Over-expression in cis of the midgut P450 CYP392A16 contributes to abamectin resistance in *Tetranychus urticae*.

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Abstract

Cytochrome P450 mediated metabolism is a well-known mechanism of insecticide resistance. However, to what extent qualitative or quantitative changes are responsible for increased metabolism, is not well understood. Increased expression of P450 genes is most often reported, but the underlying regulatory mechanisms remain widely unclear. In this study, we investigate CYP392A16, a P450 from the polyphagous and major agricultural pest *Tetranychus urticae*. High expression levels of *CYP392A16* and *in vitro* metabolism assays have previously associated this P450 with abamectin resistance. Here, we show that CYP392A16 is primarily localized in the midgut epithelial cells, as indicated by immunofluorescence analysis, a finding also supported by a comparison between feeding and contact toxicity bioassays. Silencing via RNAi of *CYP392A16* in a highly resistant *T. urticae* population reduced insecticide resistance levels from 3400- to 1900- fold, compared to the susceptible reference strain. Marker-assisted backcrossing, using a single nucleotide polymorphism (SNP) found in the *CYP392A16* allele from the resistant population, was subsequently performed to create congenic lines bearing this gene in a susceptible genetic background. Toxicity assays indicated that the allele derived from the resistant strain confers 3.6-fold abamectin resistance compared to the lines with susceptible genetic background. *CYP392A16* is over-expressed at the same levels in these lines, pointing to *cis*-regulation of gene expression. In support of that, functional analysis of the putative promoter region from the resistant and susceptible parental strains revealed a higher reporter gene expression, confirming the presence of *cis*-acting regulatory mechanisms.

Key words

P450, marker assisted backcrossing, immunolocalization, *cis*-regulation, RNAi
1. Introduction

Cytochrome P450-monooxygenases (P450s) are a superfamily of heme-thiolate enzymes involved in the oxidative transformation of endogenous substrates and xenobiotics, including pesticides (Dermauw et al., 2020b; Feyereisen, 2012). The recent explosion in available P450 sequences, due to the expansion of invertebrate sequenced genomes, facilitated the study of their functional relevance in insecticide and acaricide resistance, which is of critical importance for agriculture and public health. For example, members of the CYP6 and CYP9 families have been associated with high levels of insecticide resistance in mosquitoes (Chandor-Proust et al., 2013; Liu et al., 2015; Moyes et al., 2017; Smith et al., 2019); CYP6BQ23 with pyrethroid resistance in the pollen beetle Meligethes aeneus and CYP6CM1 with neonicotinoid resistance in Bemisia tabaci (Hamada et al., 2019; Karunker et al., 2009; Zimmer et al., 2014). Several individual P450s from Tetranychus urticae, one of the most notorious agricultural pests worldwide (Van Leeuwen et al., 2015) have been functionally associated with acaricide resistance. Specifically, CYP392A16 is capable of metabolizing abamectin and pyflubumide in vitro; CYP392A11 metabolizes fenpyroximate and cyenopyrafen while CYP392E10 metabolizes spirodiclofen (Demaeght et al., 2013; Fotoukkiaii et al., 2021; Riga et al., 2015; Riga et al., 2014).

The often high expression levels of a P450 gene observed in resistant mites, together with functional expression studies showing metabolism of insecticides in vitro, provide good indication for its involvement in resistance, however the biological relevance of these findings still remains elusive. In particular, the physiological role and tissue-specificity, together with uncertainties on the extent of additive contributions in complex insecticide/acaricide resistance phenotypes and finally the regulation mechanisms of gene expression, is far from understood (Nauen et al., 2022; Vontas et al., 2020).

Several studies that explored the tissue localization pattern of P450 metabolizers in several insects, have revealed that the main detoxification tissues are the midgut, Malpighian tubules and fat body (Chung et al., 2009; Hu et al., 2014; Ingham et al., 2017). For example, the Anopheles gambiae CYP6M2 is detected in the midgut and Malpighian tubules (Ingham et al., 2017), whereas CYP321E1 from Plutella xylostella is highly expressed in the midgut, fat body and epidermis (Hu et al., 2014). Other studies implicated alternative tissues in detoxification, such as the case of CYP6BQ9, which is mainly expressed in the brain of a pyrethroid resistant strain of Tribolium castaneum (Zhu et al., 2010). However, the location of P450 expression in
Acari, including mites and ticks, has not yet been determined, and therefore the principal
detoxification tissues are far from characterized in these notorious pests and disease vectors.

The molecular genetic mechanisms of P450-mediated resistance include over-expression, due
to gene amplification and/or regulatory variation or enhanced metabolism due to variation of
coding sequence or gene conversion (Feyereisen et al., 2015; Ibrahim et al., 2015; Joussen et
al., 2012; Nauen et al., 2022; Zimmer et al., 2018). Over the last years, increased expression of
P450s due to transcriptional regulation by trans and/or cis factors, as well as gene amplification,
have been associated with resistance (Bass et al., 2013; Ingham et al., 2017; Kalsi and Palli,
2015, 2017; Lucas et al., 2019; Mugenzi et al., 2019). For example: cis regulatory elements
have been associated with the over-expression of CYP6P9a in Anopheles funestus (Weedall et
al., 2019). Both cis and trans factors regulate the expression of CYP6BQs in T. castaneum
(Kalsi and Palli, 2015), Cncc-Maf regulate the expression of three P450 genes in Tetranychus
cinnabarinus (Shi et al., 2017), CREB transcription factor regulates the expression of
CYP6CM1 in B. tabaci (Yang et al., 2020) and finally, CYP6CY3 has several copies and a
dinucleotide microsatellite in the promoter region of a resistant strain of Myzus persicae (Bass
et al., 2013). Recently, a QTL analysis based on bulk segregant analysis (BSA) revealed that,
most likely, cis regulatory changes underlie the over-expression of CYP392A16 associated with
pyflubumide resistance in T. urticae (Fotoukkiaii et al., 2021). Nevertheless, the underlying
 genetic changes at the basis of this effect were not uncovered and the gene expression regulation
mechanisms of P450s in T. urticae remain to be explored.

Forward and reverse genetics, i.e. in vivo over-expression, RNAi or knock-out of P450s in
model and non-model species, have provided different levels of functional validation for the
contribution of these enzymes in the resistance phenotype. RNAi-based knock-down of the
deltamethrin metabolizer CYP6BQ9 in T. castaneum substantially suppressed resistance levels
to deltamethrin by approximately 100-fold (Zhu et al., 2010). Administration of dsRNA
targeting CYP389C16 from T. cinnabarinus, a P450 that is over-expressed in a cyflumetofen
resistant strain, only slightly decreased resistance to cyflumetofen (Feng et al., 2020). Although
RNAi is a relatively fast method, it is not always precise enough due to cross-silencing of related
genes, and it does not work equally well across different pests (Cooper et al., 2019).

A precise way to determine the role of a resistance allele in vivo is by engineering it in a defined
 genetic background utilizing genome editing techniques in species where this approach is
applicable (Douris et al., 2020). The most common technique to functionally link a P450 with
a resistant phenotype in vivo, is by ectopic over-expression of individual P450s in the model
organism *Drosophila melanogaster*. However, the system has limitations in determining the relative importance and strength of the phenotype (Daborn et al., 2012; Douris et al., 2020; McLeman et al., 2020; Nauen et al., 2022). An alternative option is to generate transgenic organisms of the species of interest, although not trivial. For example, mosquitoes over-expressing certain P450s in specific tissues in a susceptible genetic background have been created, thus allowing a more clear determination of P450’s functional role in resistance to insecticides (Adolfi et al., 2019). The applicability of CRISPR/Cas9 technique in model and non-model organisms potentially provides a better tool for the study of the functional role of P450s. Deletion of a cluster of nine *Helicoverpa armigera* P450 genes that belong to the CYP6AE subfamily by CRISPR technology, demonstrated their involvement in resistance to esfenvalerate, yet this deletion does not show any apparent fitness cost (Wang et al., 2018).

In species where genome editing tools are not yet available or the efficiency is still very low (Dermauw et al., 2020a), an alternative that has been explored is to repeatedly backcross resistant individuals with susceptible ones via the assistance of a marker for the gene of interest. Marker-assisted backcrossing provides a straight-forward method to uncouple a genomic locus that contains the gene of interest from other loci that contribute to resistance, on the condition that there is sufficient genetic distance between them allowing recombination. This way, near isogenic strains can be generated differing only in the “inserted” region on the chromosome and the impact of a locus can be analyzed. This approach has been employed in *T. urticae* for studying the contribution of target-site mutations in acaricide resistance (Bajda et al., 2017; Riga et al., 2017), but not yet for genes involved in metabolic resistance.

In this study, immunolocalization experiments and targeted bioassays were employed in order to identify the tissue specificity and physiology of the CYP392A16 - based detoxification. Additionally, the contribution of CYP392A16 in resistance to abamectin in *T. urticae* was determined by RNAi. Marker-assisted backcrossing of the CYP392A16 allele in a susceptible background confirmed its role in resistance and pointed towards strong cis-regulatory effects, which were corroborated with promoter analysis via gene reporter assays.

### 2. Materials and Methods

#### 2.1 *T. urticae* strains

The London inbred strain (Wybouw et al., 2019) originates from the London strain which was used to construct the reference mite genome (Grbic et al., 2011) and was subjected to an additional six mother-son crosses as previously described (Bryon et al., 2017). The London
strain originates from Ontario. The abamectin resistant strain Mar-ab was collected from a rose-greenhouse in Marathonas (Greece), as has been previously described (Dermauw et al., 2012). Mar-ab was kept under 70 ppm selection of abamectin (Vertimec 1.8 EC) under laboratory conditions. London inbred was reared without any selection pressure. The laboratory conditions were 25 °C, 50-60% relative humidity, 16:8, light: dark.

2.2 Toxicity assays

Three different toxicity assays were performed that reflect different exposure modes: i) both feeding and contact, ii) feeding and iii) contact. For the feeding and contact toxicity assays, 20 adult female mites were transferred on the upper side of square bean leaves and wet tissue was used for preventing mites to escape. Five to six concentrations of abamectin were applied per biological replicate and each concentration consisted of three replicates. Water served as control. Plates were individually sprayed with 1 ml of aqueous solution of abamectin at 1 bar pressure using a Potter Spray Tower (Burkhard Scientific) to obtain a homogenous spray film (2mg/cm²). Mortality was assessed after 48 h and mites were considered dead if they could not walk twice the distance of their body. LC₅₀ values, slopes, RRs and 95% confidence intervals (CIs) were calculated by probit analysis (POLO, LeOra Software, Berkeley, USA). Resistance ratio (RR) was considered significant if the value one was not included in the 95% confidence intervals (CI) or in case of RNAi, if the 95% CI of LC₅₀ values did not overlap (Robertson et al., 2007).

Feeding toxicity assays were performed as the feeding and contact assay with some modifications. Abamectin has translaminar activity, thus it can penetrate the leaf tissue and form a reservoir of the active ingredient within the leaf (Rugg et al., 2005). A square bean leaf was placed with the upper surface facing the wet cotton wool and abamectin was applied to the lower surface. When abamectin was adsorbed, the adult female mites were transferred to the upper surface of the leaf. Contact toxicity assays were performed on glass petri dishes. The surface was sprayed with abamectin and after drying, 20 adult females were transferred and wet cotton wool restricted the mites from escaping. Four to five concentrations of abamectin were applied in both toxicity assay types per biological replicate with three replicates each. Mortality was scored after 72h and 24h for feeding and contact toxicity assays, respectively, and analyzed as described above. In case 4000 mg L⁻¹ did not cause 50% mortality, no further attempts were undertaken to determine LC₅₀ levels.
2.3 Western blot analysis

Two antibodies were designed and raised against CYP392A P450s. The first antibody was designed for CYP392A16 (hereafter α-CYP392A16), while the second one was designed for the CYP392A subfamily (hereafter α-CYP392A). The amino acid sequence of CYP392A16 was aligned with P450s of T. urticae that share high sequence similarity, which allowed to identify i) a region in CYP392A16 (aa 161-178) that is the most differentiated and ii) a region that is shared by the phylogenetically closely related P450s. A hydrophobicity test was also carried out in order to ensure that the selected peptides do not correspond to a transmembrane domain. The peptides SALENNGKPADFEKCISH and KCFQIVDSHIQDEIDKHQEK which correspond to α-CYP392A16 and α-CYP392A, respectively, were chemically synthesized and used to raise antibodies in rabbits and were affinity purified (Davids Biotechnologie, GmbH, Germany).

Five μg of bacterial membrane samples expressing CYP392A16, CYP392A11 and CYP392A12 were treated with Laemmli Buffer (1x) at 95°C for 5min. The treated samples were loaded on 10% SDS polyacrylamide gel and proteins were transferred on a PVDF membrane, pre-activated with methanol. After transfer, the membrane was blocked with blocking solution (5% milk in 1xTBS-Tween) for 1h at room temperature and then incubated with α-CYP392A16 (1:1000 dilution in 3% milk) or α-CYP392A (1:5000 dilution in 3% milk), by shaking at 4°C overnight. Antibody binding was detected with 1:10000 dilution of goat anti-rabbit IgG coupled to horse-radish peroxidase (Cell Signaling) and chemiluminescence was determined by using ECL detection system (BioRad).

2.4 Immunofluorescence and confocal microscopy

Immunolocalization studies were performed on mites collected from Mar-ab strain and fixed in 4% formaldehyde (methanol free; Thermo Fisher Scientific) solution for 1 h, RT. Then, the samples were cryo-protected in 30% w/v sucrose/PBS at 4°C for 12 to 16 h, immobilized in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura) and stored at -80°C until use. Immunofluorescence analysis, followed by confocal microscopy, was performed on sections of frozen mite specimens. Specifically, 6 μm sections, were obtained in a cryostat with Ultraviolet C (UVC) disinfection (Leica CM1850UV), mounted on microscope slides (SuperFrost Plus microscope slides, O. Kindler GmbH D-79110) and stored at -20°C until use. Fluorescence signals were detected after antigen retrieval at acidic conditions (pH= 4.0), when indicated, (or not), followed by permeabilization with 0.2% v/v Triton/PBS and blocking for 1
(5% w/v bovine serum albumin in 0.2% v/v Triton/PBS). Subsequently, sections were stained with rabbit primary antibody raised against CYP392A16 in blocking solution for 12 to 16 h at 4°C (1:250 dilution), followed by goat anti-rabbit secondary antibody (Alexa Fluor 488, Molecular Probes) in 1:1000 dilution. DNA was stained using DAPI (PanReac AppliChem). Finally, sections of frozen specimens without antigen retrieval, were stained with α-CYP392A16 and the pre-immune serum served as a control (both, in 1:500 dilution). Imaging was conducted in a Leica SP8 laser-scanning microscope, using the 40x – objective.

2.5 Nucleic acid extraction, cDNA synthesis and quantitative PCR (qPCR)

Genomic DNA was extracted from pools of 50 mites, as well as individual mites, from both London inbred and Mar-ab strains following the CTAB method as previously described (Navajas et al., 1999). Total RNA was extracted using the GeneJet RNA purification kit (Thermo Fisher Scientific) from pools of 150 adult female mites or Trizol (MRC) from pools of 15-30 adult female mites in case of RNAi experiments, according to manufacturer’s instructions. Extracted RNA samples were treated with Turbo DNase (Ambion) and one μg of RNA was used for gene specific cDNA synthesis using the reverse transcriptase kit from Minotech (Minotech, Heraklion, Crete), according to the manufacturer’s instructions. The expression levels of CYP392A16 were determined by qPCR amplification on CFX Connect, Real-Time PCR Detection System (Biorad). Reactions consisted of 0.4 μM of each primer, 1.5 μl cDNA and 7.5 μl KapaSYBR Green (Kapa Biosystems) to a final volume of 15 μl. The primers for CYP392A16 (tetur06g04520), as well as for Rp49 (tetur18g03590) and ubiquitin (tetur03g06910) are listed in Table S1. A 5-fold dilution series of pooled cDNA was used to assess the efficiency of the qPCR reaction for each gene-specific primer pair. A no template control (NTC) was also included to detect possible contamination. qPCR reactions were performed with 5 biological and 3 technical replicates and relative expression levels were calculated according to Pfaffl (2002) (Pfaffl et al., 2002).

2.6 dsRNA synthesis and delivery via soaking

cDNA was used as template for PCR based dsRNA synthesis with primers that include the T7 promoter sequence on their 5’ ends (Table S1). PCR products were purified from agarose gel with the MinElute Gel Extraction kit (QIAGEN). One hundred ng of purified PCR product was used as template for in vitro dsRNA synthesis reaction with HiScribe RNA synthesis kit (New England Biolabs) according to manufacturer’s instructions for CYP392A16 and LacZ, which served as negative control (Riga et al., 2020). dsRNAs were purified by phenol-chloroform extraction followed by ethanol precipitation according to manufacturer’s instructions. The
dsRNAs were dissolved in nuclease free water and quantified using NanoDrop. The quality of synthesized dsRNAs was inspected visually on 1% agarose gel. The synthesized dsRNAs were delivered to newly molted adult female mites from London inbred and Mar-ab strains via the soaking method, as previously described (Suzuki et al., 2017). The blue dye erioglaucine (McCormick, Sparks Glencoe, MD) was used at 3% (v/v) and post soaking, mites with visible blue dye in the posterior midgut were transferred onto bean leaf discs after being washed with double distilled water and kept at 26°C, 16:8 h light : dark photoperiod and 50% RH. Toxicity assays were performed on mites from Mar-ab as well as London inbred strain, after treatment with dsLacZ and dsCYP392A16. The expression levels of CYP392A16 after 3 and 5 days post dsRNA treatment, as well as CYP392A11 (tetur03g00970), CYP392A12 (tetur03g00830) and CYP392A15 (tetur03g09941) for possible off-target effects after 5 days post dsRNA treatment, were evaluated with 5 biological replicates of 15-30 females and cDNA synthesis and qPCR analysis were performed as described in section 2.5.

2.7 Identification of gDNA marker and backcrossing

Genomic DNA from resistant and susceptible mites was used as template for the amplification of the full-length sequence (1512bp) of the intronless CYP392A16 gene with specific primers, based on London genome (Table S1). PCR was performed with 1 unit of Kapa Taq DNA Polymerase (Kapa Biosystems) and the conditions were 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 40 s, extension at 72 °C for 2 min, followed by a final extension step for 4 min. The amplified PCR product was extracted from 1% agarose gel and purified with Macherey Nagel Nucleospin kit according to the manufacturer’s instructions. The purified amplicons were sequenced bi-directionally with two pair of primers by GeneWiz (Germany). The sequences and the corresponding chromatograms were inspected visually with BioEdit v. 7.0.1 (Hall, 1999). Another set of primers (Table S1) that amplifies a 382 bp region was used for the development of a PCR-RFLP diagnostic assay. The Mar-ab CYP392A16 allele bears a mutation (A to T) at 882 bp that leads to the absence of the restriction site recognized by the SspI restriction enzyme, while this restriction site is present in the susceptible strain. In order to set up the PCR-RFLP assay, gDNAs from susceptible and resistant strains, as well as an artificial gDNA mix of resistant and susceptible parents mimicking heterozygotes, served as templates for the PCR. Reactions were performed with 1 unit of Kapa Taq DNA Polymerase (Kapa Biosystems) and the conditions were 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, followed by a final extension step for 5 min. The enzymatic
reaction with SspI (Minotech, Heraklion, Crete) was carried out in 30 μl reaction volume using 10 units of SspI enzyme, incubated for 2h at 37 °C and followed by 10 min enzyme inactivation at 65 °C. The reaction products were visualized in 2% agarose gel.

The crossing procedure was previously outlined in Bajda, et al. 2017 (Bajda et al., 2017). Briefly, a haploid male of the resistant strain was crossed with a virgin female of the susceptible strain. The resulting heterozygous virgin females were back-crossed to susceptible males and the heterozygous genotypes were identified by PCR-RFLP. The backcrossing procedure was repeated for seven generations. In the last generation, the backcrossed heterozygous virgin females were crossed with their first-born son representing either a resistant (the resistant CYP392A16 allele, T nucleotide mutation) or a susceptible (the susceptible CYP392A16 allele, A nucleotide) genotype. These crosses resulted in the generation of congenic homozygous lines that either bear the Mar-ab allele or the wild-type allele of CYP392A16. The final crosses were performed as follows: ♀CYP392A16_T/CYP392A16_A x ♂CYP392A16_T or ♂CYP392A16_A were crossed to obtain the homozygous congenic lines CYP392A16_L1-L3 and CYP392A16_C, respectively.

2.8 Cloning of the upstream sequence of CYP392A16

One kb upstream of the start codon of CYP392A16, which is expected to at least partially include the gene promoter, was amplified from the resistant and susceptible strains, with specific primers based on the London reference genome sequence using Phusion Polymerase (New England Biolabs) (Table S1). PCR reactions were performed as follows: 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 45 s, followed by final extension at 72 °C for 5 min. PCR products were purified with Nucleospin Gel and PCR clean-up kit (Macherey-Nagel) according to manufacturer’s instructions and were sequenced bi-directionally by GeneWiz (Germany). The sequences and the corresponding chromatograms were inspected visually with BioEdit v. 7.0.1 (Hall, 1999). The software PROMO-ALGGEN and JASPAR within Insecta group were used for the identification of possible Transcription Factor Binding Sites (TFBS).

Promoter truncations were obtained using the full promoter as template under the same conditions mentioned above. The chimeric promoters were generated by overlap extension PCR in case of C-133 or by conventional PCR for C-10. Briefly, for the C-133 promoter, the 887bp region of the susceptible promoter and the fragment of 133bp (upstream of the start codon) of the resistant promoter were amplified with the primer pairs Fsus-MluI/R1 and F1/Rres-NcoI respectively (Figure S1). The C-10 promoter was generated by the replacement of the 5bp of
the susceptible with the 10bp of the resistant promoter and synthesized with PCR using the
primer pair Fsus-MluI/R3. All promoter sequences were inserted into pGL3 basic vector (kindly
provided by Dr. George Skavdis, DUTH) in between the MluI and NcoI cut sites. Constructs
were purified with NucleoSpin Plasmid kit (Mancherey-Nagel) and verified by sequencing.

2.9 Functional promoter analysis in insect cell lines

Luciferase gene reporter assays with the putative promoters were performed in two insect cell
lines, Hi5 and Sf9. Both cell lines were maintained as suspension and adherent cultures
respectively, in the insect serum free medium SF-900 II SFM (Thermo Fisher Scientific) with
100U ml\(^{-1}\) of penicillin and 0.1mg ml\(^{-1}\) streptomycin, while the medium of Sf9 cells was
additionally supplemented with 10% FBS (Gibco, Thermo Fisher Scientific). 10\(^5\) cells/well
were seeded in 24-well plates and incubated for 24h prior to transfection at 27°C. Next day the
cells were washed and incubated with incomplete medium. The cells were co-transfected with
250ng DNA, including the experimental reporter constructs pGL3 vector and BmAc3-Renilla
construct (Samantsidis et al., 2021) at molecular ratio 500:1, using the ESCORT IV
Transfection Reagent (Sigma-Aldrich). After six hours, cells were supplemented with complete
medium and incubated overnight. Sixteen hours post-transfection the medium was replaced
with 500 μl of complete medium. Cells were harvested 72h post-transfection and lysed with
100 μl of passive lysis buffer (Promega). The promoter activity was measured with the Dual-
Luciferase Reporter Assay System (Promega). The same methodology was followed for
reporter assays with the mutant promoters (truncations / deletions and chimeric) but only in Sf9
cells. Relative luciferase activity (RLA) was calculated as a ratio of Firefly luciferase against
the Renilla luciferase values. The fold change activity was calculated relative to pGL3 activity
and expressed as mean ± SD of three independent transfections. Luciferase assay data were
analyzed for significant differences among the promoters with two-way (full length promoters)
or one-way ANOVA (truncated and chimeric promoters) and Tukey test was applied for
pairwise comparisons, using the software GraphPad Prism 8.0.2.
3. Results

3.1 Antibody specificity and expression of CYP392A16 in the midgut

The antibody specificity was assessed by western blot on isolated bacterial membranes expressing the closely related *T. urticae* P450s CYP392A11, CYP392A12 and CYP392A16 and immunostained by α-CYP392A16 or α-CYP392A. As depicted in Figure S2, the recombinant CYP392A16 expressed in bacterial membranes is recognized by the α-CYP392A16 antibody and cross reactivity to the other recombinant P450 proteins is not observed, supporting the specificity of the α-CYP392A16. On the other hand, all three recombinant proteins are recognized by α-CYP392A that has been designed in a shared protein region. Thus, α-CYP392A16 was used in immunolocalization.

Immunolocalization of CYP392A16 revealed that the primary site of expression of this P450 is the midgut, which composes the larger part of *T. urticae* digestive system, occupying almost the entire opisthosoma (Figure 1 A-C, Figure S3). A direct comparison between stained and virtual cross sections at the level of *T. urticae* midgut, obtained by immunostaining and X-ray sub-micron computed tomography respectively, clarified mite’s internal anatomy and tissue compartmentalization. Evidently, midgut epithelial cells that form the midgut wall are being very close to the chlorophyll-rich plant cells in the lumen, while the lower part of the mite’s body consists of the ovary. In our cryosections, we managed to observe a filament-like green fluorescence signal inside the midgut cells that corresponds to CYP392A16 (Figure 1 D-F) and in proximity the autofluorescence of chlorophyll which is depicted red, strongly indicate that the enzyme is expressed in the midgut tissue. Finally, an artificial green signal, lining up both sides of the chitin-rich part of the cuticle was observed, when both antibody or pre-immune serum was used (Figure S3, panels B and D, merge with BF, respectively).

To further explore if CYP392A16 is expressed in the midgut, we conducted feeding and contact toxicity assays. Indeed, Mar-ab showed higher resistance levels to abamectin when subjected to feeding (RR> 1100, Table 1) when compared to contact assay (RR: 880-fold, Table 1). Although the difference in RR between the feeding and contact assays seems limited, it is probably an underestimate as the highest abamectin dose administered through feeding (4000 mg L\(^{-1}\)) did not cause 50% mortality in the resistant strain. Therefore, the actual LC\(_{50}\) might be much higher, which may increase the difference in RRs between both assays. Nevertheless, these data suggest that CYP392A16-mediated metabolism of abamectin is taking place predominantly in the gut, upon ingestion of the insecticide during feeding.
3.2 RNAi of CYP392A16 partially alleviates abamectin resistance

CYP392A16 expression levels were significantly down-regulated in the resistant strain, (Mar-ab), 3 and 5 days post-treatment with dsCYP392A16, by 42% and 48% respectively, in comparison to mites (Mar-ab) treated with dsLacZ (Figure 2). In contrast, CYP392A16 expression levels were also affected (47.5%) in the susceptible strain (London inbred), but only 3 days post-treatment (Figure 2). Additionally, off-target effects on closely related cytochrome P450s according to phylogenetic analysis (Gbric et al., 2011) and sequence similarity were not observed in either strain, as evidenced by the expression levels of CYP392A11, CYP392A12 and CYP392A15 (Figure S4). Administration of dsRNA targeting the CYP392A16 transcript reduced the LC$_{50}$ value from 600 to 280 mg L$^{-1}$, approximately, in the resistant strain, while similar LC$_{50}$ values were obtained in London inbred upon administration of dsCYP392A16 compared to dsLacZ. Thus, the resistant factor of the Mar-ab population decreased from 3400-1900-fold, in comparison with the susceptible strain, whereas the application of dsLacZ did not affect either strain (Table 2).

3.3 Marker assisted back-crossing of the locus containing the CYP392A16 allele and analysis of expression levels in congenic lines.

The Mar-ab CYP392A16 allele bears a non-silent mutation (A to T) at base pair position 882 in the open reading frame that leads to the absence of the restriction site recognized by the SspI restriction enzyme (AATATT), whereas the enzyme restriction site is present in the susceptible strain (Figure S5A). Digestion of the amplified products produce distinct bands upon enzymatic reaction in presence of SspI with the following patterns: one fragment of 382 bp for the resistant allele, two fragments of 238 bp and 144 bp for the susceptible allele and the heterozygote consists of all three fragments (Figure S5B). After this validation, the marker was subsequently used for the establishment of the congenic lines in which the CYP392A16 allele from the resistant parental strain was introgressed in the susceptible genetic background of London inbred.

The initial crosses between the parental resistant and susceptible strains as well as the backcrossing methodology/procedure are outlined in Figure S5C. Finally, homozygous backcrossed near isogenic lines carrying the CYP392A16 allele from the Mar-ab strain (CYP392A16_L1-L3) and their respective congenic control line (CYP392A16_C) were generated.
Examination of the expression levels of \textit{CYP392A16} in the generated lines, as well as the parental strains, indicated significant over-expression in the near isogenic lines bearing the \textit{CYP392A16} resistant allele and Mar-ab by approximately 2.8-fold, when compared to either the London inbred strain or the congenic \textit{CYP392A16} \_C line (Figure 3).

Toxicity assays were also performed with the congenic lines in order to identify the contribution of \textit{CYP392A16} to abamectin resistance \textit{in vivo}. Specifically, the \textit{CYP392A16} \_C line showed similar response levels compared to the parental susceptible strain, while the congenic lines \textit{CYP392A16} \_L1-L3 exhibited moderate resistance to abamectin with RR values of 3.6-fold, approximately (Table 2).

\textbf{3.4 Promoter analysis}

The putative (partial) promoter region (one kb upstream of the start codon) of \textit{CYP392A16} from Mar-ab, London inbred and the generated near isogenic lines was amplified by PCR. Sequence alignment indicated the existence of multiple SNPs and insertions – deletions (indels) in Mar-ab and \textit{CYP392A16} \_L1-L2 lines compared to the London genome reference sequence and the sequences obtained from London inbred and \textit{CYP392A16} \_C line (Figure S6). Since, as expected, the promoter sequences of the congenic lines were identical to the ones from their respective parental strains, subsequent analysis was focused on promoter regions obtained from the parental strains. The prediction tools used for the identification of potential transcription factors based on Insecta group did not identify any differentially enriched TFBSs in the putative \textit{CYP392A16} promoter region of the two strains. Moreover, the analysis did not indicate the existence of motifs that correspond to previously reported TFBSs associated with P450 regulation.

The promoter region was inserted into pGL3 basic vector and subjected to functional analysis in Hi5 and Sf9 insect cells. The luciferase assays indicated that the putative promoter sequence of the resistant strain drives almost 9-fold greater reporter gene expression than the one of the London inbred strain, in both Hi5 and Sf9 cells. The difference in the activity between the promoters of the two strains, indicates the existence of sequence elements that are localized in the promoter sequence of Mar-ab (Figure 4A). To narrow down the region of the resistant promoter that will retain the highest transcriptional activity, a series of deletions (-826bp, -607bp, -350bp, -206bp, -133bp in respect with the length upstream of the start codon as well as the Δ-10bp which corresponds to the full resistant promoter lacking the final 10bp) were analyzed. Truncation analysis revealed that deletions led to slightly higher, but not significantly
different activity compared to the full-length promoter. Moreover, the high activity is retained by the -133bp of the promoter. On the contrary, the Δ-10 deletion, displayed a significant reduction in the activity, almost at the half of the full-length promoter (Figure 4B).

Given that the -133bp fragment of the resistant strain displayed similar levels of activity with the full-length promoter, it was hypothesized that this region encompasses cis-regulatory elements. Moreover, the 10bp sequence upstream of the start codon, which resides only in the resistant strain, seemed to be necessary for the activity of the promoter highlighting its activity as a putative positive cis-acting element. To further validate these hypotheses, two chimeric promoters were constructed and analyzed. The susceptible chimeric promoter encompassing the -133bp of the resistant promoter (C-133) displayed a 6-fold increase of luciferase activity compared to the full-length susceptible promoter. Moreover, replacement of the 5bp upstream of the start codon of the susceptible promoter with the 10bp (5’-GTTGAAAAAG-3’, C-10) of the resistant one lead to a 5-fold increase. Although both chimeric promoters did not exhibit the same levels of activity as the full resistant promoter, functional analysis designated that the 10bp sequence is sufficient to increase the activity of the susceptible promoter significantly, hence it seems to be partially responsible for CYP392A16 regulation (Figure 4C and D).

4. Discussion

Since more than 20 years, abamectin is a widely used insecticide/acaricide and resistance cases have been reported in insect and mite pests (Çağatay et al., 2018; Dermauw et al., 2012; Kwon et al., 2010; Wang et al., 2016). In *T. urticae*, reported abamectin resistance mechanisms include: i) target site resistance mutations in glutamate gated chloride channels (GluCls) and their combined effect provides moderate resistance in congenic *T. urticae* lines (Dermauw et al., 2012; Kwon et al., 2010; Riga et al., 2017) and ii) resistance by increased abamectin metabolism. In the latter case, the cytochrome P450 CYP392A16 and UDP-glycosyltransferases (UGTs) have been reported (Riga et al., 2014; Snoeck et al., 2019; Xue et al., 2020) to metabolize abamectin in vitro. Cytochrome P450s play a significant role in the metabolism of xenobiotics in all organisms. Several studies have identified tissue-specific expression patterns and investigated their contribution to resistance and, to a lesser extent, in some cases the molecular genetic mechanisms of increased expression have been studied in insects (Adolfi et al., 2019; Bass et al., 2013; Mugenzi et al., 2019; Yang et al., 2020; Zhu et al., 2010). However, in mites and ticks, far little is known. Therefore, we aimed in this study to answer some outstanding questions by studying the P450 CYP392A16 from a highly abamectin resistant strain of *Tetranychus urticae* strain (Mar-ab). Over-expression of CYP392A16 has
been previously associated with high levels of resistance in this strain and was shown to metabolize abamectin in vitro (Riga et al., 2014).

In insects, cytochrome P450s involved in detoxification are primarily expressed in midgut, fat body and Malpighian tubules (Chung et al., 2009; Hu et al., 2014; Ingham et al., 2017). However, the lack of fat body and Malpighian tubules in some mite species as T. urticae, together with an extensive midgut epithelium that occupies almost the entire opisthosoma, points towards the midgut as the most likely tissue of detoxification in this pest (Bensoussan et al., 2018). Indeed, immunolocalization data from this study revealed that CYP392A16 is expressed in the midgut. This is the first time that tissue-specific expression of a mite P450, or any other detoxification enzyme, has been described. One of the factors contributing to this lack of knowledge is the extreme small size of the mite that makes tissue dissections unfeasible and complicates protocols for sectioning, staining and imaging. Nevertheless, we managed to detect a reproducible, filament-like green fluorescent signal inside the cells. Based on the recent description of the mite alimentary tract (Bensoussan et al., 2018) and the detailed described internal anatomy with high resolution of T. urticae, using X-ray sub-micron computed tomography (Jonckheere et al., 2016), it is clear that the cells expressing CYP392A16 correspond to midgut cells, in particular the gut wall epithelium. The filamentous pattern of CYP392A16 could be associated with the cisternae of the endoplasmic reticulum (ER), which is in agreement with the fact that the majority of cytochrome P450s are located mainly the ER compartment (Feyereisen, 2012). Within the midgut lumen, we also observed a variety of free-floating red auto-fluorescent cells of different sizes, which presumably correspond to digestive cells, having phagocytosed the chlorophyll-rich plant cell contents. In addition, an artificial fluorescent signal in the cuticle was detected both in mites treated with the antigen retrieval method and the non-treated samples, with less intensity.

It is likely that the expression of CYP392A16 in the midgut provides a first line of defense against the ingestion of insecticides during feeding. To obtain further evidence for the metabolic role of CYP392A16 in the midgut, we examined the mite susceptibility to abamectin, either ingested during feeding or absorbed by cuticle tarsal contact. Although the intensity of resistance was high in both feeding and contact bioassays, the RR in feeding toxicity assay was higher, which further suggests that abamectin resistance is in part specifically linked with the digestive system of this pest. The remaining but not negligible abamectin resistance observed in the contact bioassay, could be explained by additional resistance mechanisms linked to either the cuticle itself, or the overall different route of internalization of the insecticide and transport.
to the target-site. Even if the physiological role of CYP392A16 in the midgut needs further investigation, it is clear that the potential of CYP392A16 to metabolize the incoming insecticide ingested with the food, could at least partially protect mite from abamectin toxicity.

Next, we further corroborated the functional role and contribution of CYP392A16 in abamectin resistance by silencing the gene via RNAi. Knock down of CYP392A16 in a highly resistant T. urticae population (Mar-ab) reduced resistance levels (RR) from 3400- to 1900- fold (i.e. 1.8-fold contribution to resistance), compared to the susceptible strain, indicating a functional but moderate role of CYP392A16 in abamectin resistance. The fact that the RR is reduced by 2-folds only, could reflect insufficient silencing, as indicated in several studies (Feng et al., 2020; Pang et al., 2016). Alternatively, the presence of additional resistance factors is probably more likely, as also the congenic lines that over-express CYP392A16 by 2.8-fold, similar to the highly resistant parent, only displayed 3.6-fold abamectin resistance, in line with the RNAi data. Notably, the resistance levels obtained here are of the same magnitude as in case of isogenic lines bearing the known GluCl point mutations separately, approximately 3-fold, while the combination of the two mutations in GluCls, which are both present in the parental Mar-ab strain, result in up to 12-fold resistance in the near-isogenic T. urticae lines (Riga et al., 2017).

It is clear that the relative contribution of each mechanism (target site mutations and P450-based) provides low to moderate resistance levels to abamectin, while the abamectin RR in the parental resistant Mar-ab strain mounts to >3000-fold. It therefore remains to be investigated whether the observed resistance levels in the parental strain stems from the synergistic effect of these mechanisms. It has been previously suggested (Smith et al., 2019) and recently functionally demonstrated in Drosophila (Samantsidis et al., 2020) that the synergistic effect of sodium channel mutation and P450 over-expression lead to higher pyrethroid resistance levels. Alternatively, complex interactions between additional genes involved in resistance, such as UGTs (Xue et al., 2020) and/ or GSTs (Pavlidi et al., 2015), might contribute to the phenotype in a similar multiplicative way (Samantsidis et al., 2020).

The congenic lines bearing the CYP392A16 allele from the resistant population over-express the CYP392A16 gene at similar levels compared to the resistant parental donor strain. Thus, the regulation of CYP392A16 is possibly driven by cis-regulatory changes or regulated by nearby trans-acting factors that were not uncoupled by the backcrossing experiment (Guerrero et al., 2016).

To test this likely cis-regulation hypothesis, the putative promoter sequence (defined as the one kb upstream of the translation start site) was sequenced from the parental strains and the
generated congenic lines. *In silico* analysis did not identify differentially enriched TFBSs between the resistant and susceptible strains or TFBS motifs that had been previously associated with P450 regulation. It should be noted that the analysis was performed on the Insecta group, since computational tools relying on mite motif databases are currently lacking. The identified SNPs and indels could be potential sites for the binding of transcription factors, yet this information is currently unknown for mites. Therefore, *in vitro* assays with the putative promoter sequences from the parental strains were performed in a comparative reporter luciferase assay in insect cell lines, even though transcriptional regulation may differ between insect cell lines and the mite body. Despite the evolutionary distance between *T. urticae* and Lepidoptera and in absence of available mite-derived cell lines, we used Sf9 and Hi5 cell lines. The putative promoter of Mar-ab strain displayed higher activity in both insect cell lines, compared to the respective region of London inbred, indicating the existence of *cis*-elements in the sequence of Mar-ab promoter that are recognized by conserved endogenous factors of Sf9 and Hi5 cells.

Truncation assays of the promoter from the resistant strain indicated higher activity even when only 133bp upstream of the start codon were retained, while the absence of the 10bp proximal to ATG lead to decreased activity but not at the same levels of the full promoter of the susceptible strain. In order to identify the region accounting for enhanced activity, chimeric promoters were constructed and analyzed. Luciferase assays indicated that the 10bp region provides significantly higher gene reporter expression compared to the susceptible promoter, revealing the necessity and sufficiency of this region to confer the higher activity observed with the promoter of the resistant strain. Similar results were obtained from the reporter gene assay experiments of *CYP321A8* from *Spodoptera exigua*, which revealed that P450 over-expression is partially due to a mutation in the promoter leading to the binding of the nuclear receptor *knirps* (Hu et al., 2021). In our case, the promoter sequence between the strains was highly variable, yet the 10bp of the resistant promoter most probably accounts for enhanced activity. Our results support a role of *cis*-acting elements and is also in accordance with a recent study where *CYP392A16* was identified as one of the three causal genes for pyflubumide resistance. The presence of *CYP392A16* under one of the QTL peaks, together with the observed over-expression and pyflubumide metabolism, points towards *cis*-regulatory variation as resistance factor (Fotoukkiaii et al., 2021). It is also in line with other studies indicating a role for *cis*-elements in regulation of P450s expression, such as *CYP6P9a* of *An. funestus* (Weedall et al., 2019) and *CYP9M10* of *Culex quinquefasciatus* (Itokawa et al., 2015; Wilding et al., 2012).
However, other studies link P450 over-expression with trans-acting factors in insects (Carino et al., 1994; Sabourault et al., 2001; Yang et al., 2020) and mites (Shi et al., 2017). Given that the promoter assays of this study are performed in insect cell lines, with potentially evolutionary divergent transcription factors from T. urticae, the biological significance is not clear, and the combined effect of both cis and trans-acting factors cannot be completely ruled out at this point. Therefore, further studies are required to elucidate the precise causative cis-acting genetic change(s) involved in the regulation of the CYP392A16 in T. urticae, for example with recent gene editing tools developed for T. urticae (Dermauw et al., 2020a).

In summary, this study provides evidence for the tissue specific expression pattern of a mite metabolic enzyme, shedding light on acaricide metabolism and physiology. Additionally, RNAi and marker assisted backcrosses provided evidence for the functional role and contribution of CYP392A16 in abamectin resistance, while the synergistic or cumulative effect with target site mutations or other genes remains to be elucidated. Finally, the expression levels of the introgressed CYP392A16 in the constructed congenic lines, clearly point towards cis-regulation, which was further corroborated with gene reporter assays and promoter truncation analysis. Together, insights into the localization and regulation of this T. urticae P450 can further increase our understanding of acaricide resistance in this notorious pest.

Competing interests

The authors have declared that no competing interests exist.

Author contributions:

JV, TVL and MR designed the research. KMP performed the RNAi, multiple backcrossing, promoter sequencing, qPCR experiments, toxicity assays and the respective statistical analysis. GRS performed the generation of promoter constructs, reporter assays and the respective statistical analysis. ES and VB performed immunostaining experiments. MR performed the contact and feeding toxicity assays. All authors wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

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References


### Table 1: Feeding and contact toxicity assays of abamectin in parental susceptible and resistant strains of *T. urticae*.

<table>
<thead>
<tr>
<th>Assay type</th>
<th>Strain</th>
<th>LC₅₀ in mg l⁻¹ (95% CI)</th>
<th>Slope</th>
<th>χ² (df)</th>
<th>RR (95% CI)</th>
<th>RR (95% CI) vs London inbred</th>
</tr>
</thead>
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<td>Feeding</td>
<td>London inbred</td>
<td>3.35 (2.24-4.39)</td>
<td>2.34±0.41</td>
<td>5.2 (6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mar-ab</td>
<td>&gt;4000</td>
<td>-</td>
<td>-</td>
<td>&gt;1100</td>
<td>-</td>
</tr>
<tr>
<td>Contact</td>
<td>London inbred</td>
<td>3.07 (2.74-3.38)</td>
<td>6.20±0.96</td>
<td>15.1 (16)</td>
<td>877.1 (755 – 1018.8)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mar-ab</td>
<td>2692.26 (2194.8-3060.8)</td>
<td>6.46±0.95</td>
<td>20.3 (13)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Scoring mortality after 24h and 72h for contact and feeding assays, respectively

### Table 2: Toxicity assays with abamectin of dsRNA treated mites, congenic lines carrying the *CYP392A16* allele and the respective parental strains. The feeding and contact toxicity assay type was performed, as described in section 2.2. of Materials and Methods.

<table>
<thead>
<tr>
<th>Method / Experiment</th>
<th>Strain</th>
<th>LC₅₀ in mg l⁻¹ (95% CI)</th>
<th>Slope</th>
<th>χ² (df)</th>
<th>RR* (95% CI) vs London inbred</th>
<th>RR (95% CI) vs CYP392A16_C#</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAi</td>
<td>London inbred (dslacZ)</td>
<td>0.18 (0.15-0.21)</td>
<td>3.18±0.36</td>
<td>3.94 (13)</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>London inbred (dsA16)</td>
<td>0.15 (0.12-0.17)</td>
<td>2.57±0.28</td>
<td>8.42 (16)</td>
<td>-</td>
<td>-</td>
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<td></td>
<td>Mar-ab (dslacZ)</td>
<td>601.73 (531.15-681.94)</td>
<td>3.88±0.51</td>
<td>10.32 (12)</td>
<td>3400.19</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2781.76-4156.11)</td>
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<tr>
<td></td>
<td>Mar-ab (dsA16)</td>
<td>278.86 (217.93-330.19)</td>
<td>2.81±0.31</td>
<td>15.69 (15)</td>
<td>1904.89</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1474.08-2461.61)</td>
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<tr>
<td>Marker assisted</td>
<td>London inbred</td>
<td>0.19 (0.17-0.21)</td>
<td>5.75±0.79</td>
<td>11.56 (13)</td>
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<tr>
<td>backcrossing</td>
<td>Mar-ab</td>
<td>606.01 (541.5-662.22)</td>
<td>8.59±1.45</td>
<td>5.97 (12)</td>
<td>3181.34</td>
<td>3184.76 (2583.39-3926.11)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>(2731.97-3704.61)</td>
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<td></td>
<td>CYP392A16_C</td>
<td>0.19 (0.15-0.22)</td>
<td>2.55±0.28</td>
<td>5.37 (13)</td>
<td>3181.34</td>
<td>3184.76 (2583.39-3926.11)</td>
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<tr>
<td></td>
<td>CYP392A16_L1</td>
<td>0.69 (0.59-0.75)</td>
<td>8.29±1.9</td>
<td>7.95 (16)</td>
<td>3.64 (3.13-4.25)</td>
<td>3.65 (2.96-4.50)</td>
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<td>CYP392A16_L2</td>
<td>0.70 (0.61-0.76)</td>
<td>7.15±1.45</td>
<td>9.64 (16)</td>
<td>3.67 (3.15-4.27)</td>
<td>3.67 (2.98-4.53)</td>
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<td>CYP392A16_L3</td>
<td>0.69 (0.60-0.75)</td>
<td>6.61±1.26</td>
<td>10.71 (16)</td>
<td>3.61 (3.09-4.21)</td>
<td>3.61 (2.92-4.46)</td>
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</tbody>
</table>


#RR: Resistance ratio was calculated compared to CYP392A16_C in experimental design of marker assisted backcrosses and it is not applicable for the RNAi method.
Figure Legends

Figure 1: Localization of CYP392A16. A. Virtual cross section at the level of *T. urticae* midgut, obtained by X-ray sub-micron computed tomography from Jonckheere and co-workers (Jonckheere et al., 2016) is presented. B. A representative image of a mite cross-section from Mar-ab strain stained after antigen retrieval with α-CYP392A16 (green) is shown. An artificial signal in the cuticle was also observed. C. Schematic representation of the cross section from B is shown. D-F. Higher magnification image focusing on midgut cells in the white frame (Panel B). DAPI stained the nuclei blue and auto-fluorescence of chlorophyll is colored red. Picture was obtained using confocal microscope (40x). Scale bar: 30 μm

Figure 2: Relative expression of *CYP392A16* after silencing in susceptible (London inbred) and resistant (Mar-ab) strains. The expression levels of *CYP392A16* transcript after treatment with dsLacZ (control) and dsCYP392A16 are depicted in A. 3 days post-treatment and B. 5 days post-treatment. The error bars represent standard error (SE). Stars indicate statistically significant down-regulation of *CYP392A16* in Mar-ab strain treated with dsRNA targeting *CYP392A16* (p-value <0.0332 (*) and <0.0021 (**)) when compared to Mar-ab strain treated with dsLacZ.

Figure 3: *CYP392A16* transcript levels in the congenic lines. The relative expression levels of *CYP392A16* is presented in the congenic lines CYP392A16_L1-L3 and C, as well as the parental susceptible (London inbred) and resistant (Mar-ab) strains. Stars indicate statistically significant expression (p-value <0.0332 (*) and <0.0021 (**)) when compared to the susceptible parental strain, London inbred. The error bars represent standard error (SE).

Figure 4: Functional analysis of the full length, truncated and chimeric promoters. A. Functional analysis of the putative promoter sequences of *CYP392A16* from resistant (Mar-ab) and susceptible (London inbred) strains in Sf9 and Hi5 insect cell lines. B. Truncation analysis of the putative promoter of the resistant strain (“-” indicates the length of each fragment upstream of the start codon of *CYP392A16*). C. Schematic representation of C-133 and C-10 construction. For details concerning the cloning strategy of C-133 advise also Figure S1. Yellow box represents the overlapping area between the two promoter sequences used for the construction of C-133. In C-10, the dark grey area represents the 5bp of the susceptible promoter that was replaced by PCR with the white area (10bp) of the resistant promoter. D. Functional analysis of chimeric promoters C-133 and C-10. Stars indicate statistically
significant fold change activity when compared to full length susceptible (A and D: ****p<0.0001) and resistant promoter (B: **p<0.0033).

Supplementary Figure legends

Figure S1: Cloning strategy for the generation of C-133. A schematic diagram presents the cloning strategy by overlapping extension PCR performed for the construction of C-133 promoter. The conditions are also outlined.

Figure S2: Immunoblot of bacterial membranes with α-CYP392A16 or α-CYP392A. Five μg of bacterial membranes expressing CYP392A16, CYP392A11 and CYP392A12 were loaded on 10% SDS polyacrylamide gel and the blot was probed with α-CYP39A16 (left part) or α-CYP39A2 (right part).

Figure S3: Immunostaining with pre-immune serum and α-CYP392A16. Pre-immune serum was used as negative control (green) (A and B), in parallel with α-CYP392A16 (green) (C and D). DAPI stained the nuclei blue and auto-fluorescence of chlorophyll is colored red. All pictures were obtained using confocal microscope (40x). Scale bar: 30 μm. BF, bright field.

Figure S4: Relative expression of CYP392A11, CYP392A12 and CYP392A15 after silencing of CYP392A16 in susceptible (London inbred) and resistant (Mar-ab) strains. The expression levels of each transcript after treatment with dsLacZ (control) and dsCYP392A16 are depicted. The error bars represent standard error (SE). Ns indicates no statistically significant down-regulation of CYP392As in Mar-ab strain treated with dsRNA targeting CYP392A16 when compared to Mar-ab strain treated with dsLacZ.

Figure S5: PCR-RFLP diagnostic assay of the CYP392A16 PCR fragments with SspI and schematic representation of marker-assisted backcrosses. A. Assay design of the diagnostic PCR-RFLP for CYP392A16 sequence. The nucleotide polymorphism between the Mar-ab and London inbred allele is depicted in bold. The upper sequence corresponds to the Mar-ab allele, whereas the lower sequence depicts the CYP392A16 from the London inbred strain. B. PCR-RFLP of the 382 bp amplicon upon enzymatic reaction with SspI. Lanes 1,2,3 and 4 represent Marker (100bp), wild type/susceptible homozygous, resistant homozygous and heterozygous genotypes after the enzymatic reaction with SspI. C. Construction of congenic lines. The susceptible genotype is depicted by green-colored chromosomes, while the resistant genotype is depicted in yellow. CYP392A16 allele from the resistant strain, bearing SNP that used for detection of CYP392A16 allele originating from Mar-ab by PCR-RFLP, is depicted in red color.
Figure S6: Promoter sequence alignment of 1 kb upstream of the start codon between the two parental strains and congenic lines. The insertions and deletions (indels) in the putative promoter region of Mar-ab and CYP392A16_L1-L2 are depicted in green boxes. Minimal promoter sequence (TATA box) is depicted in red box. Start codon of the gene (ATG) is double underlined.

Supplementary Tables

Table S1: Primers used for the determination of CYP392A16 expression levels, sequencing and functional analysis of the promoter region, as well as dsRNA synthesis.

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Figure S5
Figure S6
### Table S1

Table S1: Primers used for the determination of CYP392A16 expression levels, sequencing and functional analysis of the promoter region, as well as dsRNA synthesis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tetur ID</th>
<th>Primer name</th>
<th>Sequence (5'-3')</th>
<th>Product size (bp)</th>
<th>Template</th>
<th>Assay</th>
<th>Reference</th>
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<tbody>
<tr>
<td>CYP392A16</td>
<td>tenor06g04520</td>
<td>A16ext_F</td>
<td>CGTCTGACACTCATCTGAAAGTTA</td>
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<td>gDNA</td>
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**Underlined sequences denote restriction enzymes recognition sites.**

[T] denotes the T7 promoter sequence (TAATACGACTCACTATAG GGGAGA) used for the in vitro transcription of dsRNAs.