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Expression of 20-hydroxyecdysone-induced genes in the silkworm brain and their functional analysis in post-embryonic development

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Abstract

The insect brain is the center of developmental control, from which ecdysone governs brain morphogenesis and regulates gene-expression cascades associated with molting and metamorphosis. In order to identify the 20-hydroxyecdysone (20E)-inducible genes responsible for molting and metamorphosis, we constructed a 20E-induced subtraction complementary DNA library from the fifth instar larval brain of the silkworm *Bombyx mori*. We isolated 10 genes, designated as *bombeil-1* to *bombeil-10*, three of which did not show any sequence similarity to previously identified *Bombyx* genes. Whole-mount *in situ* hybridization revealed that all of these *bombeil* messenger RNAs were exclusively located in two pairs of lateral neurosecretory cells in the larval brain, known as prothoracotropic hormone (PTTH)-producing cells. RNA-interference knockdown targeting *bombeil-2* resulted in larval–pupal molt defects, and adult wing and leg malformations. These results, together with the cell-specific co-localization of *bombeil* transcripts with *PTTH*, suggest that *bombeil* genes play important roles during larval–pupal–adult development.

Keywords: 20-hydroxyecdysone; brain; metamorphosis; molting; prothoracotropic hormone; RNA interference; silkworm.

1. Introduction

Ecdysone is an insect steroid hormone that is synthesized in, and released from, the prothoracic glands (PGs) upon stimulation by the brain neuropeptide, prothoracicotropic hormone (PTTH) (Gilbert, et al., 2002; Kawakami et al., 1990). The active form of ecdysone, 20-hydroxyecdysone (20E), controls various physiological and developmental processes underlying insect molting and metamorphosis (Riddiford, 1993; Riddiford et al., 2003). PTTH, which is the primary stimulator of ecdysteroidogenesis, is produced by two pairs of lateral neurosecretory cells (LNCs) in the brain (Agui et al., 1979; Kawakami et al., 1990; Mizoguchi et al., 1990). Besides PTTH, the brain produces a variety of other neuropeptides that control molting and metamorphosis. During insect post-embryonic development, 20E governs the morphological changes of the brain (Champlin and Truman, 1998; Kraft et al., 1998) by regulating the gene-expression cascade associated with molting and metamorphosis (Beckstead et al., 2005). The insect brain is thus the control center of insect growth and development, and plays a key role in post-embryonic development.

The 20E signal is transduced by the ecdysteroid receptor (EcR)–ultraspiracle (USP) complex (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1992, 1993). This receptor complex directly induces the transcription of the primary-response genes, known as early genes (Dibello et al., 1991; Segraves and Hogness, 1990; Thummel, 1995; Thummel et al., 1990). The early-gene products transduce and amplify the 20E signal by controlling the expression of the secondary-response late genes (Jiang et al., 2000; Thummel, 1996; White et al., 1997); these, in turn, appear to direct appropriate stage-specific and tissue-specific developmental responses to 20E. We recently demonstrated exclusive expression of *EcR* in the PTTH cells of the *Bombyx* brain (Hossain et al., 2006). A comprehensive analysis of ecdysone-inducible gene expression in the brain should therefore clarify the mechanisms of ecdysone action in development. To identify ecdysone-inducible genes in the brain, we prepared a brain-subtraction complementary DNA (cDNA) library, and identified 10 novel genes, all of which were expressed in the PTTH cells of

the brain. We also applied the RNA-interference (RNAi) gene-knockdown technique to elucidate the role of the 20E-induced genes identified.

2. Materials and methods

2.1. Animals and hormones

Bombyx eggs of the racial hybrid Kinshu × Showa were obtained from Ueda Sanshu (Ueda, Japan). The larvae were reared on an artificial diet (Silkmate II, Nihon Nousan Kogyo, Yokohama, Japan) under a 12-h light/12-h dark photoperiod at 25 ± 1°C. Newly molted fifth instar larvae were fed from the beginning of the photophase following the scotophase during which they molted to fifth instars. The 24-h period of the photophase following the scotophase during which the fourth instar larva molted was designated day 0 of the fifth instar (V0). Similarly, the 24-h period of the photophase following the scotophase during which the fifth instar larva pupated was designated day 0 of the pupal stage (P0). Days 1–10 after V0 and days 1–2 after P0 were denoted V1–V10 and P1–P2, respectively. The 20E solution (Sigma, St. Louis, MO, USA) for injection was prepared as described elsewhere (Hossain et al., 2006).

2.2. Subtraction of cDNA and construction of cDNA library

Half of V2 larvae were injected with 0.5 μg of 20E and the other half of V2 larvae were injected with 1 μg of 20E. We took two doses of 20E for the construction of the subtraction library to cover all 20E-induced genes. Poly(A)$^+$ RNA was prepared from brains dissected 2 h after injection of the 20E or control insect Ringer's solution (130 mM NaCl, 4.7 mM KCl, and 1.9 mM $CaCl_2$) using a Quick Prep Micro mRNA purification kit (GE Healthcare, Buckinghamshire, England). Subtraction of cDNA was performed with a PCR-Select cDNA subtraction kit (Clontech, Mountain View, CA). The subtracted cDNA was cloned into the pGEM-T vector (Promega, Madison, WI). About 10,000 clones of the resulting cDNA library were screened by

colony hybridization with reverse-transcribed poly(A)$^+$ RNA prepared from 20E-treated V2 brains as a probe, and 1,736 clones were isolated. About 5,000 clones were screened with the subtracted cDNA as a probe, and 451 clones were isolated. A second screening against the 2,187 positive clones was performed by Southern hybridization with the same subtracted cDNA as a probe. The inserts of the 1,442 positive clones were sequenced, and the positive clones were found to consist of more than 300 independent clones. RT-PCR against these clones identified 10 genes up-regulated by 20E in the brain.

2.3. RNA isolation and semi-quantitative RT-PCR

Total RNA was purified from V2 brains 2 h after injection of 20E (1 μg/larva; +20E) and insect Ringer's solution (–20E), as described previously (Tsuzuki et al., 2001). Total RNA was purified successively from V0 to V10 larval brains and from P0 to P2 pupal brains. Semi-quantitative RT-PCR was performed as described previously (Hossain et al., 2006) using gene-specific primers and the annealing temperatures shown in Table 1.

PCR involved 20–40 cycles of amplifications in a thermo-cycler (Gene Amp PCR System 9700, Applied Biosystems, Foster City, CA, USA) using a thermal cycle of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. The PCR products were separated on a 1.5% (w/v) agarose gel and visualized by ethidium bromide staining. No amplification occurred in the absence of reverse transcriptase even after 40 cycles of PCR (data not shown).

2.4. Whole-mount in situ hybridization

Whole-mount *in situ* hybridization of brains was performed as described previously (Hossain et al., 2006; Iwami et al., 1996). In brief, after with the application of fixative (85% [v/v] ethanol, 4% [w/v] formaldehyde, and 5% [v/v] acetic acid) on ice for 40 min, the brains were treated with proteinase K (0.05 mg/mL) at 37°C for 40 min, followed by re-fixation with 3% (w/v) paraformaldehyde at room temperature for 20 min. The brains were hybridized with 60 ng

bombeil probes and *PTTH* probes 3′-labeled with digoxigenin (Table 1, reverse sequences) using a Dig-labeling kit (Roche Diagnostics, Mannheim, Germany). Hybridization was carried out at 37°C for 20–48 h in 100 µl hybridization solution (50% [v/v] formamide, 5× standard sodium citrate [SSC; 0.15 m NaCl, and 0.015 M sodium citrate], and 5% (w/v) dextran sulfate. After several washes at room temperature, the brains were treated with 5% (v/v) sheep serum (Rockland, Gilbertsville, PA, USA) at 4°C for 15–20 h, followed by 2 h incubation with alkaline phosphatase-conjugated anti-digoxigenin immunoglobulin G (IgG; Roche Diagnostics) at room temperature. Color was developed with 4-nitroblue-tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate in the presence of 1 mM levamisole, as described elsewhere (Hossain et al., 2006). Negative controls omitted the labeled probes and used sense dig-labeled probes, and no signals were detected (data not shown).

2.5. Preparation of double-stranded RNA (dsRNA) and RNAi

Plasmid DNA was purified from a clone containing a 482 base pair (bp) fragment of the *bombeil-2* transcript isolated from the subtraction cDNA library. The plasmid was linearized with *Pst*I and *Nco*I. Sense and antisense RNAs were synthesized *in vitro* using T7 RNA polymerase (Takara, Otsu, Japan) and SP6 RNA polymerase (Takara), respectively. To generate dsRNA, equal amounts of sense and antisense RNAs were mixed, heated at 95°C for 5 min, and gradually cooled to 25°C. The solution was then treated with RNaseA (Nacalai Tesque, Kyoto, Japan) and DNase RQ1 (Promega) for 45 min at 37°C. The dsRNA was purified with phenol/chloroform followed by ethanol precipitation, and then dissolved in water. A 722-bp fragment of dsEGFP was used as a control. The enhanced green fluorescent protein (EGFP) sequence was derived from the pEGFP-N3 vector (Clontech, Palo Alto, CA), sub-cloned into the pGEM-7Zf(+) vector (Promega), and the dsRNA was prepared according to the protocol described above.

A 5-µg sample of the dsRNA of *bombeil-2* (dsbombeil-2) in 10 µl insect Ringer's solution was injected into V2 larvae through the first pro-leg. Ringer's solution and dsEGFP (5 µg) in Ringer's

solution were injected as controls. After injection, the larvae were maintained under normal rearing conditions until adult eclosion. To examine the effects of *bombeil-2* RNAi on brain development during the larval–pupal transformation, the brains were dissected at stage P2. To evaluate the knockdown effect of RNAi, total RNA was purified from the brains, the wing discs (WDs), and leg discs (LDs) of V4 larvae. A 1-μg sample of total RNA was reverse-transcribed, and the resulting cDNA was amplified for 30 or 35 cycles using a thermal cycle as follows: 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for *EcR-A*, *EcR-B1,* and *RpL32*; 94°C for 30 s, 64°C for 30 s, and 72°C for 30 s for *bombeil-2*; and 94°C for 30 s, 59°C for 30 s, and 72°C for 45 s for *PTTH*. The primers for PCR were designed from the nucleotide sequences (Table 1). There was no amplification without reverse transcriptase, even after 40 cycles of PCR (data not shown).

3. Results

3.1. 20E-induced gene expression in the brain

We identified 10 20E-induced genes, designated as *bombeil-1* to *bombeil-10* (GenBank accession nos. **AB372866**, **AB372867**, **AB372868**, **AB372869**, **AB372870**, **AB372871**, **AB372872**, **AB372873**, **AB372874**, and **AB372875**), in a brain-subtraction cDNA library. The *bombeil* genes differed in their responses to 20E (Fig. 1). Transcripts of *bombeil-5* and *bombeil-8* were detected after 20 cycles of amplification, indicating relatively high levels of induction by 20E (+20E) compared with the control (–20E). *Bombeil-1, bombeil-2, bombeil-3, bombeil-4, bombeil-6,* and *bombeil-7* transcripts were detected after 30 cycles of amplification, indicating moderate levels of induction. *Bombeil-9* and *bombeil-10* transcripts only appeared after 40 cycles of amplification, indicating low levels of induction. Ribosomal protein L32 (*RpL32*) was used as an internal standard, and showed no differences of expression in the +20E and –20E samples.

3.2. Cell-specific expression of bombeil gene

We determined the spatial localization of the *bombeil* transcripts by whole-mount *in situ* hybridization in the larval brains at V2, day 2 of the fifth instar. (Fig. 2). All of the transcripts were restricted to two pairs of LNCs. The localization of the *bombeil*-positive cells appeared to be the same as that of the PTTH cells (Fig. 2K). To confirm this finding, we performed *in situ* hybridization using a mixture of *bombeil* and *PTTH* probes. Hybridization signals were detected exclusively in the two pairs of LNCs (Fig. 2L-U), indicating that the *bombeil* genes were expressed in the PTTH cells of the fifth instar larval brain.

3.3. Stage-specific expression of bombeil genes during development

All *bombeil* gene expression in the brain (Fig. 3A) was found to be correlated with the endogenous hemolymph ecdysone titer (Fig. 3B): the fluctuations of *bombeil* expression levels in larval brains were correlated with the changes in 20E titer. *Bombeil-1* expression occurred transiently at V4, began to rise at V7, and reached a maximum level at V9 and V10, followed by a sharp decrease at P0. *Bombeil-2, bombeil-5, bombeil-6, bombeil-7,* and *bombeil-8* showed similar expression profiles, and were not expressed at the pupal stage. *Bombeil-3* expression was observed initially at V7 and continued until V10. By contrast, *bombeil-4* expression was detected at V4 and was maintained at a similar level until V10, with the exception of a drop at V9. The expression profiles of *bombeil-9* and *bombeil-10* differed from those of the other genes. *Bombeil-9* and *bombeil-10* were expressed only at V9 and V10, respectively. No expression of *bombeil* genes was detected at P0 and P2 (Fig. 3).

3.4. RNAi of bombeil-2

DsRNA synthesized from *bombeil-2* cDNA was injected into V2 larvae. After 2 days, the semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) revealed a reduced level of the transcript in the RNAi sample compared with the control (Ringer's solution; Fig. 4A). This indicated that the injected dsRNA effectively silenced *bombeil-2* in the brain.

The *bombeil-2* RNAi caused abnormal molts in 36 of 90 larvae (40%) at pupation (Fig. 4B). The remainder (60%) of the larvae pupated successfully and underwent adult eclosion. However, these adults exhibited prominent defects of the legs and wings and, to a lesser extent, of the cuticle (Fig. 4E, F). The controls developed into normal adults (Fig. 4G for Ringer's solution, and Figure 4H for dsRNA of dsEGFP). *Bombeil-2* RNAi also affected brain development. Among 60 P2 brains, 46 (77%) showed normal morphology (Fig. 4I), while 14 (23%) had abnormalities, including lateral compression and compaction (Fig. 4J). This result indicates that *bombeil-2* RNAi might also influence brain morphogenesis during the larval–pupal transformation.

Because *EcR* is expressed exclusively in V2 larval brains (Hossain et al., 2006) and *bombeil-2* expression is localized in PTTH cells, it was possible that down-regulation of *bombeil-2* influenced the expression of *EcR* and *PTTH*. To address this issue, we injected *bombeil-2* dsRNA into V2 larvae, and examined *PTTH* and *EcR* messenger RNA (mRNA) levels in their brains (Fig. 4). The dsRNA caused a dramatic decrease in the *EcR-A* mRNA level, and weakly affected *EcR-B1* expression (Fig. 4A). *RpL32* expression was not altered by the dsRNA.

RNAi of *bombeil-2* led to adult wing and leg defects, suggesting the involvement of *bombeil-2* in the differentiation of these appendages. We therefore measured endogenous *bombeil-2* expression levels by RT-PCR using total RNA from WDs and LDs of V4 larvae. We observed a low level of expression of *bombeil-2* in the WDs and a high level in the LDs (Fig. 4L). Injection of *bombeil-2* dsRNA resulted in reduced expression of *bombeil-2* in both WDs and LDs, indicating that it effectively silenced *bombeil-2* in these organs (Fig. 4M). RNAi did not affect *RpL32* expression (Fig. 4M). Among the EcR isoforms in the WDs and LDs, *bombeil-2* RNAi caused a large decrease in the *EcR-B1* mRNA level only in the LDs; no such decrease was noted for *EcR-A* in WDs and LDs, or for *EcR-B1* in WDs (Fig. 4M).

4. Discussion

We isolated 10 20E-induced genes, designated as *bombeil-1* to *bombeil-10*, from a subtraction cDNA library of the *Bombyx* larval brain. The expression of these genes was restricted exclusively to the PTTH cells (Fig. 2). In addition, the knockdown of *bombeil-2* by RNAi suggested that it played a critical role in larval–pupal–adult development (Fig. 4).

The fluctuations of *bombeil* expression levels in larval brains (Fig. 3) were correlated with the changes in 20E titer (Kaneko et al., 2006). *Bombeil-9* and *bombeil-10* were transcribed at a low level, while the other *bombeil* genes were transcribed at a high level (Fig. 1). *Bombeil-2, bombeil-4, bombeil-5, bombeil-6, bombeil-7,* and *bombeil-8* were expressed at a detectable level at V4, which is a crucial stage when dynamic cell proliferation occurs in WDs (Kawasaki et al., 2004). All of the *bombeil* genes, with the exceptions of *bombeil-9* and *bombeil-10*, were expressed at V7 (Fig. 3); this suggested their involvement in cell proliferation and differentiation at a time when the growth of many of the internal tissues and imaginal discs depends not on feeding but on hormone-regulated events (Nijhout et al., 2006).

BLAST analysis of the available silkworm databases, KAIKOBLAST (http://kaikoblast.dna.affrc.go.jp/) and SilkBase (http://morus.ab.a.u-tokyo.ac.jp/), revealed homologs of *bombeil-1, bombeil-4, bombeil-6, bombeil-7, bombeil-8, bombeil-9,* and *bombeil-10,* but not *bombeil-2, bombeil-3,* and *bombeil-5*, indicating that the last three are novel genes. The open reading frame of the isolated *Bombeil-1* clone encodes a 175 amino acids protein. This region of *bombeil-1* showed 98% identity with a clone (wds30400 of silkbase) that had 65% identity with a cytochrome P450 18a1 of *Drosophila*. This gene is thought to encode ecdysone 26-hydroxylase and to be involved in the 20E-inactivation pathway in *Drosophila* (Bassett et al., 1997). The most conserved region of cytochrome P450 is its heme-binding domain (PFXXGXRXCXG). A homology search for the P450 signature domain of *bombeil-1* revealed 100% identity with *CYP18a1* of *Drosophila* and *Spodoptera* (Fig. 5). *Bombeil-1* showed a low degree of identity with the *Bombyx CYP306a1*, *CYP302a1*, *CYP315a1*, and *CYP314a1* genes, which are involved in ecdysteroid biosynthesis (Figure 5), indicating that *bombeil-1* encodes a

26-hydroxylase-like enzyme. Moreover, its unique expression in PTTH cells, where 20E acts (Hossain et al., 2006), and its temporal expression profile in the brain (that is, elevation at V9 and V10), strongly indicate its involvement in the 20E-inactivation pathway (Bassett et al., 1997; Davies et al., 2006). *Bombeil-4, bombeil-6, bombeil-8,* and *bombeil-10* showed high similarity with four clones in the Silkworm database, although the functions of these homologous genes is not known. *Bombeil-7* showed 98% identity with a clone (SilkBase FWDP07_FL5_B21) with sequence identity to glutaminyl-transfer RNA (tRNA) synthetase, and *bombeil-9* showed significant identity with several clones (SilkBase FWDP14_FL5_C24, FWDP16_FL5_C11, pg-0985.Seq, and tesS0580) related to lysyl-tRNA synthetase.

Bombeil-2 might be involved in larval–pupal development. RNAi against *bombeil-2* resulted in defects in larval–pupal molt, and malformations of adult wings and legs (Fig. 4). 20E plays critical roles during development, and its precursor is synthesized in, and released from, PTTH-stimulated PGs (Gilbert et al., 2002). In the brain, *bombeil-2* RNAi reduced *PTTH* and *EcR* expression (Fig. 4A), which was indicative of the down-regulation of PTTH synthesis, resulting in an inadequate arrest of stimulation of PGs and thereby causing a shortage of ecdysteroids. As a consequence, down-regulation of *EcR* expression might occur in the brain, and could ultimately lead to the defects in the larval–pupal transition observed in the present study (Fig. 4). In *Drosophila*, mutations of the brain tumor gene *brat* resulted in defects in several ecdysone-regulated developmental processes, and altered the expression of key 20E-regulatory genes (Beckstead et al., 2005; Sonoda and Wharton, 2001). This pattern of gene regulation was similar to the present findings for *bombeil-2*. By contrast, the knockdown of *BR-C* by RNAi in *Bombyx* caused the complete failure of pupation, or in later the morphogenetic defects, including differentiation of adult compound eyes, legs and wings from their larval progenitors (Uhlirova et al., 2003). The effects of *BR-C* RNAi were consistent with those of *bombeil-2* RNAi, with the exception of the eye defects in adults (Fig. 4). The morphogenesis of adult organs derived from the imaginal discs is regulated by 20E and 20E-inducible gene products (Bayer et al., 2003;

Natzle, 1993; Oberlander, 1985), as well as by brain-derived factors (Nijhout et al., 2006). In the present study, *bombeil-2* RNAi not only lowered the number of *bombeil-2* transcripts in the WDs and LDs, but also affected *EcR* gene expression (Fig. 4M); this indicated that *bombeil-2* might act up-stream of steroid-dependent gene regulation in the imaginal discs (Kawasaki et al., 2004; Kamimura et al., 1996; Matsuoka and Fujiwara, 2000; Ote et al., 2004; Zhao et al., 2001).

During larval–pupal development, tremendous morphogenetic changes are involved in the transition from the larval brain to the adult brain, including the formation of the optic lobes and mushroom bodies, as well as adult-specific sensory structures derived by neurogenesis (Champlin and Truman, 1998; Farris et al., 1999; Kraft et al., 1998; Vafopoulou et al., 2007). In the present study, RNAi of *bombeil-2* caused pupal brain morphological changes (23%) during the larval–pupal transformation (Figure 4J, K). Most of these events are regulated by 20E that induces a wide range of cellular responses including neuronal differentiation, maturation, and cell death. 20E thus leads a remodeling of larval neurons into their adult forms during metamorphic reorganization in the insect post-embryonic development (Beckstead et al., 2005; Champlin and Truman, 1998; Farris et al., 1999; Kraft et al., 1998; Schubiger et al., 1998; Vafopoulou et al., 2007). Moreover, we show that *bombeil-2* RNAi reduced *PTTH* and *EcR* expressions in the brain (Fig. 4A), which was indicative of the down-regulation of PTTH synthesis and the shortage of ecdysteroids. As a consequence, the down-regulation of *EcR* expression might occur in the brain and could ultimately lead to the defects in the brain morphology, as observed in the previous studies (Schubiger et al., 1998; Truman et al., 1994). Until now, little information has been available about gene expression in the brain in relation to adult development. Hence, functional analysis of the 10 *bombeil* genes identified in the present study should improve our understanding of the complex gene hierarchies underlying pupal–adult development.

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Figure Legends

Fig. 1. RT-PCR analysis of 20E-induced gene expression in day-2 fifth instar larval brains following injection of 20E (20E+) or Ringer's solution (20E–). The housekeeping gene *RpL32* was used as a control. The number of PCR cycles is indicated above the panel.

Fig. 2. Localization of *bombeil* mRNAs revealed by whole-mount *in situ* hybridization. *Bombeil-1* to *bombeil-10* mRNAs were detected in two pairs of LNCs in the day-2 fifth instar larval brain (A–J, respectively; black arrows). *PTTH* mRNA was detected in day-2 fifth instar larval brains (K; black arrows). *Bombeil* and *PTTH* probes were used for simultaneous hybridization, and the positive signals were detected exclusively in two pairs of LNCs of the day-2 fifth instar larval brains (L-U; black arrows). Panels show only the anterior portions of the larval brains. Scale bar = 100 μm.

Fig. 3 Temporal expression of bombeil genes. A) RT-PCR was performed on brain RNAs from fifth instar larvae to pupae. RpL32 was used as an internal standard. The number of PCR cycles was 30 in all cases except bombeil-9 and bombeil-10, which underwent 35 cycles. B) Developmental changes of ecdysteroid titer in hemolymph ecdysteroid concentration from V5 to P0 depicted from Kaneko et al. (2006). The titer from V0 to V4 is kept lower than that at V5. Concentrations are expressed in μM 20E-equivalents. Each data point (every 6 h interval) represents the mean ±SD (n=5).

Fig. 4. Knockdown effects of *bombeil-2* RNAi on development. A: RT-PCR was performed on brain RNAs from the day-4 fifth instar larval brains after the injection of dsbombeil-2 or Ringer's solution into day-2 fifth instar larvae. Effects of *bombeil-2* RNAi; Phenotype of pupae (B: dsbombeil-2; C: Ringer's solution, D: dsEGFP) and adults (E, F: dsbombeil-2, G: Ringer's

solution, H: dsEGFP) injected as day-2 fifth instar larvae with dsbombeil-2, Ringer's solution, or dsEGFP. The adult phenotypes of the dsbombeil-2-treated organisms included severe wing defects (E and F, red arrows) and undeveloped prothoracic legs (F, black arrow), as well as malformed tarsi (F, bracket). Morphology of day-2 pupal brains that were injected with either dsbombeil-2 (I, J) or Ringer's solution (K) as day-2 fifth instar larvae. RT-PCR analysis of *bombeil-2* expression in WDs and LDs in day-4 fifth instar larvae; (L) *Bombeil-2* endogenous expression; (M) Expression of *bombeil-2* and *EcR* in dsbombeil-2 and control (Ringer's solution) samples. *RpL32* was used as an internal standard.

Fig. 5. Comparison of the amino-acid sequences of the heme-binding domain (P450-signature) of bombeil-1 and the P450 enzymes of *Bombyx* and other insects. Conserved amino acids are underlined. The percentage amino-acid identity to bombeil-1 is indicated in parenthes.

Table 1. Oligonucleotide primers for PCR and *in situ* hybridization

Gene	Forward	Reverse	Annealing temp (°C)
Bombeil-1	CGTCACCTTCAGCCATTTCC	TCCGAATCTCTGGGATGAACC	63
Bombeil-2	TCAATTAATCCGTGCTCTCGC	GAAGCGCGTGCATTTAG	64
Bombeil-3	CGTCTCTCCGCGACTTCATAT	TCCCCTAGCCCAGCTAACAG	63
Bombeil-4	CAGGTACACCCGCAAATGGT	CGCAGCTCGGTTTTGAGAAT	63
Bombeil-5	TTTTTCGAGACCGCTACTCCA	CGCGGAGCCTATATACACAGC	64
Bombeil-6	TCCTGCGAAGCAAGAGAGTCTT	CAGACTCATCACCATCTTCCTCG	60
Bombeil-7	TGGGACCTAAGACTGAGGCTG	TCTCCCCTGGTGCATGAAAG	60
Bombeil-8	TCGTATACATGGTGTCCGCGT	TTACGGAAACGTTGCGAGC	64
Bombeil-9	GGCCGAGGTACCATACCAGAC	AGGTACATGCGAATAGCTCCTGA	60
Bombeil-10	ACATGGCGCATTACGAAGC	TTGTCACTGGCGGATTTTTG	64
EcR-A	TGGAGCTGAAACACGAGGTGGC	TCCCATTAGGGCTGTACGGACC	60
EcR-B1	ATAACGGTGGCTTCCCGCTGCG	CGGTGTTGTGGGAGGCATTGGTA	60
PTTH	CAGTTGAGTTATCCAGCATTCCC	CGGAACAAATCATCAGGCG	59
RpL32	GAGGACGAAGAGATTTATCAGGCA	CGAAGAGACACCATGAGCGAT	60

Fig. 1

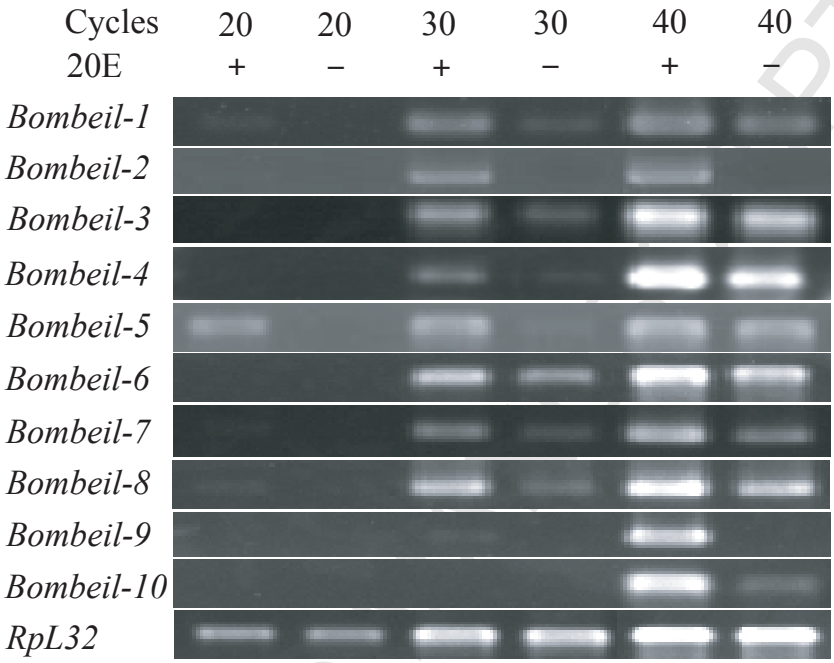

Fig. 2

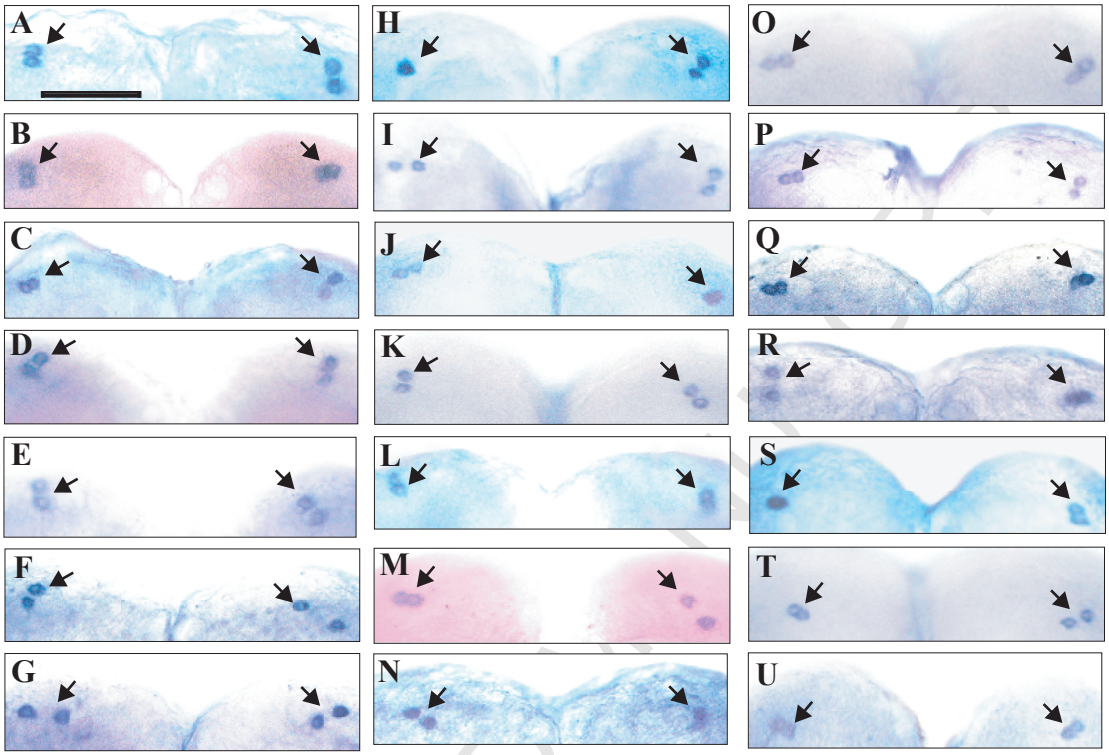

Fig. 3

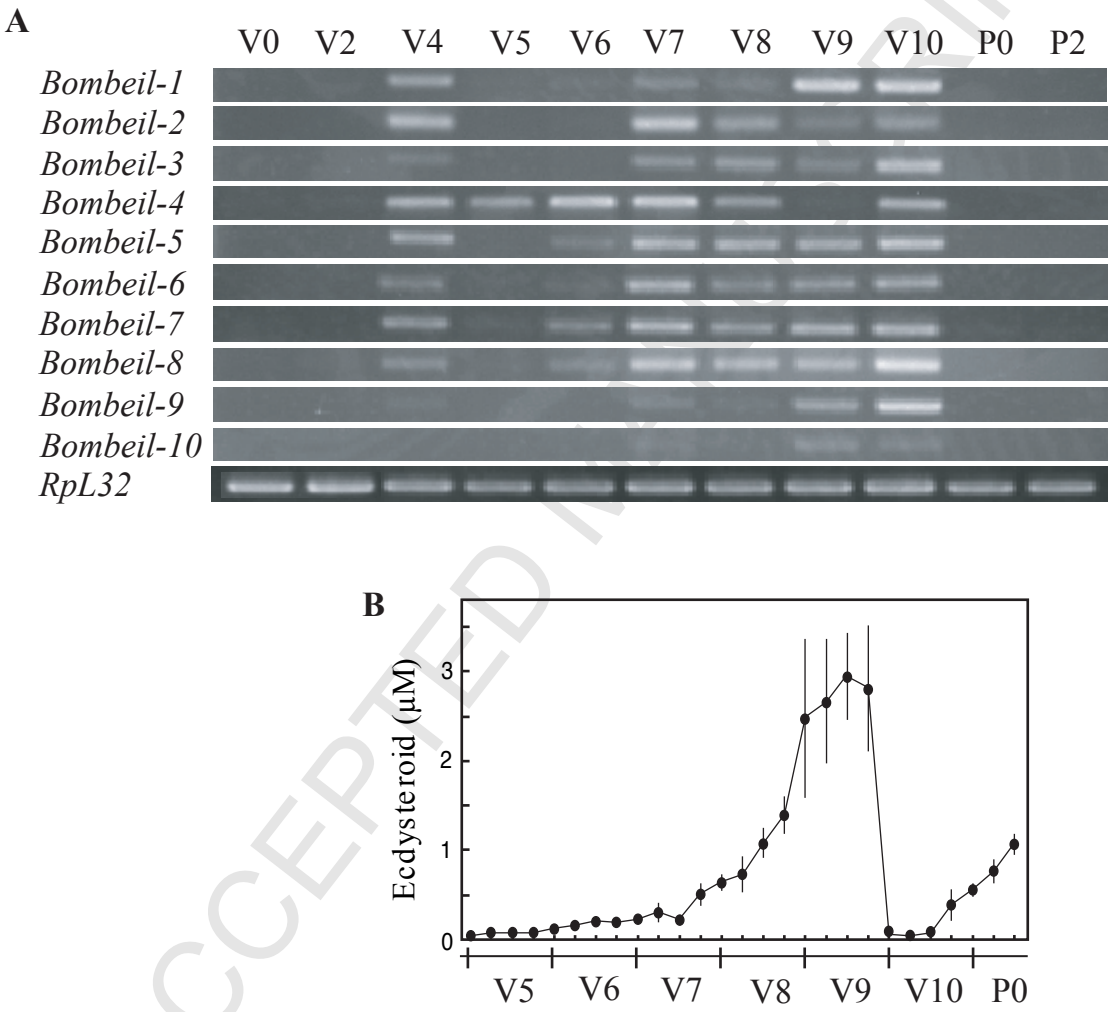

Fig. 4

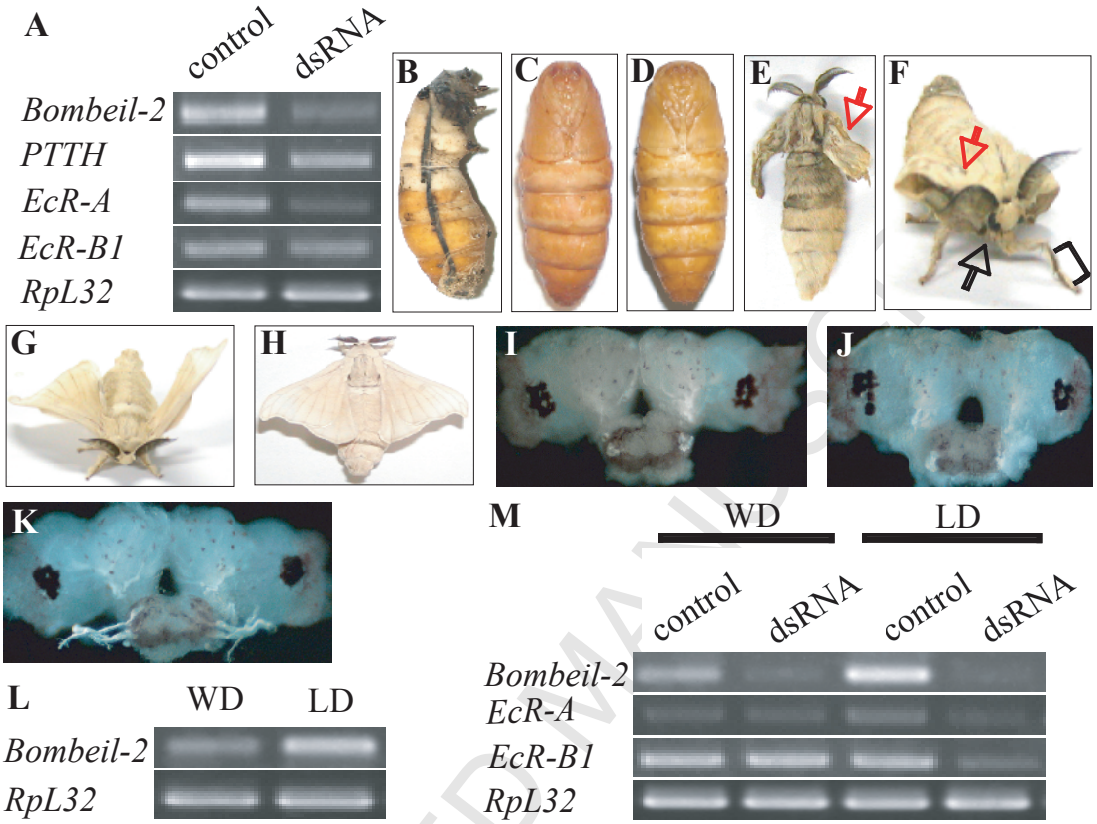

Fig. 5

Bombeil-1	*(Bombyx)*	PFGVGRRMCLGDVLAR	
CYP18a1	*(Drosophila)*	PFGVGRRMCLGDVLAR	(100%)
CYP18a1	*(Spodoptera)*	PFGVGRRMCLGDVLAR	(100%)
CYP306a1	*(Bombyx)*	PFQTGKRMCPGDELSR	(62%)
CYP302a1	*(Bombyx)*	PFGHGPRSCIARRLAE	(50%)
CYP315a1	*(Bombyx)*	PFAFGARSCIGKKMAM	(44%)
CYP314a1	*(Bombyx)*	PFGRGRRMCPGKRFVE	(50%)