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著者	Niwa Ryusuke, Sakudoh Takashi, Matsuya Takeshi, Namiki Toshiki, Kasai Shinji, Tomita Takashi, Kataoka Hiroshi
journal or publication title	Applied entomology and zoology
volume	46
number	4
page range	533-543
year	2011-11
権利	(C) The Japanese Society of Applied Entomology and Zoology 2011.The original publication is available at www.springerlink.com
URL	http://hdl.handle.net/2241/114738

doi: 10.1007/s13355-011-0074-6

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Ryusuke Niwa · Takashi Sakudoh · Takeshi Matsuya · Toshiki Namiki · Shinji Kasai · Takashi Tomita · Hiroshi Kataoka

R. Niwa (corresponding author)
Initiative for the Promotion of Young Scientists' Independent Research, Graduate School of Life and Environmental Sciences, University of Tsukuba, Seinou-tou B411, Tennoudai 1-1-1, Tsukuba, Ibaraki 305-8572, Japan
TEL: +81-29-853-6652, FAX: +81-29-853-6614
Email: ryusuke-niwa@umin.ac.jp

T. Sakudoh
Division of Radiological Protection and Biology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku Tokyo 162-8640, Japan

T. Matsuya, T. Namiki, H. Kataoka
Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan

S. Kasai, T. Tomita
Department of Medical Entomology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan

Abstract

Here we describe the expression profiles of the cytochromoe P450 monooxygease gene *Cyp4g1* in the fruit fly, *Drosophila melanogaster* Meigen and its homolog in the silkworm *Bombyx mori* L. We identified *Cyp4g1* by a microarray analysis to examine the expression levels of 86 predicted *D. melanogaster* P450 genes in the ring gland that contains the prothoracic gland (PG), an endocrine organ responsible for synthesizing ecdysteroids. *B. mori Cyp4g25* is a closely-related homolog of *D. melanogaster Cyp4g1* and is also expressed in the PG. A developmental expression pattern of *Cyp4g25* in the PG is positively correlated with a fluctuation in hemolymph ecdysteroid titer in the late stage of the final instar. Moreover, the expression of *Cyp4g25* in cultured PGs is significantly induced by the addition of prothoracicotropic hormone (PTTH), a neuropeptide hormone that stimulates the synthesis and release of ecdysone. We propose that *Cyp4g1* and *Cyp4g25* are the candidates that play a role in regulating PG function and control ecdysteroid production and/or metabolism during insect development.

Keywords

cytochrome P450 monooxygenase, prothoracic gland, *Bombyx mori*, *Drosophila melanogaster*

Introduction

In arthropods, steroid hormones designated as ecdysteroids, such as ecdysone and its derivative 20-hydroxyecdysone (20E), are essential for precise progression through development (Thummel, 2001; Gilbert et al., 2002; Spindler et al., 2009). Ecdysone is synthesized from dietary cholesterol via a series of hydroxylation and oxidation steps in the prothoracic gland (PG) during postembryonic development (Gilbert et al., 2002). Ecdysone is subsequently converted to 20E by the 20-hydroxylase present in the peripheral tissues (Gilbert et al., 2002).

Recently, molecular genetic studies using the fruit fly *Drosophila melanogaster* Meigen and the silkworm *Bombyx mori* L. have successfully identified several genes crucial for intermediate steps in ecdysone biosynthesis. The dehydrogenation of cholesterol to 7-hydrocholesterol (7dC), the first step in synthesizing ecdysone, is catalyzed by the Rieske-domain enzyme Neverland (Nvd) (Yoshiyama et al., 2006; Niwa and Niwa, 2011; Yoshiyama-Yanagawa et al., 2011). The conversion of 7dC to 5β-ketodiol is commonly referred to as a “Black Box” since no stable intermediate has been identified (Gilbert et al., 2002). Recent studies have demonstrated that the cytochrome P450 monooxygenases, CYP307A1/Spook (Spo) and CYP307A2/Spookier (Spok), and the short-chain dehydrogenase/reductase Non-molting glossy/Shroud are involved in the Black Box reaction (Namiki et al., 2005; Ono et al., 2006; Niwa et al., 2010). The terminal hydroxylation steps from 5β-ketodiol to ecdysone in the PG are catalyzed by three cytochrome P450 monooxygenases: CYP306A1/Phantom (Phm), CYP302A1/Disembodied (Dib) and CYP315A1/Shadow (Sad) (Chávez et al., 2000; Warren et al., 2002; Niwa et al., 2004; Warren et al., 2004; Niwa et al., 2005). The conversion of ecdysone to 20E is also mediated by a P450 monooxygenase, CYP314A1/Shade (Shd), in the peripheral tissues (Petryk et al., 2003).

Shroud and the P450 enzymes described above were identified from embryonic lethal mutants, known as the Halloween mutants, that exhibit embryonic ecdysone deficiency (Chávez et al., 2000). The recent discovery of these ecdysteroidogenic enzymes greatly advances our knowledge of ecdysone biosynthesis at the molecular level. However, it has not yet been proven whether the enzymes identified thus far are sufficient for the conversion of cholesterol to 20-hydroxyecdysone. Therefore, it is unclear whether there are still unidentified enzyme(s) that are responsible for ecdysone biosynthesis.

Here, we report that another P450 gene, *Cyp4g1*, is highly expressed in the PG in *D. melanogaster*. A closely-related homolog of *Cyp4g1* from *B. mori*, *Cyp4g25*, is also expressed in the PG, and its expression profile is positively correlated with a change in ecdysteroid titer in the hemolymph during the late stage of the last larval instar. Furthermore, we show that in cultured PGs, the expression of *Cyp4g25* is significantly induced by the addition of the prothoracicotropic hormone (PTTH), which is a crucial neuropeptide that stimulates the synthesis and release of ecdysone (Gilbert et al., 2002). These results suggest that *Cyp4g1* and *Cyp4g25* play a role in regulating the PG function during insect development.

Materials and Methods

Insects

Silkworms, *B. mori* (KINSYU x SHOWA F1 hybrid), were reared on an artificial diet (Silkmate, Nihon-Nosan-Kogyo, Japan) at 25 ºC under a 16 h light/8 h dark cycle. The first days corresponding to the developmental stages of the 4th to 5th larval ecdysis, wandering and pupation were designated as V0, W0 and P0, respectively. *D.*

melanogaster flies were reared on standard agar-cornmeal medium at 25°C under a 12 h light/12 h dark cycle. Oregon R was used as the wild-type fly.

Microarray analysis

We created a customized cDNA microarray, which contained DNA fragments corresponding to 86 predicted *D. melanogaster* P450 gene that were chose in our previous study (Kasai and Tomita, 2003). A DNA fragment corresponding to each of the 86 P450 genes was amplified by PCR as previously described (Kasai and Tomita, 2003). Gene specific primers used for PCR are listed in Table 1. The DNA fragments of the 86 P450 genes were approximately 500-600 bp in length (Table 1). PCR products were purified by agarose gel electrophoresis and then subcloned to pCR2.1 (Invitrogen). After DNA sequences of each of the PCR products were verified, we re-performed PCR using pCR2.1 plasmids containing sequence-verified P450 fragments with the gene specific primers (Table 1). These PCR products were spotted onto microarrays. Total RNA from the ring glands and the brain-ventral nerve cord complex of the wandering 3rd instar *D. melanogaster* larvae were prepared using TRIzol reagent (Invitrogen). Spotting, cRNA amplification, fluorescent labeling, hybridization, detection and analysis were conducted by Bio Matrix Research, Inc., Kashiwa, Japan. One customized microarray contained 4 spots for each of the P450 genes. Spot intensities were normalized using a summation of total spot intensities in the hybridization experiments.

RNA in situ hybridization

Synthesis of DIG-labeled RNA probes and RNA *in situ* hybridizations were performed as previously described (Lehmann and Tautz, 1994). To synthesize sense and antisense RNA probes for *Cyp4g1* and *Cyp310a1*, EST cDNA clones of the Berkeley *Drosophila*

genome project GH05567 and LD44491, respectively (Stapleton et al., 2002), were used as templates. To generate a template for synthesizing sense and antisense *Cyp12e1* RNA probes, the ORF region of *Cyp12e1* was amplified by PCR with primers (5′-ATGTTGTCAACGCAGTGGAACGCAAATAAA-3′ and 5′-AAACCCGATCTTAAAGTTTCTTACCAACCG-3′) using wild-type genomic DNA as template and subcloned into pBluescript.

Quantitative reverse-transcription PCR (qRT-PCR)

Single-stranded cDNA synthesis was performed as previously described (Niwa et al., 2004). qRT-PCR was performed using a real-time thermal Smart Cycler System (Cepheid) with the SYBR Premix ExTaq (TaKaRa). Specific primers used in this study were the following: Cyp4g1-forward (5′-CGGTCCTGGGATTCAGTCCTATG-3′), Cyp4g1-reverse (5′-CATCACCGAACCAGGGCTTGAAG-3′), Cyp4g25-forward (5′-TCGTCGGTGGATCTGCTGACATCTTC-3′), Cyp4g25-reverse (5′-CGATGAGACCTCCATTTTTGACCAGTACTG-3′), rp49-forward (5′-CGGATCGATATGCTAAGCTGT-3′), rp49-reverse (5′-GCGCTTGTTCGATCCGTA-3′), rpL3-forward (5′-CGTCGTCATCGTGGTAAGGTCAAG-3′) and rpL3-reverse (5′-GGTCTCAATGTATCCAACAACACCGACAC-3′). Serial dilutions of plasmids containing cDNAs of *Cyp4g1, Cyp4g25*, *rp49* and *rpL3* were used as standards. The plasmid containing *Cyp4g25* cDNA was *B. mori* EST clone prgv0895 (Mita et al., 2003), which was a gift from Kazuei Mita. PCR was performed with 40 cycles of 94 ºC for 5 s and 60 ºC for 20 s. The amount of each transcript was calculated based on crossing point analysis, with standard curves generated from the standard plasmids.

Transcript levels of *Cyp4g1* and *Cyp4g25* were normalized to transcript levels of *rp49* and *rpL3*, respectively, in the same samples.

In vitro culture of PGs

The *in vitro* culture of PGs was performed as previously described (Niwa et al., 2005; Yamanaka et al., 2007). Recombinant PTTH (rPTTH) was prepared as previously described (Ishibashi et al., 1994). V4 silkworms were anaesthetized by water submersion for 5 min. The PGs were dissected rapidly in sterile saline and pre-incubated in 100 μl of Grace's Insect Medium (Sigma). After 20 min, each single PG was transferred into 100 μl of medium in the presence or absence of 10 nM rPTTH, because ecdysone release from the PGs is at its highest in 10 nM rPTTH (Yamanaka et al., 2005). After incubation for 30 min, 2 h, 4 h or 6 h, each PG was removed, frozen at -80 ºC and analyzed by quantitative RT-PCR. For experiments in which transcription was inhibited, the transcriptional inhibitor, α-amanitin (1 μg/ml) (Sigma) was used as previously described (Niwa et al., 2005). The inhibitor was added to the pre-incubation medium 15 min before incubation with rPTTH. The inhibitors were also included during the incubation period (2 h). For experiments using ecdysteroids, the PG was cultured in the presence or absence of 74 nM of ecdysone (Sigma), as the amount of ecdysone released from a single cultured PG at 2 h post-PTTH stimulation was estimated at 3.44 ng in 100 μl medium, i.e. 74 nM, as previously reported (Niwa et al., 2005).

Results

Microarray analysis for P450 expression in the *D. melanogaster* ring gland

To examine which P450 genes are predominantly expressed in the PG of *D. melanogaster*, we used our customized microarray on which non-redundant DNA fragments corresponding to 86 predicted P450 genes (Kasai and Tomita, 2003) were spotted. We compared gene expression levels of all the predicted P450 genes in the ring glands containing the PG cells compared to the brain-ventral nerve cord (VNC) complex. Both the ring glands and the brain-VNC complexes were isolated from wandering 3rd instar larvae. The microarray data obtained from 2 independent experiments yielded 7 cDNAs showing a more than a 2-fold increase in expression in the ring gland when compared with expression in the brain-VNC complex. These 7 genes included all of the previously identified P450 genes known to be predominantly expressed in the PG and corpora allata of the ring gland (Fig. 1 and Table 2), such as *sad* (Warren et al., 2002), *dib* (Chávez et al., 2000), *phm* (Niwa et al., 2004; Warren et al., 2004) and *Cyp6g2* (Chung et al., 2009). S*pok* (Ono et al., 2006) was not identified from our micorarray analysis simply because the *spok* probe was not included in our customized microarray. These results demonstrate the reliability of the microarray analysis.

D. melanogaster Cyp4g1 is strongly expressed in the prothoracic gland

In addition to *dib*, *sad*, *phm* and *Cyp6g2*, we found that 3 other P450 genes, *Cyp4g1* (GenBank accession no. NM_080292), *Cyp12e1* (NM_141746) and *Cyp310a1* (NM_136047), exhibited a more than 2-fold expression change in the ring gland when compared to expression in the brain-VNC complex (Fig. 1 and Table 2). To confirm the gene expression in the ring gland, we performed RNA *in situ* hybridization. Whereas neither *Cyp12e1* nor *Cyp310a1* was strongly expressed in the ring gland (data not shown), we found that *Cyp4g1* was predominantly expressed in the PG cells of the ring

gland in the wandering 3rd instar larva (Fig. 2a-e). The *Cyp4g1* transcript was exclusively observed in the PG, but not in other endocrine organs in the ring gland, such as the corpus allatum or corpus cardiacum. Curiously, the expression of *Cyp4g1* was detected in a subset, but not all, of the PG cells (Fig. 2b-e). In addition, spatial distributions of the *Cyp4g1*-expressing cells in the PGs were different among specimens (Fig. 2b-e). Features of the spatial expression pattern of *Cyp4g1* were unique and unlike the expression patterns of the previously identified ecdysteroidogenic genes (Chávez et al., 2000; Warren et al., 2002; Niwa et al., 2004; Warren et al., 2004; Niwa et al., 2010). While it has been reported that *Cyp4g1* is the most highly expressed P450 gene in the adult stage (Daborn et al., 2002; Kasai and Tomita, 2003) and is also expressed in larval oenocytes (Gutierrez et al., 2007), our work is the first report that the expression of *Cyp4g1* in the PG cells.

We also examined the spatial expression profile of *Cyp4g1* using qRT-PCR. In addition to high expression in the PG, *Cyp4g1* was also highly expressed in the epidermis (Fig. 2f). The epidermal expression of *Cyp4g1* was thought to reflect the expression in oenocytes because strong *in situ* signals were detected in seven pairs of the oenocytes (Fig. 2g) as reported in previous studies (Simpson, 1997; Tarès et al., 2000; Tomancak et al., 2002; Gutierrez et al., 2007; Chung et al., 2009).

B. mori Cyp4g25, the closely related genes to _D. melanogaster Cyp4g1_, is also expressed in the prothoracic gland

We next examined whether a gene closely related to *D. melanogaster Cyp4g1* was also expressed in the PG in another model insect, the silkworm *B. mori*. A BLAST search revealed that *D. melanogaster Cyp4g1* is most similar to the *B. mori* gene *Cyp4g25* (GenBank accession no. ABF51415) among all of the predicted genes in the *B.*

mori genome. The deduced amino acid sequence of *B. mori* CYP4G25 compared to that of *D. melanogaster* CYP4G1 shows 49.6 % identity, with an additional 16.0 % of the amino acids judged to be similar. The *Cyp4g25* transcript was detected in the PG of *B. mori*, as expected, as well as in other tissues including the salivary gland (Fig. 3a). We also found that *Cyp4g25* expression in the PG fluctuated in 5th instar larvae (Fig. 3b). This fluctuation was especially prevalent in the wandering larvae in the late 5th instar larval stage; this change in the *Cyp4g25* PG expression level correlated well with the change in the hemolymph ecdysteroid titer during development (Fig. 3b).Around the wandering stage, the PTTH titer is elevated in the *B. mori* hemolymph (Mizoguchi et al., 2001; Mizoguchi et al., 2002). We have previously reported that *B. mori dib* (*dib-Bm*) expression also dramatically increases in the wandering stage and is transcriptionally regulated by PTTH (Niwa et al., 2005), raising the possibility that *Cyp4g25* transcription is also regulated by PTTH.

The expression level of *B. mori Cyp4g25* is increased by the prothoracicotropic hormone in the cultured prothoracic gland

To address the question of whether PTTH regulates the expression of *Cyp4g25* in the PG, we incubated PGs with 10 nM recombinant PTTH (rPTTH). In this study, we used the PGs from V4 stage 5th instar larvae. PGs from V4 stage silkworms are highly sensitive to treatment with PTTH, as shown by elevated glandular cAMP levels and ecdysone secretion (Yamanaka et al., 2005). Under our culture conditions, there was a significant induction of ecdysteroid production (Niwa et al., 2005). We found that *Cyp4g25* expression was significantly induced within 2 h in 4 independently isolated PGs (Fig. 4a). After 2 h of treatment with rPTTH, *Cyp4g25* mRNA levels showed more than an eight-fold increase in expression over *Cyp4g25* levels at the beginning of the

245 incubation. The elevation of *Cyp4g25* mRNA by rPTTH was significantly inhibited by
246 the presence of α-amanitin, an inhibitor of RNA polymerase II-dependent transcription
247 (Fig. 4b), suggesting that PTTH regulates *Cyp4g25* mRNA at the level of transcription.
248 Indeed, the elevation of *Cyp4g25* mRNA levels by rPTTH was more rapidly and
249 drastically induced compared to that of *dib-Bm* (Niwa et al., 2005).
250 It is possible that the *Cyp4g1* mRNA is induced by ecdysone, which is
251 produced in and secreted from the PG by the PTTH stimulation. In order to test this
252 hypothesis, we applied ecdysone at a concentration of 74 nM (see Materials and
253 methods) to cultured PGs in place of rPTTH. No significant increase of *Cyp4g25*
254 mRNA level was observed after 2 h of incubation with ecdysone as compared to rPTTH
255 (Fig. 4C). These data suggest that *Cyp4g25* is specifically transcriptionally regulated by
256 PTTH rather than ecdysone during ecdysteroid biosynthesis in the PG.
257
258 **Discussion**
259 In this study, we identified *D. melanogaster Cyp4g1* and showed that *Cyp4g1* is highly
260 expressed in the PG during embryonic and larval development. In addition, we
261 demonstrated that the expression of the *B. mori* homolog of *Cyp4g1*, designated
262 *Cyp4g25*, was in concert with the changes in ecdysone titer during the wandering stage
263 of 5th instar larvae. We also showed that the expression of *Cyp4g25* in cultured PGs is
264 dramatically induced by treatment with PTTH. It should be noted that the increase of
265 *Cyp4g25* mRNA level was more rapid and drastic when compared to that of *dib-Bm*,
266 which encodes a crucial enzyme for ecdysone biosynthesis (Niwa et al., 2005).
267 Considering that vertebrate neuropeptides that regulate steroidogenesis also affect the
268 transcriptional regulation of steroidogenic enzymes (Kagawa et al., 1999; Sewer and
269 Waterman, 2003), we propose that CYP4G1/CYP4G25 might play an important role in

ecdysone biosynthesis in the PG in insects. A previous study showed that another P450 enzyme gene belonging to the CYP4 family, *Cyp4c15*, is specifically expressed in the steroidogenic gland in the crayfish, *Orconectes limosus* Rafinesque (Aragon et al., 2002), suggesting that some of the CYP4 family members play a role in ecdysone biosynthesis not only in insects but also in other arthropods.

It has not yet been elucidated whether CYP4G1/CYP4G25 contributes to ecdysone biosynthesis in the PG during development. A recent study demonstrates that *Cyp4g1* expression in oenocytes is crucial for regulating the lipid composition of the fat body (Gutierrez et al., 2007). Complete loss-of-function mutants of *Cyp4g1* develop normally through larval and early pupal stages, but arrest during mid-to-late pupal stages; many fail during adult eclosion due to abnormal lipid metabolism (Gutierrez et al., 2007). Further analysis is needed to examine whether the pupal arrest phenotype of the *Cyp4g1* mutants is partly due to a defect in ecdysone biosynthesis in the PG. However, these data indicate that *Cyp4g1* is not necessary for embryonic and larval ecdysis at least in *D. melanogaster*.

We have not identified a specific enzymatic activity or any substrate for CYP4G1/CYP4G25. It is known that the mammalian CYP4 family includes a group of over 60 members that ω-hydroxylate the terminal carbon of fatty acids (Hardwick, 2008). *D. melanogaster Cyp4g1* is also thought to act as a fatty acid ω-hydroxylase because flies with mutant *Cyp4g1* exhibit abnormal lipid metabolism in oenocytes, as described above (Gutierrez et al., 2007). Therefore, CYP4G1/CYP4G25 might be involved in lipid metabolism in the PG and may indirectly regulate ecdysone biosynthesis. It would be interesting to examine whether specific lipid and fat depositions occur in the PG during development and whether lipid and fat contents affect ecdysone biosynthesis. Alternatively, it is possible that CYP4G1/CYP4G25

295 catalyzes a specific intermediate of the ecdysone biosynthesis pathway. Recent studies
296 have revealed that the first and last 3 conversion steps of ecdysone biosynthesis are
297 mediated by specific ecdysteroidogenic enzymes, namely, Nvd, Phm, Dib and Sad
298 (Warren et al., 2002; Niwa et al., 2004; Warren et al., 2004; Yoshiyama-Yanagawa et al.,
299 2011). We also examined whether the CYP4G1/CYP4G25 protein can convert
300 substrates in these known steps (cholesterol, 5β-ketodiol, 5β-ketotoriol and
301 2-deoxyecdysone) using a S2 cell system that was previously utilized in biochemical
302 studies of ecdysteroidogenic enzymes (Niwa et al., 2004; Niwa et al., 2005;
303 Yoshiyama-Yanagawa et al., 2011). However, no metabolites have yet been detected
304 (data not shown). Thus, it is likely that CYP4G1/CYP4G25 is involved in the currently
305 uncharacterized, intervening conversion steps from 7dC to 5β-ketodiol, known as the
306 Black Box (Gilbert et al., 2002). Another possibility is that CYP4G1/CYP25G1
307 negatively regulates ecdysone biosynthesis or inactivates ecdysteroids in the PG. In fact,
308 the late pupal lethality, which occurs in *Cyp4g1* null mutants (Gutierrez et al., 2007), is
309 also observed in loss-of-function mutants of *Cyp18a1*, which encodes a P450 gene that
310 inactivates ecdysteroids in peripheral tissues (Rewitz et al., 2010; Guittard et al., 2011).
311 It is also noteworthy that both *B. mori Cyp4g25* (Fig. 3A) and *D. melanogaster*
312 *Cyp18a1* (Guittard et al., 2011) show strong expression in the salivary gland, one of
313 tissues that are thought to play a role in inactivating ecdysteroids. Further biochemical
314 studies on CYP4G1/CYP4G25 will shed light on the molecular mechanisms controlling
315 insect development.
316
317 **Acknowledgements**
318 We thank Kaoru Saigo, Kuniaki Takahashi and Ryu Ueda for their support in the initial
319 stages of this work. We also thank Kazuei Mita for providing us with the *B. mori* EST

clone; Ken Watanabe and Mariko Fujishita for technical assistance; and Takuji Yoshiyama-Yanagawa and Yuko Shimada-Niwa for comments on the manuscript. We are also grateful to Katsuo Furukubo-Tokunaga for allowing R.N. to use his space and equipment. This work was supported in part by Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese Government and by grants to H.K. from Grants-in-Aid for Scientific Research (19380034) from JSPS and the Program for Promotion of Basic Research Activities for Innovative Biosciences.

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459 **Figure Legends**

460

461 **Fig. 1.** Expression profile of *D. melanogaster*'s 86 P450 genes in the ring gland and the
462 brain-ventral nerve cord (VNC) complex in wandering 3rd instar larvae. X- and Y-axes
463 represent the intensity of the microarray spots hybridized with fluorescently-labeled
464 reverse-transcribed probes prepared from RNAs from the brain-VNC and the ring gland,
465 respectively, in logarithmic scales. Each dot indicates the spot intensity level of each
466 P450 gene. A solid line indicates the same gene expression level between the ring gland
467 and the brain-VNC complex. Genes represented by red spots above a dashed line are the
468 P450 genes showing more than a 2-fold increase in expression in the ring gland
469 compared to the expression in the brain-VNC complex.

470

471 **Fig. 2.** *Cyp4g1* expression in *D. melanogaster.* (**a-e**) *In situ* expression of *Cyp4g1* in the
472 ring gland and the brain-VNC complex of the wandering stage of *D. melanogaster* 3rd
473 instar larva. The ring glands are marked by arrowheads. (**a**) Signals from samples
474 hybridized with sense (control) RNA probe. (**b-e**) Four independent signals from
475 samples hybridized with antisense RNA probe. Note that expression of *Cyp4g1* was
476 detected in a subset but not all of the PG cells. Moreover, the distribution of the
477 *Cyp4g1*-expressing cells in the ring gland was not uniform among specimens. (**f**) The
478 *Cyp4g1* transcript levels in several larval tissues from wandering third instar larvae of *D.*
479 *melanogaster*. RG, ring gland; BR, brain; ID, imaginal discs; IT, intestine; EP,
480 epidermis. The normalized *Cyp4g1* mRNA level in the ring gland is set as 1. (**g**) *In situ*
481 expression of *Cyp4g1* in a stage 16 embryo. Arrowheads and arrows indicate the ring
482 gland and oenocytes, respectively.

483

Fig. 3. The expression pattern of *B. mori Cyp4g25*. (**a**) qRT-PCR analysis of the *Cyp4g25* transcript in several tissues from W1 wandering fifth instar larvae. BR, brain; PG, prothoracic gland; SG, salivary gland; AS, anterior silk gland; MS, middle silk gland; PS, posterior silk gland; MG, midgut; HG, hindgut; TR, trachea; MT, Malpighian tubules; FB, fat body; MS, muscle; OV, ovary; TE, testis. The normalized *Cyp4g25* mRNA level in the salivary gland is set as 1. (**b**) The temporal expression profile of *Cyp4g25* in the PG during the fifth larval (V) and pupal stages. The periods (in days) corresponding to the developmental stages of the fourth to fifth larval ecdysis, wandering, and pupation were designated as V0, W0, and P0, respectively. The dashed line is a schematic representation of developmental changes in hemolymph ecdysteroid titer based on the data previously described (Kiguchi and Agui, 1981; Kiguchi et al., 1985). Each error bar represents the standard deviation from three independent samples. The normalized average *Cyp4g25* mRNA level in W4 wandering *B. mori* larvae is set as 1.

Fig. 4. *Cyp4g25* expression is induced by treatment with rPTTH in cultured PGs. (**a**) Changes in mRNA expression levels of *Cyp4g25* in cultured PGs in the presence (circular dots and solid lines) or absence (square dots and dashed lines) of 10 nM rPTTH. Each horizontal axis represents the time of the incubation periods in the presence or absence of rPTTH. Each vertical axis indicates the fold-increase in transcript levels compared to each mRNA amount at the incubation time 0 hour (h). Each value is an average of the fold increase ± SE ($N = 4$). A long, dashed line represents the changes of *dib-Bm* transcript levels in cultured PGs in the presence of 10 nM rPTTH based on the data described in our previous study (Niwa et al., 2005). (**b**) Treatment with 1 μg/ml α-amanitin inhibits *Cyp4g25* transcription after 2 h of

509 incubation with rPTTH. Each value on the vertical axis is an average of the fold
510 increase ± SE ($N = 4$). The gene expression level of the sample in the absence of both
511 rPTTH and α-amanitin is represented as 1. Asterisk (*) indicates a statistical
512 significance of $P < 0.05$ using the Student's *t*-test. **(c)** Ecdysone (E) does not cause a
513 significant increase of *Cyp4g25* mRNA level. The grey and white bars represent the
514 *Cyp4g25* mRNA amounts (±SE; $N = 4$) in the 2 h treatment with and without 74 nM
515 ecdysone, respectively. The expression level of *Cyp4g25* in the absence of the reagent is
516 represented as 1 on the vertical axis.
517
518

Fig. 1

Niwa et al.

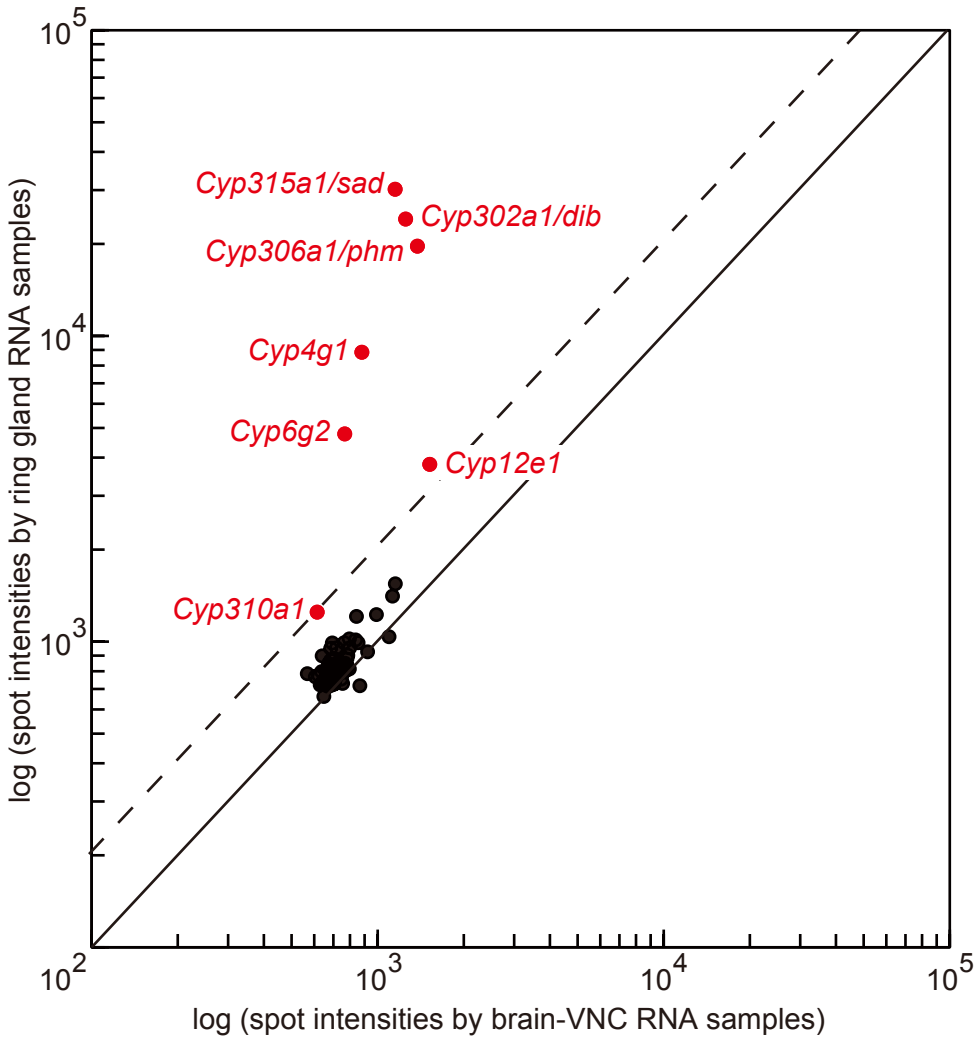

Fig. 2

Niwa et al.

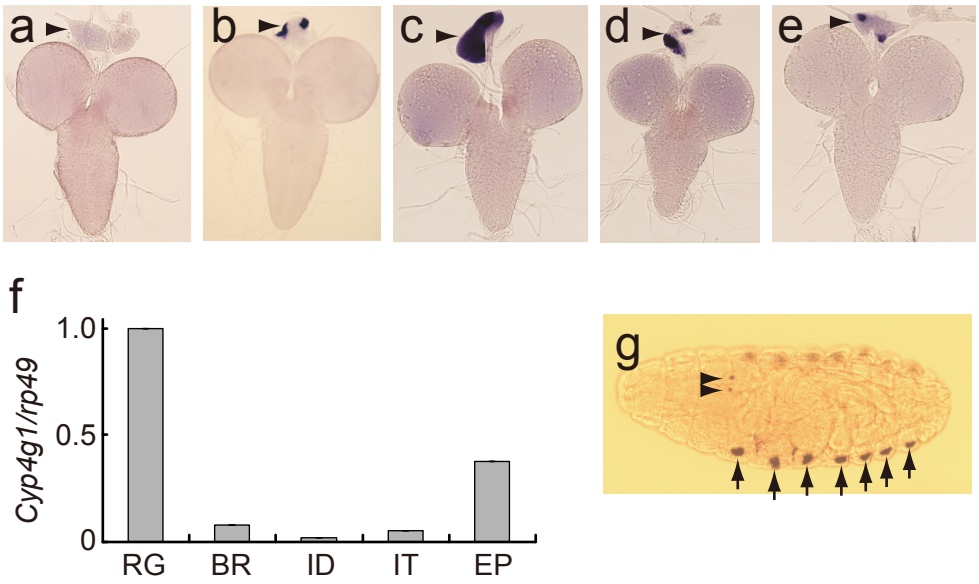

Fig. 3

Niwa et al.

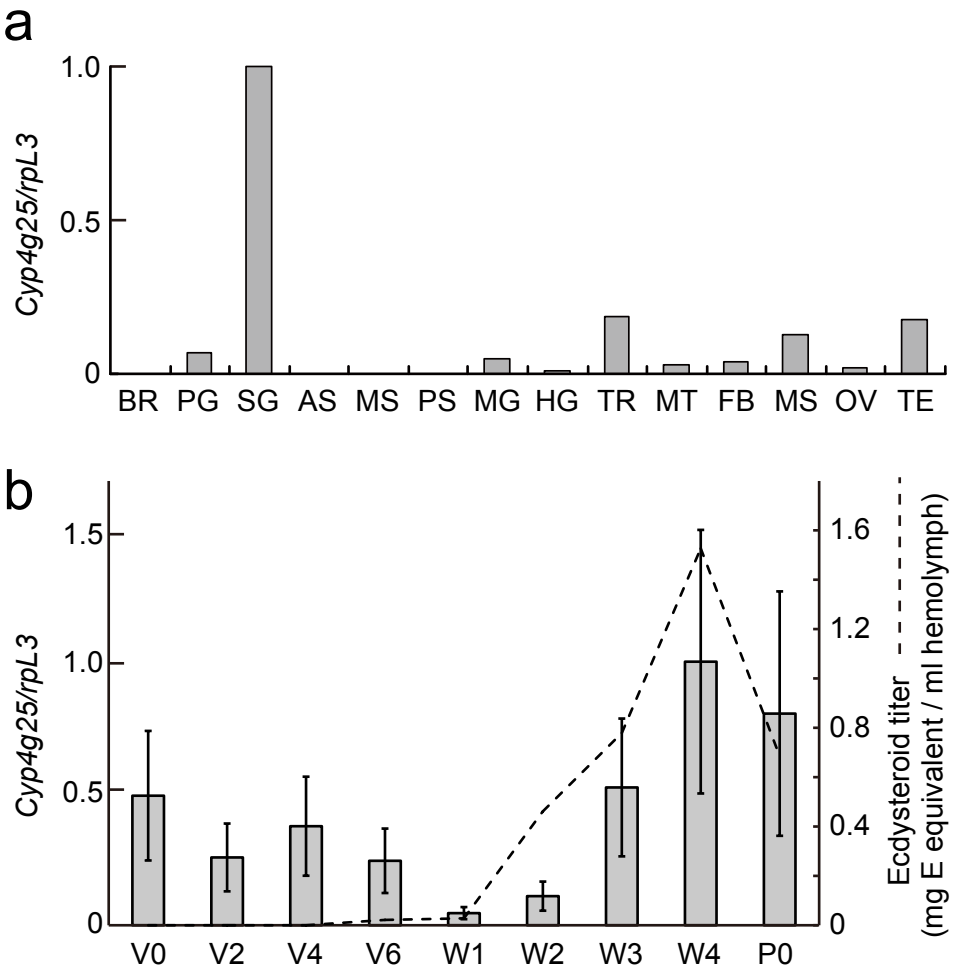

Fig. 4

Niwa et al.

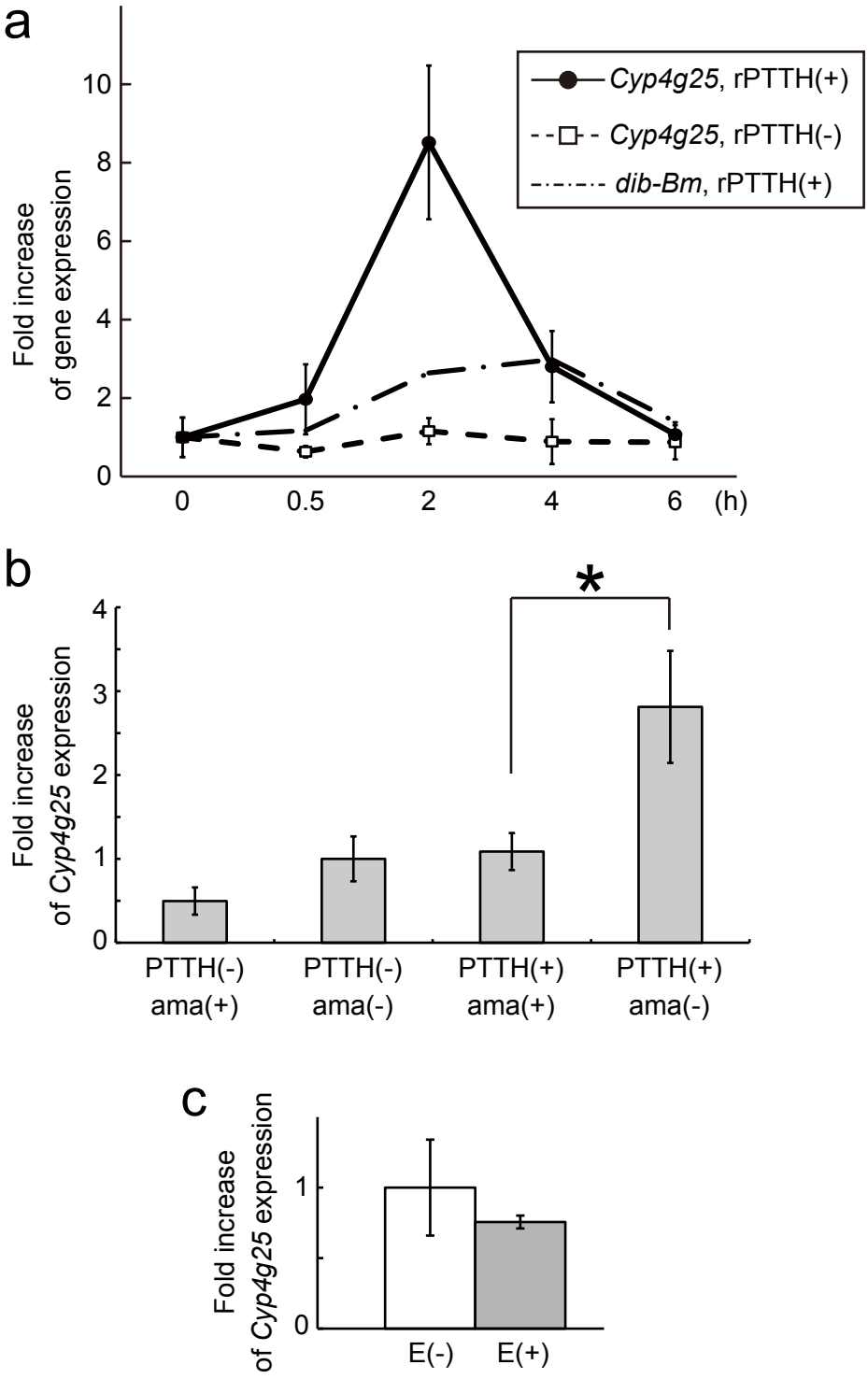

Table 1. Primers used to amplify DNA fragments corresponding to the 86 validated and predicted *D. melanogaster* P450 genes that were spotted on our customized microarray.

Name	Forward (5′ > 3′)	Reverse (5′ > 3′)	Length
Cyp4c3	TGAATGTGGATCACGACGAG	CTCTGGTGGAGCTTGTACT	573
Cyp4d1	ATGTTTCTGGTCATCGG	GCAGATCGTGTCCATGGT	564
Cyp4d1alt	ATGTGGCTCCTACTATCG	GCAAATGGCGTCCAGAGC	537
Cyp4d2	TGGATTCTCCACCAGTTGG	GTTGTTAACCAGCGTTTCACG	571
Cyp4d8	AGCATCTGGTGAAGCATCC	AGTGGACATCAGCAGGACGT	581
Cyp4d14	GATATGCAGTTCCGACTGA	GTCGTGCATGTTCTTCACG	570
Cyp4d20	AAGGGTCAACTCTACGAGT	AGAGCCATCTGCGACTTGCT	560
Cyp4d21	AAAGCTCACCTCTACCGAT	GTCCAGCAAAGTCATCTTAGC	567
Cyp4e1	TCCACTGTTCTTGGTGACC	CTTGCACAACGGAGGAACTT	578
Cyp4e2	ACCACTGCTGCTGGTTGCA	TGCACAATGGAAGAGCTG	576
Cyp4e3	GCCACTGATCACATTGGTG	GGACAATGGAGGAGTCAC	575
Cyp4g1	TAGTTCAGGAGACGCTGCAA	AGGATGTCAACCGTGGTCT	598
Cyp4g15	ATGGAGGTGCTGAAGAAGG	AGAATCTCCACGGTTGCCT	575
Cyp4p1	ATCTTGTGGCTGATTCTGG	CGTGTAACGTTATGGTTACC	541
Cyp4p2	CCATACTTGTGGTCATCCAC	TCTCTGCCATTTCATCCAGT	593
Cyp4p3	GTGGATCTATAGGCTGAACAG	CCATCTCGTCCAGCTTCACA	572
Cyp4s3	GCAACGAATGGAAACCAGAAG	TAGCTTCTCAGGAGCATCG	601
Cyp4aa1	GCTATGCTCCATTCTGATCC	ATGGCCACATCCTGACCTC	594
Cyp4ac1	CGGTCCTAACGCTTCTTCTA	ATCCAGCTTCACACCCAGA	578
Cyp4ac2	TTCGCAAGTTATGGGCTCA	CCTCCGACAAGTCATCAAGT	585
Cyp4ac3	GCTCCTGCTGAGACAACT	CCTTTCTGTACTCGTTTCCT	599

Cyp4ad1	TTGGTGTTCAAGGGAGTGAG	AAGTCCTTGATGGCTCCATG	590
Cyp4ae1	GGCACGATGTACTTTGCCT	GCTTGTCAGTCAATGGTTGC	571
Cyp6a2	TACCTGTTGATCGCGATCTC	CAGCGTGTTACACTCAATGC	582
Cyp6a8	AGGGATTCCCTTCGTTGCAC	AGAGTCCCATGTCTCTTGTC	570
Cyp6a9	AGTGCAGACCAGTCGATCA	TTTCATGTGCAGTCTGCGTG	574
Cyp6a13	ACAGCTACTGGAGCAGAAG	CTCCTGGGTCACTGATCG	560
Cyp6a14	AGGTGTTCCACACGAGACA	TAGCGTGGAATGACGACGT	580
Cyp6a16	TTCACCTACTGGGAACTGC	GGATACTTCTGTTGCTGTTCC	577
Cyp6a17	AATGGATAAGGTCTTCAGAAG	TTCCCTCGTAGGTGAACTCT	587
Cyp6a18	ACTCCATCGCAAACTAACG	TACCATGACGAGAATCCAGC	599
Cyp6a19	ACATTGTCATCACGGACGTG	GTCCATGAAATCGTTCCGAG	575
Cyp6a20	GTACTTCAAGAGGATGGTAG	CCACGAAATCGTGTCTCTTC	587
Cyp6a21	TTAACGAGATCTGGACGAGC	GCATGAAGAACCTTTCGATGG	588
Cyp6a22	AGACCTGTGGTCTTGGTCAC	TCCTCCCTCTGCTTCACAG	496
Cyp6a23	CCGAATGCAGAGTTTGTGAC	AATCTGTCTGGGTCATTCG	544
Cyp6d2	TCAAGGATGTGATGACCACG	CCTCGTAGGTGAACCTATC	581
Cyp6d4	CTTCAAGGAGGTGGACAT	CATCTTGTTCAGGGAATCGT	601
Cyp6d5	GAGCTGGAACTCAAGAAGCT	CCTCCAGATACTTCATGTCC	601
Cyp6g1	GCTCTACACTTGGTTCCAG	ATGCAATCGTGGCTATGCTG	539
Cyp6g2	GAACTGGTACTGCTGATCCT	TGGTGTAGAGAGCACACAG	562
Cyp6t1	GAGACGCACAAGATCTTTGC	CTCGTACAGCGCAAACGTG	573
Cyp6t3	TGGCTAAGTACCATCACTGG	AGAGAGTGAATCCCATCAGG	576
Cyp6u1	CCTTGCAGGATATCTACACC	AAGCTGTAGCAGGTTCTGC	546
Cyp6v1	GATAGTGACGATCCTGACG	CGGTGTTGTACAGATCACAC	555
Cyp6w1	GTTGTTACTGCTTCTTCTCG	TTAGGTCAGTGGTGAACCG	548

Cyp9b1	TTGTTCAAGTGGAGTACTGG	CTCATTCTCTGGGTCATCGA	579
Cyp9b2	CTCATCTACAAATGGAGCACG	TTTCGGGTTGTCGTACGAG	573
Cyp9c1	CAGCACAAGGTCTATGGAG	CCTTTCGATACTTCATGGCAC	592
Cyp9f2	AACATGCTGATGGAGGCTC	GCTTCAAGTAGAGCGAATCG	572
Cyp9h1	ATGATCGGTGGAATGCCAG	GGAGAACCTTCATCAGTCG	578
Cyp12a4	AAAGTTCGCAGTGCTCTATC	ATCTCTCAGTTCGAGAATGC	576
Cyp12a5	CCATCGTCTTCTCTGCAAG	ACCTCCTGAGTGCTGGCAT	553
Cyp12b2	GAGCACTTCGCAACACAAAC	TGCACATCGAACTGGAAGC	569
Cyp12c1	CAGATGCATCATCGTACGTC	ACTGACTCGAAGGTCAGGTG	559
Cyp12d1	AGCACAAGACCTACGATGAG	GAAGAGGGTCAATGCATCG	593
Cyp12e1	GATCTCTAGGCAGATCTACC	AGTCATCTGGCATCTCTTGC	545
Cyp18a1	TCGTGATGAGCGACTACAAG	TTGTGGTCATCGATCACGTC	538
Cyp28a5	CGTGCTGGTATGGAACTATG	GACCATCTCTGTTGTGAAGC	517
Cyp28c1	TCTATGCCTTTCTGGTCTCG	AGTGGATTGTCGGTGAAGGT	568
Cyp28d1	TAGCTACTGGAAGAAGAGG	CTTGGTCATTCCCACCATG	571
Cyp28d2	AGATCATGCCAGCACTGTC	GACCTTATCTTGCTCCTCC	581
Cyp301a1	ATACACTCCACTTCCGAGTG	GCTTCAGATTCGATTCCAGG	604
Cyp302a1	TGGCTAAGATTGCACCAAGC	CGAAAGCTAGGTGTCTCCA	581
Cyp303a1	ACTTGAAGGACAAGGTGCTG	CTCCTTGATCTCCTGAAAGG	545
Cyp304a1	AATCAGGTGTTCGATGGACG	GTAGACATCCATGAAGTTGC	588
Cyp305a1	TTCCGTAAGGAAGCTAGTGC	GATGAGATTGTAGCCAGTGC	591
Cyp306a1	ACTATTGGCTGAGTTCTCC	CGACAATCACTTGTGGTGG	547
Cyp307a1	TGGTGAACAACTTGGAGCTG	GTAGAGCATCTGTGAAGTCC	600
Cyp308a1	CAGAGCATGTCAGTTGCTC	AGAGCCACTGGATCAATCAG	552
Cyp309a1	TGGTGGACAAGTTCAGTCAC	CTGCAGCTGAATGAGATGG	578

Cyp309a2	TGCTACAAGGACTCTCTGC	AAGCTGCAGCAGATGCGAAAG	558
Cyp310a1	ACTTCAGCGAACTGAAGTGG	GATGGACAACAGTTTGTCTGC	527
Cyp311a1	TGACCATTTGGATCCTGGT	ATGGAATGCCTGGATGATGG	566
Cyp312a1	GAACATCTACACGATCATCG	CCTCTGTGAATCCGTGAAG	556
Cyp313a1	CTGATTGCCACAACAAGAGC	ACATTCGCTCTTCACATCC	593
Cyp313a2	GCGAGTCAGAACTAAAGACTG	GGAATGAAGGCGTATGGATG	505
Cyp313a3	ATAGCTGTACAGGAGATGG	GTCACCAGTTGTCTCAAAGG	590
Cyp313a4	TGTTCCTGCTCTGGATCTAC	TCCAGGATGCATTGGTATCG	589
Cyp313a5	TTTCCTGGTGACCTTACTCG	AACTCGATCCAGCTTCACT	595
Cyp313b1	TCCTCTACATCAACGATCC	GGGTTCGAGAAGCTGTTCT	524
Cyp314a1	CTTGAGGACTTCTACCATGC	AAAGTGCACACAGCTTCCAG	578
Cyp315a1	AGTTGGGACACTTGTGGATC	CAATCTGCGTGAAGTAGTCC	563
Cyp316a1	AGCCTACAGTCTGCAAACAG	CGACAATCACTTGTGGTGG	478
Cyp317a1	TGGACATTCCACACGAGAGA	TTAGGTAGCCATGTTTGTGG	593
Cyp318a1	CACTAGTGATGCACCTGAAC	GAGTACAGCTCGACTAAGCA	553

Table 2. Ratio of gene expression levels in the ring gland as compared to expression in the brain-ventral nerve cord complex. These ratios are averages of signal intensities of 8 independent microarray spots in 2 independent experiments.

Name	Ratio
Cyp315a1/sad	26.204
Cyp302a1/dib	19.237
Cyp306a1/phm	14.266
Cyp4g1	10.035
Cyp6g2	6.225
Cyp12e1	2.497
Cyp310a1	2.030
Cyp9c1	1.434
Cyp6v1	1.427
Cyp307a1/spo	1.404
Cyp303a1	1.388
Cyp313a3	1.384
Cyp4p2	1.341
Cyp6a17	1.328
Cyp4ad1	1.299
Cyp6g1	1.282
Cyp314a1	1.268
Cyp317a1	1.268
Cyp6w1	1.259
Cyp4d2	1.257

Cyp4d1alt	1.253
Cyp316a1	1.251
Cyp6u1	1.250
Cyp309a1	1.242
Cyp301a1	1.239
Cyp4e1	1.238
Cyp6d2	1.217
Cyp28d2	1.210
Cyp308a1	1.209
Cyp6a21	1.199
Cyp4e2	1.195
Cyp6a14	1.179
Cyp12a5	1.173
Cyp4s3	1.171
Cyp9b2	1.169
Cyp6d4	1.161
Cyp28c1	1.160
Cyp313b1	1.160
Cyp6a23	1.156
Cyp18a1	1.153
Cyp313a4	1.150
Cyp305a1	1.147
Cyp6a16	1.146
Cyp4c3	1.145
Cyp4ac1	1.144

Cyp6a20	1.139
Cyp6a8	1.139
Cyp4d14	1.139
Cyp304a1	1.137
Cyp311a1	1.135
Cyp4e3	1.134
Cyp313a2	1.132
Cyp6a18	1.132
Cyp4d8	1.127
Cyp318a1	1.125
Cyp6a2	1.124
Cyp4p1	1.120
Cyp4d20	1.119
Cyp6a13	1.112
Cyp9b1	1.111
Cyp4d21	1.109
Cyp4p3	1.107
Cyp6t3	1.106
Cyp4aa1	1.105
Cyp309a2	1.096
Cyp9h1	1.086
Cyp12a4	1.081
Cyp4ac3	1.076
Cyp6a19	1.072
Cyp6a22	1.068

Cyp4g15	1.066
Cyp12c1	1.063
Cyp4ae1	1.059
Cyp313a5	1.058
Cyp312a1	1.056
Cyp28d1	1.040
Cyp28a5	1.039
Cyp313a1	1.037
Cyp6a9	1.031
Cyp4ac2	1.023
Cyp6d5	1.020
Cyp4d1	1.011
Cyp6t1	1.005
Cyp12d1	0.968
Cyp9f2	0.947
Cyp12b2	0.828