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Biological and molecular effects of pyriproxyfen, insect growth regulator (IGR), on *Met* gene in susceptible wild type and *Met* mutant *Drosophila*

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ABSTRACT

Juvenile hormone (JH) signaling pathway at the molecular level is still not well understood. *Methoprene-tolerant* (*Met*) in *Drosophila melanogaster* fulfills many of the requirements for a hormone receptor gene. However, *Met* mutant flies are resistant to insect growth regulators (IGRs). In our study, the resistance to juvenile hormone agonist (JHA) in *Methoprene-tolerant* (*Met*) mutant flies was studied. *Met* mutant and susceptible wild type larvae were treated by different concentrations of pyriproxyfen at 0, 0.03, 0.1, 0.3, 1, and 3 ppm. We observed that the mean percentage mortalities in *Met* mutant were significantly ($P<0.05$) lower than that in the susceptible wild type. Also, resistance in insects to IGRs due to *Met* gene was studied using PCR and DNA sequencing analyses. We found that there are no changes in the DNA sequences of *Met* gene after the treatment with pyriproxyfen.

Keywords: pyriproxyfen- *Met* gene- *Drosophila melanogaster*

INTRODUCTION

Methoprene-tolerant (*Met*) gene, which is also known as *Resistance to Juvenile Hormone*, was discovered by Wilson and Fabian (1986) while they were screening mutagenized *Drosophila* for resistance to methoprene, a JH analog used as an insecticide. The *Met* encodes a basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) protein family member and several different studies propose MET as a component of the elusive JH receptor. Methoprene was found to be toxic when applied to late third-instar larvae of *Drosophila melanogaster* by Wilson and Fabian (1986). On the other hand, Shemshedini and Wilson (1990) found that the *Met* mutant of *Drosophila melanogaster* is highly resistant to juvenile hormone III (JH III) or its chemical analog, methoprene, an insect growth regulator. The *Methoprene-tolerant* (*Met*) mutation of *Drosophila melanogaster* results in resistance to juvenile hormone (JH) or JH analogs and appears to alter JH reception during late larval development. Several alleles of *Met* have been recovered from methoprene selection screens after mutagenesis with ethyl methanesulfonate, X-rays, or transposable genetic elements. The phenotype of flies carrying any of these alleles is similar resistance to the toxic and morphogenetic effects of methoprene. The level of resistance to either the toxic or the morphogenetic effect of methoprene was similar among flies heterozygous for either the deficiency chromosome or for any of the alleles (Wilson 1998). The results provide genetic evidence that the *Met* alleles recovered to date are amorphic and suggest that the *Met* gene may not be mutable to a more severe *Met* allele that affects the viability, development, or reproduction of the flies. JH III and pyriproxyfen had little effect on abdominal bristle or cuticle formation, but disrupted the development of the

central nervous system as in wild-type flies (Zhou and Riddiford 2008). In an attempt to study the effect of pyriproxyfen on *Met* gene, we conducted experiments to study the biological and molecular effects of pyriproxyfen, insect growth regulators (IGRs), on *Met* gene in susceptible wild type and *Met* mutant flies.

MATERIALS AND METHODS

Fly Strains:

The red-eyed *Oregon*-R (OR) strain was used as susceptible wild type control from Bloomington *Drosophila* Stock Center at Indiana University (USA). Resistant *Drosophila* fly strain (Met^{27}) was a gift from Dr. Thomas Wilson (Ohio State University, USA). All *Drosophila* strains were grown on standard cornmeal/molasses/yeast food at 25°C.

Chemicals:

Pyriproxyfen (Sigma-Aldrich, USA) is an agonist of the juvenile hormone (JH) which prevents larvae from developing into adult stage. Pyriproxyfen is a pyridine based pesticide which is found to be effective against a variety of insects.

Bioassay test:

The bioassay tests were conducted using feeding technique where five concentrations of pyriproxyfen were prepared (0.03, 0.1, 0.3, 1, and 3 ppm) using ethanol as a solvent and as a control (0 ppm). Each concentration was mixed with the liquid fly food just before solidification (55^{o}C).This experiment was performed for the wild type (Oregon-R) and the *Met* mutant flies (Met^{27}).

All fly types were reared on grape juice agar plates for the newly hatched 1st instar larvae collection. This is a firm medium that keeps well and makes a good base for larvae collecting.

Very large number (~ 300) flies of each fly type were collected in large fly bottle and allowed to lay eggs on each grape juice agar plate supplied with about 0.5g yeast paste for 3hrs. The plates were removed and kept in a separate moisturized plastic container. After 24hrs, one hundred first instar larvae were picked up from each fly type using blunt ending needle and placed in vials containing 0, 0.03, 0.1, 0.3, 1, or 3 ppm of pyriproxyfen fly food. Each concentration was replicated six times. The mean percentage mortality was calculated for each concentration corresponding to each fly type.

Toxicological effect of pyriproxyfen on *met* gene in both susceptible wild type (OR) and resistant flies (Met^{27}):

The effect of pyriproxyfen on *Met* gene was tested by applying different concentrations of pyriproxyfen (0.03, 0.1, 0.3, 1, and 3 ppm) onto the food of the parental flies and larvae of both types. About 10 males or females flies of each type were placed separately on the five concentrations independently and left to feed for 7 days. A single female fly was crossed to a single male come from the same type and the same concentration of pyriproxyfen. All crossed flies were fed on the corresponding concentration of pyriproxyfen media. After 3 to 5 days, all flies were removed from the vials where there were few eggs on the surface of the food. The larvae were left on the treated fly food until reaching the 3rd instar stage. The experiment was made as four repeats for each fly type and for each pyriproxyfen concentration independently.

Molecular effect of pyriproxyfen on *met* gene in both wild type (OR) and resistant flies (Met^{27})

DNA extraction:

DNA was extracted from the 3rd instar larvae of each type treated with each pyriproxyfen concentration independently using QIAamp DNA Mini Kit (QIAGEN, USA). We designed five different pairs of primers to amplify five different regions (fragments) of the *Met*

gene and its promoter region as described below.

Primers sequences:

F1: cgaacaggaagtcgtagctaccga.	R1: tcctgcttgcgtgtgagcgattc.
F2: ctcgatgacctgcaaatgaatagt.	R2: tgttcgctgtcgataacattgttg.
F3: gaggattacaagtggaaaacaagga.	R3: ctgcaggttagggtgaggaagca.
F4: gttcgccagcaaataccttaactct.	R4: tcgtgatcagatcattattactgtc.
F5: ctgtaatgtcaaatgtcaagaggta.	R5: catctgagccatgggcttagtga.

The extracted DNA from each fly type was independently used as a template for the PCR reactions.

The PCR mix was made as 100 µl total volume for each DNA template in 5 independent reactions using 5 pairs of primers. The mixes were amplified in the PCR machine. The PCR products and the DNA ladder Plus marker (Invitrogen, USA) were run on 1% agarose gel electrophoresis which is stained by ethidium bromide, and then purified by PCR purification kit (QIAGEN, USA). The purified fragments were sequenced and the results were compared by using NCBI Blast (http://blast.ncbi.nlm.nih.gov).

Statistical analysis:

Statistical analysis of results according to Statistical Package for the Social Sciences (SPSS, 2010) General linear model (GLM Factorial) was used for biological studies.

RESULTS AND DISCUSSION

Toxicological effect of pyriproxyfen on susceptible wild type and *Met* mutant flies:

Met gene has been proposed to be components of the elusive JH receptor. Accordingly *Met* mutant and susceptible wild type larvae were treated by different concentrations of pyriproxyfen (0, 0.03, 0.1, 0.3, 1, and 3 ppm) and the mean mortalities at the pupal stage were calculated and presented in table (1).

As shown in table (1) and figure (1), as the concentration of pyriproxyfen is increased, the mean percentage of mortality is significantly increased ($P<0.05$) in both *Met* mutant and susceptible wild type strains.

Also, Results showed the mean percentage of mortalities in *Met* mutant were significantly lower than that susceptible wild type throughout 0.1, 0.3, 1, and 3 ppm pyriproxyfen concentrations (Table 1 and Fig.1).

Table 1: Mean percentage mortality in *Met* mutant and susceptible wild type at the pupal stage after treatment by different concentrations of pyriproxyfen (IGRs).

	Mean % mortality at the pupal stage ± SE	
	Susceptible wild type	*Met* mutant
0 ppm	5.8±4.6 a	6.3±2.5 a
0.03 ppm	17.5±7.3 b	11.9±4.7 b
0.1 ppm	67.7±5.6 c *	13.7±5.8 b *
0.3 ppm	94.4±2.7 d *	16.4±6.8 b *
1 ppm	100±0 e *	26.7±4.7 c *
3 ppm	100±0 e *	43.6±6.3 d *

Mean values in vertical columns having different small letters are statistically significant ($p < 0.05$), Mean values in horizontal rows followed by asterisk (*) are statistically significant ($p < 0.05$).

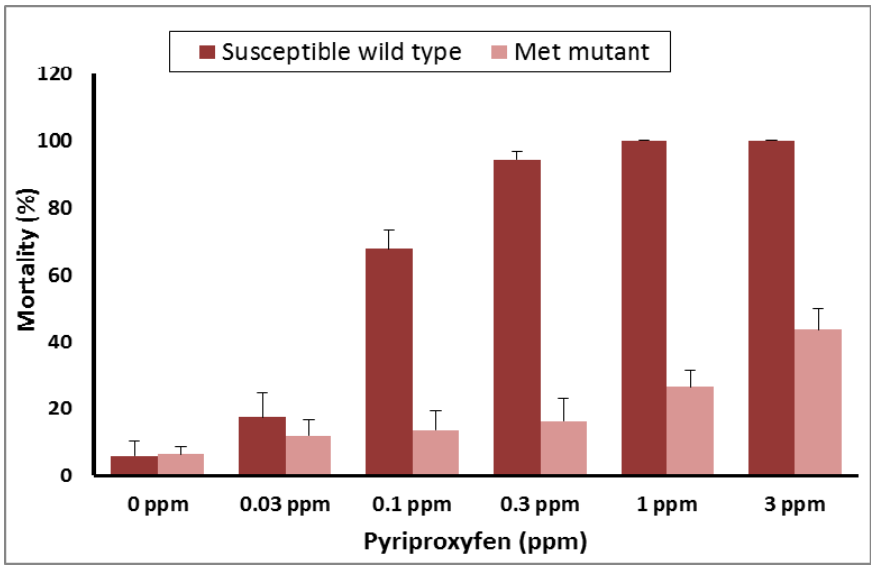

Fig. 1: Mean percentage mortality of susceptible wild type and *Met* mutant at the pupal stage after treatment with different concentrations of pyriproxyfen (IGRs).

Molecular effect of pyriproxyfen on susceptible wild type and *Met* mutant flies:

The *Met* mutant and susceptible wild type flies were treated by 5 different concentrations of pyriproxyfen (0.03, 0.1, 0.3, 1, and 3 ppm) and the molecular effect of pyriproxyfen was studied on the progenies of these flies. Five different specific primers were used to amplify 5 different regions of *Met* gene.

Figures 2, 3, 4, 5, and 6 show the specific amplified polymorphic DNA-polymerase chain reaction (SAPD-PCR) of *Met* gene amplified products of treated larvae with 0.03, 0.1, 0.3, 1, and 3 ppm pyriproxyfen, respectively.

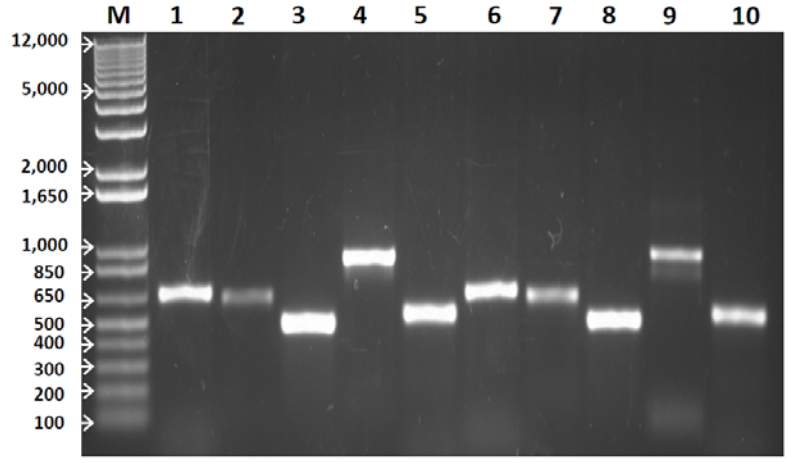

Fig. 2: Agarose gel electrophoresis of SAPD-PCR *Met* gene amplified products of *Met* mutant (lanes 1-5) and the wild type (lanes 6-10) treated larvae by 0.03 ppm pyriproxyfen using specific primers Met-F1-R1, Met-F2-R2, Met-F3-R3, Met-F4- R4, and Met-F5-R5. M: DNA ladder marker in bp.

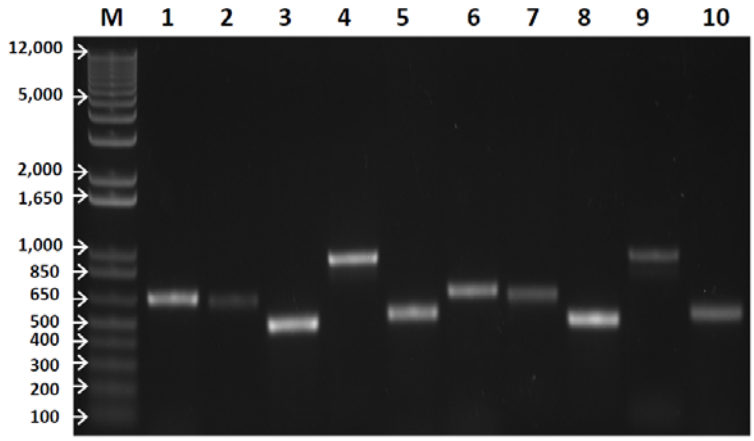

Fig. 3: Agarose gel electrophoresis of SAPD-PCR *Met* gene amplified products of *Met* mutant (lanes 1-5) and the wild type (lanes 6-10) treated larvae by 0.1 ppm pyriproxyfen using specific primers Met-F1-R1, Met-F2-R2, Met-F3-R3, Met-F4- R4, and Met-F5-R5. M: DNA ladder marker in bp.

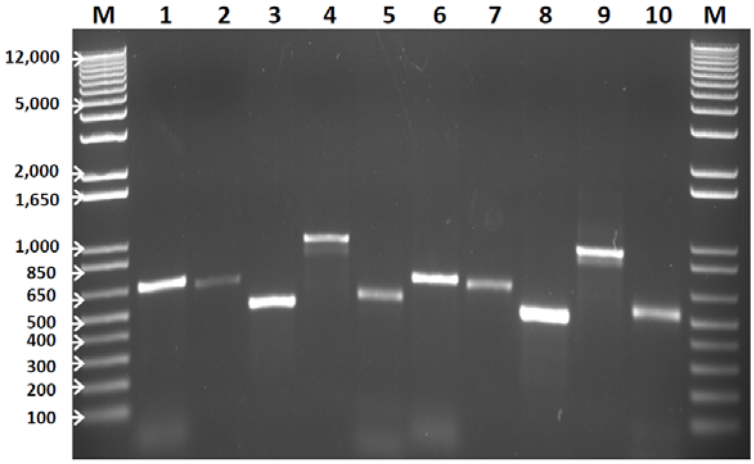

Fig. 4: Agarose gel electrophoresis of SAPD-PCR *Met* gene amplified products of *Met* mutant (lanes 1-5) and the wild type (lanes 6-10) treated larvae by 0.3 ppm pyriproxyfen using specific primers Met-F1-R1, Met-F2-R2, Met-F3-R3, Met-F4- R4, and Met-F5-R5. M: DNA ladder marker in bp.

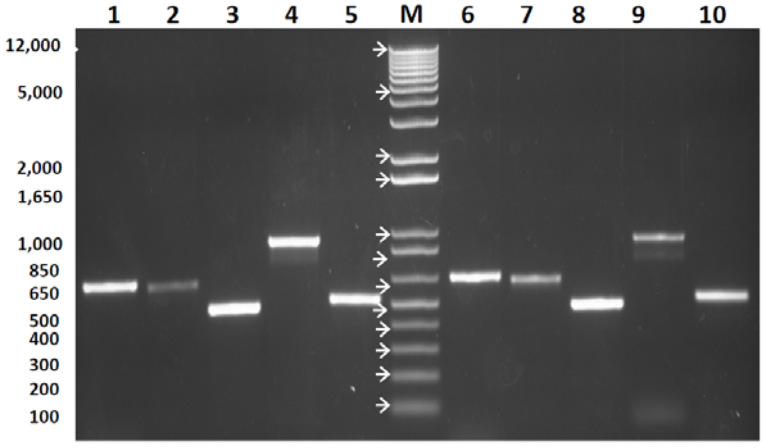

Fig. 5: Agarose gel electrophoresis of SAPD-PCR *Met* gene amplified products of *Met* mutant (lanes 1-5) and the wild type (lanes 6-10) treated larvae by 1 ppm pyriproxyfen using specific primers Met-F1-R1, Met-F2-R2, Met-F3-R3, Met-F4- R4, and Met-F5-R5. M: DNA ladder marker in bp.

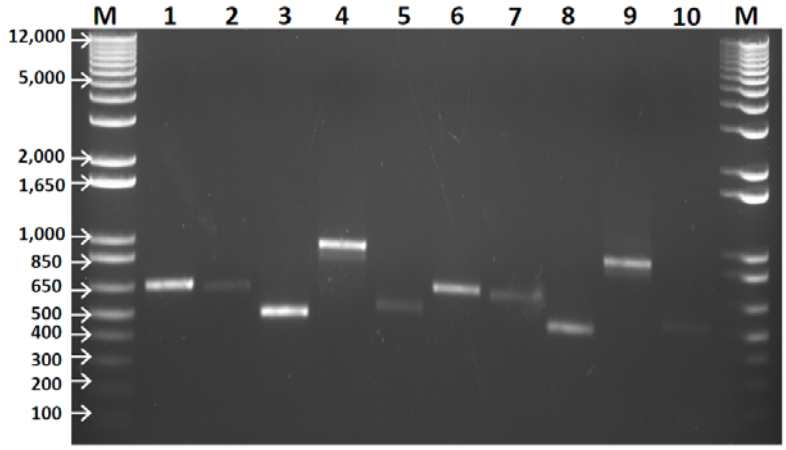

Fig. 6: Agarose gel electrophoresis of SAPD-PCR *Met* gene amplified products of *Met* mutant (lanes 1-5) and the wild type (lanes 6-10) treated larvae by 3 ppm pyriproxyfen using specific primers Met-F1-R1, Met-F2-R2, Met-F3-R3, Met-F4- R4, and Met-F5-R5. M: DNA ladder marker in bp.

We conducted a bioassay test to record the resistance in the *Met* mutant flies compared with the susceptible wild type (Oregon R) flies. Pyriproxyfen (JHA) was used in this experiment in different concentrations. Our results showed high resistance in the *Met* mutant flies even in the very high concentration such as 1 and 3 ppm. At the same two concentrations of pyriproxyfen, all susceptible wild type died at the pupal stage.

These data are consistent and in agreement with different previous findings of other authors (Wilson and Fabian, 1986; Ashok *et. al.*, 1998; Wilson and Ashok 1998; Konopova and Jindra, 2007; Baumann *et. al.*, 2010; Riddiford *et. al.*, 2010).

The effect of chemical IGRs on DNA may take the form of point mutation, deletion, insertion, or breakage at one or both primer annealing sites on the DNA strand. Inversion may also occur and separate the annealing site at a greater distance than can be amplified and the DNA damages translated into dominant lethal mutations (El-Bermawy and Abulyazid, 1998; Kubota *et al*., 2006).

In order to figure out the effect of pyriproxyfen on the genomic DNA, two types of flies were used. They are the susceptible wild type (Oregon R) and the resistant strain (*Met* mutant –*Met*^{27}).

The ability to reproduce a target section of a DNA sequence through the use of the polymerase chain reaction has facilitated a wide array of amplification techniques. Whether the objective is random sequencing, or target specific, the success of the PCR strategy is highly dependent on the small synthetic oligonucleotides that hybridize to the complementary DNA sequence. These short nucleotides function in pairs known as the forward and reverse primers, which amplify a specific DNA sequence and, more importantly, anneal exclusively to that DNA target locus (Lexa *et al.*, 2001).

In order to detect the effects of the pyriproxyfen on the *Met* gene, five pairs of specific primers were used to amplify five different fragments. These fragments are representing the promoter, open reading frame, and the 3' untranslated regions of the *Met* gene. All fragments from treated susceptible wild type and the resistant strains were subjected to sequencing analyses.

Our sequencing analyses revealed that there is no difference in the genomic sequences of either treated susceptible wild type or the resistant strain when compared with the *Drosophila melanogaster* genomic sequence

database. It was known that the resistance in the *Met* mutant flies (*Met*^{27}) is due to the mutation in this specific gene, *Met*. It was reported previously that the mutation in the *Met* gene is due to a deletion in the promoter region (Wilson and Ashok 1998).

Recently it was reported that *Met* mutant flies are resistant to the JHA such as pyriproxyfen due to the mutation in *Met* gene. RT-PCR analysis revealed that *Met* mutant flies are unable to produce Met protein due to the failure to perform transcription for the *Met* gene (Abdou *et. al.*, 2011). Our results accompanied with other authors findings concluded that the resistance in the *Met* mutant flies to the JHA is not from any deletion in any part of the *Met* gene at the DNA level and it is due to the malformation in producing the messenger RNA (mRNA).

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ARABIC SUMMERY

تأثيرات بيولوجية وجزيئية للبيروبروكسيفين ، منظمات نمو الحشرات، علي جين ال *م ي ت* في *الدروسوفيلا* المرجعية الحساسة *والدروسوفيلا* التي بها طفرة وراثية في جين ال *م ي ت*

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تعتبر آلية عمل مسار هرمون الشباب علي المستوي الجزيئي لا تزال غير مفهومة جيدا. يوفر جين ال م *ي ت* في الدروسوفيلا كثير من المتطلبات كمستقبل للهرمون. ولكن الحشرات التي بها طفرة في جين ال *م ي ت* لديها مقاومة تجاه منظمات نمو الحشرات. في هذة الدراسة تمت دراسة مقاومة الحشرات التي لديها طفرة في جين ال *م ي ت* تجاه مشابهات هرمون الشباب. تم تعريض اليرقات من الحشرات الحساسة والحشرات التي بها طفره في جين ال *م ي ت* إلي تركيزات مختلفه من البربروكسيفين وهي (0- 0,03 – 0,1 – 0,3 – 1 – 3 جزء في المليون. لوحظ ان متوسط نسبة الإماته في الحشرات التي لديها طفرة في ال *م ي ت* كانت اقل معنويا منها في الحشرات الحساسة. وكذلك تمت دراسة المقاومة في الحشرات لمنظمات نمو الحشرات لوجود جين ال م *ي ت* بإستخدام جهاز البلمرة المتسلسل وتسلسل القواعد النيتروجينية. وقد وجد انه لا يوجد اختلاف بين القواعد النيتروجينية لجين ال *م ي ت* بعد المعالجة بالبيروبروكسيفين.