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Overexpression of cytochrome P450 gene CYP6K1 is associated with pyrethroid resistance in German cockroaches (Blattodea: Ectobiidae) from California

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We investigated the expression of 4 cytochrome P450 genes (CYP4G19, CYP6J1, CYP6K1, and CYP4C21) in 4 field-collected strains (WM, RG386, CDR, and Ryan) of the German cockroach, *Blattella germanica* (L.), collected from California. The UCR susceptible strain was used as a comparison. Topical assays using a diagnostic dose (3× LD_{95}) of deltamethrin revealed decreased sensitivity in all field-collected strains with mortality ranging from 0% to 58%, and the addition of PBO before deltamethrin treatment increased mortality to 52.5%–87.5%. Using qPCR to investigate the expression levels of CYP4G19, CYP4C21, CYP6J1, and CYP6K1, we found that only CYP6K1 was significantly overexpressed (2.1–5.8× higher) in all field-collected strains when compared to the UCR strain. Next, we investigated the role of the CYP6K1 gene by performing gene knockdown using RNAi. After dsCYP6K1 treatment, the expression levels of CYP6K1 in WM and Ryan strains were significantly reduced ($P < 0.01$) by 91%–94% vs. those treated with dsEGFP (control) on the third and sixth day posttreatment. RG386, CDR, and Ryan strains were more susceptible compared to their respective controls to topically applied deltamethrin 6 days after treatment with dsCYP6K1. This study provides evidence of the involvement of the P450 CYP6K1 gene in pyrethroid resistance in some populations of German cockroaches.

Key words: deltamethrin, RNAi, CYP4G19, *Blattella germanica*

Introduction

The German cockroach, *Blattella germanica* (L.), is a major worldwide indoor public health insect pest, especially in residential premises and food preparative establishments such as restaurants, food courts, food packaging factories, etc. (Lee and Wang 2021). The negative consequences of a German cockroach infestation include mechanical transmission of pathogenic microorganisms, respiratory illness (allergy and asthma), and hygiene issues (Cohn et al. 2006, Schal and DeVries 2021). To mitigate the negative impacts of cockroach infestations, insecticides have been the preferred control method due to their quick action, low cost, and availability. Over-reliance and indiscriminate use of insecticides have led to the development of insecticide resistance in German cockroaches (Scharf and Gondhalekar 2021). This species was estimated to have developed resistance to 42 insecticide active ingredients (Zhu et al. 2016, Lee and Rust 2021, Scharf and Gondhalekar 2021).

Using comparative analysis of the *B. germanica* genome, researchers uncovered an immense expansion of the cytochrome P450 monooxygenase (P450s) gene family, which is believed to have enabled the species to evolve a broad range of resistance to toxins and pathogens (Harrison et al. 2018). Many studies have emphasized the significant role of P450s in insecticide resistance; however, only a few studies have successfully uncovered the links between different isoforms of P450s and insecticide resistance in *B. germanica* (Scharf et al. 1998, 1999, Guo et al. 2010, Chen et al. 2019). The involvement of P450s in insecticide resistance has been primarily elucidated through bioassays with and without P450 inhibitors, such as piperonyl butoxide (PBO) and MGK-264 (Atkinson et al. 1991, Lee et al. 1996, Valles and Yu 1996, Chai and Lee 2010, Hu et al. 2021, Scharf and Gondhalekar 2021). Research into the specific importance and roles of individual P450 genes in conferring insecticide resistance in German cockroaches is still in its early stages. Of the different P450 genes that have been identified, only CYP4G19 has

been shown to contribute toward insecticide detoxification and penetration resistance (Pridgeon et al. 2003, Guo et al. 2010, Chen et al. 2020, Hu et al. 2021).

Lee et al. (2022b) discovered that the P450-related metabolic resistance mechanism was a primary mechanism of pyrethroid resistance in strains of *B. germanica* collected between 2018 and 2020 in California. In the current study, we examined deltamethrin resistance and P450-mediated detoxification through topical applications in 4 field-collected strains (WM, RG386, CDR, and Ryan). We investigated the expression of four P450 genes, CYP4G19, CYP6J1, CYP6K1, and CYP4C21, and found that the CYP6K1 gene was significantly expressed in all field-collected strains. Next, we determined the expression level of the CYP6K1 gene after dsRNA treatment in WM and Ryan strains. Lastly, we investigated whether dsCYP6K1 treatment on the field-collected strains led to an increase in deltamethrin susceptibility.

Materials and Methods

Cockroach Populations

Four field-collected strains of the German cockroach (WM, RG386, CDR, and Ryan) that were collected earlier from different localities in California from 2018 to 2020 were used in this study. For details on these strains' collection sites and insecticide resistance profiles, refer to Lee et al. (2022a, 2022b). These strains were reared separately in the laboratory in 121-liter garbage bins equipped with an electrical barrier (Wagner et al. 1964) at 24 ± 2 °C, 30%–50% relative humidity, and 12-h photoperiod. Food (Purina Dog Chow, Nestlé Purina Petcare, St. Louis, MO, USA), water, and cardboard harborages were provided ad libitum. A susceptible laboratory strain (UCR), established from the Orlando Normal strain over 40 years ago, was used for comparison.

Topical Assay and Synergism

Field-collected cockroach strains were assessed for deltamethrin resistance using a diagnostic dose (0.0339 µg/insect), which is equivalent to the 3× LD_{95} generated earlier on the UCR strain (Georghiou and Mellon 1983, Mota-Sanchez et al. 2008, Lee et al. 2022a). Deltamethrin solution was prepared by diluting technical grade deltamethrin (≥98%, Sigma-Aldrich Corporation, St. Louis, MO, USA) in acetone. Adult males of 4 field-collected strains and the UCR strain were anesthetized with a brief CO_2 exposure, and 1 µl of deltamethrin solution was applied to the first and second abdominal sternites using a microapplicator (Burkard Manufacturing Co Ltd., Rickmansworth, UK). Five replicates of 10 adult males were used per treatment. Treated cockroaches were kept in a clean container with dog food, water, and cardboard harborages. Mortality was scored at 72 h posttreatment. The control sets were treated with acetone.

Evidence of P450-mediated detoxification in the field-collected strains was examined using a topical treatment of 100 µg of PBO onto the first and second abdominal sternites in the same manner described above, followed by topical application of the diagnostic dose of deltamethrin 1 h later. The discrepancy in mortality between deltamethrin alone and deltamethrin + PBO was analyzed with the Mantel–Haenszel method in R version 4.3.1.

Total RNA Extraction and cDNA Preparation

Total RNA was extracted from the whole body of adult male cockroaches (minus legs) with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Prior to RNA extraction, cockroaches were not treated with any insecticide. RNase-Free DNase I (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to digest genomic DNA from RNA preparations. RNA quality and quantity were determined by measuring the absorbance ratio of 260/280 using the Epoch 2 Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). First-strand cDNA was synthesized using the Invitrogen SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Thermo Fisher Scientific Inc., Waltham, MA, USA). Total RNA (1 µg) was used to synthesize the first cDNA strand in a 20 µl total reaction volume following the manufacturer's instructions, and the synthesized cDNAs were stored at –20 °C until use.

Quantitative Polymerase Chain Reaction (qPCR)

The transcription levels of four P450 genes (CYP4G19, CYP6J1, CYP6K1, and CYP4C21) were measured for each strain. Quantitative polymerase chain reaction (qPCR) was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) on a MyGo mini RealTime PCR system (Azura Genomics, MA, USA) according to the manufacturer's protocol. Standard curves were established using serially diluted cDNA samples to screen primer pairs with high specificity and an appropriate efficiency (E = 1.91–1.99, Supplementary Table S1). Each qPCR reaction (15 µl) included 7.5 µl of PowerUp SYBR Green Master Mix, 0.5 µl of primer mix (an equal mixture of primers from 10 µM stock solutions), 1 µl of 4-fold diluted cDNA, and 5.5 µl of DEPC-water. The negative control was a "no-template" reaction. The reaction cycle applied the following PCR program: a melting step of 50 °C for 2 min, then 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. All the reactions were run with 15 biological replicates prepared separately and 2 technical replicates for each. *Blattella germanica* actin 5c (GenBank: AJ862721) was used as a housekeeping gene for normalization (Supplementary Table S1). Kruskal–Wallis rank sum test was used to evaluate the differences of dCT values among strains, followed by a pairwise Conover-Iman test with Bonferroni correction for post hoc tests.

Synthesis of dsRNA

After observing that CYP6K1 was highly expressed in all field-collected strains (see the Results section for more details), we further utilized RNAi to examine the function of this gene. The dsRNA targeting CYP6K1 was designed based on the CYP6K1 mRNA sequence obtained from GenBank (accession number: AF281328) (Supplementary Table S2). The software siRNA-Finder version 1.2.3 (Lück et al. 2019) was used to predict the number of siRNA, and the sequence region with the highest predicted siRNAs (listed in Supplementary Table S2) was selected for dsRNA synthesis. This software was also employed for off-target analysis. The off-target search pipeline commenced by dividing the RNAi trigger sequence region into all possible x-mers, where x represents the selected length of the siRNA. These x-mers were then matched against the German cockroach cDNA sequence database. We did not identify any off-targets for the selected dsRNA region targeting CYP6K1. As an unrelated control, the enhanced green fluorescence protein (eGFP; accession number: JQ417433) sequence, which is not found in *B. germanica*, was employed (refer to Supplementary Table S2). dsRNA targeting 388 base pairs (bps) of the CYP6K1 gene (abbreviated as dsCYP6K1) and dsRNA targeting 264 bps of the eGFP gene (abbreviated as dsEGFP) were synthesized by RNA Greentech LLC, Frisco, TX, USA, followed the protocol described in Li and Zamore

(2019). All dsRNAs were diluted to a concentration of 1.5 µg/µl with RNase-free water and stored at −70 °C until use.

RNAi Experiment

WM and Ryan strains were utilized to assess the efficacy of dsRNA injections. Adult males were briefly chilled on ice, and 1.5 µg of the dsRNAs specific to CYP6K1 or eGFP was injected into the abdominal intersegmental membrane with a 30G needle (BD PrecisionGlide Single-use Needles, Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) fitted on a 0.25-ml glass syringe (Fortuna All Glass Syringes, Grainger, Lake Forest, IL, USA) using an electronic microapplicator (Precision Microapplicator 900X model, Burkard Manufacturing Co. Ltd., UK). The injected cockroaches were reared in standard conditions for recovery. Samples were collected on the third and sixth day postinjection to monitor RNAi efficiency using the qPCR method mentioned previously.

Chen et al. (2020) demonstrated that gene suppression following a single dose of dsRNA injection remained effective on day 6 postinjection in the German cockroach. Based on this evidence, we selected day 6 for our topical assay for 4 field-collected strains. To assess deltamethrin susceptibility post-CYP6K1 inhibition, we conducted a topical bioassay at the diagnostic dose (0.0339 µg/insect) on day 6 post-dsRNA injection, selecting only cockroaches that exhibited normal behavior postinjection for the bioassays. Five replicates of 10 adult males were used per treatment for the topical bioassay (50 males were used in each group). Mortality rates were adjusted by subtracting the control mortality rate (dsRNA-injected males treated with acetone only). The control sets were treated with acetone. Any surviving cockroaches were collected after the topical assay to verify whether CYP6K1 inhibition remained effective. The effects of CYP6K1 inhibition on deltamethrin resistance in the WM, RG386, CDR, and Ryan strains were determined using a Mantel–Haenszel test using R version 4.3.1 by comparing the mortality of cockroaches between dsCYP6K1 and dsEGFP injection groups ($N = 50$ for each group). German cockroaches injected with either dsCYP6K1 or dsEGFP and treated with acetone alone were used as controls. Spearman's correlation of the magnitude of the impact of treatment (difference between treatment and control) between dsRNA injection and PBO topical preapplication (described earlier) was calculated in R version 4.3.1.

Results

Deltamethrin Resistance and Synergism

All field-collected strains were less sensitive to the deltamethrin diagnostic dose compared to the UCR strain, with mortality ranging from 0% to 58% vs. the UCR strain at 100% (Fig. 1; Supplementary Table S3). Susceptibility toward deltamethrin varied between strains, with the highest mortality in WM (58%), followed by RG386 and Ryan (28% and 18%, respectively), and no mortality for CDR (0%). The addition of PBO before deltamethrin treatment significantly increased the mortality in all field-collected strains (52.5%–87.5%) (Fig. 1).

Expression of 4 P450 Genes

Expression levels of CYP4G19, CYP4C21, CYP6J1, and CYP6K1 among 5 *B. germanica* strains were investigated by using qPCR (Fig. 2). CYP4G19 gene expressions in the 4 field-collected strains were not significantly different from that of the UCR strain (Fig. 2A). There was no significant difference in expression level of CYP4C21 among the 5 strains (Fig. 2B). CYP6J1 was highly expressed in RG386 but not in other field-collected strains (Fig. 2C). CYP6K1 was significantly overexpressed in all field-collected strains when compared to the UCR strain, with the overexpression ranging from 2.1 to 5.8 times higher than that of the UCR strain (Fig. 2D).

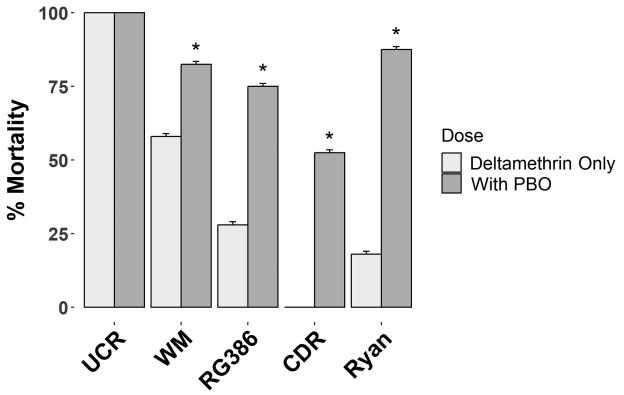

Fig. 1. Mortality of cockroaches of susceptible strain (UCR) and deltamethrin-resistant strains (WM, RG386, CDR, and Ryan) 72 h after treatment with topically applied deltamethrin (0.0339 µg/insect) or deltamethrin (0.0339 µg/insect) + PBO (100 µg/insect). An asterisk indicates a significant difference between deltamethrin and deltamethrin + PBO for the respective strain (Mantel–Haenszel test; $P < 0.05$).

Functional Analysis of CYP6K1 by RNAi

We performed an RNAi experiment to further examine the involvement of CYP6K1 in deltamethrin resistance in WM and Ryan strains. Monitoring of RNAi efficiency showed that the expression levels of CYP6K1 were significantly lower in males with dsCYP6K1 treatment than those with dsEGFP treatment on both third and sixth day posttreatment (Kruskal–Wallis rank sum tests; all χ^2 values = 8.31; all P-values < 0.01; Fig. 3). The dsCYP6K1 treatment significantly reduced CYP6K1 mRNA levels by 93% and 94% in WM strain and by 92% and 91% in Ryan strain at third and sixth days, respectively. We further tested the deltamethrin susceptibility of field-collected strains at sixth day post-dsRNA treatment. We found CYP6K1 gene knockdown increased the susceptibility to deltamethrin significantly in the RG386, CDR, and Ryan strain (Fig. 4). No significant correlation was found between dsRNA injection and PBO preapplication when comparing the magnitude of difference in mortality resulting from the 2 treatments ($\rho = 0.4$; $P = 0.75$)

Discussion

We investigated and found that the constitutive overexpression of CYP6K1 is associated with deltamethrin resistance in German cockroach populations collected from California. We demonstrated that this gene is involved in resistance, as CYP6K1 gene knockdown significantly increased deltamethrin susceptibility in 3 field-collected strains: RG386, Ryan, and CDR (Fig. 4). However because mortality under CYP6K1 knockdown treatment never reached completely susceptible levels (i.e., 100% mortality) in any strain, it is likely that other resistance mechanisms, such as target-site resistance previously reported (Lee et al. 2022b), contributed to the total deltamethrin resistance in these strains. Furthermore, the WM strain did not experience a significant difference in mortality between CYP6K1 knockdown and eGFP groups, indicating some strain-level variability in the importance of CYP6K1. This contrasts with the significantly increased mortality of all field-collected strains when treated with PBO + deltamethrin versus deltamethrin alone (Fig. 1), suggesting

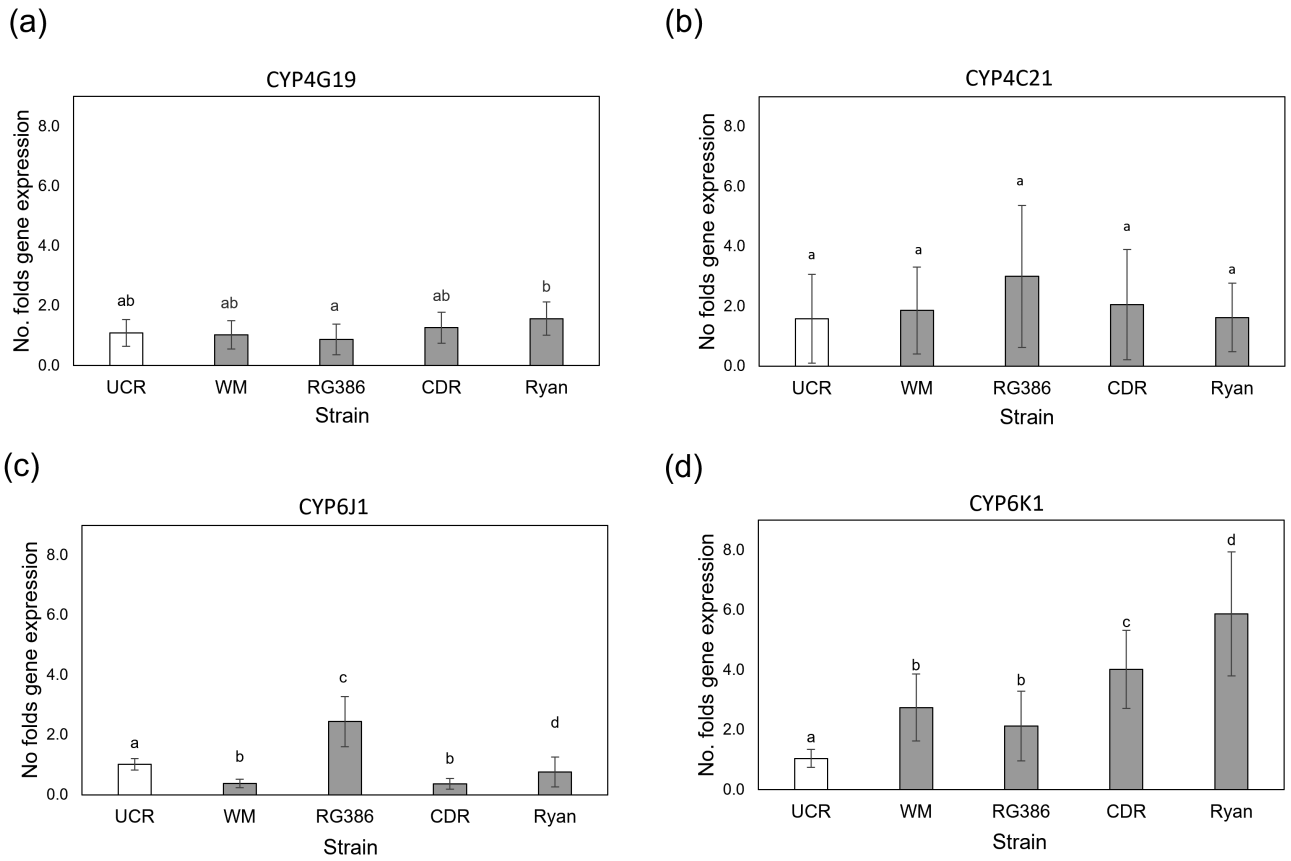

Fig. 2. Relative expression levels of A) CYP4G19, B) CYP4C21, C) CYP6J1, and D) CYP6K1 genes among 5 cockroach strains. Error bars represent standard deviations of the means ($n = 15$). Bars with different letters are significantly different (Kruskal–Wallis rank sum test followed by pairwise Conover–Iman test with Bonferroni correction; $P < 0.05$).

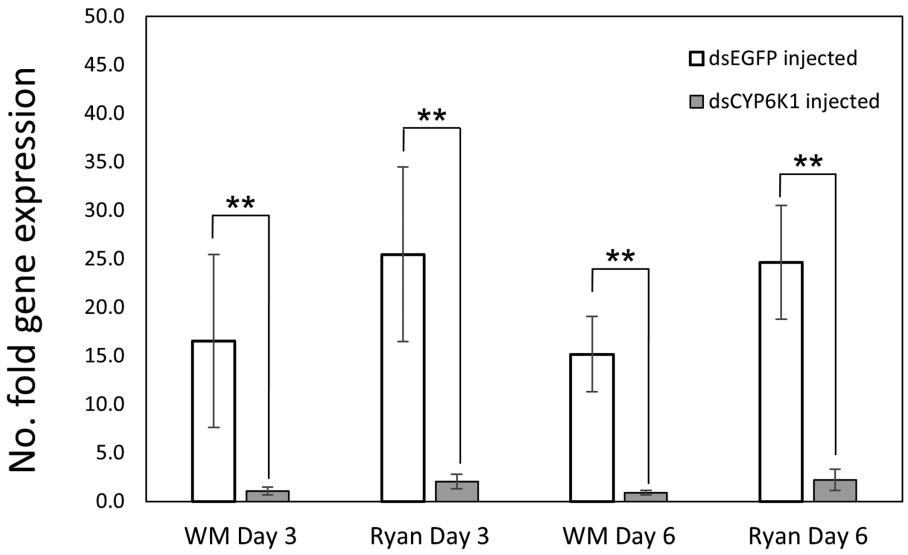

Fig. 3. Relative expression levels of CYP6K1 in deltamethrin-resistant strains (WM and Ryan) after 3 and 6 days of dsRNA injection. Error bars represent standard deviations of the means ($n = 6$). Asterisks above the bars represent statistically significant differences from Kruskal–Wallis rank sum tests (**$P < 0.01$).

that the overall contribution of P450-driven resistance can also involve other uninvestigated P450 genes. Additional factors, such as the functional differences between RNAi (which is highly specific at the mRNA level) and PBO (which is a general inhibitor at the protein level), may have contributed to discrepancies between the PBO and RNAi results.

CYP4G19 was previously linked to pyrethroid resistance and cuticular penetration-based resistance in German cockroach populations in the United States (Alabama), China (Shenzhen), and Taiwan (Pridgeon et al. 2003, Guo et al. 2010, Chen et al. 2020, Hu et al. 2021). However, CYP4G19 overexpression was only detected in one of the 4 resistant strains in California. Instead, overexpression

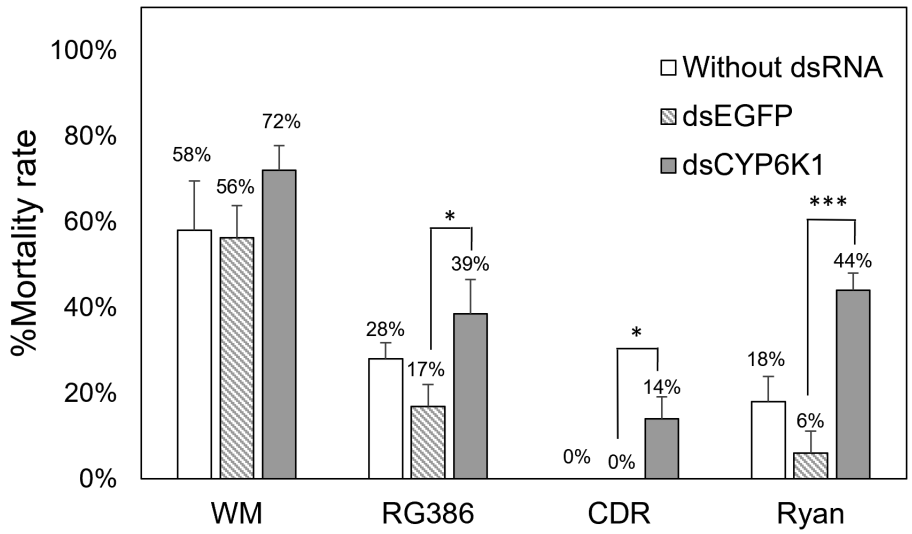

Fig. 4. Mortalities of *Blattella germanica* in response to deltamethrin following the knockdown of CYP6K in deltamethrin-resistant strains (WM, RG386, CDR, and Ryan). Mortalities were determined 72 h after topical application of deltamethrin and corrected for control mortality (dsRNA-injected males were treated with acetone only). For comparison, mortality rates without dsRNA injection are adopted from Fig. 1. Asterisks above the bars indicate statistically significant differences between the dsEGFP- and dsCYP6K1-injected groups, as determined by Mantel–Haenszel tests (*$P < 0.05$; ***$P < 0.001$).

of CYP6K1 was observed in all field-collected strains tested in this study, suggesting that the evolution of detoxification pathways of the same insecticide class can vary between populations (Nauen et al. 2022).

Cytochrome P450s are remarkable for their diverse functions, and their involvement in insecticide resistance is generally associated with metabolic resistance; this includes an increase in metabolic conversion of insecticides to less toxic metabolites and reduction of propesticide activation (Feyereisen 2012, Nauen et al. 2022, Ye et al. 2022). Besides that, some studies suggested that the overexpression of the CYP4 family also contributes to insecticide resistance by enhancing cuticular hydrocarbon production, leading to reduced insecticide penetration (Chen et al. 2020, Feyereisen 2020). In this study, the experiments were conducted on adult male German cockroaches that no longer molt. Hence, the increased deltamethrin susceptibility after CYP6K1 gene knockdown is logically the result of decreased detoxification efficiency rather than reduced cuticular penetration. CYP6 cytochrome P450 genes have also been associated with pyrethroid resistance in other insects, such as lepidopterans and culicines (Yang et al. 2006, Zhu et al. 2010, David et al. 2013, Zou et al. 2019, 2022).

Recent studies demonstrated that CYP6 enzymes could metabolize deltamethrin through several pathways, but the primary route of metabolism seems to be 4-hydroxylation, making the molecule less toxic (Zhu et al. 2010, David et al. 2013, Elzaki et al. 2018, Yang et al. 2021). CYP6-related metabolic resistance is known to result in insects developing cross-resistance to other insecticides. For example, CYP6Z1 in *Anopheles funestus* Giles overexpression contributed to cross-resistance to pyrethroids and carbamates, while CYP6M2 in *A. gambiae* Giles is capable of metabolizing pyrethroids and organochlorines (Mitchell et al. 2012, Ibrahim et al. 2018). Overexpression of CYP6K1 homologs was previously reported by Scharf et al. (2021) after 6 generations of indoxacarb selection, implying the potential of this gene to confer multiple resistance in German cockroach populations. Further investigations are warranted to confirm the role of CYP6K1 homologs in conferring indoxacarb resistance and its potential to detoxify other insecticides.

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Author Contributions

Shu-Ping Tseng (Conceptualization [lead], Data curation [lead], Formal analysis [lead], Investigation [lead], Methodology [lead], Software [lead], Validation [lead], Visualization [lead], Writing—original draft [equal], Writing—review & editing [equal]), Shao-Hung Lee (Conceptualization [supporting], Data curation [supporting], Formal analysis [supporting], Funding acquisition [supporting], Investigation [supporting], Methodology [supporting], Validation [supporting], Visualization [supporting], Writing—original draft [equal], Writing—review & editing [equal]), Dong-Hwan Choe (Validation [supporting], Writing—original draft [supporting], Writing—review & editing [supporting]), and Chow-Yang Lee (Conceptualization [supporting], Funding acquisition [lead], Project administration [lead], Resources [lead], Supervision [lead], Writing—original draft [equal], Writing—review & editing [supporting])

Supplementary Material

Supplementary material is available at *Journal of Economic Entomology* online.

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