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ABSTRACT

Spodoptera frugiperda (fall armyworm) is a highly destructive invasive pest that feeds on numerous crops including maize and rice. It has developed sophisticated mechanisms to detoxify xenobiotics such as secondary plant metabolites as well as manmade insecticides. The aim of the study was to explore the detoxification response to plant secondary metabolites and insecticides employing a *S. frugiperda* Sf9 cell model exposed to indole 3-carbinol (I3C) and methoprene. The cell Inhibitory Concentration 50 (IC_{50}) for these molecules was determined and IC_{10}, IC_{20} and IC_{30} doses were used to monitor the induction profiles of detoxification genes. Cytochrome P450 monooxygenases (P450s) of the *CYP9A* subfamily were the most inducible genes of the seven examined. Our results also showed the induction of the transcription factor Cap'n'collar isoform C (CncC). Transient transformation of Sf9 cells overexpressing CncC and its partner muscle aponeurosis fibromatosis (*Maf*) induces overexpression of *CYP4M14, CYP4M15, CYP321A9* and *GSTE1* while CYP9As were not induced. Next, we determined the capacity of recombinantly expressed CYP9A30, CYP9A31 and CYP9A32 to interact with methoprene and I3C. Fluorescence-based biochemical assays revealed an interaction of methoprene with functionally expressed CYP9A30, CYP9A31 and CYP9A32 whereas almost no interaction was detected for I3C, suggesting the ability of CYP9As to metabolize methoprene. Our results showed that Sf9 cells could be a useful model to decipher detoxification pathways of *S. frugiperda*.

1. Introduction

The fall armyworm (FAW), *Spodoptera frugiperda*, (Lepidoptera : Noctuidae), is a polyphagous, highly destructive pest feeding on numerous plants of agronomic importance such as maize, rice and sorghum (Overton et al., 2021). Although it was originally present on the Americas, it has since invaded the world, Africa in 2016, Asia from 2018 and Australia in 2020 (Acharya et al., 2021; Cook et al., 2021; Ganiger et al., 2018; Goergen et al., 2016; Nagoshi et al., 2018). Its invasion of Europe is highly likely and it was detected on the Canary Islands (Spain) in July 2020 (https://gd.eppo.int/taxon/LAPHFR/distribution (accessed on 06 Dec 2021). The Food and Agriculture Organization of the United Nations (FAO) proclaimed it in 2021 as "one of the most destructive pests jeopardizing food security across vast regions of the globe". The control of FAW largely relies on the expression of insecticidal *Bacillus thuringiensis* (Bt) proteins, particularly in transgenic maize, and the application of synthetic insecticides. However, continuous selection pressure and frequent application of insecticides resulted in the development of resistance to many classes of synthetic insecticides and Bt proteins. In fact, it is ranked in the top 15 of the globally most resistant arthropods (Sparks et al., 2020).

FAW has developed mechanisms to eliminate toxic compounds present in its host-plants and insecticides. The detoxification enzyme families, cytochrome P450 monooxygenases (P450s), esterases and glutathione S-transferases (GSTs) are part of this armament. Pioneering work in the 1980s showed, for example, that microsomal fractions, which carry P450 activity, extracted from *S. frugiperda* larvae could metabolize allelochemicals of various chemical structures such as alkaloids, indoles, furanocoumarins, and glucosinolate compounds found in various host plants (Yu, 1987). Detoxification enzymes are often expressed at a basal level and induced when the insect is exposed to toxic compounds (Yu, 1986). For example, indole 3-carbinol (I3C), which is a degradation product of glucosinolates, induced P450, GST and esterase activities in *S. frugiperda* by 5-, 4- and 1.6-fold, respectively (Yu, 1983; Yu and Hsu, 1985). *S. frugiperda* larvae feeding on maize leaves were less sensitive to certain pyrethroid insecticides such as permethrin and cypermethrin than larvae feeding on soybean (Yu, 1982). Similarly, tolerance to cypermethrin was shown to increase in other Lepidoptera (*Helicoverpa armigera* and *Spodoptera litura*) when insects were exposed to

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xanthotoxin, a furanocoumarin (Li et al., 2000; Lu et al., 2021b). Insecticides, like allelochemicals, are inducers of detoxification enzymes, but an insecticide will not necessarily induce the same genes as a secondary plant metabolite. Giraudo et al. showed that P450 expression profiles in *S. frugiperda* larvae were specific to the compound tested, *e.g.* I3C induced *CYP321A7, CYP321A8* and *CYP321A9* whereas the insecticide methoxyfenozide induced *CYP9A25, CYP9A58* and *CYP9A59* (Giraudo et al., 2015). However, a global analysis of the expression pattern of detoxification genes in response to xenobiotics in four species of the *Spodoptera* genus showed that diverse compounds could induce some genes in common (Amezian et al., 2021a). Some of these enzymes will have the ability to metabolize both plant allelochemicals and insecticides, such as CYP321A1 of the cotton bollworm, *H. armigera*, which was shown to metabolize furanocoumarins and the insecticide cypermethrin (Sasabe et al., 2004).

The signaling pathways that allow the insect, following exposure to a xenobiotic, to induce the expression of the machinery necessary to eliminate the respective toxicant are still poorly understood. Of the five main pathways identified so far (Amezian et al., 2021b), the transcription factor Cap'n'collar isoform C (CncC) and its partner of heterodimerization muscle aponeurosis fibromatosis (Maf) have been identified as « master regulators » of detoxification in several insects (Palli, 2020). Identified for the first time in *Drosophila*, CncC has been shown to control more than half of the genes regulated by phenobarbital, a barbiturate well known to induce many detoxification genes (Misra et al., 2011). Furthermore, in this study, the authors showed that *CncC* activation leads to resistance to the insecticide malathion because it induces over-expression of enzymes that degrade the insecticide. Since then, several studies have demonstrated the constitutive overexpression of *CncC* in resistant insect populations, first in laboratory-selected DDT-resistant strains of *Drosophila* (Misra et al., 2013) and more recently, for example, in lepidopterans including *Spodoptera exigua* resistant to chlorpyrifos and cypermethrin (Hu et al., 2019b) and *S. litura* resistant to indoxacarb (Shi et al., 2021a). The CncC pathway seems to play a fundamental role in adaptation to insecticides, but its role in plant allelochemical adaptations is less understood (Lu et al., 2021a, 2021b).

Sf9 cells were derived from the ovary of the *S. frugiperda* pupa and have been used as a system for the heterologous expression of proteins for several decades (Altmann et al., 1999; van Oers et al., 2015). However, they also have been employed to evaluate the potential of certain molecules to act as insecticides (Pereira et al., 2021; Ruttanaphan et al., 2020). Indeed, Sf9 cells have been shown to be a useful model responsive to xenobiotics as well as to investigate potential resistance mechanisms (Cui et al., 2020; Giraudo et al., 2011, 2015; Saleh et al., 2013).

Here, we used Sf9 cells as a model to investigate the molecular mechanisms underlying *S. frugiperda* detoxification of plant secondary metabolites and insecticides, *i.e.*, indole 3-carbinol and methoprene, respectively. The questions we tried to answer are: (i) Does exposure of cells to these compounds induce a detoxification response? (ii) Is this response mediated by the transcription factors CncC and Maf? and (iii) Are I3C and methoprene able to interact with the most inducible P450s in this study, namely CYP9As?

2. Materials & methods

2.1. Chemicals

Indole 3-carbinol (I3C) and methoprene were purchased from Sigma–Aldrich Chimie (Saint-Quentin Fallavier, France). The fluorescent probe 7-benzyloxymethoxy-4-(trifluoromethyl)-coumarin (BOMFC; CAS 277,309–33–8) was custom synthesized by Enamine Ltd. (Kiev, Ukraine) with a purity of 95%. β-Nicotinamide adenine dinucleotide 2´-phosphate (NADPH) reduced tetrasodium salt hydrate (CAS: 2646–71–1 anhydrous, purity 93%), 7-ethoxycoumarin (EC; CAS: 31,005–02–4, purity > 99%), 7-methoxy-4-trifluoromethylcoumarin (MFC; CAS: 575–04–2, purity 99%), 7-Ethoxy-4-trifluoromethylcoumarin (EFC; CAS: 115,453–82–2, purity 98%), 7-benzyloxy-4-trifluoromethylcoumarin (BFC; CAS: 220,001–53–6, purity 99%), 7-hydroxy-coumarin (HC; CAS: 93–35–6, purity 99%), 7-hydroxy-4-trifluoromethylcoumarin (HFC; CAS: 575–03–1, purity 98%), 7-methoxyresorufin (MR; CAS: 5725–89–3, purity 98%) were purchased from Sigma-Aldrich/Merck (Darmstadt, Germany). All other chemicals were of analytical grade and obtained from Sigma-Aldrich unless otherwise mentioned.

2.2. Cell culture

Sf9 cells were purchased from Invitrogen (Cat. No. B82501; Thermo Fisher Scientific, France). Cells were maintained as adherent cultures at 27 °C in Insect-XPRESS™ protein-free insect cell medium (Lonza, France) and passaged every third day. Cell density was determined by Malassez haemocytometer (Marienfeld, Germany) counts and cell viability was evaluated by Trypan blue (1 mg/ml, v/v) staining. High-Five cells (*Trichoplusia ni*, ovary) employed for protein expression were purchased from Invitrogen (Cat. No. B85502; Thermo Fisher Scientific, Germany) and maintained according to manufacturer instructions.

2.3. Cell viability assay and induction treatments

Viable cells were determined by measuring the conversion of the tetrazolium salt MTT to formazan, as previously described (Fautrel et al., 1991). Cells were seeded onto 96-well plates (Techno Plastic Products AG, Switzerland) at 2×10^5 cells/ml and incubated for 24 h at 27 °C. Cells were then treated in triplicates for 24 h with different concentrations of I3C (10, 50, 100, 200, 350, 500 μM) and methoprene (25, 75, 100, 150, 300 μM) or dimethyl sulfoxide (DMSO) as a control. The medium was then removed and cells were loaded with MTT (5 mg/ml) and incubated at 27 °C for 2 h. Cell homogenates were solubilized in 100 μl DMSO and used to measure absorbance at 570 nm using a microplate reader (SpectraMax 382, Molecular Devices, USA). For induction of gene expression studies, cells were seeded onto six-well plates at 2×10^5 cells/ml and then treated for 24 h with sublethal doses (corresponded to inhibition concentration 10, 20 and 30) of I3C (40, 58 and 74 μM) and methoprene (50, 65 and 74 μM) or the equal volume of DMSO, which served as control.

2.4. Transient transfection

2.4.1. Plasmid constructions

RNA extracted from *S. frugiperda* fifth instar larvae midguts (five pooled individuals) was used to synthesize cDNA which served as template for *CncC* (GenBank no. OL781184) and *Maf* (GenBank no. OL781185) amplification. Two primer pairs were designed to amplify *CncC*, and one pair to amplify *Maf*, using the genomic sequences retrieved from the genome (v3.0) on BIPAA (bipaa.genouest.org, (Gouin et al., 2017)) and customized to introduce restriction enzymes sites (Table S1). *CncC* was amplified with two successive runs using overlapping primers and a high fidelity PrimeSTAR® polymerase (Takara Bio Europe, France) on a MJ Research Tetrad PTC-225 Thermal Cycler (GMI, USA). PCR products were purified using the GenElute™ PCR Clean-Up kit (Sigma-Aldrich, France) and the purity was assessed using NanoDrop™. The BglII/NotI digested PCR amplicons of *CncC* and *Maf* were subcloned into the BglII/NotI linearized pBiEx™ Expression Vector (Novagen, Germany) at 20:1 and 3:1 (w/w) ratios using a T4 DNA Ligase (Roche, Germany). The pBiEx-Maf and pBiEx-CncC products were subsequently transformed into Subcloning Efficiency™ DH5α™ Competent Cells (Life Technologies, Germany) according to the supplier's instruction. Successfully transformed bacterial colonies were screened by direct PCR using GoTaq® DNA Polymerase (Promega, France) (Table S1). Finally, plasmids were isolated using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, France). Recombinant constructs were confirmed by Sanger sequencing (GENEWIZ, Germany).

2.4.2. Transient expression of cncc and maf in Sf9 cells

Transient expression of target genes was performed by transfection of the expression vector pBiEx™ using FUGENE® transfection reagent (Life Technologies, Germany) according to the manufacturer's instructions. Briefly, Sf9 cells were seeded onto six-well plates at 1×10^6 cells/ml and incubated at 27 °C for 24 h prior to transfection. In each well, adherent cells were transfected with 2 μg expression vector DNA. The plasmid DNA and 3 μl FUGENE® transfection reagent were incubated 15 min in 100 μl of Insect-XPRESS medium at room temperature prior to be diluted to a final volume of 1 ml of Insect-XPRESS medium supplemented with 10% fetal bovine serum (FBS) from Invitrogen (Villebon-sur-Yvette, France). Cells were transfected with either a single expression plasmid construct, *i.e.,* pBiEx:CncC or pBiEx:Maf, or transfected with both expression vectors in equal proportions. An empty vector was used to transfect control cell lines. Each transformation condition was replicated three times. Cells were incubated for 24, 48 and 72 h at 27 °C before RNA extraction.

2.5. RNA extraction, cDNA synthesis and real-time quantitative pcr (RT-qPCR)

RNA was extracted either from cells treated by IC_{10}, IC_{20} and IC_{30} of I3C and methoprene or from cells transiently expressing CncC and Maf at 24, 48 and 72 h post-transfection. Cells from each well were washed with 1 ml DPBS and total RNA was extracted using 1 ml TRIzol Reagent (Life Technologies, Germany) according to the manufacturer's protocol.

Total RNA (500 ng) was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad, France) following the manufacturer's guidelines. Quantitative real-time (qRT)-PCR reactions were carried out on an AriaMx Real-Time PCR system (Agilent technologies, USA) using qPCR Mastermix plus for SYBR Green I no ROX (Eurogentec, Belgium). The PCR conditions were as follows: 10 min at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 60 °C and followed by a melting curve step, except for CncC for which conditions were slightly different: 40 cycles of 5 s at 95 °C and 30 s at 60 °C and 20 s at 72 °C. Each reaction was performed in duplicate and the mean of three independent, biological replicates was calculated. For monitoring the induction of detoxification genes following exposure of Sf9 cells to IC_{10}, IC_{20} and IC_{30} of I3C and methoprene, the data were normalized using the mRNA level of three reference genes (ribosomal protein L4, ribosomal protein L10 and ribosomal protein L17) and setting the control condition corresponding to DMSO-treated cells to 1. The raw data of Ct values as well as the relative expression values calculated using SATqPCR (satqpcr.sophia.inra.fr) (Rancurel et al., 2019) are available at (https://figshare.com/articles/dataset/_/19236600). For monitoring the expression of transcription factors and detoxification genes following transient transfection of CncC and Maf at 24, 48 and 72 h post-transfection, the data were normalized using the mRNA level of three reference genes (ribosomal protein L4, glucose-6-phosphate dehydrogenase and ribosomal L18) and setting the control condition corresponding to the cells transfected with the empty vector pBiEx to 1. The raw data of Ct values as well as the relative expression values calculated using SATqPCR are available at (https://figshare.com/articles/dataset/_/19236699). Primers were designed using Primer3 (v0.4.0); sequences and efficiencies are listed in Table S2.

2.6. Recombinant expression of cyp9a genes in high-5 cells

Functional expression of P450 enzymes together with cytochrome P450-reductase (CPR) was conducted utilizing the Bac-to-Bac baculovirus (ThermoFisher Scientific, Waltham, USA) expression procedure recently described by Manjon et al. (2018) with the following changes. Sequences of *CYP9A30, CYP9A31* and *CYP9A32* as well as *S. frugiperda CPR* were based on transcriptomic data of an insecticide resistant FAW population (Boaventura et al., 2020a, 2020b). Sequences were codon optimized for expression in High five cells (Thermo Fisher Scientific) and produced by gene synthesis (GeneArt, Thermo Fisher Scientific) including subsequent cloning into pFastBac1 vector. Baculovirus production was performed in ExpiSf9 cells (Gibco) according to manufacturer's instructions. Each *CYP* gene was co-transfected independently into High-5 cells in combination with *CPR* at a MOI (multiplicity of infection) ratio of 1:0.5. Microsomal fractions of cell pellets were prepared as described previously (Haas and Nauen, 2021; Janmohamed et al., 2006) and protein concentrations were measured using Bradford reagent (Bio-Rad) (Bradford, 1976) with bovine serum albumin (BSA) as standard.

2.7. Enzyme assays

CYP9A30–32 activity on coumarin based model substrates was tested in enzymatic assays as previously described (Manjon et al., 2018; Nolden et al., 2021; Zimmer et al., 2014). Twenty-five μl of microsomal preparations diluted to 0.2 μg/μl in resuspension buffer (0.1 M K_2HPO_4, 1 mM DTT, 0.1 mM EDTA, 5% glycerol, pH 7.6) were used to yield a final protein amount of 5 μg per 50 μl reaction mix. Model substrates were dissolved in DMSO and further diluted in 0.1 M K_2HPO_4, pH 7.6. Twenty-five μl were added to microsome dilutions to a final concentration of 50 μM for BFC, MFC, EFC and EC, and 10 μM for BOMFC, and 100 μM for PC. Assays were conducted with or without 250 μM NADPH in black flat-bottom 384-well plates (Greiner bio-one) and replicated four times. Reactions without substrate or mock baculovirus infection served as negative controls. After incubation for 1 h at 20 °C, reactions were stopped with 50 μl stop solution (45% (v/v) DMSO, 45% (v/v) 0.05 M Trizma Base (pH 10), 5 mM oxidized glutathione, 0.2 U glutathione reductase). Formation of fluorescent product was measured after at least 20 min incubation in a microplate reader (Spark, Tecan) at excitation and emission wavelengths previously described for each substrate (Manjon et al., 2018).

Enzyme inhibition kinetics were conducted as described recently (Haas and Nauen, 2021) using eleven BOMFC concentrations in a 1:2 serial dilution series in 0.1 M K_2HPO_4, pH 7.6, 0.01% Zwittergent 3–10. Final concentrations of BOMFC ranged from 0.195 μM – 200 μM. I3C and methoprene were dissolved in DMSO and added as competing compounds at final concentrations of 5 μM, 15 μM and 50 μM. Recombinantly expressed CYP9A30–32 were diluted in resuspension buffer supplemented with 0.01% Zwittergent 3–10 and 0.05% BSA. After incubation of 25 μl microsomal solution with 25 μl substrate dilutions with or without 250 μM NADPH for 20 min at 20 °C, the reaction was stopped with 50 μl stopping solution (45% (v/v) DMSO, 45% (v/v) 0.05 M Trizma Base (pH 10), 5 mM oxidized glutathione, 0.05 U glutathione reductase). Fluorescence was measured after at least 20 min incubation in a microplate reader (Spark, Tecan). Standard curves were obtained for the reaction products 7-hydroxy-coumarin (HC) and 7-hydroxy-4-trifluoromethylcoumarin (HFC) to enable calculation of product formation in pmol/(mg*min). The data were further analyzed using Prism 9.0.2 software (GraphPad, CA, USA).

2.8. Statistical analysis

Dose-response assays were analyzed with GraphPad Software (V9.2.0) using nonlinear regression (four-parameters logistic (4PL) regression model). RT-qPCR data was analyzed using SATqPCR (Rancurel et al., 2019) and all biological replicates were compared using a pairwise *t*-test with a pooled standard error (satqpcr.sophia.inra.fr).

3. Results

3.1. Cytotoxicity of indole 3-carbinol and methoprene on Sf9 cells

The cytotoxicity of I3C and methoprene was determined on Sf9 cells after exposure for 24 h to increasing concentrations resulting in IC_{50} values of 103.4 (CL95%: 84.4–126.7) μM and 87.6 (CL95%: 81.1–94.5) μM for I3C and methoprene, respectively (Fig 1). Resulting IC_{10}, IC_{20}

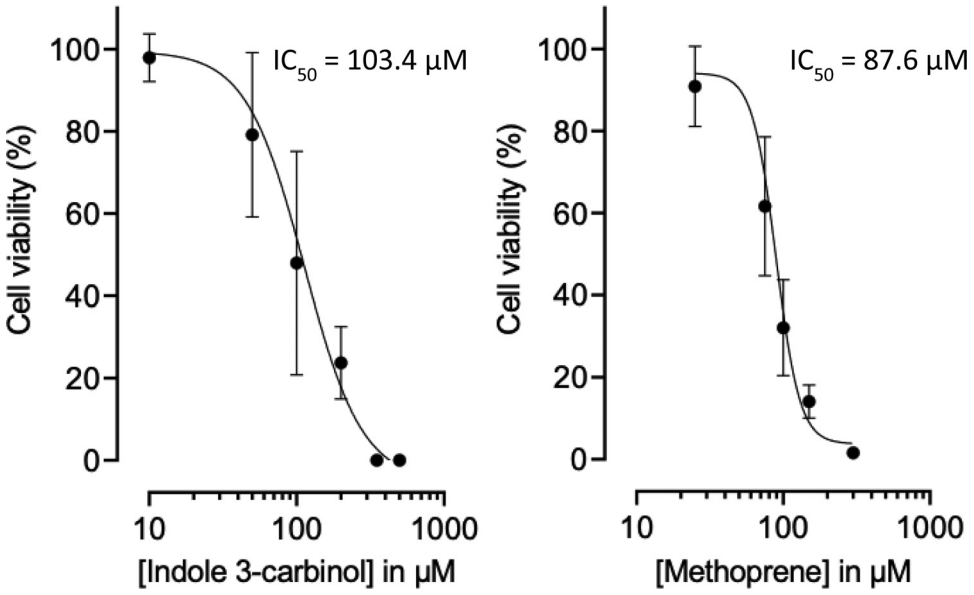

Fig. 1. Dose-response curves of methoprene and indole 3-carbinol in insect cell viability bioassays. Toxicity of Indole 3-carbinol (left) and Methoprene (right) towards Sf9 cells obtained by cell viability bioassays. Each point was expressed as a percentage of the maximum viability (DMSO treatment). Curves were obtained by nonlinear regressions (sigmoidal, 4PL) and error bars represent standard deviation (SD) of mean values from three biological replicates.

and IC_{30} values were 39.7, 57.8 and 73.8 μM for I3C and 50.7, 65.3 and 74 μM for methoprene.

3.2. Induction of detoxification gene expression by indole 3-carbinol and methoprene

The adaptive capacity of the cells to respond to these two xenobiotics was assessed by measuring the expression of selected detoxification genes by quantitative PCR. The choice of P450s is based on one of our previous studies (Giraudo et al., 2015) while GSTE1 has been shown to metabolize I3C in *S. litura* (Zou et al., 2016). In order to determine if this response is dose-dependent, Sf9 cells were treated with IC_{10}, IC_{20} and IC_{30} of I3C and methoprene. Fig. 2 shows that the expression of seven P450s (*CYP4M14, CYP4M15, CYP9A24, CYP9A30, CYP9A31, CYP9A32* and *CYP321A9*) and one GST (*GSTE1*) were induced at the IC_{30} of I3C and methoprene. This induction was dose-dependent. Some genes such as *CYP9A31* were strongly induced (expression fold-change 101.48 at methoprene IC_{30}, $p = 0.0014$) while for others this induction is either weak or not significant as for *CYP4M15* and *CYP321A9* at I3C IC_{10}. The levels of gene induction between the I3C and methoprene treatments were different but of the same order of magnitude except for *CYP9A31* where the levels of induction for methoprene are between 3 and 5 times higher than for I3C depending on the treatment considered.

3.3. Induction of cncc and maf, transcription factors involved in the regulation of detoxification gene expression

To test whether CncC and Maf transcription factors were also inducible in Sf9 cells by sublethal doses of I3C and methoprene, RT-qPCR experiments were performed. Results presented in Fig. 2 show that the induction of *CncC* by I3C and methoprene was dose-dependent, while the expression levels of *Maf* varied in a dose-independent manner. I3C had the least potent effect on the expression of *CncC* with a 3-fold increase at IC_{30} as compared to methoprene (6.82-fold, n.s., $p = 0.069$).

3.4. Effect of transient overexpression of cncc and maf on detoxification gene expression

To assess whether there is a causal link between the transcriptional upregulation of *CncC* and *Maf* and the activation of detoxification genes, the transcription factors were transiently overexpressed in Sf9 cells. Three types of cell lines were obtained, one line that overexpressed *CncC* alone, one that overexpressed *Maf* alone and finally one that overexpressed both transcription factors. The expression levels of *CncC* and *Maf* were monitored at 24, 48 and 72 h after transfection in each transformed line (Fig. 3A-C). *CncC* and *Maf* were strongly upregulated in transformants as compared to the control cells. The highest expression fold-change was obtained at 48 h post-transfection: 1012- and 1053-fold change respectively in *CncC* and *Maf* single-gene transformants and 1142- / 643-fold change in the double-gene transformants (Fig. 3B).

The expression of detoxification genes was assessed 48 h post-transfection. The overexpression of *CncC* and *Maf* genes led to significant upregulation of most detoxification genes monitored such as *CYP4M14, CYP4M15, CYP9A24, CYP321A9* and *GSTE1* while the expression of *CYP9A30, CYP9A31* and *CYP9A32* were not affected, suggesting no regulatory role of CncC/Maf for their expression upon transient overexpression of these transcription factors. The co-transfection of *CncC* and *Maf* had a stronger impact on the induction of detoxification genes, for example the expression of *CYP4M15* was 2.5-fold higher in pCncC:Maf cells than in pCncC and pMaf cells.

3.5. Activities of recombinant CYP9A30, CYP9A31 and CYP9A32 on P450 model substrates

CYP9A31 was the most induced gene following xenobiotic exposure. It is part of a cluster that contains 12 CYP9As, with *CYP9A32* and *CYP9A30* on either side of its position in the genome (Hilliou et al., 2021). We chose to heterologously express these three P450s and to investigate their capacity to metabolize diverse coumarin fluorescent probe substrates. All three P450s were successfully expressed in High-5 cells as indicated by their ability to oxidize fluorescent coumarin model substrates. Of the six coumarin derivatives tested, BFC and BOMFC were the best substrates, with CYP9A31 being the most active P450, followed by CYP9A32 and CYP9A30 (Fig. 4). Whereas the alkylated coumarin derivates provided rather low activity, suggesting a preference for O-debenzylation reactions rather than dealkylations.

3.6. I3C and methoprene interactions with CYP9As employing a fluorescent probe assay

Next, we tested whether these recombinantly expressed CYP9A P450s interact with I3C and methoprene by inhibiting the formation of HFC in a fluorescent probe assay utilizing BOMFC. Indeed, BOMFC metabolism by CYP9A30 and CYP9A32 was significantly inhibited with

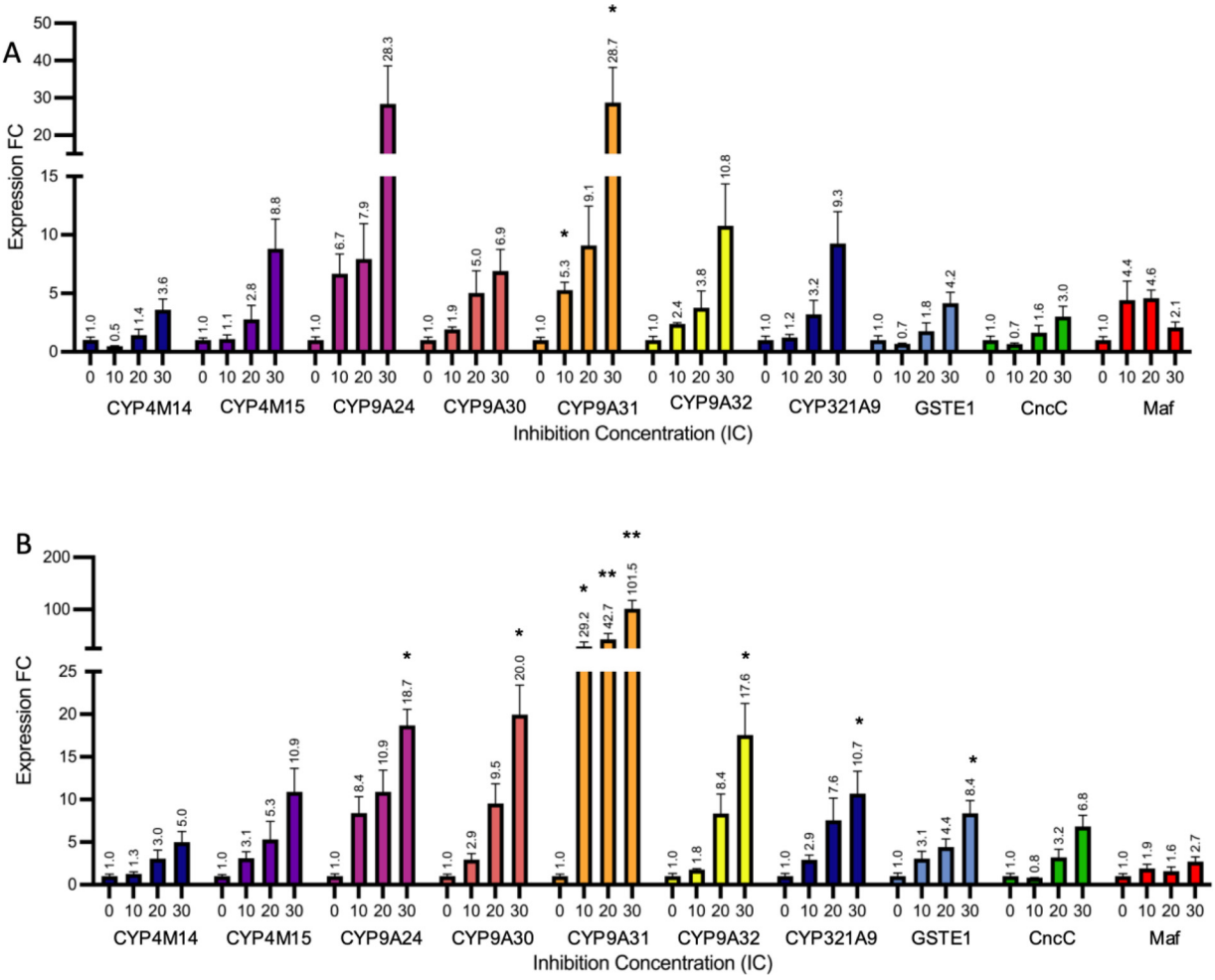

Fig. 2. Induction of detoxification genes, CncC and Maf in Sf9 cells. Expression levels of eight detoxification genes as well as CncC and Maf were monitored in Sf9 cells exposed to IC10, IC20 and IC30 I3C (A) and methoprene (B). DMSO was used as control treatment (IC0). Gene expression was normalized using the expression of the ribosomal protein L4, L10 and L17 reference genes and shown as fold-change relative to the expression of cell lines treated with DMSO. Data are mean values of three biological replicates ± SEM. A pairwise *t*-test was performed to establish the statistical significance of induction by I3C and methoprene as compared to DMSO (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

increasing concentrations of methoprene (Fig. 5A,C) whereas the effect on CYP9A31 was less pronounced and only observed at the highest concentration of 50 μM methoprene (Fig. 5B). No concentration-dependent inhibition of HFC formation was detected with I3C, suggesting that it did not directly compete with BOMFC at the active site of the recombinantly expressed CYP9A enzymes (Fig. 5).

4. Discussion

Herbivorous pest insects with a broad host range such as FAW are exposed to a wide variety of xenobiotics present in their environment such as secondary plant metabolites but also insecticides used to keep them under damage thresholds. The inducibility of detoxifying enzymes upon exposure to xenobiotics allow them to provide a timely and coordinated response to external stimuli that would otherwise be costly to implement permanently. In this study, we demonstrated that I3C and methoprene induced several detoxification genes as well as the transcription factor *CncC* in Sf9 cells employed as a model system. These induction profiles were obtained after exposure to sublethal concentrations of I3C and methoprene to detect specific adaptative responses of detoxification genes avoiding nonspecific general stress responses usually elicited with higher doses. Transient overexpression of the transcription factors *CncC* and *Maf* induced the over-expression of *CYP4M14, CYP4M15, CYP9A24, CYP321A9* and *GSTE1* while no effect was observed on the expression of *CYP9A30, CYP9A31* and *CYP9A32*, suggesting that another signaling pathway is involved in controlling their expression. The functional expression of three FAW P450s: CYP9A30, CYP9A31 and CYP9A32 demonstrated the ability of these detoxification enzymes to metabolize diverse fluorescent coumarin substrates. The ability of CYP9A30 and CYP9A32 to interact with methoprene but not I3C was demonstrated by a fluorescence-based assay recently developed (and validated) to biochemically investigate pesticide/pesticide interactions with *Apis mellifera* P450s to inform bee pollinator pesticide risk assessment (Haas and Nauen, 2021). Our results on methoprene suggest a potential for both CYP9A30 and CYP9A32 to interact with methoprene and possibly facilitating its degradation.

CYP9As were strongly induced by both xenobiotics (up to 100-fold for *CYP9A31* by methoprene), which may suggest that they play a role in the metabolism of these compounds. CYP9As were reported inducible in the genus *Spodoptera* (*S. exigua, S. frugiperda* and *S. litura*) by plant secondary metabolites of various structures like terpenoids and glucosinolates, but also synthetic insecticides (reviewed in (Amezian et al., 2021a). In *S. frugiperda*, their role in insecticide resistance is supported by the fact that they are overexpressed in several field populations resistant to insecticides such as pyrethroids (Boaventura et al., 2020a; Gimenez et al., 2020). These studies associated resistance with the

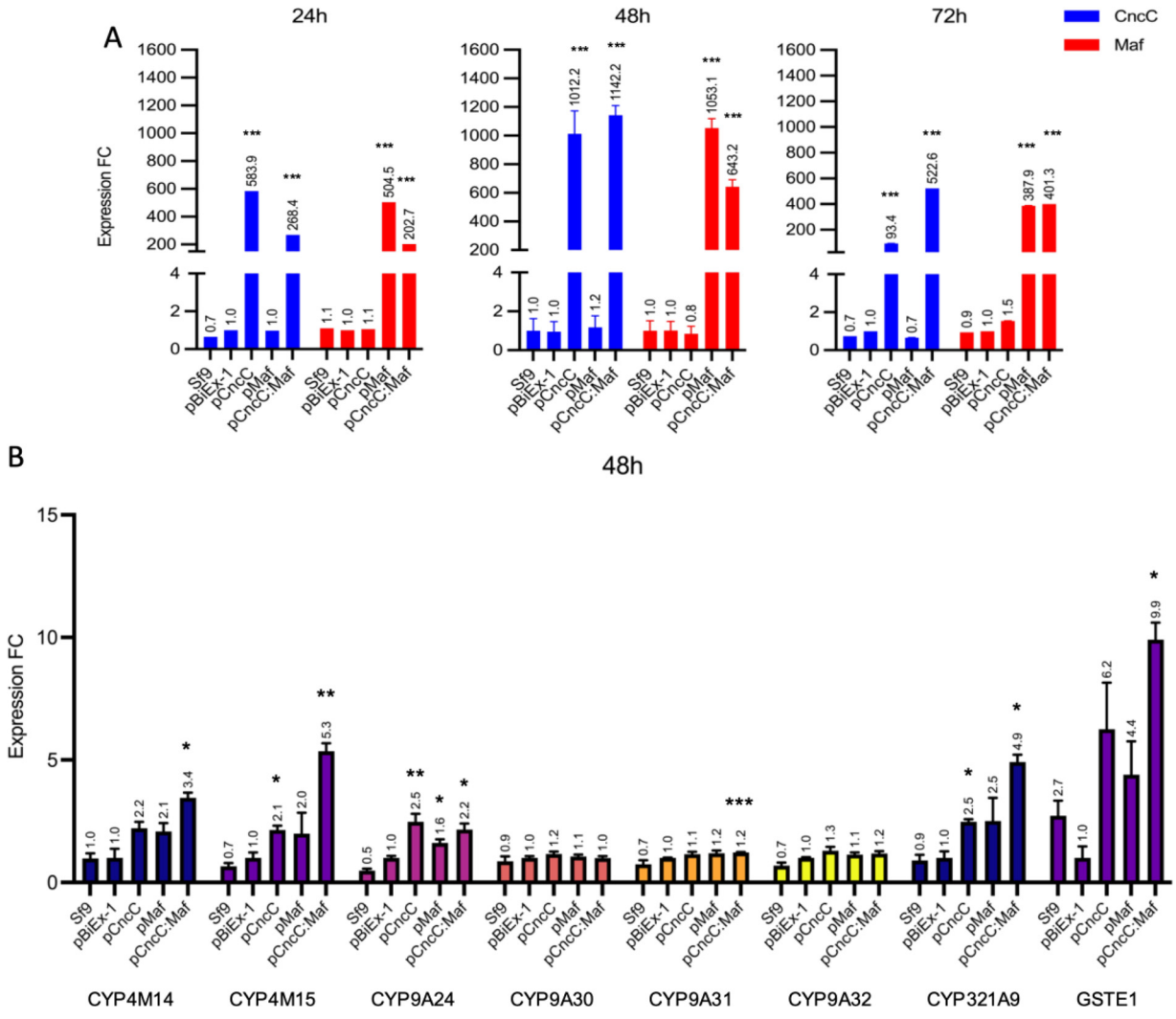

Fig. 3. A) Transcript levels of CncC and Maf in transiently transformed Sf9 cells at 24, 48 and 72 h post-transfection and B) Transcript levels of detoxification genes in transiently transformed Sf9 cells 48 h post-transfection. Expression of detoxification genes was monitored in cell lines transfected with either CncC (pCncC), Maf (pMaf) or both transcription factors (pCncC:Maf). Gene expression was normalized using the expression of the ribosomal protein L4, L18 and G6PDH reference genes and shown as fold-change relative to the expression of cell lines transfected with an "empty" plasmid, pBiEx-1. Data are mean values of three biological replicates ± SEM. A pairwise *t*-test was performed to establish the statistical significance of gene overexpression (A) and gene up-regulation (B) in transformed cell lines as compared to the control (pBiEx-1) (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

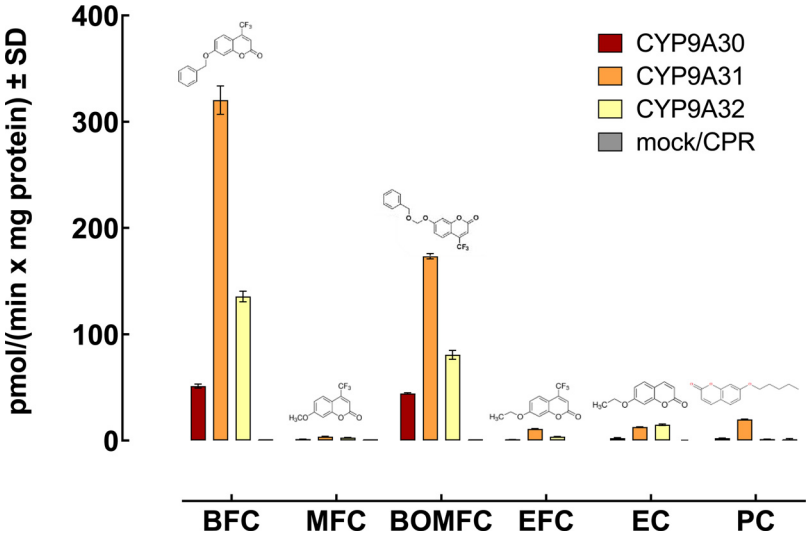

Fig. 4. Metabolism of coumarin fluorescent probe substrates by recombinantly expressed CYP9A30, CYP9A31 and CYP9A32 of Spodoptera frugiperda. Data are mean values ± SD ($n = 4$). Abbreviations: BFC, 7-benzyloxy-4-trifluoromethyl coumarin; MFC, 7-methoxy-4-trifluoromethyl coumarin; EFC, 7-ethoxy-4-trifluoromethyl coumarin; BOMFC, 7-benzyloxymethoxy-4-trifluoromethyl coumarin; PC, 7-n-pentoxy coumarin; EC, 7-ethoxy coumarin.

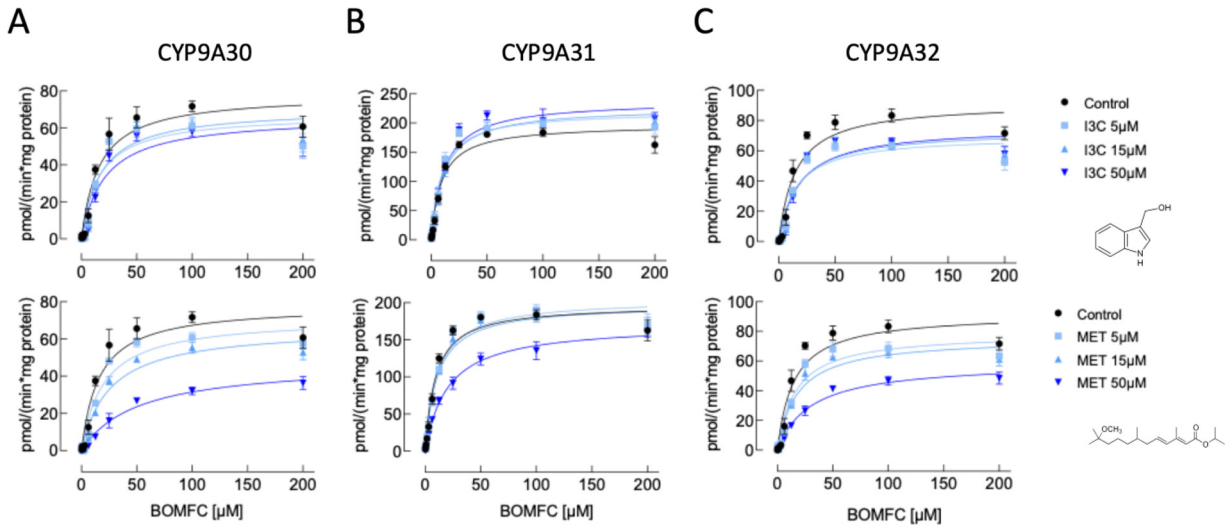

Fig. 5. Steady-state kinetics of 7-hydroxy-4-(trifluoromethyl)coumarin (HC) formation using BOMFC as a substrate by recombinantly expressed Spodoptera frugiperda (A) CYP9A30, (B) CYP9A31 and (C) CYP9A32 in the presence of different concentrations of either methoprene (MET) or indole-3-carbinol (I3C). Data are mean values ± SD ($n = 4$).

overexpression of CYP9As, however there is little functional evidence demonstrating their ability to metabolize an insecticide or plant secondary metabolites. In *S. exigua,* CYP9A186 was shown to play a major role in resistance to abamectin and emamectin benzoate. Heterologous expression of CYP9A186 revealed its capacity to metabolize these insecticides into hydroxy- and O-desmethyl-metabolites (Zuo et al., 2021). In *H. armigera*, several CYP9As (CYP9A3, CYP9A14, CYP9A15, CYP9A16, CYP9A12/17, CYP9A23) were heterologously expressed in either yeast or insect cells and were shown to metabolize esfenvalerate into hydroxy-metabolites (Shi et al., 2021b; Yang et al., 2008). These results show that there is functional redundancy between the six members of *H. armigera* CYP9A, each of which can metabolize pyrethroids. Our results show that three of the twelve *S. frugiperda* CYP9As, namely CYP9A30, CYP9A31 and CYP9A32 have the ability to interact with methoprene, albeit with variations as shown for CYP9A31. These results suggest, as for the *H. armigera* CYP9As, a certain redundancy in the capacity of these enzymes to metabolize insecticides. However, further experiments are needed to determine whether this redundancy extends to additional members of the cluster and whether *S. frugiperda* CYP9As are capable of metabolizing pyrethroids.

CYP321A9 was induced by both I3C and methoprene suggesting it may be involved in the tolerance of Sf9 cells to these compounds. This P450 is also part of a gene cluster of which the synteny (*CYP321A9-CYP321A7-CYP321A8-CYP321A10*) is conserved within the noctuid lineage (Cheng et al., 2017). Although *CYP321A9* is the only member of the CYP321A subfamily to be expressed in Sf9 cells (Giraudo et al., 2015), genes of this cluster were reported to be induced and to metabolize xenobiotics (Cheng et al., 2017; Giraudo et al., 2015; Hu et al., 2019c; Jia et al., 2020; Wang et al., 2017). For example, a recent study in *S. frugiperda* larvae showed that transgenic overexpression of *CYP321A8* increased tolerance of insects to deltamethrin by 10.3-fold based on LC_{50} value (Chen and Palli, 2021). In *S. exigua,* CYP321A16 was shown to metabolize chlorpyrifos, whereas CYP321A8 degrades chlorpyrifos, cypermethrin and deltamethrin (Hu et al., 2021, 2020).

The data currently available show that members of both CYP9A and CYP321A subfamilies could metabolize certain insecticides, but their action on secondary plant metabolites remains to be tested. In our case, we did not see any interaction between the three recombinantly expressed CYP9As and I3C, suggesting that this compound is not a substrate of these enzymes. Insecticides metabolized by CYP9A or CYP321A P450 enzymes include pyrethroids, avermectins and an organophosphate. To our knowledge no other studies yet indicated CYP9A interaction with the juvenile hormone analogue methoprene. Whether methoprene is indeed metabolized by CYP9A30–32 warrants further studies with subsequent metabolite analysis.

In addition to the P450s, our study showed that *GSTE1* was inducible. The fact that the expression of this enzyme was induced by I3C was not surprising, because it was shown to be induced in a related species, *S. litura*, feeding on *Brassica juncea* leaves, a plant containing glucosinolates, of which one of the degradation products is I3C (Zou et al., 2016). The same authors have shown that GSTE1 indeed metabolizes I3C and allyl isothiocyanate. The involvement of GSTs in the metabolism of glucosinolates present in the food of *Spodoptera* species had also been highlighted by the identification of glutathione conjugates of aliphatic and aromatic isothiocyanates in their frass (Schramm et al., 2012).

In our study, the induction of *CYP4M14, CYP4M15, CYP321A9* and *GSTE1* by xenobiotics correlated well with their upregulation after transient overexpression of *CncC* and *Maf*. For all these genes the expression was also higher when *CncC* and *Maf* were co-expressed, which supports the assumption that these two transcription factors act as heterodimers. These results confirmed previous studies using ectopic expression of *CncC* and *Maf* in *Tribolium castaneum* (Kalsi and Palli, 2017) and *S. exigua* (Hu et al., 2019b, 2020). Indeed it seems well established that this GST is a target gene of the CncC:Maf pathway in several species [*D. melanogaster:* (Deng and Kerppola, 2013); *S. exigua*: (Hu et al., 2019a, 2019b); *S. litura*: (Chen et al., 2018); *T. castaneum*: (Kalsi and Palli, 2017)].

On the other hand, *CYP9A30, CYP9A31* and *CYP9A32* were not overexpressed in any of the *CncC* and *Maf* over-expressing cell lines we tested. This clearly demonstrates that methoprene and I3C induction of these genes does not rely on the activation of the CncC/Maf pathway and is likely controlled by other factors, in any case in Sf9 cells and further *in vivo* experiments are needed to confirm these data in the insect. Several xenobiotic responsive pathways have been identified in recent years (Amezian et al., 2021b), and one of the pathways potentially involved in the regulation of *SfCYP9As* could be the G-protein-coupled receptor (GPCR) pathway. Work of Li and Liu (2019) on the mosquito *Culex quinquefasciatus* highlighted the role of a GPCR pathway and of the intracellular effectors G-protein alpha subunit (Gs), adenylate cyclase (AC) and protein kinase A in the development of insecticide resistance by regulating the expression of certain P450s. Interestingly, this work showed that heterologous expression of these mosquito effectors in Sf9 cells resulted in the over-expression of *SfCYP9A32* (Li and Liu, 2019). Thus, these results suggest that the GPCR pathway regulates *CYP9A32* expres-

sion and that there is conservation between the cis-regulatory consensus sequences across species. However, further experiments are necessary to better understand the role of GPCRs in the regulation of CYP9A expression. The different lines of Sf9 cells generated in this study are a good starting point and model to further characterize the factors involved in xenobiotic mediated overexpression of detoxification genes.

5. Conclusion

Our results show that Sf9 cells can serve as a model to study the impact of xenobiotics on detoxification gene expression, and thus helping to investigate the genetic adaptation mechanisms of *S. frugiperda* to its chemical environment. Molecules of different chemical origin can induce the same detoxification genes, but induction does not imply direct involvement in the metabolism of the respective substance. Indeed, as our results show, selected CYP9As are able to interact with methoprene but not with I3C, despite the observation that they are significantly induced in Sf9 cells. Multiple signaling pathways lead to a detoxification response and our data suggest that those CYP9As investigated here do not appear to be regulated by the CncC/Maf pathway.

Declaration of Competing Interest

None.

CRediT authorship contribution statement

Dries Amezian: Investigation, Methodology, Formal analysis, Visualization, Validation, Writing – original draft, Writing – review & editing. **Sonja Mehlhorn:** Investigation, Methodology, Formal analysis, Visualization, Validation, Writing – review & editing. **Calypso Vacher-Chicane:** Investigation, Methodology, Formal analysis, Writing – review & editing. **Ralf Nauen:** Conceptualization, Supervision, Visualization, Writing – review & editing. **Gaëlle Le Goff:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2022.100037.

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