Juvenile hormone acid *O*-methyltransferase in *Drosophila melanogaster*

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Abstract (162 words)

Juvenile hormone (JH) acid *O*-methyltransferase (JHAMT) is the enzyme that transfers a methyl group from S-adenosyl-L-methionine (SAM) to the carboxyl group of JH acids to produce active JHs in the corpora allata. While the *JHAMT* gene was originally identified and characterized in the silkworm *Bombyx mori*, no orthologs from other insects have been studied until now. Here we report on the functional characterization of the *CG17330/DmJHAMT* gene in the fruit fly *Drosophila melanogaster*. Recombinant DmJHAMT protein expressed in *E. coli* catalyzes the conversion of farnesoic acid and JH III acid to their cognate methyl esters in the presence of SAM. *DmJHAMT* is predominantly expressed in corpora allata, and its developmental expression profile correlates with changes in the JH titer. While a transgenic RNA interference against *DmJHAMT* has no visible effect, overexpression of *DmJHAMT* results in a pharate adult lethal phenotype, similar to that obtained with application of JH analogs, suggesting that the temporal regulation of *DmJHAMT* is critical for *Drosophila* development.

Key words

corpora allata, *Drosophila melanogaster,* JHAMT, juvenile hormone, methyltransferase

Introduction

Juvenile hormones (JHs) are a family of sesquiterpenoid hormones that play a central role in the control of many of biological processes in insects, including development, growth and reproduction (Gilbert et al., 2000). JHs are synthesized *de novo* in specialized endocrine glands, the corpora allata (CA). A strict regulation of JH titer throughout insect's life is critical to its successful development and reproduction, and the biosynthetic activity in the CA is generally considered to be a major factor in the regulation of JH titer (Gilbert et al., 2000).

A number of genes responsible for the biosynthetic activity in the CA have been characterized (Shinoda and Itoyama 2003; Helvig et al., 2004; Noriega et al., 2006; Kinjoh et al., 2007). Among them, the *JHAMT* gene encodes the *S*-adenosyl-L-methionine (SAM)-dependent JH acid *O*-methyltransferase, which is the enzyme catalyzing the final step of the JH biosynthesis pathway in Lepidoptera (Shinoda and Itoyama 2003). It has been shown that JHAMT of the silkworm *Bombyx mori* (BmJHAMT) methylates the carboxyl group of JH I, II, and III acids (JHAs) to generate hormonally active JHs (Shinoda and Itoyama 2003). BmJHAMT also catalyzes the methylation of farnesoic acid (FA) to methylfarnesoate (MF), the putative JH in crustaceans and in some insect species, including the fruit fly *Drosophila melanogaster* (Jones and Jones 2007). *BmJHAMT* is specifically expressed in the CA, and the temporal gene expression profile of *BmJHAMT* strongly correlates to the JH biosynthetic activity of the CA, suggesting that the transcriptional control of the *BmJHAMT* gene is critical for the regulation of JH biosynthesis in the CA (Shinoda and Itoyama 2003; Kinjoh et al., 2007). As putative orthologs of *JHAMT* have been found in several insects (Shinoda and Itoyama 2003), the *JHAMT* genes appear to have a conserved role among species.

However, direct evidence for the significance of *JHAMT* in the regulation of JH biosynthesis in insects other than *B. mori* is still missing.

Toward understanding the roles of JHAMT in insect development via the regulation of JH biosynthesis *in vivo*, here we report on the identification and the functional characterization of the *Drosophila melanogaster* ortholog of *JHAMT*. We show that purified recombinant DmJHAMT, like BmJHAMT, catalyzes the conversion of JHA III and FA to JH III and MF, respectively, in the presence of SAM. *DmJHAMT* is predominantly expressed in the CA, and its developmental expression profile correlates well with changes in the JH titer during *Drosophila* development. Furthermore, overexpression of *DmJHAMT* leads to pupal lethality and a misorientation of male genitalia, which are similar to phenotypes obtained following application of JH analogs. These results suggest that the temporal control of *DmJHAMT* activity is critical for *Drosophila* development.

Materials and methods

Animal strains and culture

All *Drosophila melanogaster* flies were reared on a standard medium at 25 ºC under a 12-h light/12-h dark photoperiod. The *D. melanogaster* strains, *AUG21-GAL4* (Siegmund and Korge 2001) and *Actin5c-GAL4* (originally established by Dr. Y. Hiromi), were provided from Dr. G. Korge and the Bloomington stock center, respectively.

Chemicals

Racemic JH III was purchased from Sigma and purified by HPLC before use as described

(Shinoda and Itoyama 2003). JH III acid (JHA III) was prepared from the purified racemic JH III as described (Goodman and Adams 1984). Farnesoic acid (FA; (*2E,6E,10E*)-3,7,11-trimethyldodecatri-2,6,10-eneoic acid) and methyl farnesoate (MF; Methyl-(*2E,6E,10E*)-3,7,11-trimethyldecatri-2,6,10-eneoate) were purchased from Echeron Biosciences. A synthetic JH analog, methoprene, was a gift from Dr. Ehrenstorfer-Schafers (Augsburg, Germany). JH I was purchased from SciTech (Prague, Czech Republic). Other reagents were purchased from commercial suppliers as described (Shinoda and Itoyama 2003).

Molecular cloning

Drosophila JHAMT (*DmJHAMT*) was identified from the *Drosophila* genome sequence by a BLAST search and corresponds to the gene annotated as *CG17330* (www.FlyBase.org). The cDNA containing the entire open reading frame (ORF) for *DmJHAMT* was amplified by RT-PCR. RNA was extracted from the Oregon R strain using the RNAeasy Mini kit (Qiagen). After a reverse-transcription reaction using First-strand cDNA synthesis kit (Amersham-Pharmacia) with an oligo dT primer, PCR was performed using the following primers: DmJHAMT-F, 5′-AAACATATGAATCAGGCCTCTCTATATCAG-3′; DmJHAMT-R, 5′-AACTCGAGGACTCTGTTAACAAATGCAATTACTG-3′. The PCR product was cloned into a TA-cloning vector, pDrive (Qiagen). The DNA sequence of *DmJHAMT* was deposited in GenBank (Accession no. **AB113579**).

Protein expression

The fragment containing the *DmJHAMT* ORF was excised from a *Nde*I/*Xho*I-digested *DmJHAMT*-pDrive and then cloned into a *Nde*I/*Xho*I-digested pET28a(+) (Novagen).

BL21(DE3) *E. coli* cells (Novagen) were then transformed with *DmJHAMT*-pET28a(+) for protein expression. Preparation and purification of recombinant 6xHis-tagged protein was performed essentially as described (Shinoda and Itoyama 2003). After the protein purification with a HiTrap Chelating column HP (GE Healthcare), glycerol was added to the enzyme solution (final concentration 25%), and the sample was frozen immediately in liquid N_2 and stored at –80 ºC until use.

Antiserum and western blot

A polyclonal antiserum against the DmJHAMT was prepared by immunizing a rabbit with 0.2 mg of the purified recombinant DmJHAMT protein by 6 injections. Western blot analysis was performed with the anti-DmJHAMT antiserum (1:5000 dilution) and peroxidase-conjugated anti-Rabbit IgG (Pierce; 1:10,000 dilution), followed by detection using the TMB Membrane Peroxidase Substrate System (KPL). Precision plus protein standards (BIO-RAD) were used as molecular markers.

Enzyme assay and analysis

Enzyme assays were performed essentially as described with some modifications (Shinoda and Itoyama 2003). Briefly, JHA III (50 μM), FA (50 μM), lauric acid (LA; 100 μM), or palmitic acid (PA; 100 μM) were dissolved individually in 500 μl of Tris-Cl buffer (50 mM, pH 7.5), with or without 500 μM SAM in a siliconized glass tube (12 x 75 mm). The enzymatic reactions were started by the addition of 2 μg (for JHA III and FA) or 10 μg (for LA and PA) of the purified DmJHAMT protein. After incubation at 25 ºC for 5 min (for JHA III and FA) or 60 min (for LA and PA), the reactions were stopped by the addition of 500 μl of CH_3CN. Under these conditions, the rate of product formation was linear during the assays (data not shown). The reaction mixture was centrifuged at 4,200

g for 5 min, and the supernatant was subjected to qualitative and quantitative analyses of the products by HPLC and GC-MS.

JH III and MF generated from JHA III and FA, respectively, were analyzed by reversed-phase (RP)-HPLC with a Shimadzu LC10 apparatus and a Shiseido ODS UG80 column (3 x 150 mm). The conditions were: mobile phase, 60% CH_3CN for JH III (Fig. 2, A-C) and 80% CH_3CN for MF (Fig. 2, D-F); flow, 0.5 ml/min; detector, UV at 219 nm. Stereospecificity of the enzymatically produced JH III was analyzed by a chiral-HPLC (Ichikawa et al., 2007) using a HP1100 series HPLC system (Hewlett-Packard) and a chiral-column, Chiralpack IA (4.6 x 250 mm, DAICEL). Supernatant from the reaction with JHA III was extracted with hexane and concentrated under N_2 gas stream and subjected to chiral-HPLC. The HPLC conditions were: mobile phase, hexane:EtOH, 99:1; flow, 0.5 ml/min; detector, UV at 219 nm.

Products from LA and PA were analyzed by GC-MS. Supernatants of the reactions with LA and PA were extracted with hexane containing 5 μg/ml methyltridecanoate as an internal standard, concentrated under N_2 gas stream and analyzed with GCMS-QP2010 (Shimadzu) equipped with a GC column DB-35MS (30 m x 0.25 mm I.D., 0.25 μm film, J&W Scientific). The carrier gas was helium (1.2 ml/min), and the injector port temperature was 280 ºC. The samples were introduced by split injection. The column oven temperature was programmed at 120 ºC for 2 min before being elevated to 240 ºC at 12 ºC increase per min and then held for 10 min. EI-MS detector was set at 70-eV ionization with selective ion monitoring (SIM) mode on m/z at 74.

Reverse transcription (RT)-PCR analysis

Total RNA preparation derived from whole fly bodies and single-stranded cDNA

synthesis were performed as previously described (Niwa et al., 2004). Specific primers for *DmJHAMT* (forward, 5′-GACCATGTCACCTCGTTCTACTGC-3′; reverse, 5′-GAAGTCATCCAGGAACTGTTCATGC-3′) and *juvenile hormone esterase* (*jhe*) (forward, 5′-GGTGAACATTCTGGGCAATGAGACG-3′; reverse, 5′-GTGACTGGAGCACCTCAATGGAG-3′) were used. *rp49* was used as a loading control (Niwa et al., 2004). Quantitative RT-PCR was performed as previously described (Shinoda and Itoyama 2003). PCR conditions for data shown in Fig. 3B, 3I and 4A were 94 ºC for 5 min, followed by 34 cycles of 94 ºC for 30 sec, 57 ºC for 30 sec, and 72 ºC for 1 min.

Transgenic flies

Overexpression and RNA interference (RNAi) studies were performed using the GAL4/UAS system (Brand and Perrimon 1993). The construct for *DmJHAMT* overexpression (*UAS-DmJHAMT*) was generated by first amplifying an ORF from *DmJHAMT*-pDrive by PCR using the primers DmJHAMT-F/BglII (5'-AAAAGATCTATGAATCAGGCCTCTCTATATCAG-3') and DmJHAMT-R, and ligating the product into the *Bgl*II/*Xho*I site of the pUAST vector (Brand and Perrimon 1993). The construct for transgenic RNAi (Kennerdell and Carthew 2000) against *DmJHAMT* was generated from the 4-677 bp region of exon and intron of the *DmJHAMT* gene (nucleotide numbering as in **AB113579**). The cDNA region was amplified by PCR using the primers DmJHAMT[655-635]/XhoI (5′-AAACTCGAGCTTTCAGAGTCCTTACACCT-3′) and DmJHAMT[7-26]/XbaI (5′-AAATCTAGAAATCAGGCCTCTCTATATCA-3′). The genomic region, containing a part of the 3′ end of the first intron, was amplified by PCR using primers: DmJHAMT[7-26]/BglII (5′-AAAAGATCAATCAGGCCTCTCTATATCA-3′) and

DmJHAMT[744-725]/XhoI (5′-AAACTCGAGAAAGGACAAATGGCCTTTAC-3′). The cDNA and genomic fragments were digested by *Xho*I/*Xba*I and *Bgl*II/*Xho*I, respectively, and then ligated into pUAST digested with *Bgl*II/*Xba*I. *Drosophila* transformants were obtained using standard protocols (Spradling 1986).

Topical application of JH and its analog

Methoprene (5 ng) or JH I (25 ng) diluted in 100% ethanol was applied to wandering Oregon R 3rd instar larvae, as the only sensitive period for application is during late larval-early pupal development (Postlethwait and Weiser 1973).

Histological analyses

In situ RNA hybridization and immunostainng were performed as described (Niwa et al., 2004). Digoxigenin (DIG)-labeled RNA probes were synthesized from *DmJHAMT*-pDrive using a DIG RNA labeling kit (Roche) and T3 or SP6 RNA polymerase (Invitrogen). Under our conditions, no signal was detected using a sense RNA probe (data not shown). For immunostaining, the rabbit anti-JHAMT antiserum was applied at a 1:200 dilution in PBS at 4 ºC and incubated overnight. The signal was visualized using the Alexa488-coupled secondary antibody (Molecular Probes) at a 1:200 dilution. Alexa568-conjugated phalloidin (Molecular Probes) was also used. Specimens were observed under a laser scanning confocal microscope LSM510 (Zeiss). For observations using the scanning electron microscope (SEM), pharate adults were dissected from pupara, glued on a SEM stage with a regular bond, and then observed with a S-3000N SEM (Hitachi) under a low vacuum condition.

Results and Discussion

CG17330 encodes functional SAM-dependent JH acid *O*-methyltransferase (DmJHAMT)

A tBlastn search has revealed that *CG17330* in *Drosophila melanogaster* is the only gene encoding a predicted protein with substantial similarity to the BmJHAMT protein (41%) (Shinoda and Itoyama 2003). The predicted protein CG17330 contains a SAM binding motif (LLDIGCGSG) that is commonly found in members of SAM-dependent methyltrasnferases (Wu et al., 1992). We hereafter refer to this gene as *DmJHAMT (Drosophila melanogaster homolog of JHAMT)*.

The *D. melanogaster* CA produces JH III, JH III bisepoxide (JHB_3), and MF as major products (Jones and Jones 2007). We investigated whether DmJHAMT introduced the methyl group to the immediate precursors of JH III and MF, namely JHA III and FA, respectively, *in vitro*. We expressed His-tagged recombinant DmJHAMT protein in *E. coli* and prepared a purified protein using a Ni-column (Fig. 1A). When JHA III and FA were incubated with the purified DmJHAMT protein in the presence of SAM, a major peak was observed in the reactions by reverse-phase (RP)-HPLC (Fig. 2, B and E). The retention times of the major metabolites of JHA III and FA corresponded to the standard JH III and MF, respectively (Fig. 2C and F). These peaks were further confirmed to be JH III and MF, respectively, by GC-MS (data not shown). In contrast, these peaks were not observed in the same reactions but lacking SAM (Fig. 2A and D). The catalytic activity of DmJHAMT against JHA III and FA were nearly equal (Table I). Preliminary kinetic analyses revealed that *Km* (μM) and *Vmax* (mol product/mol enzyme/min) values were 3.32±2.28 and 5.58±1.66 against racemic JHA III, and 0.18±0.03 and 5.77±0.90 against FA (mean±SD, n=3). When the DmJHAMT protein was incubated with saturated

long-chain fatty acids, laurate acid (LA) and palmitate acid (PA), in the presence of SAM, the production of low levels of methyl laurate and methyl palmitate was detected by GC-MS, respectively. However, the catalytic activities of DmJHAMT for LA and PA were less than 1% that for FA (Table I). These results suggest that, like BmJHAMT (Shinoda and Itoyama 2003), DmJHAMT is highly specific to farnesoid acids, such as FA and JHA III, but not to non-branched fatty acids.

We also examined whether the DmJHAMT protein was enantioselective. Such enantioselectivity has been documented in a study on an JHAMT activity in *Cecropia* (Peter et al., 1981), whereas it has been found absent in *Locusta* has been reported (Hamnett et al., 1981). In our experiment, we used racemic JHA III prepared by an alkaline hydrolysis method (Goodman and Adams 1984) from racemic JH III (Fig. 2G). However, chiral-HPLC analysis revealed that the metabolite from the JHA III racemate (25 μM for each *R* and *S* isomer) by DmJHAMT contained *(10S)*- and *(10R)*-JH III isomers at a ratio of 20:80 (Fig. 2H). Under the same conditions, recombinant BmJHAMT generated *(10S)*- and *(10R)*-JH III isomers at a ratio of 2:98 (Shinoda, unpublished data). This result indicates that DmJHAMT has moderate enantioselectivity to *(10R)*-JHA III. Although the absolute configuration of JH III in *D. melanogaster* remains to be determined, JHB_3 produced by another Dipteran, *Lucilia cuprina*, has the absolute configuration 10*R* (Herlt et al., 1993). The stereospecificity of DmJHAMT is consistent with an idea that the *(10R)*-isomer is also the natural form of JH III in *D. melanogaster*.

These results demonstrate that *DmJHAMT/CG17330* encodes a functional JHAMT involved in the production of JH III and MF from JHA III and FA, respectively. JHB_3, the major JH in *Drosophila*, is expected to be synthesized by the methylation of 6,7;10,11-bisepoxy farnesoic acid (JHB_3 acid) to JHB_3, rather than by the epoxidation of

MF and JH III (Moshitzky and Applebaum 1995). DmJHAMT is thus likely to also catalyze the methylation of JHB_3 acid to JHB_3.

Tissue- and stage-specific expression of *DmJHAMT*

The temporal expression profile of *DmJHAMT* was examined by quantitative real-time RT-PCR analysis. The expression level of *DmJHAMT* was high in the 1st instar larvae and then gradually decreased during larval development, while the significant re-increase of the *DmJHAMT* expression was observed in the wandering 3rd larval stage (Fig. 3A). The lowest amount of expression was observed in the early- and mid-pupal stages (Fig. 3A). After this, the expression increased in the late pupal stage in both male and female adults (Fig. 3A). This overall change in the mRNA expression level of *DmJHAMT* positively correlated with changes in hemolymph JH titers during *Drosophila* development (Riddiford 1993). RT-PCR analysis also revealed that *DmJHAMT* was predominantly expressed in larval tissues containing CA, and a trace amount was also detected in the testis (Fig. 3B).

To further analyze spatial localization of *DmJHAMT* during development, *in situ* RNA hybridization and immunostaining were performed. In embryogenesis, no *DmJHAMT* expression was detected in the blastoderm, gastrulation and germ band elongation stages (data not shown). *DmJHAMT* expression was first seen at the germ band retraction stage (stage 13) in somatic muscles (Fig. 3C). Then *DmJHAMT* disappeared in all tissues and reappeared in primordial CA at stage 17 (Fig. 3D). In addition to being expressed in late embryogenesis, *DmJHAMT* mRNA was expressed specifically in larval CA cells but not in the prothoracic gland or corpora cardiaca cells of the ring gland, or other tissues examined except the testis (Fig. 3B and E). For immunostaining, we generated a specific antiserum against DmJHAMT protein (Fig. 1B).

Using the antiserum, DmJHAMT was also observed predominantly in the CA in both the larval and adult stages (Fig. 3F and H). These results indicate that the expression of *DmJHAMT* coincides with JH biosynthesis in both a spatial and temporal manner, which is similar to the characteristics of *BmJHAMT* (Shinoda and Itoyama 2003). It is also of note that, to our knowledge, this is the first identification of a gene expressed predominantly in the CA of *D. melanogaster*.

Overexpression of *DmJHAMT* causes a pupal lethality and a rotation defect on male genitalia

To assess the importance of *DmJHAMT* during *Drosophila* development, we examined gain-of-function phenotypes in developing flies. We found that the overexpression of *DmJHAMT* specifically in the CA using flies carrying *UAS-DmJHAMT* and *AUG21-GAL4*, which is active in the CA cells (Siegmund and Korge 2001; Adám et al., 2003), showed no irregular phenotype. In contrast, we found that individuals overexpressing *DmJHAMT* using a ubiquitous *Actin5c* promoter failed to develop into adults. The flies carrying *Actin5c-GAL4* and *UAS-DmJHAMT* (*Actin5c>DmJHAMT*) consistently exhibited higher expression of *DmJHAMT* through the wandering larval and pupal stages, as compared to wild type (Fig. 4A). Under our experimental conditions, wild type flies took 4 days from puparium formation to eclosion, and there was no significant mortality at the pharate adult stage (P4) in wild type animals (<1%; n=99). In contrast, the *Actin5c>DmJHAMT* animals completed embryogenesis and larval development normally (data not shown), but showed a prolonged pupal development. The majority (97%; n=110) of the *Actin5c>DmJHAMT* flies became pharate adults 9 days after puparium formation (P9) and all died before eclosion (100%; n=107). The phenotype of the *Actin5c>DmJHAMT* animals were reminiscent of wild type animals

topically treated with JH analogs, which also exhibit no effect during the larval stage but show lethality at the pharate adult stage (Riddiford and Ashburner 1991). To address the question whether the *Actin5c>DmJHAMT* animals exhibited another class of phenotypes that is related to higher doses of JH, male genitalia of the *Actin5c>DmJHAMT* animals were also examined. It has been shown that either the application of high doses of JH analogs or the presence of mutation presumed to result in higher JH titer *in vivo* could cause a rotation defect of male genitalia (Riddiford and Ashburner 1991; Adám et al., 2003). Interestingly, the *Actin5c>DmJHAMT* animals (70%; n=10) also displayed rotation defects (Fig. 4, B and C) that are very similar to those observed in flies with high doses of JH I (Fig. 4D) or methoprene (data not shown). These observations support the idea that the forcible expression of *DmJHAMT* causes the overproduction of active JH and results in abnormalities during pupal-adult development, a time when JH must be absent.

Our data demonstrate that overproduction of DmJHAMT in the whole body, but not CA, affects *Drosophila* pupal development. The expression of hemolymph JH esterase (JHE), which catalyzes the catabolism of active JHs into JHAs, significantly increases during the pupal stage (Fig. 3I) (Kethidi et al., 2005). Therefore, one possible interpretation is that, even if the CA of the *AUG-21>DmJHAMT* flies continue to synthesize active JHs in the wandering and pupal stages, the hemolymph JHE is sufficient to inactivate JHs before they reach to the target tissues. On the other hand, the *Actin5c>DmJHMAT* animals express *DmJHAMT* in almost all somatic cells. Therefore, even after JHE inactivates JHs and produces JHAs in hemolymph, DmJHAMT in the peripheral target tissues can convert JHAs to active JHs, resulting in a phenotype that is similar to phenotypes caused by JH application. In addition, our results suggest another possibility that JHE plays a more important role than JH epoxide hydrolase (JHEH) in the

control of JH titer at the pupal stage as previously reported (Khlebodarova et al., 1996), because JH acids generated by JHE, but not JH diols metabolized by JHEH, can be converted to active JHs.

Knock-down of *DmJHAMT* by transgenic RNAi

Since a *DmJHAMT* genetic mutant is not currently available, we examined the effects of the knock-down of *DmJHAMT* using a transgenic RNAi technique that is known to be effective in degrading endogenous target mRNA in *Drosophila* (Kennerdell and Carthew 2000). We established transgenic lines in which double-stranded RNA molecules corresponding to *DmJHAMT* were generated using an inverted repeat construct under the control of the *UAS* promoter. To knock down *DmJHAMT* specifically in the CA, the *UAS* lines were crossed with *GAL4* lines, *AUG21-GAL4*, in which the *GAL4* transgene is active in the CA cells (Siegmund and Korge 2001; Adám et al., 2003). The DmJHAMT protein level in the CA of the RNAi larvae significantly decreased and was undetectable by immunostaining with anti-DmJHAMT antibody (Fig. 3G). The *DmJHAMT* RNAi animals, however, exhibited no visible effect on *Drosophila* development (data not shown). We did not find any irregular phenotype in *Actin5c-GAL4* and *UAS-RNAi* constructs either (data not shown). The absence of a phenotypic effect from the RNAi treatment was puzzling given that the experimental removal of JH causes premature metamorphosis in insects (Riddiford 1996). It is possible that this is due to a peculiarity of the cyclorrhaphous Diptera, including *Drosophila*, where exogenous JH does not show the typical "status quo" effect on larval development that normally occurs in other insects (Gilbert et al., 2000). Alternatively, it is important to point out that RNAi, in general, results in partial, but not complete, loss-of-function animals. Therefore, even though DmJHAMT protein was undetectable by immunostaining in the CA of the RNAi animals,

it is still possible that the residual DmJHAMT activity is enough to produce small but sufficient amount of JH to maintain larval status in *Drosophila*. This hypothesis is supported by the fact that flies overexpressing JHE under the *DmJHAMT* RNAi background cause premature wandering behavior, which may indicate early metamorphosis (E. Gervasio and J.-P. Charles, unpublished data). Isolation and study of genetic null mutants of *DmJHAMT* are necessary in order to determine the function of *DmJHAMT* in more detail.

Conclusion

Our study demonstrated that the *CG17330/DmJHAMT* gene encodes a JH acid *O*-methyltransferase that is specifically expressed in the CA and functions to produce MF and JH III, and most likely JHB_3. This is the first *JHAMT* gene to be functionally characterized in Diptera. Furthermore, transgenic flies overexpressing *DmJHAMT* suggest that the proper temporal regulation of this gene is critical for *Drosophila* development. Transgenic flies overproducing or reducing *DmJHAMT* have promise to reveal hidden functions of JH in this species and greatly contribute to the dissection of the molecular mode of JH action.

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Figure legends

Fig. 1. Preparation of recombinant DmJHAMT protein expressed in *E. coli*

(A) Coomassie brilliant blue (CBB) staining of recombinant DmJHAMT expressed in *E. coli*. Samples were: lane 1, crude supernatant from BL21(DE3)/pET28a (empty vector); lane 2, crude supernatant from BL21(DE3)/pET28a/DmJHAMT; and lane 3, DmJHAMT (marked by arrowhead) purified from the product shown in lane 2 by Ni-column. The amounts loaded were equivalent to 100 μl of *E. coli* culture in lane 1 and 2; and 0.3 μg of the purified DmJHAMT in lane 3. M, molecular size marker. (B) Western blot using anti-DmJHAMT antiserum. 1/10 volume of the amounts shown in A were loaded. The specific signal of DmJHAMT is marked with an arrowhead.

Fig. 2. Recombinant DmJHAMT methylates JHA III and FA *in vitro*

(A-F) RP-HPLC analyses were performed after the incubation of the purified recombinant DmJHAMT protein and substrates. Vertical axes represent UV absorption at 219 nm. Metabolites generated from JHA III and FA by recombinant DmJHAMT, in the absence (A and D, respectively) and presence of SAM (B and E, respectively) are shown. Arrows and arrowheads indicate JH III and MF peaks, respectively. The retention times of enzymatically produced JHs (11.5 min for JH III and 11.7 min for MF) were identical to those of standards (C and F). (G, H) Chiral-HPLC analysis was performed with racemic JH III (G) and metabolites obtained from incubation of racemic JHA III with purified DmJHAMT protein and SAM after a 4 min reaction (H). It should be noted that the *S:R* ratio of the racemic JHA III used as substrate was ~50:50 (data not shown), as the JHA III was synthesized by alkaline hydrolysis from the racemic JH III shown in (G) (Goodman

and Adams 1984). Arrows and arrowheads indicate (*10S*)-JH III (retention time: 14.0 min) and (*10R*)-JH III (retention time: 16.4 min), respectively.

Fig. 3. Spatiotemporal expression pattern of *DmJHAMT* mRNA and protein

(A) Quantitative RT-PCR analysis showing temporal expression profile of *DmJHAMT* and a reference gene, *rp49*. *DmJHAMT/rp49* indicates the levels of *DmJHAMT* mRNA normalized to the levels of internal *rp49* mRNA. Total RNAs were extracted from whole fly bodies in each stage. Embryos (E) and 1st (L1), 2nd (L2) and 3rd (L3) instar larvae were collected at 12 h intervals from 0 hours after egg laying (AEL) to 144 h AEL. Pupae (P) were also collected at 12 h intervals from 0 h after puparium formation (APF) to 96 h APF. Adults (Ad) were collected 0-12 h after eclosion. (B) RT-PCR analysis showing tissue expression profile in wandering 3rd instar larvae. (C-E) *in situ* RNA hybridization of *DmJHAMT*. (C, D) Embryonic expression. Signals were detected in somatic muscles at stage 13 (C) and in embryonic CA at stage 17 (D, arrowhead). (E) Brain-ventral nerve cord-ring gland complex of the wandering stage of 3rd instar larvae. Expression was detected only in the region of the CA (arrowhead). (F-H) Imunostaining with anti-DmJHAMT antibody (green). Overall morphology of tissues was visualized with fluorescence-phalloidin (purple). Arrowheads indicate CA. (F, G) 3rd instar larval ring gland. (F) DmJHAMT protein was localized in the CA of a control animal (*yw;AUG21-GAL4/+*). (G) No signal was detected in the CA of *DmJHAMT* RNAi animals (*yw; AUG21-GAL4; UAS-DmJHAMT-IR*). (H) CA in wild type adult males. es, esophagus; pv, proventriculus. (I) RT-PCR analysis showing the expression profile of *JH esterase (jhe)* and *rp49*. The samples are the same as shown in A.

Fig. 4. Overexpression of *DmJHAMT* causes rotation defect of male genitalia

yw; UAS-DmJHAMT/+; Actin5c-GAL4/+ was used as the overexpressor of *DmJHMAT* (*DmJHAMT* o/e). (A) RT-PCR analysis showing the expression of *DmJHAMT* and a reference gene *rp49* in control animals, *yw; UAS-DmJHAMT/+* (left), and *DmJHAMT* o/e (right). (B-D) Scanning electron micrographs of male abdomen (ventral view; posterior is downwards). The direction of genitalia rotation in each sample is marked by an arrow, whose starting point is at the position of the penis. (B) A control animal (*yw; UAS-DmJHAMT*). (C) The *DmJHAMT* o/e animal. (D) A wild type (Oregon R) with topical application of 25 ng JH I at wandering 3rd instar larval stage.

Fig. 1
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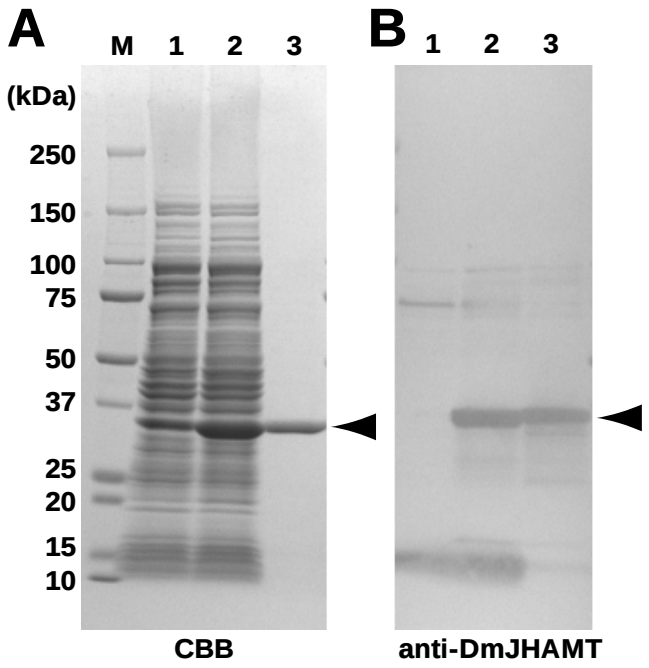

Figure 2

Fig. 2
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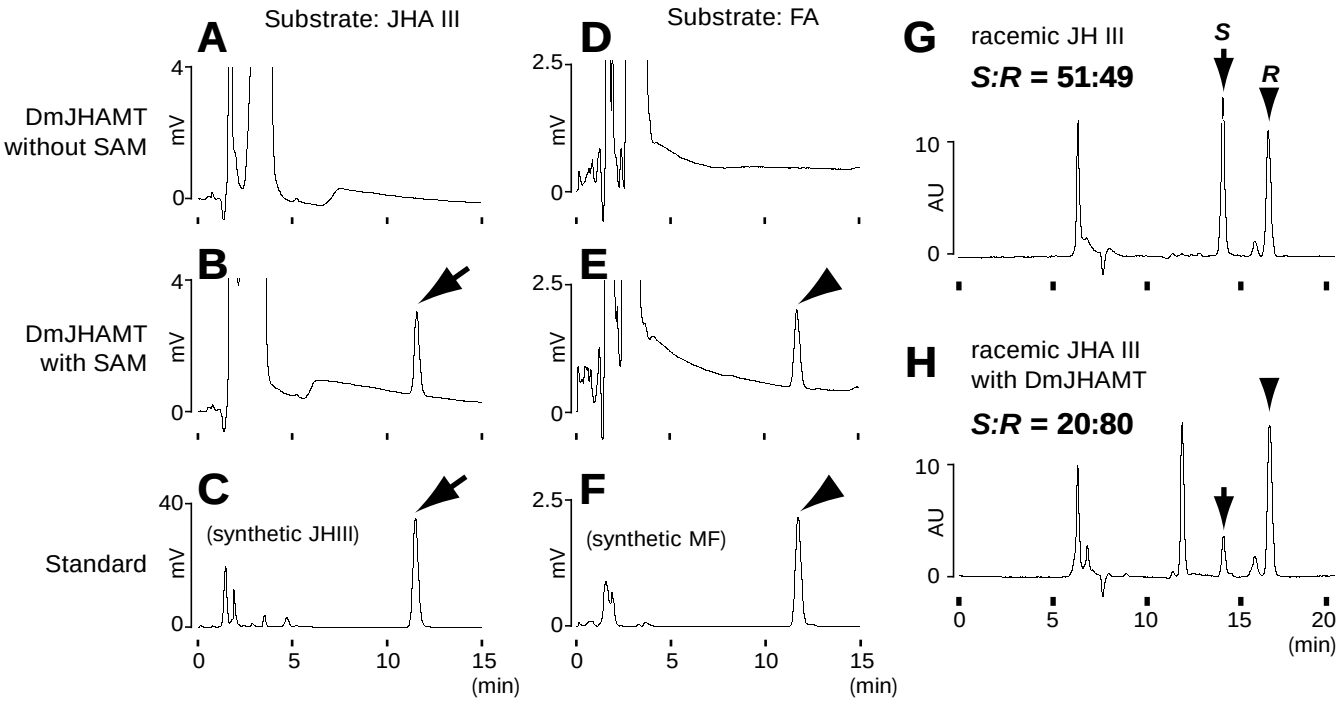

Figure 3

Fig. 3
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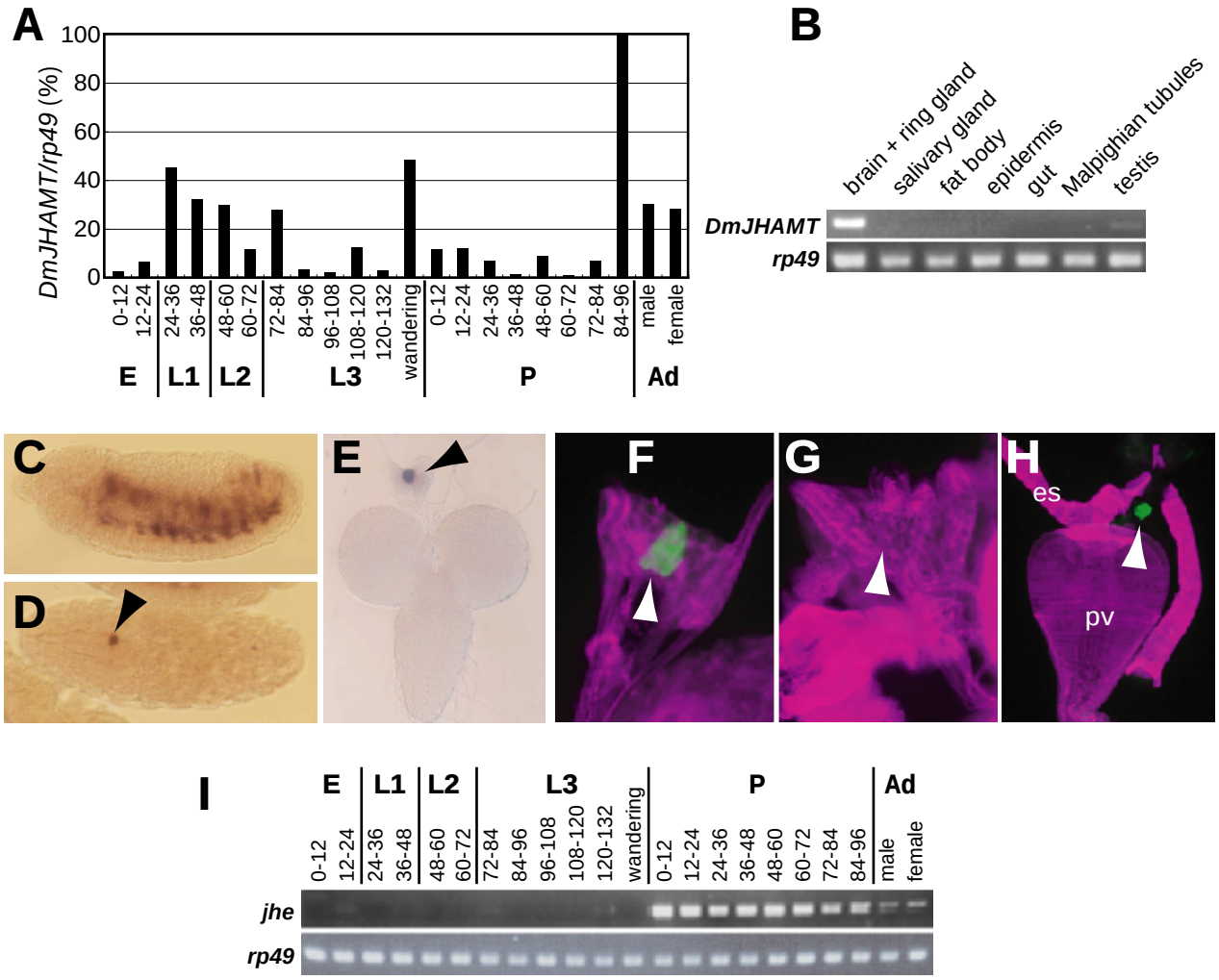

Figure 4

Fig. 4
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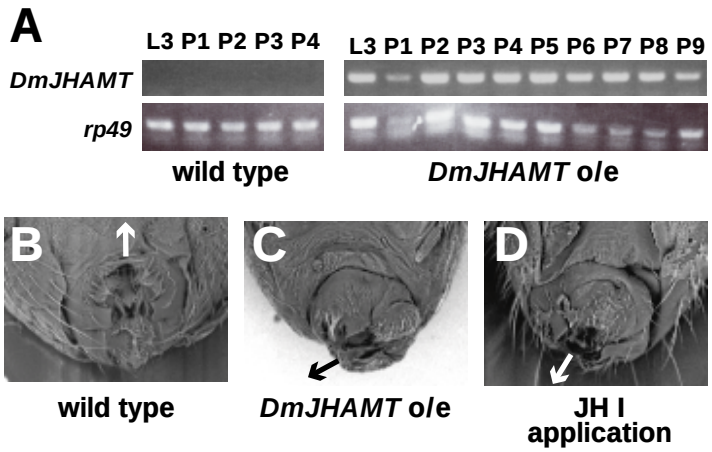

Table I. Enzymatic activity of recombinant DmJHAMT on JH III, FA and fatty acids

Substrates	k_{cat} (min^{-1}) (mean±SD, n=3)
JH III acid (racemate)	7.7 ± 0.4
Farnesoic acid	10.1 ± 0.5
Laurate acid	0.103 ± 0.007
Palmitate acid	0.041 ± 0.008