTGGCCTACCCGAACACG	TGCTTGGGCGAGAACATCA
BmJHBPd5	CAGATCCAAACCTCAACGAATG	CGGGAACTGCTCCGAAAA
BmJHBPd6	TTAAACATGGTGTTGCCAGAGG	AATTGGATCGCCAAAGTCTTCT
BmJHBPd7	AGACACCGCACCTATGTAAGT	CTGTTAATGGAACTTGCGTCA
BmJHBPd8	TTGTGCTTCGGCTTATGTTGA	AAATACGCCAGTTTTCGTTCAT
BmJHBPd9	TCTTCGGTTTAATTGTTTTCGG	TTTTCCATTTCCTTCGCCA
BmJHBPd10	ATACCGGAGTTCTCGAAAGGTC	CGTTCAATAGCTCGTCGTAAGG
BmJHBPd11	TCTCGAAGGGTATTCCGTCC	ACGGACCAGCTCATCGACA
BmJHBPd12	GACATTCCGACATTAGACCCG	AACTCTTCCGCAACTGTTTTCC
BmJHBPd13	ACAGGTGTACCGGAAATGGG	CTCCAGATGTCCGTGAAAGCT
BmJHBPd14	TCATATGCATCGAGGACCCC	CATTGCGAAACGGAAAGC
BmJHBPd15	GCTGGTGATGGAAAACTCTGC	ATGCTTGGCGATTGACGC
BmJHBPd16	ATGCGATGCCCGTCTTCTT	TAGCGTCCATAATTGGTCTTCC
BmJHBPd17	TGCCATACCCATTTTTGCT	CTTCAACGGGAATCGCTT
BmJHBPd18	AGCAGTGCGACAGAGCAGTTTT	CAAAGGTTCCATATCGGGTTC

Primers were used in RT-PCR

"*F*" forward primers sequences, "*R*" reverse primer sequences

xylostella JHBP genes, 29 *D. melanogaster JHBP* genes, and 17 *A. mellifera JHBP* genes were identified (Fig. 3). Phylogenetic tree analysis showed that *BmJHBP* genes could be classified into two major branches and four subfamilies (Fig. 3). Expression pattern analysis revealed that *BmJHBP* genes exhibit various expression patterns in different tissues, periods, and sexes (Fig. 5). BmJHBPd2 was demonstrate to be highly expressed in the PSG, coinciding with *fib-H* (Figs. 6, 7). The connection among BmJHBPd2, *fib-H*, and silk gland development need more

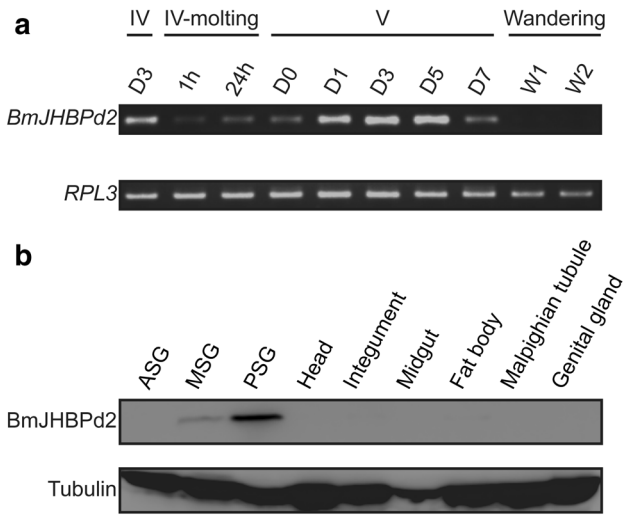


Fig. 6 BmJHBPd2 tissue distribution and expression pattern across various developmental periods. **a** *BmJHBPd2* expression in PSG across different stages was investigated by RT-PCR. *RPL3* was used as an internal control. Development stages are as follows: IV, fourth instar; V, fifth instar (day 0, 1, 3, 5, and 7); and wandering (day 1, and 2). **b** Tissue-specific distribution of BmJHBPd2 in larvae on day 3 of the fifth instar. Tissues were isolated, and the proteins were then incubated with BmJHBPd2 antibody. Tubulin was used as an internal control

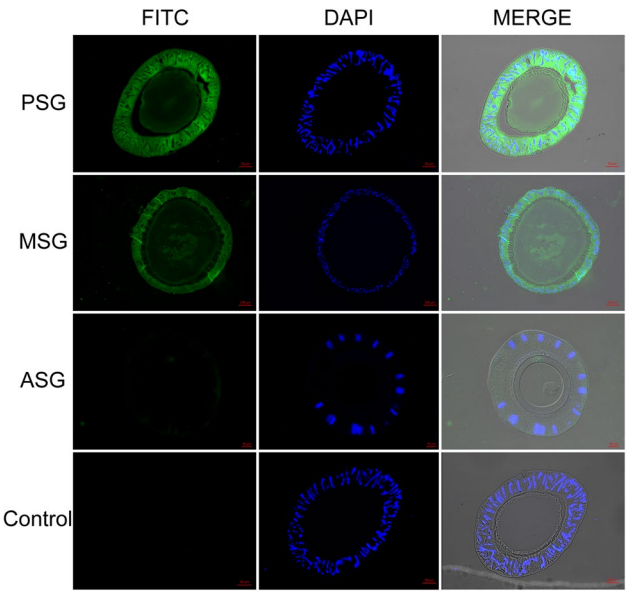


Fig. 7 Localization of BmJHBPd2 in the silk gland with immunofluorescence. Immunofluorescence was used to locate expression site in paraffin sections on day 3 of the fifth instar. BmJHBPd2 was localized in the cell cytoplasm in the PSG and to a lesser extent in the MSG. *Green* positive signal, *blue* DAPI signal (nuclei) (colour figure online)

research to explain. In addition, the reason why 5 JHBPs encode Grp7_allergen domains except for JHBP domains is still unknown (Supplementary Fig. 1). Our findings will help researchers to understand the JHBP family, providing potential applications in further function research.

Acknowledgments We thank M.S. Duolian Liu, and M.S. Li Peng for assistance in data processing.

Compliance with ethical standards

Funding This work was funded by the National Natural Science Foundation of China (31530071), the National Natural Science Foundation of China (31372380), and Chongqing Fundamental and Frontier Research Program (cstc2013jcyjA0500).

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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