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Drosophila CG10527 mutants are resistant to juvenile hormone and its analog methoprene

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ABSTRACT

Juvenile hormone (JH) is critical for development, metamorphosis, and reproduction in insects. While the physiological importance of JH has been appreciated for decades, its biosynthetic pathway and molecular action remain poorly understood. *Drosophila CG10527* encodes a protein with high homology to crustacean farnesoic acid methyltransferase (FAMeT) that converts farnesoic acid to methyl farnesoate (MF), a precursor of JH, but its *in vivo* functions remain unclear. Here we report that *CG10527* is expressed widely in secondary cells in the male accessory glands, in ovarian follicle cells, and in glial cells in the nervous system. Furthermore, *CG10527* is expressed abundantly in the corpora allata where JH is synthesized. To understand the physiological functions of *CG10527*, we generated specific *CG10527* deletions. Phenotypic analysis showed that *CG10527* null mutants are fully viable and fertile in both sexes, indicating that *CG10527* is not essential for survival and fertility. Surprisingly, *CG10527* mutants showed no defects in the biosynthesis of MF and JH. However, *CG10527* mutants were 3–5 times more resistant than wild-type flies to topically applied MF and JH as well as the JH analog methoprene at both sub-lethal and lethal doses. Taken together, our data indicate that *Drosophila CG10527* plays little, if any, role in JH biosynthesis but may participate in the JH signaling pathway.

1. Introduction

Juvenile hormone (JH) is a sesquiterpenoid hormone in insects secreted by endocrine glands called corpora allata (CA). JH affects a vast array of physiological processes including growth, development, metamorphosis, reproduction and behavior (for reviews, see [1–3]). There are several forms of JH-like compounds such as JH III and JH3 bisepoxide (JHB_3) produced in insects. Additionally, methyl farnesoate (MF), a precursor of JH III lacking the epoxide group, first isolated from spider crabs [4], is present in many species of crustaceans [5,6], where it plays a critical role similar to that of JH in insects. MF also has JH activity in insects such as the fruit fly *Drosophila melanogaster* [7,8]. Due to their importance in development and reproduction, chemically synthesized JH analogs have been developed to control insect pests. For example, methoprene, a JH analog and agonist, has been widely used to control insect pests by disrupting their metamorphosis [2].

There are two alternative biosynthetic pathways from farnesoic acid (FA) to JH in insects [2]. In the major pathway, which accounts for 95% of the total JH titer, JH acid methyltransferase (JHAMT) converts JH acid to active JH [9–11]. Alternatively, FA methyltransferase (FAMeT) converts FA to MF, which is then transformed to active JH by JH epoxidase [2]. In *Drosophila*, *CG17330* encodes a protein with JHAMT activity [9,10], while *CG10527* encodes a protein with high homology to crustacean FAMeT [12,13]. CG10527 shares 46.5% and 48.9% similarity with FAMeTs of shrimp (NCBI protein entry number AAZ22180) and lobster (AAA67081), respectively. The gene encoding FAMeT was first identified in shrimp [14] and subsequently in many other crustaceans [6,15]. However, whether *Drosophila CG10527* encodes a protein with FAMeT activity has not been determined.

To uncover the *in vivo* functions of *CG10527*, we generated CG10527-specific antibodies and found by immunostaining that *CG10527* is widely expressed with high enrichment in the CA in which JH is synthesized. Phenotypic analysis revealed for the first time that *CG10527* null mutants show no defects in the biosynthesis of MF and JH indicating that CG10527 may not be involved in the biosynthesis of JH. However, we found that *CG10527* mutants show mild resistance to JH, MF, and the JH analog methoprene

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compared to the well characterized *methoprene tolerant* (*Met*) mutants. *Met* encodes a putative transcriptional regulator which may function as a JH receptor [16–19]. Taken together, our data show that *CG10527* plays little, if any, role in JH biosynthesis but may participate in JH signaling. The exact functions of *CG10527* remain to be elucidated.

2. Materials and methods

2.1. Drosophila stocks and husbandry

All *Drosophila* cultures were grown at 25 °C on a standard cornmeal medium. The deficiency line *ED3755* was provided by John Roote at Cambridge University. Glial-cell-specific *repo-Gal4* was from Dr. K. Ito at Tokyo University. All other stocks including *UAS-GFP*, Met^3, and *KG05615* which has a *p*-element insertion in the 5′ regulatory region of *CG10527*, were obtained from the Bloomington *Drosophila* Stock Center.

2.2. Generation of CG10527 mutants

P-element-mediated excision was used to generate a series of deletions in or near *CG10527* following a standard protocol. The original stock *KG05615* from Bloomington has a *p*-element insertion 194 bp upstream of the transcription start site (Fig. 1A). Before mobilizing this *p* element using *p* transposase △2–3 we isogenized chromosome 2 of the original stock. Candidate w^- deletion lines in which the *p* insertion had been excised either precisely or imprecisely were initially screened by PCR followed by sequencing, in conjunction with immunochemical analysis to confirm the mutants at the protein level.

2.3. Generation of specific antibodies against CG10527

For production of antibodies against CG10527, highly purified, His-tagged full-length fusion proteins, produced in *Escherichia coli*, were used to immunize rat and Balb/c mice according to standard procedures. A week after the third booster injection, the antibody titer was monitored by ELISA. Rat polyclonal antiserum with a high titer of antibodies was prepared by centrifugation at 800 g for 15 min and the supernatants were stored at −80 °C in aliquots. For production of monoclonal antibodies, spleen cells from immunized mice with a high titer of antiserum were isolated and fused with SP2/0 tumor cells to isolate and establish monoclonal antibody-producing cell lines. Two positive cell lines, 10E7c7 and 10E7c12, were identified for the production of large quantities of monoclonal antibodies from ascites. The specificities of the antibodies against CG10527 were tested by Western blotting (Fig. 1) and immunostaining (Fig. 2).

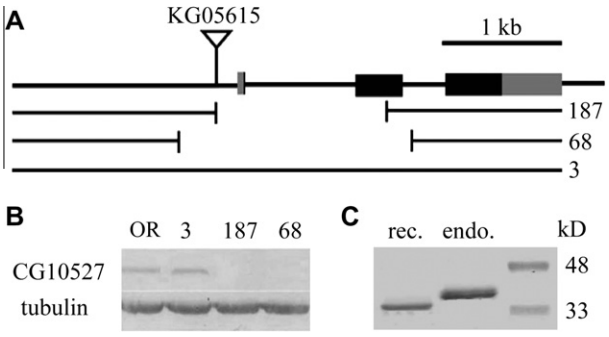

Fig. 1. Characterization of *CG10527* mutants at the DNA and protein levels. (A) Genomic structure of *CG10527*. The black box indicates the coding region while the gray box denotes the non-coding exon. A *p*-element insertion, *KG05615*, is inserted 194 bp upstream of the transcription start site. Two deletion lines are depicted by horizontal lines with gaps delineating the deleted regions. Line *3* is a precise excision line with an intact *CG10527*. (B) Precise excision line *3* has CG10527 expression comparable to the wild type (OR), but the two imprecise excision lines showed no detectable expression of CG10527. Tubulin was used as a loading control. (C) Endogenous CG10527 from wild-type flies is markedly larger than the full-length recombinant protein produced in *E. coli*.

2.4. Immunochemistry and confocal microscopy

Larval tissues were dissected in PBS and fixed for 40 min to 1 h in 4% paraformaldehyde in PBS as described elsewhere [20,21]. After three rinses with 0.1% Triton X-100 in PBS (PBST), the tissues were blocked for 30 min in 5% blocking solution (2% BSA and 3% normal horse serum in PBST). The primary monoclonal anti-CG10527 mouse antibody was used at a dilution of 1:200. FITC- or Texas red-conjugated secondary antibodies were diluted 1:2000. Propidium iodide (1.25 μg/ml) was applied for 20 min at RT to stain nuclei, and stained samples were examined with a Zeiss LSM 510 Meta laser-scanning microscope. For Western analyses, the rat polyclonal antibody against CG10527 was used at a dilution of 1:10,000, while the anti-α-tubulin monoclonal antibody (mAb B-5-1-2 from Sigma) was used at a dilution of 1:70,000 as a loading control.

2.5. Bioassay of JH III, MF and methoprene in mutants

JH III (Cat. No. J2000) and methoprene (Cat. No. 33375) were purchased from Sigma, St. Louis, MO. MF (Cat. No. S-0153) was obtained from Echelon Biosciences Inc., Salt Lake City, UT. All three chemicals were dissolved separately in acetone at different concentrations. Individual larvae were immobilized on ice and treated topically with 0.5 μl JH III, MF or methoprene at different concentrations following the protocol described by Ashburner [22]. The treated *Drosophila* larvae were kept on ice for about 10 min to allow efficient absorption of the compounds, and were subsequently allowed to complete their development at 25 °C. More than 25 late third instar larvae of different genotypes including genetic controls (wild type Oregon R and a precise excision line *3*), hypomorphic Met^3, and *CG10527* deletions were assayed for their responses to topically applied JH III, MF, and methoprene. Each treatment was repeated at least three times. Surviving adults were examined for defects in bristle and male genitalia development. For calculation of LD_{50} values, surviving adults were scored 6 days after treatment with JH, MF, and methoprene. Adult mortality was plotted as a function of dose of JH, MF or methoprene by GraphPad PRISM 5 and LD_{50} values were inferred from the dose-mortality curves.

3. Results

3.1. Generation and characterization of CG10527 mutants

As there were no previously-characterized null mutants of *CG10527* available, we generated mutations of *CG10527* via *p*-element-mediated excision to dissect the *in vivo* functions of *CG10527*. PCR and subsequent DNA sequencing identified two imprecise excisions with intragenic *CG10527* deletions (Fig. 1A). Western blotting and immunohistochemical analyses confirmed that these two lines had no detectable expression of *CG10527* (Fig. 1B and Fig. 2C). Thus, these deletions are probable null alleles of *CG10527*. For phenotypic analysis, we examined the two deletion lines and the precise excision line *3* that was used as a genetic control, as it shares the same isogenized parental chromosome as the deletion lines, but has intact *CG10527*.

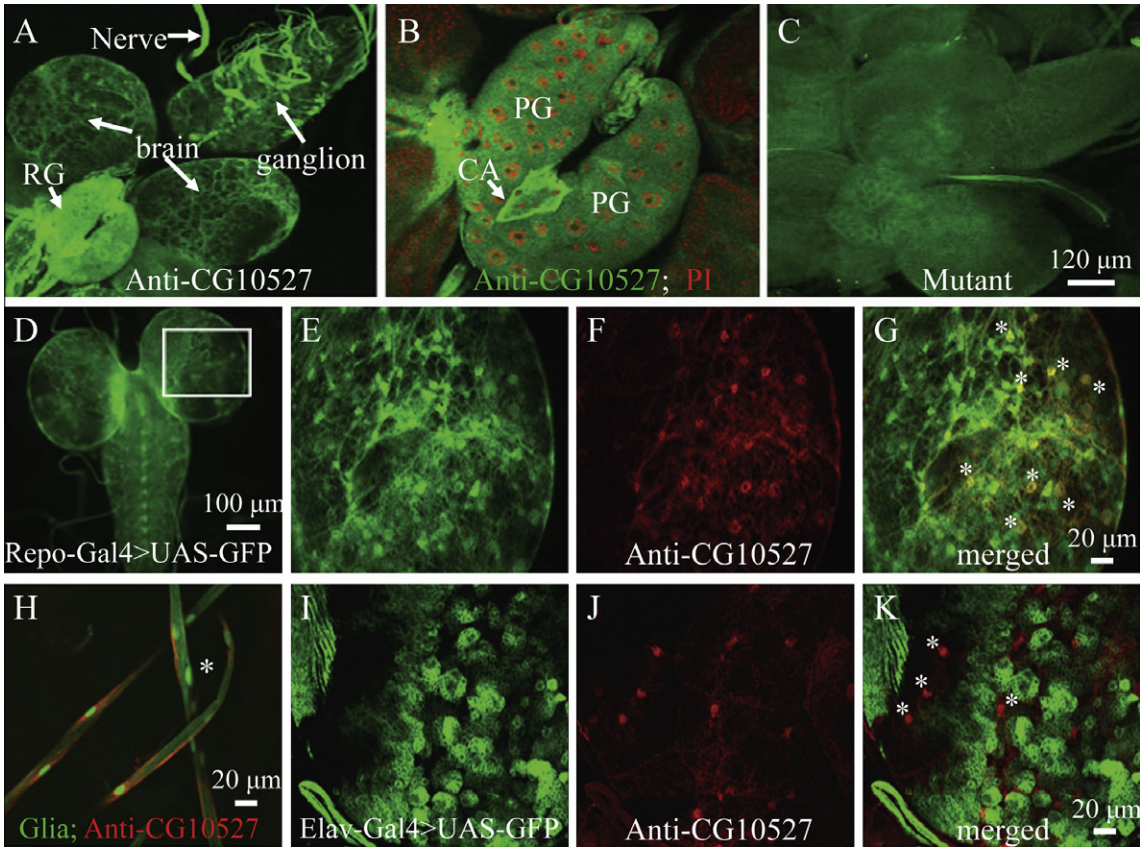

Fig. 2. CG10527 is highly expressed in the ring gland and glial cells in the nervous system. (A) CG10527 is highly expressed in the ring gland (RG) as well as in the central and peripheral nervous system, including the brain, the ganglion, and nerves. (B) CG10527 is present at higher levels in the corpus allatum (CA) than in the prothoracic gland (PG). Nuclei are labeled with propidium iodide (PI; in red). CG10527 is exclusively cytoplasmic. (C) Mutant *187* larvae show a low background level of CG10527 expression. (D–G) CG10527 is expressed in a subset of glia in the central brain. Glial cells are labeled with *repo-Gal4>UAS-GFP* (D and E). Anti-CG10527 staining is shown in red (F). (G) Double-labeling showed that only a subset of glia (indicated by asterisks) express CG10527. (H) CG10527 is also expressed in some but not all peripheral glia. The asterisk in H indicates a peripheral glia with a large nucleus that does not express CG10527. (I–K) CG10527 is not expressed in neurons. Neurons are labeled with *elav-Gal4>UAS-GFP* (I). Anti-CG10527 staining is shown in red (J). No expression of CG10527 is present in GFP-positive neurons; asterisks denote CG10527-positive glial cells in the brain (K).

3.2. CG10527 is expressed widely with high enrichment in the corpora allata and in the glial cells of the nervous system

To study the expression pattern and sub-cellular localization of CG10527, we generated both rat polyclonal and mouse monoclonal antibodies against CG10527. The rat polyclonal antibody recognized a single 35 kDa protein in the wild type but not in the deletion mutants (Fig. 1B). Interestingly, the size of endogenous CG10527 was obviously greater than the full-length recombinant protein produced in *E. coli* (Fig. 1C), suggesting a post-translational modification of the endogenous protein.

In addition to the rat polyclonal antibody, we produced a mouse monoclonal antibody, 10E7c7, suitable for immunostaining analysis (Fig. 2). Systematic examination of expression patterns showed that CG10527 is expressed widely in many tissues including the ring gland, the glial cells in the nervous system, and in multiple reproductive organs in wild-type flies, but there was no appreciable expression in mutants, confirming the specificity of the antibody (Fig. 2 and Supplementary Fig. 1). Within the ring gland, particularly high CG10527 expression was observed in the corpora allata (Fig. 2B), consistent with a previous report [13]. Double-labeling with anti-CG10527 and propidium iodide, a dye used to visualize nuclei, demonstrated that CG10527 is cytoplasmic in all cell types examined (Fig. 2B). To find out if *CG10527* is expressed in neurons or glial cells in the nervous system, we conducted double-labeling analysis. Glial cells and neurons were labeled with *repo-Gal4* and pan-neuronal *elav-Gal4*, respectively, using GFP as a reporter (Fig. 2D–K). Results showed that *CG10527* is expressed specifically in a subset of cortex glia in the brain (Fig. 2G, indicated by asterisks; [23]) and in peripheral nerve glia with small nuclei but not in those with large nuclei (Fig. 2H). No appreciable expression of *CG10527* was observed in neurons (Fig. 2I–K). Substantial expression of *CG10527* specifically in the glial cells of the nervous system is consistent with the high levels of expression of this protein found in the brain in our previous proteomics analysis [24]. In addition to the high expression in the ring gland and nervous system, *CG10527* was also expressed in reproductive organs in both males and females (Supplementary Fig. 1).

3.3. CG10527 mutants are resistant to topically applied JH III, MF, and methoprene

Since *CG1057* encodes a putative FAMeT and it is highly expressed in the CA, we reasoned that it might have something to do with JH biosynthesis or JH action. Unexpectedly, phenotypic analysis showed normal biosynthesis of JH III and MF in *CG10527* mutants (Supplementary Fig. 2) suggesting that CG10527 may not have FAMeT activity. However, *CG10527* mutants are resistant to JH, MF, and methoprene. It was firmly established more than three decades ago that topical exposure of *Drosophila* late third instar larvae or early pupae to exogenous JH at sub-lethal doses leads to specific developmental defects including arrest of

metamorphosis, abnormal bristles, and malformed male genitalia [16,22,25]. To determine if *CG10527* is involved in JH signaling, we topically applied methoprene, JH, and MF to *CG10527* mutants at different doses, using wild type and the best characterized JH-resistant *Met* mutants as controls (Fig. 3 and Table 1). All animals treated with the solvent acetone alone eclosed normally with no discernible defects (Fig. 3 and Supplementary Fig. 3), consistent with previous reports [22,25]. However, a large number of progeny (at least 76%) were lethal or eclosed with developmental defects when individual wild-type larvae were treated with 0.005 μg methoprene, 0.01 μg JH III or 2.5 μg MF, whereas normal adult flies emerged from all treated *Met* mutant larvae (Table 1). Topical treatment of control flies (w^{1118} and precise excision line *3*) with methoprene, JH III, or MF at sub-lethal doses exhibited pronounced defects in bristles including shorter and fewer bristles (Fig. 3) and malformation of genitalia (Supplementary Fig. 3), but strong and mild resistance was observed in Met and *CG10527* mutants, respectively, when treated in the same way as wild type controls (Fig. 4 and Table 1). Toxicity increased with increasing doses of the three compounds applied (Table 1). Homozygous and hemizygous mutants of null alleles *68* and *187* showed similar resistance to MF treatment at 2.5 μg/larva, indicating that the resistance is caused specifically by the *CG10527* mutations (Fig. 3). All these results showed convincingly that *CG10527* mutants are resistant to the three compounds. The increased resistance of *CG10527* mutants to JH and methoprene suggests that *CG10527* may participate in the JH signaling pathway.

In order to quantify the resistance of *CG10527* mutants, we determined and compared the LD_{50} values of methoprene and JH III for different genotypes. LD_{50} is the dosage at which half of the animals tested are killed. We plotted adult mortality as a function of the doses of methoprene and JH III applied to the larvae of different genotypes (Fig. 4). Our results showed that, for methoprene, *Met* mutants had the highest LD_{50} (0.117 μg/larva), while the wild type had the lowest LD_{50} (0.004 μg/larva), consistent with previous reports [16]. The LD_{50} for *CG10527* mutants was intermediate, at 0.019 μg/larva. The LD_{50} of methoprene for *CG10527* and *Met* mutants was about 5 and 30 times higher than that of the wild type, respectively (Fig. 4A). Similarly, the LD_{50} of JH III for *CG10527* and *Met* mutants was about 3 (0.242 versus 0.075 μg/larva) and 112 (8.429 versus 0.075 μg/larva) times higher than that of the wild type control, respectively (Fig. 4B). These results reveal that *CG10527* and *Met* mutants are more resistant to JH and methoprene than the wild type, though *CG10527* mutants have a much lower resistance to JH and methoprene than *Met* mutants.

4. Discussion

Growth regulating insecticides, including methoprene, are chemical analogs of JH and interfere with the development of certain insects. The first methoprene resistant mutant, *methoprene-tolerant* (*Met*), was isolated by Wilson and colleagues using classical mutagenesis [16]. The *Met* mutants tested here were 112 and 30 times more resistant than wild-type flies to JH III and

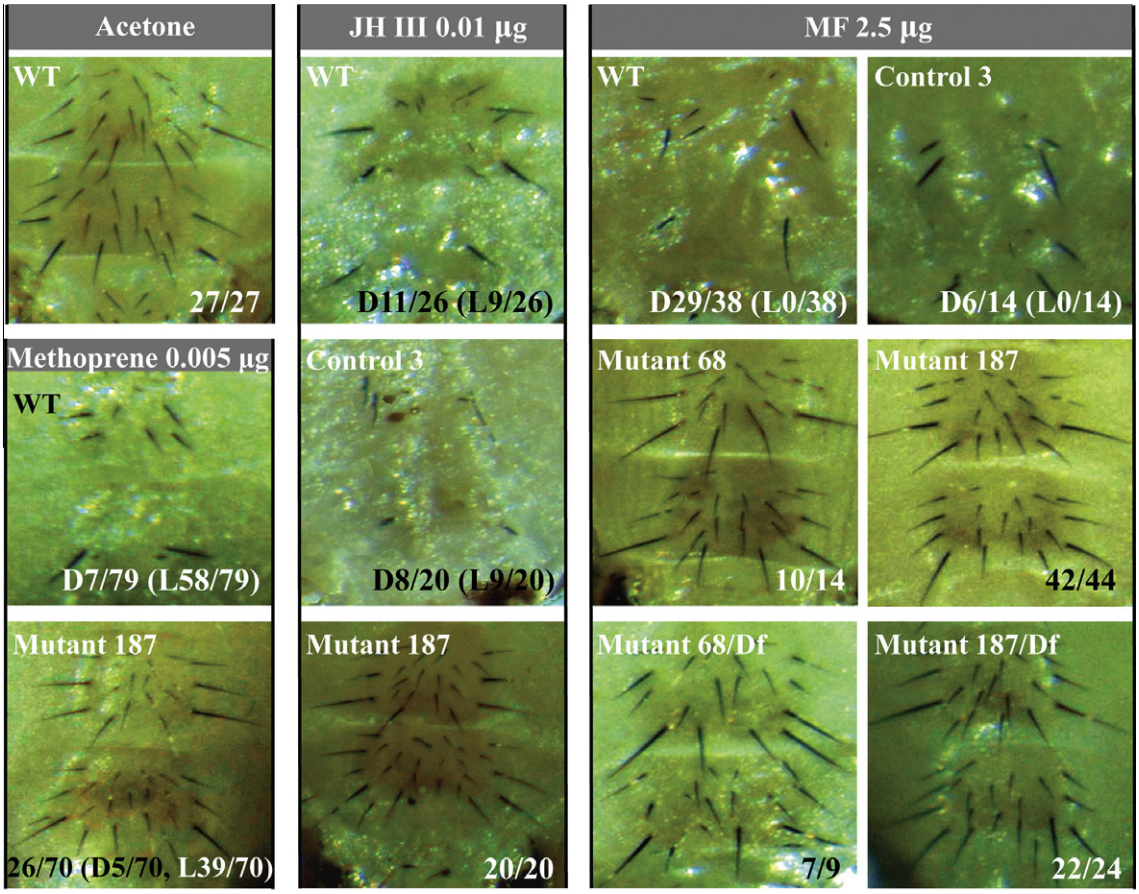

Fig. 3. *CG10527* mutants are resistant to methoprene, JH, and MF. Bristle development in *CG10527* mutants is resistant to topical treatment of JH, MF, and methoprene. Treatment of wild type (w^{1118}) larvae with the solvent acetone produced no visible phenotypes. However, abnormal bristles and scar-like cuticles were obvious when wild-type larvae were treated with 0.005 μg methoprene, 0.01 μg JH III, or 2.5 μg MF. In contrast, mutant flies of different deletions (lines *68* and *187*) exhibited largely normal development of bristles and cuticles when treated in the same way as the wild type. Line *3* is a genetic wild type control in which *CG10527* is intact. Df indicates the deficiency *ED3755* which uncovers *CG10527*. The numbers of animals displaying developmental defects (D = shorter and fewer bristles; L = adult lethality) or normal morphology out of the total number of animals tested are indicated.

Table 1
Lethality and developmental defects caused by topical treatments of methoprene, JH III, and MF.

	Dose[a]	Wild type	Met	CG10527
Meth.	0.001	0 (71)	0 (62)	0 (66)
	0.005	82 (79)	0 (57)	63 (70)
	0.025	100 (36)	3.6 (29)	100 (37)
	0.25	100 (23)	57 (35)	100 (24)
JH III	0.001	0 (60)	0 (50)	0 (58)
	0.01	77 (26)	0 (40)	0 (20)
	0.25	92 (125)	0 (42)	92 (108)
	5	100 (52)	2 (42)	100 (57)
	10	100 (26)	75 (24)	100 (26)
MF	0.1	2 (49)	0 (45)	0 (43)
	2.5	76 (38)	0 (25)	4.5 (44)
	25	70 (40)	0 (41)	53 (34)
	100	100 (21)	8 (25)	96 (23)
	200	100 (9)	100 (8)	100 (8)

[a] Indicates doses (μg/larva) of the three compounds applied. The three genotypes tested were the wild type OR, *Met*^{3}, and *CG10527* deletion line *187*. Numbers represent the percentage of animals showing lethality and defects in bristle and male genitalia development. The total number of animals tested is given in parentheses.

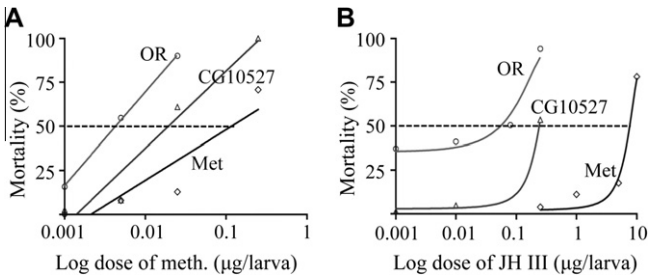

Fig. 4. Responses of larvae to topical treatment with methoprene and JH. Mortality vs dosage relationships for topical treatments of >25 larvae of different genotypes (wild type OR, *CG10527* deletion line *187*, and *Met*^{3}) with methoprene (A) and JH III (B). Each data point represents the mortality percentage at each dose. The x-axis represents the different log doses of compounds applied, while the y-axis shows the mortality percentage.

methoprene (Fig. 4), respectively, in agreement with the original report [16]. Compared with the *Met* mutants, *CG10527* mutants showed a mild 3 and 5 times higher resistance than wild-type flies against JH III and methoprene, respectively (Fig. 4). *Met* encodes a member of the basic helix-loop-helix bHLH-PAS family of transcriptional regulators [18] and may function as a JH receptor, initiating the transcriptional regulation of JH target genes [17]. In addition to JH resistance, *Met* mutants have dramatically reduced oviposition [26], but *CG10527* mutants showed normal fertility (data not shown). CG10527 is also distinct from Met in the cell types in which it is expressed and in its sub-cellular localization. CG10527 is highly expressed in the CA and glial cells of the nervous system where Met is not observed [27]. Met is found exclusively in the nucleus [27], whereas we have shown that CG10527 is cytoplasmic (Fig. 2). It will be of great interest to elucidate the mechanism by which *CG10527* mutations lead to increased JH resistance.

Given a potential role of *CG10527* in the biosynthesis of JH, and the critical role of JH in development and reproduction of *Drosophila*, we were surprised to find that *CG10527* null mutants did not show expected phenotypes such as reduced fertility (data not shown; [26]). If *CG10527* encodes FAMeT, we would expect no FAMeT activity and no MF production in *CG10527* null mutants. However, the biosynthesis of JH III and MF was normal in *CG10527* mutants (Supplementary Fig. 2), indicating that CG10527 is not involved in the process. These results are consistent with our previous finding that FAMeT activity of recombinant CG10527 produced in *E. coli* could not be detected by a radiochemical assay [13]. A possible explanation for the normal biosynthesis of MF and JH in *CG10527* mutants is that *CG17330* plays a major role in the process [9,10]. It is worth pointing out that JHAMT encoded by *CG17330* also catalyzes the methylation of FA to MF, and thus functions as a FAMeT, though at lower efficiency [10]. In other words, the function of CG10527 may be compensated for by CG17330. However, *CG10527* mutants are resistant to JH and methoprene, indicating that CG10527 is involved in JH signaling rather than biosynthesis. This putative role for CG10527 in the JH signaling pathway remains to be clarified.

In conclusion, this study reports for the time that *CG10527* null mutants show no phenotypes related to FAMeT-mediated JH biosynthesis, but are resistant to topical treatments of JH, indicating that *CG10527* is involved in JH signaling. The function of *Drosophila CG10527*, encoding a protein with high homology to crustacean FAMeT, remains unclear at present. Further characterization of CG10527 may uncover novel mechanisms of JH action.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.09.019.

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