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Communication

Co-expression of a homologous cytochrome P450 reductase is required for *in vivo* validation of the *Tetranychus urticae* CYP392A16 based abamectin resistance in *Drosophila*

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Simple Summary: The two-spotted spider mite *Tetranychus urticae* is feeding in over 1100 plant species and causes extensive damage to several crops, being one of the most damaging agricultural pests world-wide. In order to control this pest, the use of chemical acaricides remains the most widely used strategy. However, *T. urticae* has developed significant resistance to numerous acaricide compounds, due to certain features of mite biology and extensive acaricide applications that lead to the selection of resistant pests and emergence of populations where several molecular/genetic mechanisms may contribute to highly resistant phenotypes. Such mechanisms frequently involve expression of detoxification enzymes named P450s, which act together with a partner protein named CPR. In this study, we investigated the potential of a mite P450 enzyme, CYP392A16, to confer resistance to the acaricide abamectin *in vivo*, when expressed in tissues of the model fruit fly *Drosophila melanogaster*. We confirm that expression of this enzyme can indeed contribute to abamectin resistance in the fruit fly model, but only when a homologous mite CPR is co-expressed. Our findings indicate that the *Drosophila* model system can be engineered to facilitate validation of candidate mite P450s, in order to elucidate resistance mechanisms and their underlying interactions.

Abstract: Overexpression of the cytochrome P450 monooxygenase CYP392A16 has been previously associated with abamectin resistance in the two-spotted spider mite *Tetranychus urticae*, an important pest species worldwide; however, this association has not been functionally validated *in vivo* despite the demonstrated ability of CYP392A16 to metabolize abamectin *in vitro*. We expressed CYP392A16 *in vivo* via a GAL4/UAS system in *Drosophila melanogaster* flies, driving expression with detoxification tissue-specific drivers. We demonstrate that CYP392A16 expression confers meaningful abamectin resistance in toxicity bioassays in *Drosophila* only when its homologous redox partner, cytochrome P450 reductase (TuCPR) is co-expressed in transgenic flies. Our study shows that the *Drosophila* model can be further improved, to facilitate the functional analysis of insecticide resistance mechanisms acting alone or in combination.

Keywords: Detoxification; Cytochrome P450; Cytochrome P450 reductase; abamectin; *Tetranychus*; transgenic *Drosophila*

1. Introduction

The two-spotted spider mite *Tetranychus urticae* (Koch), utilizing over 1100 plant species as hosts [1,2], is one of the most damaging agricultural pests world-wide. Its control has been based mostly on the use of chemical acaricides for several years [3]. However, *T. urticae* has developed significant resistance to numerous acaricide compounds, due both to its biology (arrhenotokous reproduction, short life-cycle and high fecundity) and to strong acaricide selection pressure [4,5]. Acaricide resistance in *T. urticae* has been associated with different mechanisms, including target site mutations [6-19] as well as enhanced detoxification [20] through the overexpression of different classes of metabolic proteins such as cytochrome P450s [17,19, 21-24], glutathione S-transferases [25, 26], carboxylesterases [27], ABC transporters [28], and UDP-glycosyltransferases [17,29].

One of the most commonly used acaricides in recent years is abamectin, a compound that belongs to the avermectin subfamily of macrocyclic lactones. Avermectins were registered and widely used for several decades as antiparasitic drugs for animal health applications. Abamectin has been later developed as an acaricide/insecticide, since it has a broad spectrum of activity against arthropods, including major pests from several insect orders, and some mite species including *T. urticae* [30]. The mode of action of abamectin is the activation of a specific to invertebrates type of channels, the glutamate-gated chloride channels (GluCls) [31]. Target-site resistance to abamectin in *T. urticae* has been attributed to point mutations in different members of the cys-loop ligand gated chloride channel family [9,11,17]. However, genetic evidence revealed a polygenic nature of resistance, and subsequent detailed genetic analyses indicate that additional mechanisms operating in highly resistant strains might contribute to the phenotype [14,32]; these mechanisms most notably implicate overexpression of cytochrome P450s [33].

Cytochrome P450s belong to a diverse family of heme-containing enzymes that catalyze the mono-oxygenation of xenobiotics and endogenous compounds, and have been implicated in metabolic resistance in several pests [34]. Eighty-six cytochrome P450 (CYP) genes were detected in the *T. urticae* genome [35], several of which were shown to be associated with the abamectin resistance phenotype of the exceptionally resistant Mar-ab strain [11,20]. CYP function depends on NADPH-dependent cytochrome P450 reductase (CPR) as a co-factor that provides electrons from NADPH to the heme center of P450s [34]. In contrast to the huge variation of the cytochrome P450 gene family, only one CPR gene is identified in each arthropod species. In *T. urticae* the contribution of TuCPR to acaricide resistance has only recently been investigated; recent bulk segregant analysis genomic mapping studies in acaricide resistant *T. urticae* strains [36,37] implicated TuCPR within a potential genomic locus associated with resistance to spirodiclofen, pyridaben, and tebufenpyrad, while RNA interference (RNAi) was used to investigate the role of CPR in resistance to abamectin, bifenthrin, and fenpyroximate [38].

In recent years, functional expression of candidate CYP genes is routinely used for the investigation of their catalytic properties and substrate specificities. Moreover, the integration of the molecular genetics toolbox developed in model systems like *Drosophila* into insecticide resistance research (reviewed in [39-41]) has facilitated novel approaches that have significantly contributed to the validation of several candidate mutations conferring target-site resistance [42] and P450 genes potentially conferring metabolic resistance [43] or their synergistic interactions [44], in the absence of confounding genetic effects.

In previous studies regarding *T. urticae* candidate CYP genes, we have successfully used a combination of functional expression in bacteria along with heterologous expression in *Drosophila* to demonstrate that CYP392A11 can metabolize cyenopyrafen and other acaricides *in vitro* and confer resistance to fenpyroximate *in vivo* [23]. Furthermore, we have functionally expressed CYP392A16 *in vitro* and demonstrated that it metabolizes abamectin to a substantially less toxic compound [22].

In this study, using transgenic heterologous expression in *Drosophila*, we demonstrate that *T. urticae* CYP392A16 is able to confer abamectin resistance *in vivo*; importantly however, a significant resistance phenotype is manifest only in the context of TuCPR co-expression, indicating that a homologous CPR may be required for *in vivo* functional expression of spider mite CYP genes in *Drosophila*.

2. Materials and Methods

2.1. Chemicals

Abamectin formulation (Vertimec 18EC, Syngenta) was used in feeding bioassays.

2.2. Insects

Drosophila strain yellow white (*yw*) and balancer lines *yw* ; *Cy*O/*Sco* (for the 2nd chromosome) and *yw* ; TM3 *Sb e* / TM6b *Tb e* are part of the IMBB/FORTH fly facility collection and were provided by Christos Delidakis (IMBB/FORTH and University of Crete) while the multiple balancer strains [*w*; *If*/*Cy*O*wg*lacZ; MKRS *Sb e* / TM6 *Tb e* and +;+/T(2,3)*Cy*/*Tb*] were provided by Maria Monastirioti (IMBB/FORTH). The HR-GAL4 driver line is described in [45]. All flies were typically maintained at 25°C, 60-70% humidity, with a 12/12-h photoperiod in standard fly diet.

2.3. Extraction of RNA, cDNA synthesis and RT-PCR

Total RNA was extracted from pools of 20 adult *Drosophila* flies using Trizol reagent (MRC, Cincinnati, OH, USA), according to the manufacturer's instructions. Extracted RNA samples were treated with Turbo DNase (Ambion, Foster City, CA, USA) to remove genomic DNA and the treated RNA was used to generate first strand cDNA using oligo-dT20 primers with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Reverse transcription PCR was performed in order to confirm transgenic expression in the progeny. One microliter of cDNA was used in the PCR reaction using specific transgene primers as well as primers for RPL11 (ribosomal protein L11) which served as a reference gene (Table 1). The conditions of the reactions were 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and final extension for 2 min.

Table 1. Primers used in this study

Primer Name	Sequence (5′ – 3′)	Product size (bp)	Reference
CYP392A16_F	AAATACCGAGGTCGGACGTA	117	[20]
CYP392A16_R	AAGCACTTTTTCAATCTGGTCAC		
RPL11_Dm_F	CGATCCCTCCATCGGTATCT	120	[45]
RPL11_Dm_R	AACCACTTCATGGCATCCTC		
pUASTF	TATGTCACACCACAGAAGTAAG	n/a	[46]
pUASTR	CAAGTAAATCAACTGCAACTACTG		

2.4. Generation of pUAST.CYP392A16

The cDNA sequences encoding CYP392A16 (TeturID: tetur06g04520) and cytochrome P450 reductase (CPR) (TeturID: tetur18g03390) were isolated as previously described [22]. A BglII fragment from pCW_CYP392A16 [22] containing the CYP392A16 ORF was subcloned into the unique BamHI site of the pUAST vector as previously described [23] to generate pUAST.CYP392A16, and clones with the correct orientation were sequence-verified using primers pUASTF and pUASTR (Table 1).

2.5. Construction of the transgenic fly strains

A pUAST.CYP392A16 clone with verified sequence was selected in order to inject preblastoderm embryos of *Drosophila melanogaster yellow-white* (*yw*) strain using standard transformation techniques. Several independent transformed lines were generated and crossed with balancers stocks for the 2nd (*yw*; *Cy*O/*Sco*) and the 3rd chromosome (*yw*; TM3 *Sb e* / TM6b *Tb e*) and different homozygous lines with insertion of the transgene were established and mapped in the

relevant chromosome. Generation of transgenic lines bearing the CPR of *T. urticae* was described previously [23].

In order to generate homozygous transgenic strains that would conditionally express both *CYP392A16* and *TuCPR*, we used lines bearing UAS-CYP392A16 in the 3rd chromosome and lines bearing UAS-TuCPR in the 2nd chromosome and crossed homozygous males with a strain carrying multiple balancer chromosomes [*w*; *If*/*Cy*O*wg*lacZ; MKRS *Sbe*/TM6 *Tbe*] and used standard downstream genetic crosses in order to eventually bring both transgenes against a double-balancer chromosome (Figure 1); these were inter-crossed in order to obtain the line carrying both transgenes in homozygous state. A similar approach was used to generate lines bearing both HR-GAL4 (at the 2nd chromosome) and UAS-CYP392A16 (at the 3rd chromosome).

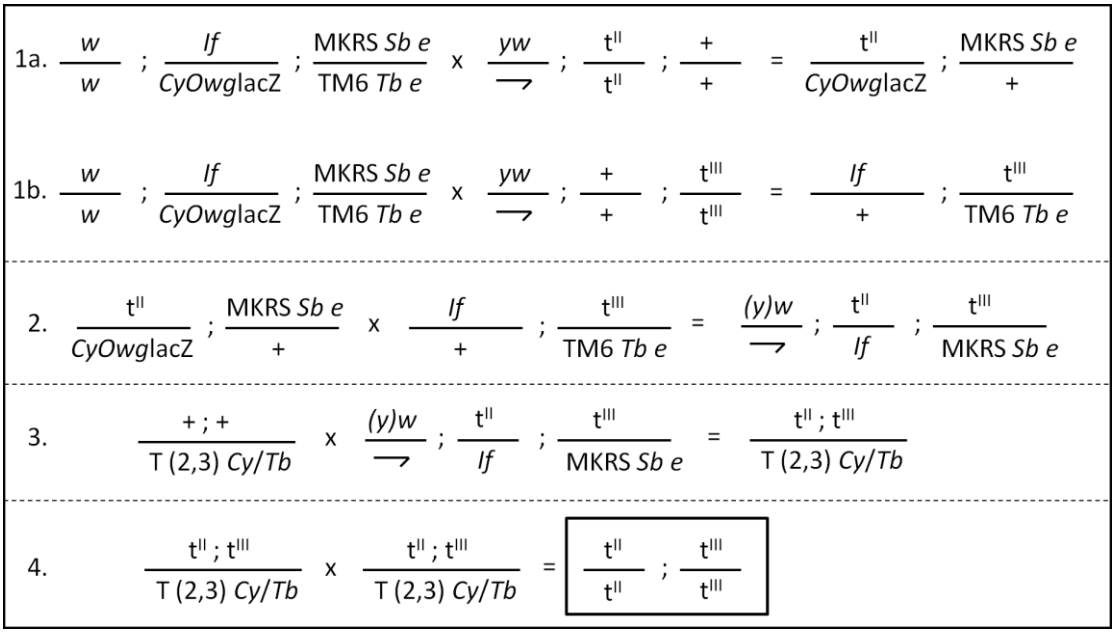

Figure 1. Generic crossing scheme for the generation of strains bearing two transgenes (either HR-GAL4 and UAS-CYP392A16 or UAS-CYP392A16 and UAS-TuCPR) in the 2nd (t^{II}) or 3rd (t^{III}) chromosome, respectively. Since all types of transgenic flies were originally generated by P-element mediated transgenesis at random (unknown) positions, crosses among several different lines were performed to account for position effects. Virgin multiple-balancer females were crossed with homozygous transgenic males bearing the relevant transgenes at the 2nd chromosome (cross 1a) or the 3rd chromosome (cross 1b) and the progeny with the indicated genotype from each cross was used for cross 2 in order to bring both transgenes opposite to selected markers *If* and *Sb* (note that the chromosomes bearing these markers are not balancers). Male progeny (not undergoing recombination) was crossed with virgin females from a double-balancer strain bearing a rearranged T(2,3) chromosome marked with both *Cy* and *Tb* (cross 3) and the progeny was selected *against If* and *Sb* to identify individuals expected to have both transgenes opposite to the T(2,3)*Cy*/*Tb* balancer. These were intercrossed (cross 4) to provide the homozygous strains (shown in box) bearing both UAS-CYP392A16 and UAS-TuCPR or both HR-GAL4 and UAS-CYP392A16, following selection *against Cy*/*Tb* markers. Note that in the HR-GAL4 line available, the transgene is located at the 2nd chromosome.

2.6. Expression of CYP392A16 and/or TuCPR in D. melanogaster

We employed the GAL4/UAS system to express *CYP392A16* and *TuCPR* in the transgenic flies, as previously described [23, 46]. We used the HR-GAL4 driver [45] in order to drive the expression of *CYP392A16* and/or *TuCPR* in specific tissues (malpighian tubules, midgut and fat body) [47]. Transgenic UAS-CYP392A16 and/or UAS-TuCPR;UAS-CYP392A16 virgin females were crossed

with HR-GAL4 or HR-GAL4;UAS-CYP392A16 males and the progeny was used in toxicity bioassays (see below). Progeny from the cross of *yw* virgin females with HR-GAL4 males (i.e. not driving transgene expression) served as control.

2.7 Toxicity bioassays

We performed an "adult feeding" bioassay as described in [23]. In brief, in order to investigate response to acaricides in *Drosophila*, 20 adult flies (10 males and 10 females) aged 2-4 days per replicate were used for the toxicity assay. Flies were collected in plastic vials and the insecticide was provided to them through wettex sponge (or cloth). The insecticide was diluted in 5% sucrose. Each dose was tested in 3 replicates and 5% sucrose alone served as control. Mortality was scored after 24 h. Five to six concentrations that cause 5-95% mortality were used.

A Chi-squared test was used to assess how well the individual LC_{50} values observed in the bioassays agreed with the calculated linear regression lines, and the results were analyzed with PoloPlus (LeOra Software, Berkeley, CA). The LC_{50} values and RR (resistance ratio) were considered significant if the 95% fiducial limits (FL) did not include 1 [48].

3. Results

3.1. Generation of transgenic lines bearing UAS-CYP392A16 and UAS-TuCPR

Several lines containing single insertions of the relevant transgenes were generated by standard P-element transgenesis. Among different transgenic lines, two UAS-TuCPR lines (#32, #92) and two UAS-CYP392A16 lines (#9, #71) demonstrating strong white phenotype (using eye color intensity as a proxy for overall transgene expression level variability due to insertion position effects) were selected for the generation of transgenic flies that express ectopically both CYP392A16 and TuCPR.

A series of genetic crosses (as per general outline shown in Figure 1) was performed to generate double-responder lines bearing a UAS-TuCPR transgene at chromosome 2, along with a UAS-CYP392A16 transgene at chromosome 3. Thus, four 'double-responder' lines were generated, i.e. lines UAS-TuCPR32;UAS-CYP392A16.9, UAS-TuCPR32;UAS-CYP392A16.71, UAS-TuCPR92; UAS-CYP392A16.9 and UAS-TuCPR92;UAS-CYP392A16.71.

In a similar fashion, two 'driver-responder' lines were generated bearing the HR-GAl4 driver at chromosome 2 along with a UAS-CYP392A16 transgene at chromosome 3, i.e. lines HR-GAL4;UAS-CYP392A16.9 and HR-GAL4;UAS-CYP392A16.71.

3.2. GAL4/UAS transgenic co-expression of CYP392A16 and TuCPR confers resistance to abamectin

Responder (UAS) lines were crossed with HR-GAL4 driver in order to drive the expression of both *CYP392A16* and *TuCPR* in the midgut, malpigian tubules and fat body. Successful expression in the progeny was confirmed by reverse transcription PCR indicating the presence of the *CYP392A16* transcript in whole adult flies (indicative gels shown in Figure 2).

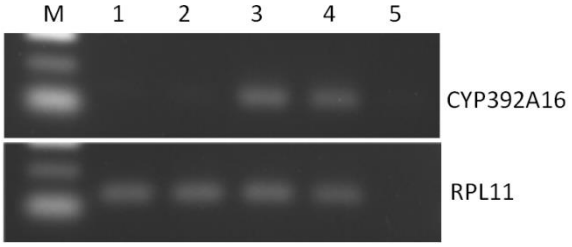

Figure 2. Confirmation of CYP392A16 expression in transgenic *Drosophila* melanogaster by PCR amplification of cDNA. Lanes 1 and 2 represent biological replicates of progeny from the cross *yw* x HR-GAL4 (not expressing CYP392A16) while lanes 4 and 5 are progeny from the cross UAS-TuCPR32; UAS-CYP392A16.71 x HR-GAL4, while lane 5 is a non-template negative control.

The top gel represents products amplified with primers CYP392A16F/R (Table 1) while the bottom gel represents products amplified with primers for RPL11 housekeeping gene.

A series of "feeding" bioassays were conducted using adult progeny (2-4 days post eclosion) from crosses involving different combinations of driver/responder/double-responder lines that correspond to different "dosage" as shown in Table 2. The survival at different concentrations of abamectin was monitored and the resistance ratio of each line combination versus the yw x HR-GAL4 negative control with the same genetic background is shown in Table 2.

Table 1. Abamectin toxicity bioassay responses of transgenic flies expressing CYP392A16 coupled with endogenous *Drosophila* CPR or along TuCPR, with different transgene copy number (dosage) combinations, compared to the control cross *yw* x HR-GAL4 of the same genetic background.

Cross			Transgene dosage			LC_{50} (95% FL) (mg/L)	Slope	χ^2 (df)	RR[1] (95% FL)
Female	x	Male	Gal4	CPR	A16				
yw		HR-GAL4	1	-	-	45.5 (33.3 – 56.9)	1.9 ± 0.35	12.1 (16)	
UAS-CYP392A16.71		HR-GAL4	1	-	1	53.4 (38.7 – 76.2)	1.316 ± 0.25	17.8 (16)	1.17 (0.81 – 1.71)
UAS-TuCPR92; UAS-CYP392A16.71		HR-GAL4	1	1	1	85.2 (78.6 – 92.7)	7.1 ± 1.6	10.9 (16)	**1.88** (1.44 – 2.44)
UAS-TuCPR32; UAS-CYP392A16.71		HR-GAL4	1	1	1	101.8 (88.6 – 127.6)	3.7 ± 0.62	10.2 (15)	**2.24** (1.65 – 3.04)
HR-GAL4; UAS-CYP392A16.71		HRGAL4; UAS-CYP392A16.71	2	-	2	45.07 (37.85 – 51.91)	3.41 ± 0.43	7.98 (10)	0.99 (0.74 – 1.33)
UAS-TuCPR32; UAS-CYP392A16.71		HR-GAL4; UAS-CYP392A16.71	1	1	2	77.01 (65.99 – 89.73)	4.37 ± 0.47	14.4 (10)	**1.69** (1.29 – 2.23)
UAS-CYP392A16.9		HR-GAL4	1	-	1	28.3 (10.3 – 42)	2.1 ± 0.4	40.3 (15)	0.62 (0.42 – 0.93)
UAS-TuCPR92; UAS-CYP392A16.9		HR-GAL4	1	1	1	54.4 (40.1 – 64.3)	4.9 ± 0.8	30.3 (16)	1.19 (0.89 – 1.59)
UAS-TuCPR32; UAS-CYP392A16.9		HR-GAL4	1	1	1	82.7 (61.7 – 117.5)	3.1 ± 0.4	49.6 (13)	**1.82** (1.37 – 2.42)
HR-GAL4; UAS-CYP392A16.9		HR-GAL4; UAS-CYP392A16.9	2	-	2	31.06 (18.82 – 40.36)	3.2 ± 0.5	14.88 (9)	0.68 (0.79 – 0.96)
UAS-TuCPR32; UAS-CYP392A16.9		HR-GAL4; UAS-CYP392A16.9	1	1	2	105.29 (79.91 – 135.06)	4.6 ± 0.6	17.4 (8)	**2.32** (1.74 – 3.08)

[1] Statistically significant resistance ratios are shown in bold.

The effect of CYP392A16 expression in abamectin-induced mortality was assessed, showing statistically significant difference (95% fiducial limits not including 1) between certain fly genotypes that overexpress CYP392A16 and the control genotype (Table 2). As shown, CYP392A16 expression is able to confer significant resistance only when TuCPR is co-expressed. Though TuCPR co-expression is not sufficient for significant resistance in every transgene combination, expression of CYP392A16 alone in the absence of TuCPR (i.e. coupling with the endogenous *Drosophila* CPR) does not produce resistant phenotypes at any transgene combination, even with two copies of HR-GAL4 driver and/or UAS-CYP392A16 responder.

4. Discussion

Our results indicate that *CYP392A16*, a P450 gene that has been associated to abamectin resistance in *T. urticae* and is capable of metabolizing abamectin *in vitro* [22], is also capable to confer resistance *in vivo*, following expression in detoxification-related tissues in transgenic *Drosophila*. This powerful and versatile system has been used frequently in recent years to test candidate genes for their potential to confer resistance, taking advantage of its unique properties [39-41].

In order to systematically assess the role of TuCPR and CYP392A16 *in vivo* in the absence of confounding resistance mechanisms, we used *Drosophila melanogaster* as a model to induce conditional (GAL4/UAS) expression. We generated a number of strains conditionally expressing CYP392A16 in the presence of endogenous *Drosophila* CPR as redox partner, or along with TuCPR, and crossed them with HR-GAL4 drivers driving expression in tissues relevant to detoxification, using different driver or responder transgene dosage. We confirmed that the progeny of UAS-CYP392A16 x HR-GAL4 successfully express CYP392A16 and toxicity bioassays indicated that the transgenic expression of CYP392A16 in *Drosophila* confers abamectin resistance in comparison to control flies with the same genetic background, but *only* when TuCPR was co-expressed. While TuCPR co-expression does not confer significant resistance in every possible transgene combination, it is possibly critical for the generation of functional and efficient monooxygenase complex.

Heterologous expression of P450s, both *in vitro* and *in vivo* requires high yields of stable and active P450 enzyme in a functional monooxygenase complex with its redox partner. Several insect P450s have been functionally expressed *in vitro* in the presence of CPR originating from different insect species/orders [49-52] and demonstrating efficient *in vitro* metabolism. In principle, CPRs originating from different organisms (mammalian, yeast, insect) perform the same function and should be interchangeable in heterologous expression systems [34]. Nevertheless, in certain cases like CYP392A16 and CYP392A11 from *T. urticae*, employment of a "generic" insect CPR like the relevant enzyme of *Anopheles gambiae* (AgCPR) that has a demonstrated ability to form active complexes with insect P450s [49] was not advantageous, while co-expression of the homologous CPR leads to active P450 enzyme complexes [22,23].

Studies involving *in vivo* functional validation of insect P450s in *Drosophila melanogaster* using its endogenous CPR as redox partner, indicate in most cases a resistant phenotype [43, 46, 50-57] and enable functional validation of candidate P450s. However, the obtained resistance ratios are quite lower than those normally observed in insect pest populations and the absence of the homologous/cognate CPR is a potential system drawback [43].

In the case of *T. urticae*, functional expression of mite P450s *in vivo* showed resistant phenotype only in the presence of the homologous TuCPR both *in vitro* [22,23] and *in vivo* [23 and this study]. Thus, it is possible that the endogenous *D. melanogaster* CPR might not be able to provide strong resistance phenotypes facilitating efficient validation of mite P450s, presumably due to suboptimal coupling. The potential to form functional and efficient complexes with mite P450s may be compromised given the significant evolutionary distance between insects and mites.

Our findings provide further functional evidence for the role of CYP392A16 in abamectin resistance and show that this approach can be a useful tool for validating candidate spider mite resistance genes, provided that a functional redox partner like TuCPR is provided and active enzyme complex reconstitution is facilitated. It must be noted however that even with this approach, observed resistance ratios among different strains vs control, vary from 1.69 to 2.32 at the maximum (Table 2). While such ratios are statistically significant, they only represent a small fraction of observable abamectin resistance in the field [11, 17, 58-60]. This fact either represents an inherent limitation of the *Drosophila* model in order to fully recapitulate field conditions (also relevant in the assessment of target-site resistance [42]) or reflects the synergistic action of multiple molecular mechanisms in resistant pest populations, perhaps involving target-site abamectin resistance [14], in order to generate the resistant phenotype.

Indeed, research involving investigation of synergistic interactions of enzyme overexpression and/or target-site mutations within a *Drosophila*-engineered unbiased framework has indicated that synergistic action of different molecular mechanisms has a multiplicative effect in phenotype

manifestation, at least for pyrethroids [44]. This implies that such an experimental system can be readily engineered, for example by stably integrating a TuCPR expressing transgene together with an attP landing site for ΦC31 integrase or equivalent, that would minimize 'noise' induced by position effects. Further development and optimization of *Drosophila*-based systems for efficient validation of spider mite P450s and assessment of their synergistic action with co-existing resistance mechanisms holds potential for significant insights, towards the elucidation of the complex resistance phenotypes found in pest populations. .

5. Conclusions

In conclusion, this study has established that *CYP392A16*, a cytochrome P450 from the two-spotted spider mite *Tetranychus urticae* which is capable of metabolizing abamectin *in vitro* is also able to confer resistance *in vivo* as shown by transgenic expression in *Drosophila*. We have also demonstrated that the resistant phenotype is manifest only in the context of TuCPR co-expression, indicating that an evolutionary less divergent partner may be more appropriate for the generation of functional and efficient monooxygenase complex. Although other resistance mechanisms also have roles in resistance phenotypes found in field populations of *T. urticae*, this information is valuable towards the development of a research framework involving investigation of synergistic interactions of enzyme overexpression and/or target-site mutations within a *Drosophila*-engineered, unbiased context..

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