Response of a lepidopteran pest to xenobiotics : exploring transduction pathways by a transcriptomic approach

Maeva Giraudo

▶ **To cite this version:**

Maeva Giraudo. Response of a lepidopteran pest to xenobiotics : exploring transduction pathways by a transcriptomic approach. Life Sciences [q-bio]. Université Côte D'Azur, 2010. English. NNT : . tel-04447503

HAL Id: tel-04447503
https://hal.inrae.fr/tel-04447503

Submitted on 8 Feb 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Public Domain

UNIVERSITE DE NICE-SOPHIA ANTIPOLIS – UFR SCIENCES
Ecole Doctorale des Sciences de la Vie et de la Santé

THESE

Présentée pour obtenir le titre de

DOCTEUR en SCIENCES
De l'Université de Nice-Sophia Antipolis

Spécialité: Aspects Moléculaires et Cellulaires de la Biologie

Par

Maeva GIRAUDO

Réponse d'un lépidoptère ravageur polyphage aux xénobiotiques: exploration des mécanismes de transduction par une approche transcriptomique

Travail réalisé sous la co-direction du Dr René Feyereisen et du Dr Gaëlle Le Goff
Équipe Evolution et Spécificité des Interactions Multitrophiques
UMR 1301 INRA-CNRS-Université de Nice Sophia Antipolis

Soutenue publiquement en anglais le 22 juin 2010, devant un jury composé de:

Pr Richard FFRENCH-CONSTANT	University of Exeter, UK	Rapporteur
Dr Jean-Marc PASCUSSI	INSERM, Montpellier	Rapporteur
Dr Gaëlle LE GOFF	INRA, Sophia Antipolis	Examinatrice
Dr David PAURON	INRA, Sophia Antipolis	Président
Dr René FEYEREISEN	INRA, Sophia Antipolis	Directeur de thèse

Abstract

Herbivorous insects have developed mechanisms to metabolically inactivate some of the potentially toxic xenobiotics that they ingest, such as plant allelochemicals and insecticides. These defence mechanisms involve mostly a cytochrome P450-based metabolic detoxification. A large portion of them is inducible by xenobiotics, and for vertebrates including humans, the nuclear receptors (or « xenosensors ») that control P450s induction are increasingly understood.

We have shown in Drosophila that only a small subset of P450s are inducible by xenobiotics and that the specific *Drosophila* CYP6A2 showed pattern of induction similar to that of mammalian CYP2 genes that are induced by PXR/CAR nuclear receptors. We have then extended our study to gene expression in *Spodoptera frugiperda* (Lepidoptera, Noctuidae) in response to xenobiotics. Transcriptional analysis with an oligonucleotide microarray of 10K genes revealed that the xenobiotic response in the midgut of this insect and in the derived Sf9 cells involves the regulation of many detoxification genes. The patterns of differentially transcribed genes were specific to each chemical tested, suggesting multiple, and partially overlapping transduction mechanisms. qRT-PCR analysis showed that the plant allelochemicals were the strongest inducers of a small number of P450s *in vivo*, when insecticides induced P450 expression manly *in vitro*. Specific focus on the effects of two hormone mimics, methoxyfenozide and methoprene, in Sf9 cells showed that both of these compounds induced cell cycle arrest. In order to identify the link between ingestion of the toxin and P450 gene induction, we have cloned SfHR96, a nuclear receptor ortholog of the vertebrate xenosensors CAR and PXR. We found that this receptor is not inducible by xenobiotics and is expressed constitutively in all tissues and developmental stages. Strategies to demonstrate the function of SfHR96 by inactivation in vitro by RNAi and in vivo with dominant negative mutants in transgenic silkworms have been initiated. We hypothesize that SfHR96 dimerizes with ultraspiracle (USP), the insect ortholog of vertebrate RXR.

Remerciements

Les remerciements c'est un peu l'occasion de faire « ma vie mon œuvre » et surtout d'avoir une pensée pour tous ces gens à côté, autour ou plus loin qui ont d'une manière ou d'une autre participé au déroulement et à l'accomplissement de ce travail. Parce qu'une thèse ça s'écrit tout seul, mais ça se gère à plusieurs. Et vous avez été nombreux!

My first and foremost huge thank you will go to Big Chief aka Mr P450 aka Dr. René Feyereisen, the one and only. I do not know any word that could be powerful enough to express all my gratitude. You are by far the best PhD supervisor that anyone can dream of and I feel like the luckiest PhD student to have had the opportunity to work with you for almost 4 years. Even though it tends to be annoying to have a boss who knows everything about everything about science, now how am I supposed to find a new boss half as good as you are? Thanks a lot for trusting me, for your weird and crazy sense of humour, your kindness, your modesty and your infinite scientific knowledge. You make P450 rock!

J'aimerais maintenant adresser mes remerciements à la plus géniale des co-directrices de thèse, celle sans qui rien de tout cela n'aurait été possible, celle qui a su être plus qu'une chef, une amie, que je respecte profondément pour ses qualités humaines et scientifiques, Dr. Gaëlle Le Goff. Je n'aurais pas pu imaginer de meilleures conditions de thèse si je ne t'avais pas eue à mes côtés durant ces 4 années. Merci de m'avoir fait confiance, d'être une source de motivation et d'inspiration quotidienne, d'avoir mangé des trucs bizarres avec moi en Chine, de m'avoir soutenue quand j'étais au pays des sushis, merci pour tout. Sincèrement. Si j'arrive un jour à devenir ne serait-ce que la moitié de ce que tu es, j'aurai le sentiment d'avoir réussi ma vie. Et j'envie le ou la prochain(e) étudiant(e) qui aura la chance de t'avoir comme chef parce que c'est un privilège qui donnerait presque envie de refaire une thèse. (j'ai dit presque)

I would like to deeply thank Pr. Richard Ffrench-Constant, Professor at the University of Exeter for accepting to judge this work and be part of the jury on the big day. It is a real honour to have such a brilliant scientist to judge my work, I really appreciate it. Besides, I would like to thank you for being such a nice and passionate person and for the good times in Italy.

Mes remerciements s'adressent également au Dr. Jean-Marc Pascussi, Directeur de Recherches à l'INSERM de Montpellier, pour m'avoir fait l'honneur de juger ce travail. Je vous remercie sincèrement pour votre regard critique sur mon manuscrit et mon travail de thèse et pour m'avoir fait partager vos précieux conseils au tout début de l'aventure lors du comité de thèse.

J'aimerais remercier très chaleureusement le Pr. David Pauron, Directeur de Recherches à l'INRA de Sophia-Antipolis, pour avoir accepté d'être le président de mon jury. Merci pour ton esprit critique toujours juste et honnête et sache que je suis très honorée que tu aies accepté. Te côtoyer pendant ces 4 années a rendu mes pauses café et mon quotidien au labo plus qu'agréable.

Un merci particulier aux Dr. Christine Risso et Alain Devaux pour avoir été les moteurs de mes choix par votre passion et votre dévotion, pour votre humilité, pour m'avoir permis d'être là et pour être mes maitres Jedi qui ont su me transmettre la passion pour l'écotox aquatique. J'espère avoir la chance un jour de pouvoir re-travailler avec vous.

Je remercie également David Rouquié pour m'avoir accompagnée de ses conseils avisés depuis le début de l'aventure en faisant partie de mon comité de thèse.

Un grand arigato à l'équipe du Dr. Shinoda de Tsukuba au Japon pour m'avoir accueillie pendant 3 mois, pour m'avoir fait bénéficier de leurs compétences et de leurs connaissances scientifiques et humaines et m'avoir permis de découvrir un peu plus du quotidien à la japonaise. Merci d'avoir rendu ce séjour inoubliable et d'avoir mis un peu de zénitude dans mes valises.

Je voudrais remercier du fond du cœur la dreamteam GFI, l'élite de l'équipe de choc, ceux qui ont été mon quotidien pendant tout ce temps et qui m'ont apporté tout leur soutien. Je pense particulièrement à Pascaline, Fred, Alex, Josiane et à tous les membres de l'équipe qui ont réussi à me supporter au quotidien : Hideki, Thierry, Trang, Isabelle, Leslie, Olivia et Nathalie.

Je voudrais à présent remercier les gens d'à côté, ceux qui ont été un soutien moral, physique et parfois logistique au quotidien et sans qui je n'aurais jamais réussi à atteindre la ligne d'arrivée.

Je pense bien sûr à Alice Baudot, ma louloute, mon amie, ma copine. Celle qui a essuyé toutes mes galères tout en ayant les siennes, celle qui a eu les mots qu'il fallait au moment où il fallait, celle qui m'a permis de survivre dans un quotidien japonais pas toujours facile, celle qui m'a redonné confiance en moi, tout le temps, sans relâche, celle qui est sans conteste la plus chouette rencontre de ces 4 dernières années. Merci pour ton canapé. Merci pour tout. Je n'y serais jamais arrivé sans toi. Il me faudrait 4 pages de remerciements rien que pour toi ! J'en profite pour remercier celui qui partage ta vie, Marc Ciosi, qui du haut de son plus qu'1m90 a su gérer les conditions de crises, a toujours eu la main tendue avec son cœur dessus et décroche haut la main l'oscar du mec qui m'a le plus fait rire, même quand il s'entête à me contredire. Merci à vous deux, vous avez été mon plus fort soutien et mes meilleurs copains.

Je voudrais remercier de tout mon cœur ma choup'choup' blonde, celle qui m'a accompagnée en dehors comme au boulot, Melle Laetitia Paganelli. Partager ton quotidien a été un réel bonheur pendant ces quelques années. Merci d'avoir fait le chemin avec moi, d'avoir bu des bières avec moi, d'avoir la classe américaine, d'avoir été la meilleure infirmière pour le zoziaux à la patte cassée, de m'avoir soutenue et d'avoir été une amie hors compète ! J'aimerais tellement pouvoir t'apporter la moitié du soutien que tu m'as donné quand viendra ton tour.

Un immense merci à ma Gégé nationale (c'est toi la bas dans le noir ?), pour tout ce que tu es et tout ce que tu m'as apporté et ta contribution nationale à l'appellation d'origine contrôlée Sussabi'. Pour tous tes conseils si judicieux, ta bonne humeur communicative et sans égal, pour être celle sur qui on peut compter, celle qui fait rigoler, pour m'avoir souvent sorti la tête de l'eau, pour être une amie hors du commun et m'avoir aidée au quotidien, même exilée à la capitale.

Un gigantesque merci à tous les potos du labo, ceux qui m'ont donné une réelle bouffée d'oxygène pendant les pauses déjeuner et en dehors, et ont rendu mon quotidien au labo beaucoup plus funky : Anto, Nico Hardcore, Dr Waffle, Maëlle, Marc, Pierpoljak-Didier, Eric. Je vous kiffe. Heureusement qu'il y a encore des gens comme vous à l'INRA, sans quoi ça serait vachement moins drôle.

Ludo, toi, jeune renard des sables, je te remercie infiniment pour… tout ! Et bien plus encore.

Je tiens à remercier particulièrement mon Gérard trop loin pour m'avoir accompagnée tout au long de la rédaction, ton soutien sans faille, ton OLO réconfortant. Maintenant c'est mon tour de te soutenir, futur grand avocat de la poutre (le barreau c'est surfait).

Merci à toi, Laurent T., pour ton arrivée imprévue et plus qu'appréciable dans ma vie, et avoir ainsi été une source de soutien et de motivation inestimable dans ces ultimes semaines de rédaction. You rock my world, t'as vu ?

Merci à tous les collègues de boulot passés ou encore là qui ont rendu mon quotidien tellement plus agréable, merci à Claude et ses pauses Bounty, Manuella, David, Lolo, Benoit, Laury, Etienne, Alex, Sophie, Petite SanSan, Emilie, Anaïs, Nico P. et Cédric. Un grand merci à Seb, mon Brandon adoré, pour ces quelques mois de colocation de bureau je sais que tu ne seras pas là le jour J mais que le cœur y est.

J'adresse mes plus sincères remerciements à mes meilleurs amis, ceux qui m'ont toujours soutenue et qui ont toujours cru en moi. Merci infiniment à Peggy, Cyril, Alice (et Mayeul !), Baptiste, Gaëlle (et Ambre !), Mary, Olive, Kévou et Fred. Merci d'être fiers de moi, de votre sincère amitié et d'être encore là, après toutes ces années. Merci à Eléonore, Ninie, Marion, Vanessa, Ophélie. A huge thank you to Ross for proof-reading my introduction. Un merci particulier à ma Chacha sur son îlot un peu trop loin, merci à toi et ta bordille d'être là malgré la distance. Vous manquez terriblement. Un merci en passant à Ben Mazuet, docteur à ses heures et chanteur pour les barmitzvah, ainsi qu'à tous ceux qui constituent la BO de ma thèse pour avoir accompagné mes oreilles pendant ces 4 années.

Un grand merci à Macbook Pro, aux expériences qui marchent, aux cellules pas contaminées, aux PCR niquels, aux clonages qui réussissent, aux séquençages pas foireux, et… aux insectes, mais bon pas trop non plus, hein, faut quand même pas abuser.

Je remercie sincèrement ceux qui espèrent que je vais « avoir mes partiels », qui me souhaitent de « bonnes vacances d'été » et de « trouver un cabinet bien placé », ceux qui me demandent « à quoi ça sert » ce que je fais, qui pensent que je vais être « étudiante toute ma vie » et qui m'ont souhaité bon courage pour écrirema thèse, parce que pour eux aussi « le mémoire de stage c'était dur, t'as vu ». Merci à vous, du fond du cœur, pour me faire apprécier d'autant plus ce que j'ai réalisé.

Je voudrais enfin remercier ma famille de survivants : ma maman, Agnès, Sandra et les enfants pour être ma raison de vivre et ma principale source de motivation au quotidien.

Un grand merci à vous tous pour votre soutien et m'avoir supportée pendant ces 4 années.

A mon Papa

Au club des survivants

Table of contents

INTRODUCTION 1

1. Navigating the chemical space through plant-insect interactions 3

2. Cytochrome P450s 7

2.1 History 7

2.2 Nomenclature 7

2.3 Catalytic mechanisms 10

2.4 Structure 12

2.5 Functions 14

2.6 Regulation 15

3. P450s in insects 17

3.1 Overview 17

3.2 Substrate diversity 19

3.3 Functions of insect P450s 19

3.3.1 Metabolism of endogenous substrates 20

3.3.2 Metabolisms of xenobiotics 21

3.3.2.1 Plant allelochemicals 21

3.3.2.2 insect P450s role in plant-insect interactions 22

3.3.2.3 Insecticide resistance 24

3.4 Regulation of insect P450s 25

4. Nuclear Hormone Receptors 27

4.1 General properties 27

4.2 Structure 27

4.2.1 The A/B domain 28

4.2.2 The DBD domain 29

4.2.3 The D region 30

4.2.4 The LBD domain 30

4.2.5 The F region 31

4.3 Nomenclature 32

4.4 Evolution 34

4.5 NR binding and response elements 35

4.6 Nuclear receptors and P450s regulation: example of CAR and PXR 37

5. Nuclear receptors in insects 41

5.1. The Ecdysone (20E) receptor: EcR/USP 43

5.2 Insect nuclear receptor and xenobiotic metabolism: DHR96 46

OBJECTIVES **51**

CHAPTER 1 **57**

Article : Regulation of cytochrome P450 in Drosophila : genomic insights. **57**

CHAPTER 2 **69**

Manuscript: Xenobiotic gene induction response in *Spodoptera frugiperda* midgut and Sf9 cells. **69**

CHAPTER 3 **109**

Manuscript: Effects of hormone agonists on the Sf9 cells, cellular proliferation and cell cycle arrest. **109**

CHAPTER 4 **145**

Manuscript: Molecular cloning and expression of nuclear receptors in *Spodoptera frugiperda* midgut ans Sf9 cells. **145**

1. RNAi impairment of SfHR96 181
   - 1.1 *in vivo* experiments 181
   - 1.2 *in vitro* experiments 183
2. Bombyx mori transgenic construction 186

GENERAL CONCLUSION **191**

REFERENCES **199**

List of illustrations

List of tables

Table 1 Updated number of P450 sequences identified as of August 2009.

Table 2 Number of CYP genes found in each insect P450 clade.

Table 3 Different sub-families of human nuclear receptors.

Table 4 Chemical properties of the xenobiotic compounds used as inducers in *S. frugiperda* larvae and Sf9 cells.

Table 5 Levels of P450 expression measured by qRT-PCR analysis in *S. frugiperda* larvae injected with dsRNA targeted against SfHR96 and exposed to xanthotoxin. Larvae injected with dsPAL from *A. thaliana* served as control.

List of figures

Figure 1 Major mechanisms in plant allelochemicals-insect interactions and resistance.

Figure 2 The detoxification process.

Figure 3 P450 nomenclature.

Figure 4 Single family tree of 107 P450 sequences.

Figure 5 Catalytic cycle of cytochrome P450s.

Figure 6 An example of a P450 system in the endoplasmic reticulum.

Figure 7 Monooxygenation reactions catalysed by P450s.

Figure 8 3D structure of P450BMP protein.

Figure 9 Examples of involvement of P450s in biological processes.

Figure 10 Schematic representation of activation mechanisms of AhR.

Figure 11 The four clades of insect P450 genes and relationships with major P450 families from other organisms.

Figure 12 Hormonal regulation of growth and development in insects.

Figure 13 Scheme of 20-hydroxyecdysone (20E) biosynthesis in Drosophila.

Figure 14 Graphical representation of the nuclear receptor structure.

Figure 15 Comparison of the crystal structures of the apo-retinoid X receptor-α (RXRα) ligand-binding domain (LBD) with the holo-RXRα LBD complexed with 9-*cis* retinoic acid).

Figure 16 The different types of receptor binding to their response elements.

Figure 17 PXR and CAR ligands.

Figure 18 Activation of the mammalian xenosensors PXR and CAR.

Figure 19 Classification of identified insect nuclear receptors .

Figure 20 Chemical structure of the 20-hydroxyecdysone .

Figure 21 Chemical structures of naturally occurring JHs.

Figure 22 Phylogenetic tree representing human and drosophila nuclear receptors.

Figure 23 The combinatorial problem in insect detoxification mechanism.

Figure 24 Chemical structures of the 11 xenobiotic compounds used in *S. frugiperda* induction study.

Figure 26 Preliminary results of gene expression levels of SfHR96 (A), CYP9A30 (B) and CYP9A31 (C) in Sf9 cells transfected with 3 concentrations (10, 25 and 50 nM) of siRNA targeted against SfHR96 (siHR96) and untransfected cells (ctrl) exposed to 50µM deltamethrin (delta), 250µM indole (Ind) or 25µM methoprene (Mtp).

Figure 27 The GAL4/UAS system principle

Figure 28 Restriction map of the plasmid DNA used as a vector for generating transgenic *B. mori*.

INTRODUCTION

1. Navigating the chemical space through plant-insect interactions

The efficient identification of small molecules that modulate protein function *in vitro* and *in vivo* is at the heart of chemical biology and drug discovery (Koch et al., 2005). All these "biologically relevant" molecules represent an infinitely small fraction of what is called the "chemical space". Identifying that chemical space – which encompasses all possible small organic molecules (Dobson, 2004) – would mean to navigate through an estimated number of 10^{60} molecules that populate that space, a number that could as well be infinite and limited only by the chemist's imagination (Lipinski and Hopkins, 2004). Given this, a thorough examination of all chemical space is practically impossible but can rather be focused on the biologically relevant part of this space for which limits could be defined by the specific binding of small molecules to biological molecules such as proteins, DNA or RNA (Lipinski and Hopkins, 2004).

Those biologically relevant molecules comprise all synthetic man-made compounds such as drugs, pesticides and industrial by-products as well as the plethora of natural compounds that are synthesised and used by living organisms. If we must look at the living matter of earth to identify these natural compounds producers, there is no doubt that green plants are a most voluminous and prolific sector (Zhu-Salzman et al., 2005). Plants synthesize a broad range of secondary metabolites that are essential for their growth and development but also for the defence against their natural enemies. These so-called phytotoxins or plant allelochemicals are very well documented by the immense diversity of plant secondary metabolism with some 200 000 compounds well known to pharmacologists (Hartmann, 2004) that account for a large part of the chemical space. About 25% of the drugs prescribed worldwide come from the plants (Rates, 2001). Recent developments in the field of constitutive plant allelochemicals have been extensively reviewed by (Wittstock and Gershenzon, 2002).

The plant metabolome therefore represents a well-documented part of the chemical space that we can navigate through in order to identify biologically relevant molecules. Such a task can be achieved by looking at other biological molecules that will interact with the plant metabolome, and thus intrinsically to the living organisms that harbour these molecules. When looking at plants, there is no doubt that herbivorous insects represent the organisms

that are the major interacting species. As Strong stated (Strong, 1988) “The overwhelming majority of all species interactions occur between herbivorous insects and plants, simply because these two groups comprise about half of the macroscopic species on Earth”.

Plant and insects have co-existed on land for as long as 350 million years and have therefore developed a series of close relationships, some of them mutually beneficial like pollination, but the most common interaction however involves insect predation on plants and the defence of plants against the attack of herbivorous insects (Gatehouse, 2002). The process of co-evolution between plants and insects is widely believed to have generated much of the Earth’s biological diversity (Rausher, 2001). Ehrlich and Raven in their famous paper “butterflies and plants: a study in co-evolution” have suggested back in 1964 that insects feeding on plants has been a determining factor in increasing species diversity in both herbivores and host plants. According to their co-evolution theory, evolution of plant chemical defences against their natural enemies is closely followed by biochemical adaptation in insect herbivores, and that newly evolved counter-resistance mechanism result in adaptive radiation of herbivore lineages (Wheat et al., 2007). Therefore, herbivorous insects impose natural selection that cause the evolution of a new plant resistance character and because most resistance characters reduce the survival of herbivorous insects, their evolution generates characters that circumvent the newly evolved plant resistance (Rausher, 2001).

The success of phytophagous insects as herbivores results therefore from their ability to successfully counteract the defensive strategies of their plant hosts (Gatehouse, 2002). They have developed for that matter multiple mechanisms to overcome plant chemical defences as explained in figure 1.

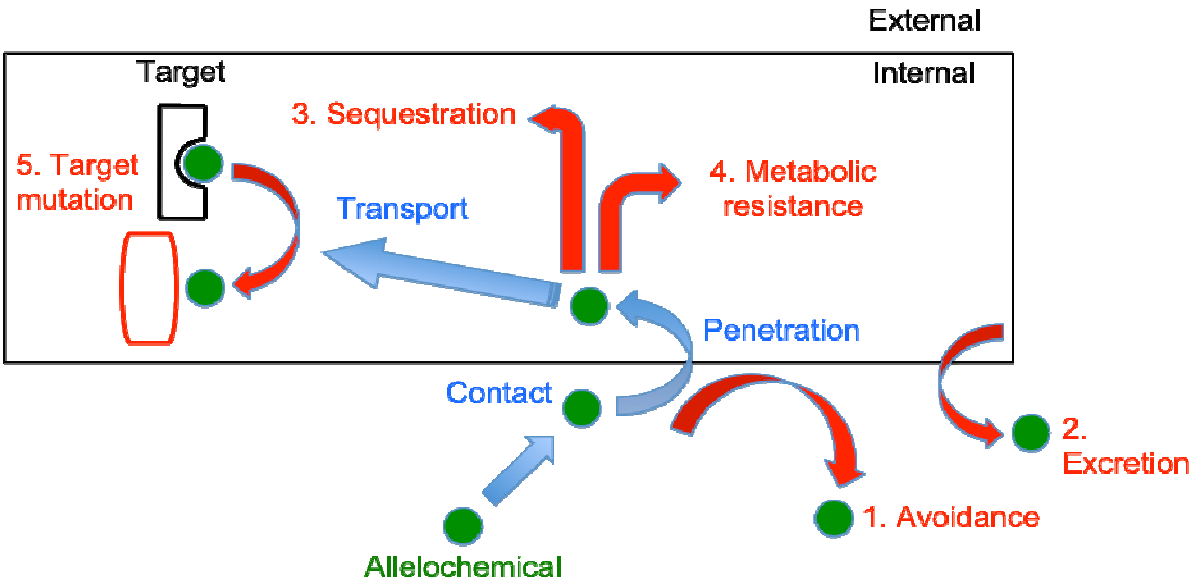


Figure 1: Major mechanisms in plant allelochemicals-insect interactions (blue arrows) and resistance (red arrows). As a first step, insects can avoid (1) contact with the plant allelochemicals (green circle), mainly by behavioural strategies. After ingestion of the allelochemical, insects have the ability to excrete (2), sequester (3) or metabolize (4) the compound before excretion. As allelochemicals reaches its molecular target, a last mechanism involves the mutation of that target so that allelochemicals effects are reduced or eliminated. (from Despres et al., 2007)

Among these mechanisms, the metabolism of plant allelochemicals (number 4 in figure 1) is one of the major weapons that insects have evolved in their co-evolutionary arms race with plants. This detoxification process allows insects to metabolically inactivate the plant toxin that they ingest and rely on two main successive steps, as is shown in figure 2 below. First, in the phase I, or the biotransformation phase, a variety of enzymes acts to introduce reactive and polar groups into their substrates. One of the most common modifications is hydroxylation catalysed by the cytochrome P450-dependent mixed-function oxidase system. These enzyme complexes act to incorporate an atom of oxygen into nonactivated hydrocarbons, which can result in either the introduction of hydroxyl groups or N-, O- and S-dealkylation of substrates (Guengerich, 2001).

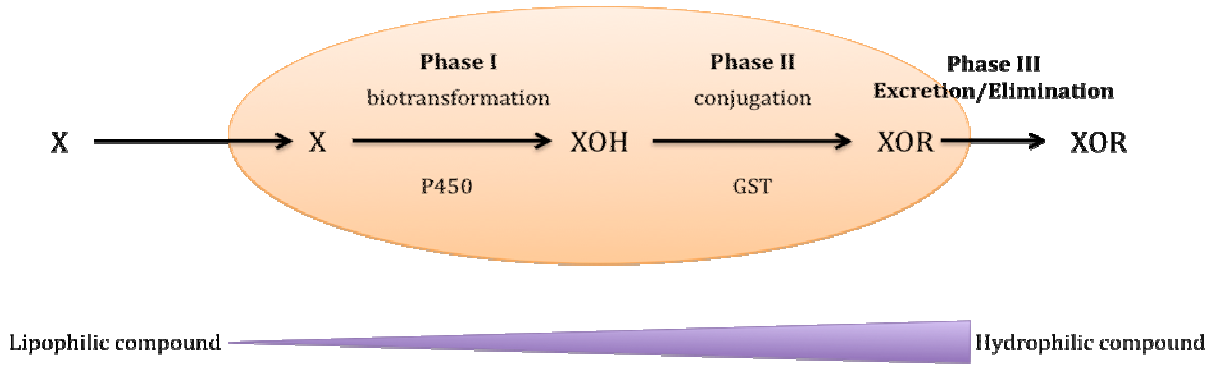


Figure 2: The detoxification process. Main enzymes involved in the phase I and phase II steps are indicated.

The substrate can then be readily excreted or goes to the second step of the detoxification pathway: the Phase II or the conjugation phase. In this phase, activated xenobiotic metabolites are conjugated with charged species such as glutathione (GSH), sulfate, glycine, or glucuronic acid. These reactions are catalysed by a large group of broad-specificity transferases, which in combination can metabolize almost any hydrophobic compound that contains nucleophilic or electrophilic groups (Jakoby and Ziegler, 1990). The most important ones of this group are the glutathione S-transferases (GSTs). The addition of large anionic groups (such as GSH) detoxifies reactive electrophiles and produces more polar metabolites that cannot diffuse across membranes, and may, therefore, be actively transported. Conjugated forms of xenobiotics can then be recognized by specific membrane-associated transporters such as ABC transporters in phase III detoxification, resulting in their vacuolar sequestration or release into the apoplasmic space via exocytosis.

Insect detoxification mechanisms rely therefore on the overexpression of specific class of enzymes, including the cytochrome P450s (Despres et al., 2007; Li et al., 2007) which will be discussed in the following chapter, after an overview of general properties and functions of these enzymes in vertebrates.

2. Cytochrome P450s

2.1 History

Cytochromes P450s are probably the most common and well-characterised detoxification enzymes that constitute one of the largest gene family with representatives in virtually all living organisms from vertebrates to insects, except in the bacteria *Escherichia coli* (Werck-Reichhart and Feyereisen, 2000).

Klinbergerg (Klingenberg, 1958) and Garfinkel (Garfinkel, 1958) were the first to report the presence of a specific pigment in rat and pig hepatocytes. This pigment showed an absorption peak at 450nm and has been later characterised by Omura and Sato (Omura and Sato, 1964) as a heme-containing protein. These proteins were rapidly identified as proteins involved in the steroid metabolism (Estabrook et al., 1963). It was then established that hepatic microsomal oxidation of xenobiotics and the increase of metabolic activity in animals treated with drugs or xenobiotics, could be the result of cytochrome P450 activity and inducibility (Remmer and Merker, 1963).

2.2 Nomenclature

As increasing numbers of P450s were isolated and named by different groups, there became more and more confusion in the literature regarding the relatedness of these isoforms. To date, more than 10 000 sequences coding for P450s have been reported (D. R. Nelson, P450 Gene Superfamily Nomenclature Committee, University of Tenessee Health Science Center, Memphis, http://drnelson.uthsc.edu/CytochromeP450.html, august 2009). This huge number of sequences identified as P450 forced the international scientific community to adopt a standardised nomenclature system: a new nomenclature was proposed in 1987 (Nebert et al., 1987) and, although subject to revisions (Nebert and Nelson, 1991; Nebert et al., 1989; Nelson et al., 1993), it remains the preferred system (Nelson et al., 1996). This nomenclature is based on percentage of protein sequence identities: two P450s that are more than 40% identical in their sequence belong to the same family, and to the same sub-family when that percentage exceeds 55%. Sequences are

named as follow: the CYP prefix (**CY**tochrome **P**450) is followed by an Arabic numeral that designate the family (e.g. CYP1), a capital letter indicating the sub-family (e.g. CYP1A) and finally an Arabic numeral if there is more than one gene in a single sub-family (e.g. CYP1A1, CYP1A2…) (figure 3).

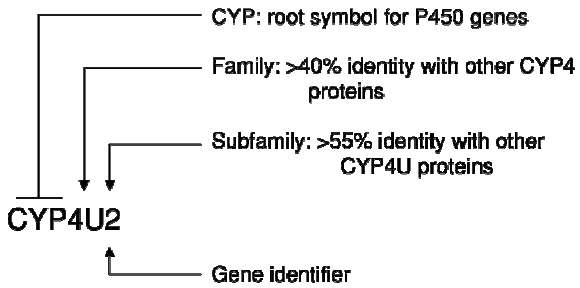


Figure 3: P450 nomenclature (from Feyereisen, 2005)

From the latest update in august 2009, there are currently 977 families of P450 identified and 2519 sub-families (see table 1).

		CYP families	CYP sub-families
Animals	3282	120	500
Insects	1675 (part of the animal total)	59	338
Animals (not insects)	1607	69	169
Plants	4266	126	464
Fungi	2570	459	1011
Protists	247	62	119
Bacteria	905	196	409
Archaea	22	12	14
Viruses	2	2	2
Total	11294	977	2519

Table 1: updated number of P450 sequences identified as of August 2009

As number of P450 families identified extended, another level of classification has been proposed based on grouping together larger families as "clans" as seen in figure 4. In this figure we can see an alignment of 107 P450 sequences, assembled by merging existing alignments. The plants have four main clans, the largest has been called the group A plant

P450s. The fungi have four clans and a few non-affiliated sequences. Animal's clans are named for a representative family as in the 2, 3, 4, and 7 clans, a unique location as in the mitochondrial clan, or for a specific organism as in the *Caenorhabditis elegans* clan. The clan number 3 has the mammalian 3 and 5 families, the insect 6 and 9 families, as well as the *C. elegans* 13 and 25 families (Nelson, 1999).

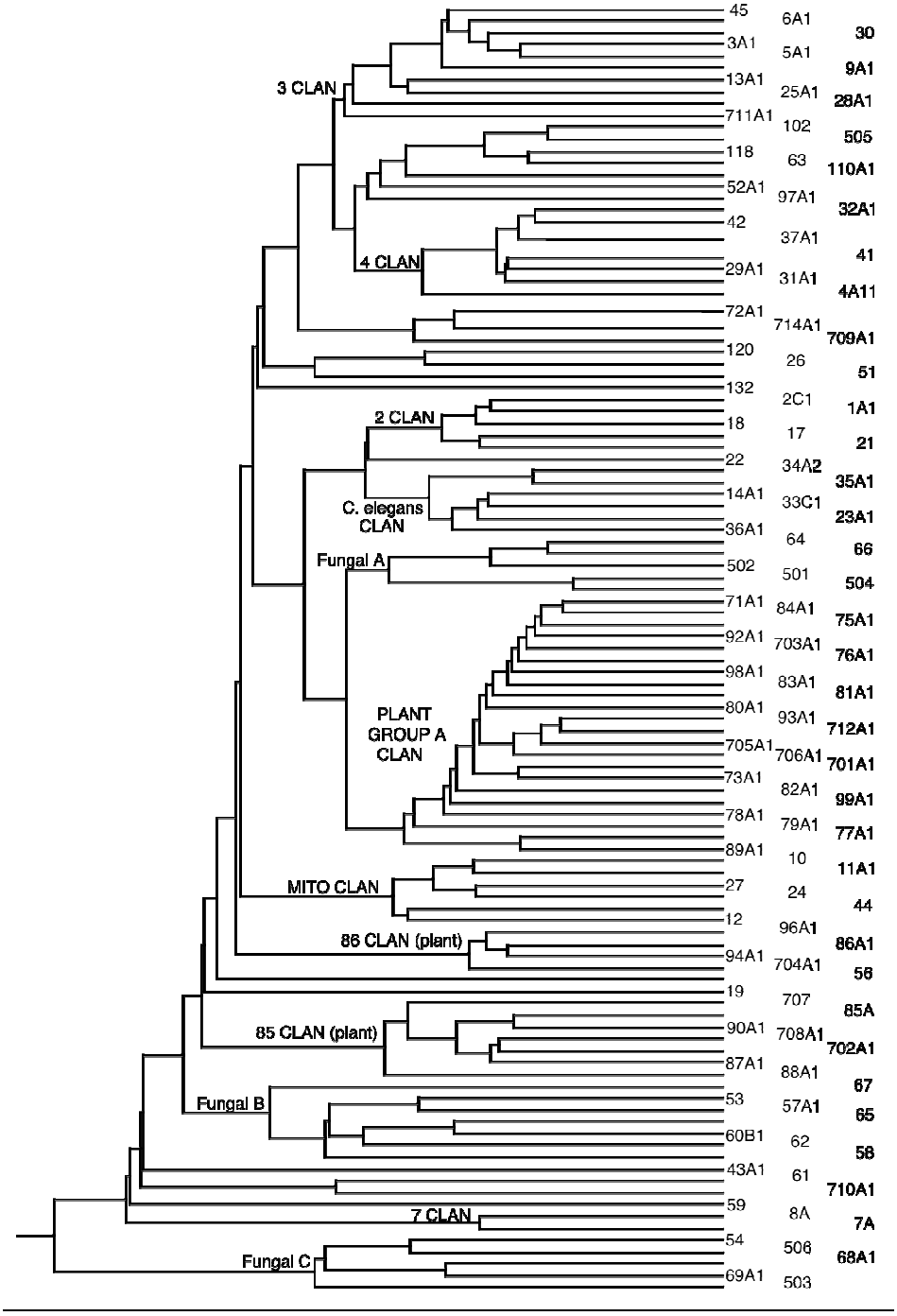


Figure 4: Single family tree of 107 P450 sequences (from http://drnelson.uthsc.edu/P450trees.html as of september 1998)

2.3 Catalytic mechanisms

Cytochrome P450s catalyse a large range of chemical reactions many of which are monooxygenation reactions, whereby they catalyse the transfer of one atom of molecular oxygen to a substrate while reducing the other to water (Feyereisen, 2005). The overall reaction of P450 monooxygenase-mediated metabolism can be expressed as follows:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

Where RH is the substrate. The different steps are represented in figure 5.

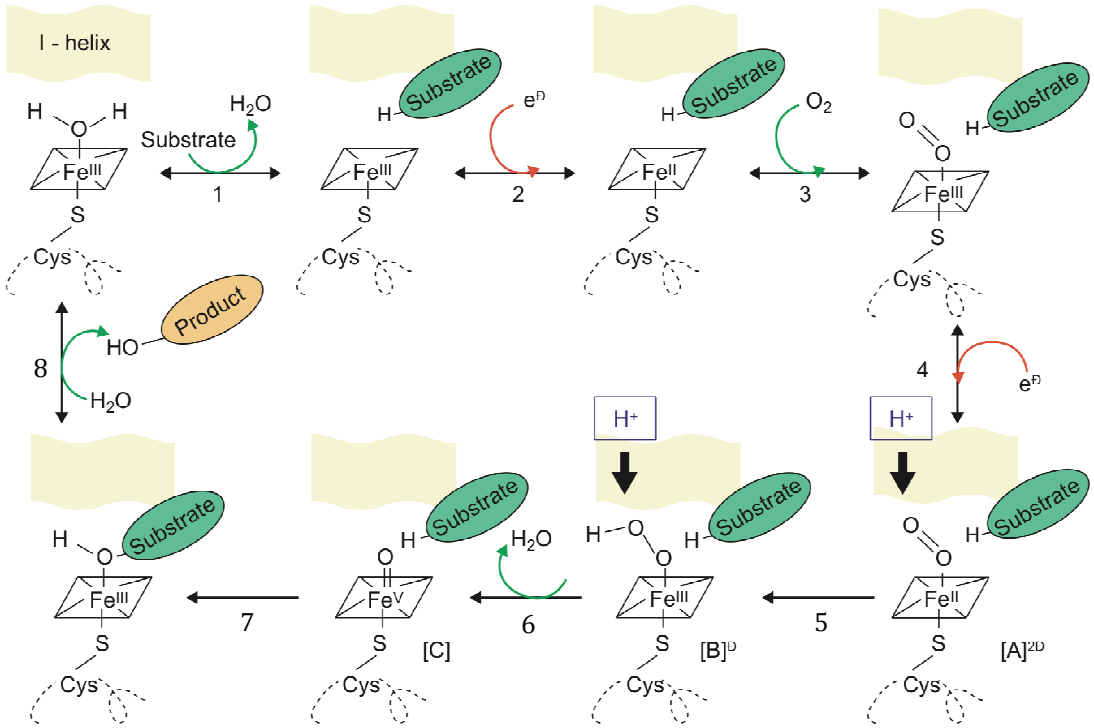


Figure 5: Catalytic cycle of cytochrome P450s (from Werck-Reichhart and Feyereisen, 2000).

In the initial step (1), the heme protein in its oxidised (Fe^{3+}) form binds a given substrate. This fixation facilitates the formation of the P450-reductase complex and transfer of a single electron from the redox partner (2). The iron reduced to Fe^{2+}, then binds oxygen (3). A second electron reduction step occurs (4) before the complex reactins in which molecular oxygen is split and an atom of oxygen is inserted into the substrate, the other one being

reduced to water. The product is then released and the P450 goes back to its initial oxidised form.

The monooxygenases reaction requires the intervention of a redox partner that is most of the time a flavoprotein but that can differ according to the substrate, the organism or the localisation. The majority of P450s in eukaryotes are located in the endoplasmic reticulum and require the flavoprotein NADPH cytochrome P450 reductase for reducing equivalents (figure 6). In addition to P450 and reductase, cytochrome b5 is sometimes needed, depending upon the substrate and/or the P450 involved. Cytochrome b5 can be important in donating the second electron from NADH to P450 or by allosterically regulating substrate binding to P450 (Murataliev et al., 2008). Mitochondrial P450s require ferredoxin and an NADPH ferredoxin reductase, and are therefore somewhat similar to the soluble P450 system found in bacteria.

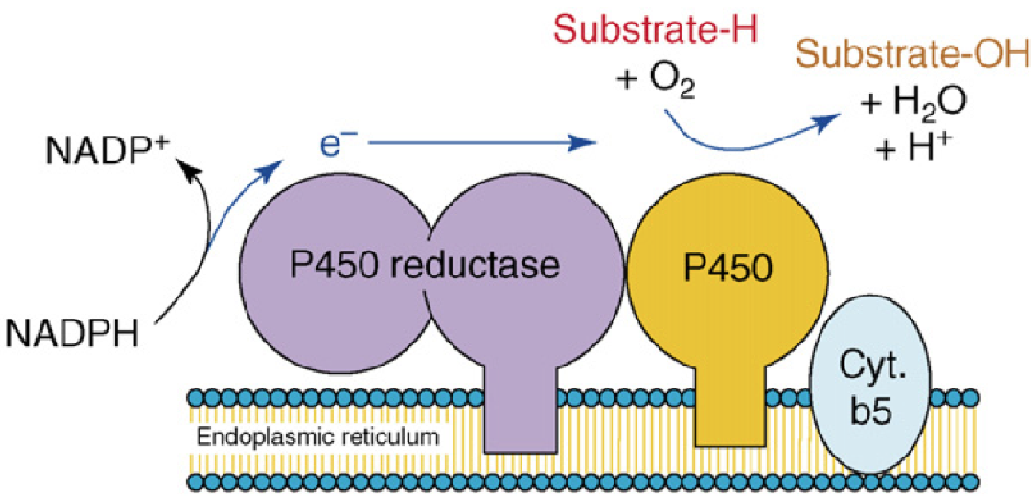


Figure 6: an example of a P450 system in the endoplasmic reticulum. (From Despres et al., 2007)

P450s can catalyse a large number of chemical reactions that have been ably reviewed in Mansuy (1998) and are not only based on the oxygen atom transfer. Indeed, P450 also show activity as oxidases, reductases, desaturases, isomerases, etc…. Figure 7 summarizes some of the main monooxygenation reactions.

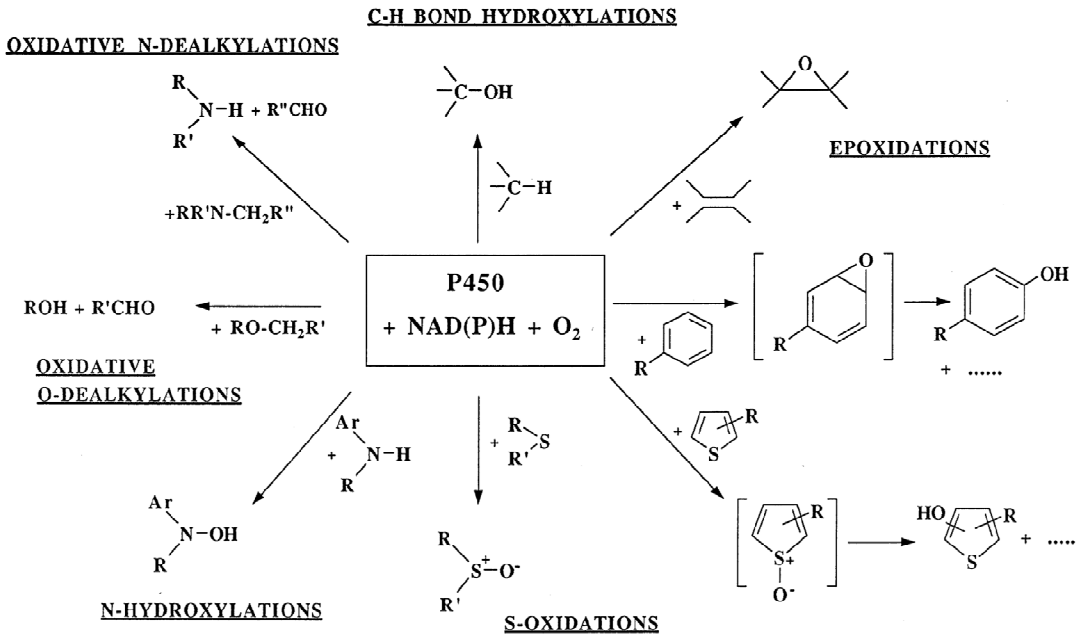


Figure 7: Monooxygenation reactions catalysed by P450s (from Mansuy, 1998)

2.4 Structure

P450s are made of an apoprotein of about 43 to 60 kD, from which one of the cysteine residue is coordinated with the heme iron atom. Three-dimensional structures of P450s are difficult to obtain in eukaryotes, as these proteins are associated with the membrane of the endoplamic reticulum or the inner mitochondrial membrane. However P450s are soluble in bacteria and allowed the determination of the first 3D structure back in 1985, $P450_{cam}$ (CYP101) from *Pseudomonas putida* (Poulos et al., 1986). It took several years before the structure of another P450 became available, that of P450BM-3 (CYP102), from *Bacillus megaterium* (Ravichandran et al., 1993). Since then, different P450s structure have been identified such as CYP120A1 from the cyanobacteria *Synechocystis sp.*(Kuhnel et al., 2008), the human CYP3A4 (Scott and Halpert, 2005) and CYP2C5 (Johnson et al., 2002). From the P450BM-3D structure, Graham and Peterson (1999) made a model of the secondary and tertiary structures of P450s as it can be seen in figure 8 (Graham and Peterson, 1999). Basically P450s are formed of two main domains: one rich in α-helix and the other one in β-sheets. The heme is located in the center of the α-domain, as part of the L helix that

contains the conserved cysteine involved in iron binding to the heme. Substrate fixation occurs through a hydrophobic canal formed by the β-sheets rich domain.

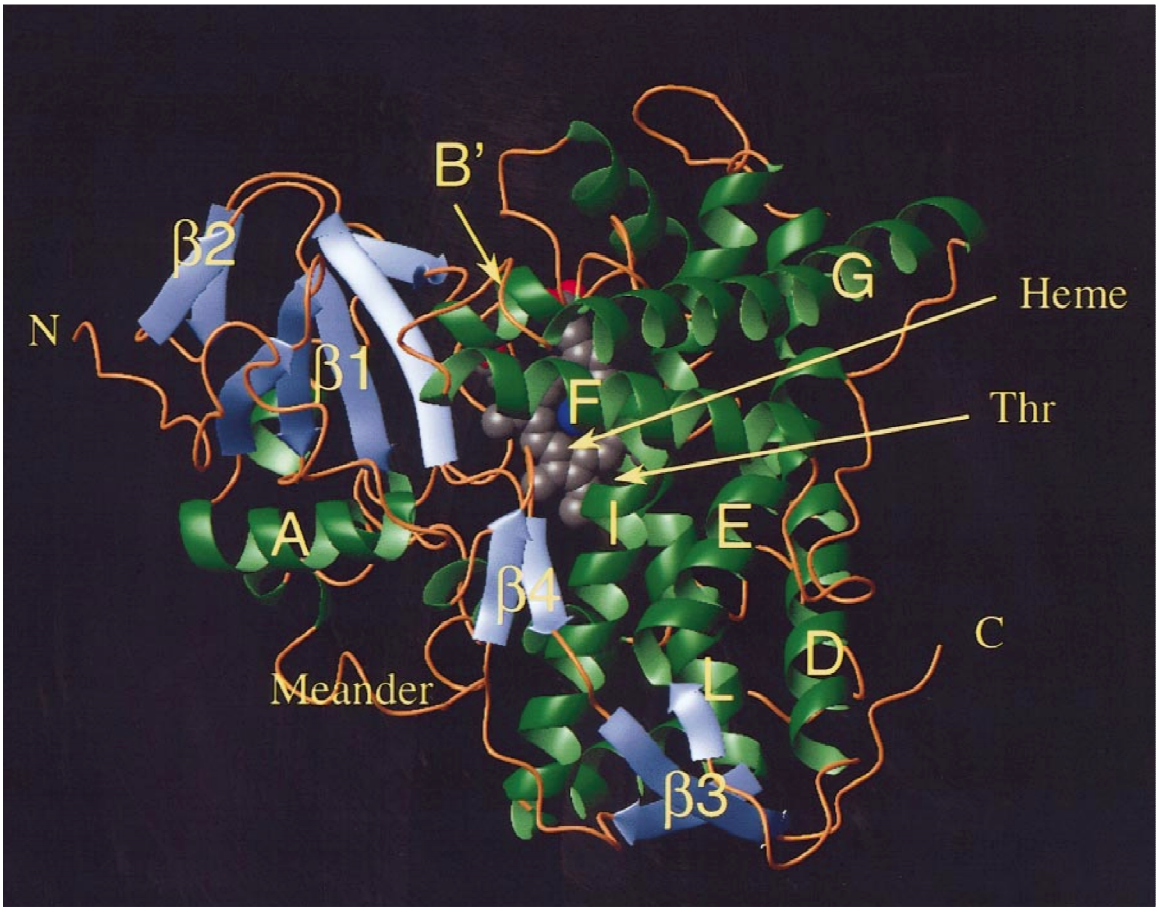


Figure 8: 3D structure of P450BMP protein. The secondary structure of P450BMP is shown from the "distal" face with helices in green coils and strands of α-sheets in blue. The random coil is shown in orange. Those elements that can be readily identified from this perspective are labelled. The N- and C-termini are labelled with N, and C, respectively. (from Graham and Peterson, 1999)

From the peptidic sequences alignment, we can point out that P450s are proteins of about 500 amino acids that can be identified by the following characteristic sequence with 4 conserved residues: FXXGXXXCXG. These 4 residues are located in the carboxyterminal region the heme binding part, the cysteine allowing the thiol-ligand binding to the heme. The N-terminal region of microsomal P450 is rich in hydrophobic AA and allows the fixation of the protein to the membrane.

2.5 Functions

Virtually any moderately to highly hydrophobic organic chemical is likely, perhaps certain, to be a substrate for one or another CYP (Stegeman and Livingstone, 1998). This large variety of potential substrates and the numerous different reactions that P450 can catalyse underline the essential and polyvalent role of P450s in the organism. Figure 9 represents how diverse is the array of biological processes into which P450s may participate.

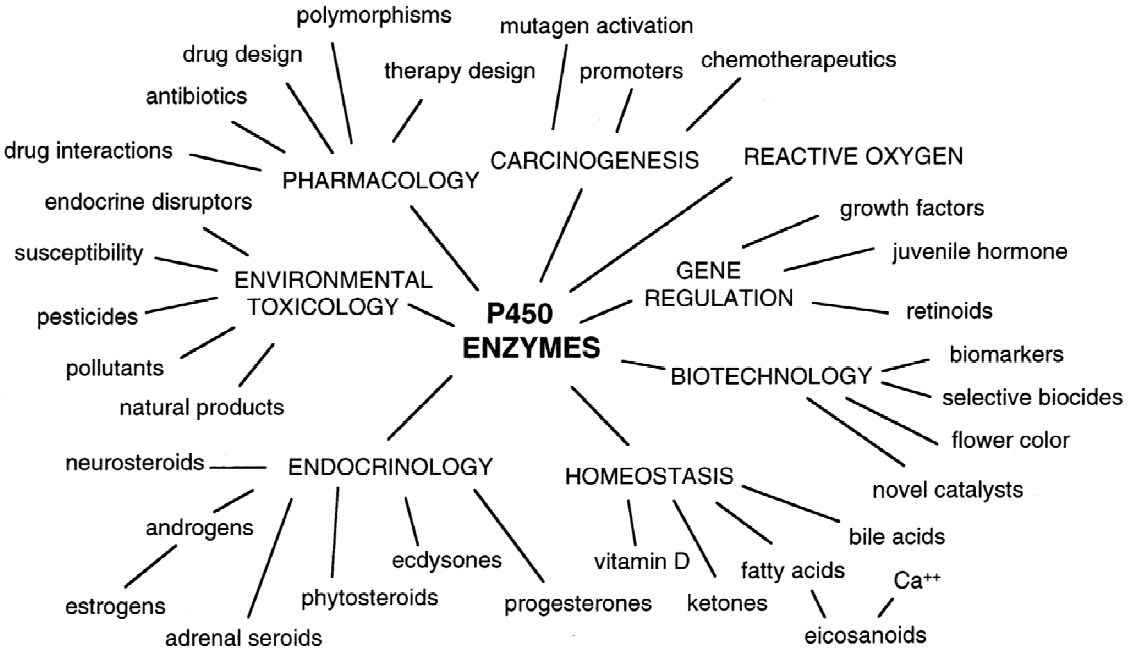


Figure 9: examples of involvement of P450s in biological processes. (from Stegeman and Livingstone, 1998)

Basically, P450s physiological functions can be divided into two mains roles: first they are also able to synthesise, activate or inactivate many endogenous compounds, some of which are toxic and some of which (e.g. steroids) are important regulatory molecules. For example, several P450s have a role in conversion of sterols to hormones, vitamin D and bile acids (Nebert and Russell, 2002). Second, they are known to be involved in the metabolism of exogenous chemicals and xenobiotics such as drugs, pollutants or natural products. From the 57 identified human P450s, at least 15 belonging essentially to the 1, 2 and 3 P450 families are involved in the detoxification of xenobiotics, including drugs (Gueguen et al., 2006). Indeed, 90% of drug metabolism is achieved by CYP activity with more than 2000 substrates

identified (Rendic, 2002). To cite a few examples, expression of the CYP1 family (including CYP1A1, 1A2 and 1B1) is induced by polycyclic aromatic hydrocarbons (PAH), such as those found in industrial incineration products, cigarette smoke, and charcoal- grilled food (Nebert and Russell, 2002; Shimada and Fujii-Kuriyama, 2004) and members of the CYP2B family are induced by the barbiturate Phenobarbital (PB) (Waxman and Azaroff, 1992) which has long been an archetypal model inducer of P450s. In addition, ethanol increases levels of CYP2E1 and peroxysome proliferators are able to induce the expression of CYP4A (Handschin and Meyer, 2003).

2.6 Regulation

Since P450s play key roles in biosynthetic and catabolic pathways of a variety of compounds, their expression must be highly regulated. Some P450s are expressed only in some tissues and specific cells within this tissue. Similarly, the expression pattern of a number of P450s is different in developmental stages and in females and males (Handschin and Meyer, 2003).

In most cases, induction of CYPs occurs by a process involving de novo RNA and protein synthesis. An alternative mechanism of CYP induction involves compounds that stabilise translation or inhibit the protein degradation pathway. For example, ethanol can induce CYP2E1 by such mechanisms leading to enhanced formation of reactive acetaminophen metabolites and enhanced hepatotoxicity (Woodcroft and Novak, 1998).

The induction of many CYPs occurs nonetheless by a similar mechanism, where ligand activation of key receptor transcription factors leads to increased transcription (Tompkins and Wallace, 2007). Such transcription factors include nuclear receptors that will be discussed later in this manuscript, as well as other receptors such as the Aryl Hydrocarbon Receptor (AhR), which will serve as an example to illustrate P450 regulation.

The molecular mechanisms governing the inducible expression of CYPs have been successfully elucidated for the CYP1 family (Fujii-Kuriyama and Mimura, 2005). Polyaromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and halogenated aromatic

hydrocarbon (HAHs) such as Benzo(a)pyrene induce CYP1A and CYP1B through binding to the aryl-hydrocarbon receptor (AhR) (Honkakoski and Negishi, 2000; Nebert et al., 2004). The induction of CYP1A1 in particular is one AhR-dependent response that has been consistently observed in most vertebrate species, and it has been used as the model system to define the mechanism by which the AhR regulates gene expression (Denison and Nagy, 2003). The typical model of AhR action is presented in Figure 10. A ligand such as TCDD binds to AhR, which then associates with its dimerisation partner, AhR nuclear translocator (Arnt), and this ligand-bound complex then translocaes into the nucleus. AhR and Arnt belong to a family of transcription factors that contain basic-helix-loop-helix (bHLH) and Per-Arnt-Sim (PAS) domains. The AhR–Arnt heterodimer binds to xenobiotic response elements (XRE) such as "TNGCGTG" in the proximal and distal promoter regions of the CYP1A gene and induces its expression (Tompkins and Wallace, 2007; Whitlock, 1999).

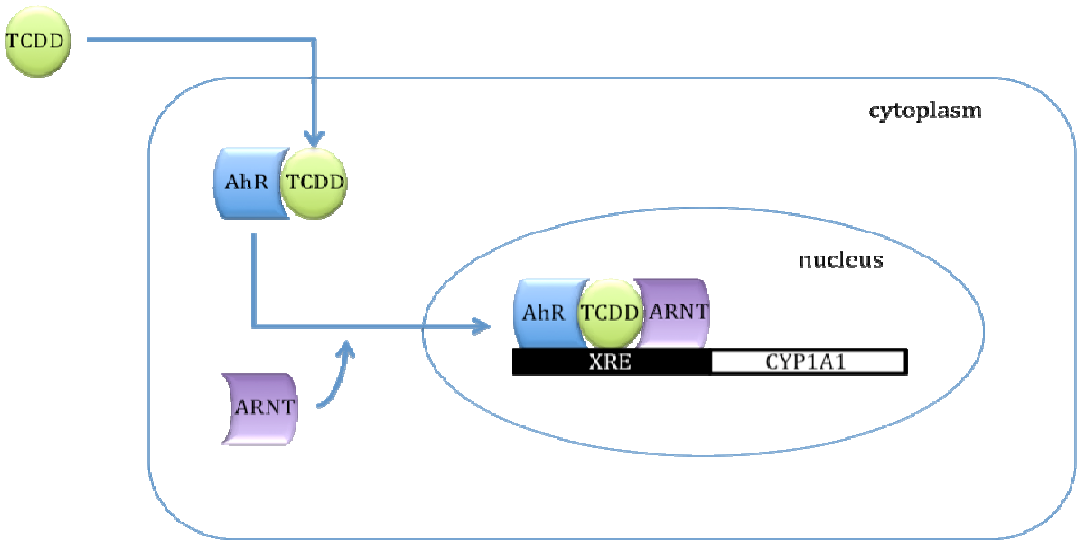


Figure 10: Schematic representation of activation mechanisms of AhR.

Transduction mechanisms of P450 induction are therefore well established in mammals. But how is the situation in insects? Insect P450s functions and regulation seem to be overall very similar to mammalian mechanisms and will be discussed in the following chapter.

3. P450s in insects

3.1 Overview

The first evidence of total P450 in insects was reported in 1967 by Ray (Ray, 1967) and the first insect P450 (CYP6A1) was cloned in 1989 by screening a cDNA expression library prepared from phenobarbital treated house flies with an antiserum raised against partially purified P450 (Feyereisen et al., 1989). Since then an increasing amount of studies have been carried out on insect P450s.

As shown in table 1, to date 59 CYP P450 families and 338 sub-families have been identified in insects, represented by 1675 sequences (http://drnelson.uthsc.edu/cytochromeP450.html). Given the tremendous diversity of insect species and the ever increasing number of fully sequenced insect genomes, there is no doubt that this number will exponentially increase in the next few years. For example, there are to date 90 fully identified sequences in the silkworm *Bombyx mori* genome, 85 active P450 genes in the fruit fly *Drosophila melanogaster* (Tijet et al., 2001), and about 143 sequences have been found in the genome of the red flour beetle *Tribolium castaneum* (Richards et al., 2008). The analysis of these available sequences revealed that insect P450s fall into 4 clades as shown in figure 11 and the summary of the number of P450 genes and P450 families found in each of these clades is presented in Table 2.

species	CYP2	mito	CYP3	CYP4	approx. total
Drosophila melanogaster	7	11	36	32	86
Anopheles gambiae	10	9	40	46	105
Aedes aegypti	12	9	82	57	160
Bombyx mori	7	12	29	33	87
Apis mellifera	8	6	28	4	46
Nasonia vitripennis	7	7	48	30	92
Tribolium castaneum	8	9	72	45	134
Pediculus humanus	8	7	12	9	36
Acyrthosiphon pisum	10	8	23	23	64

Table 2: Number of CYP genes found in each insect P450 clade (from Feyereisen, 2006, updated with insect P450 sequences as of March 2010)

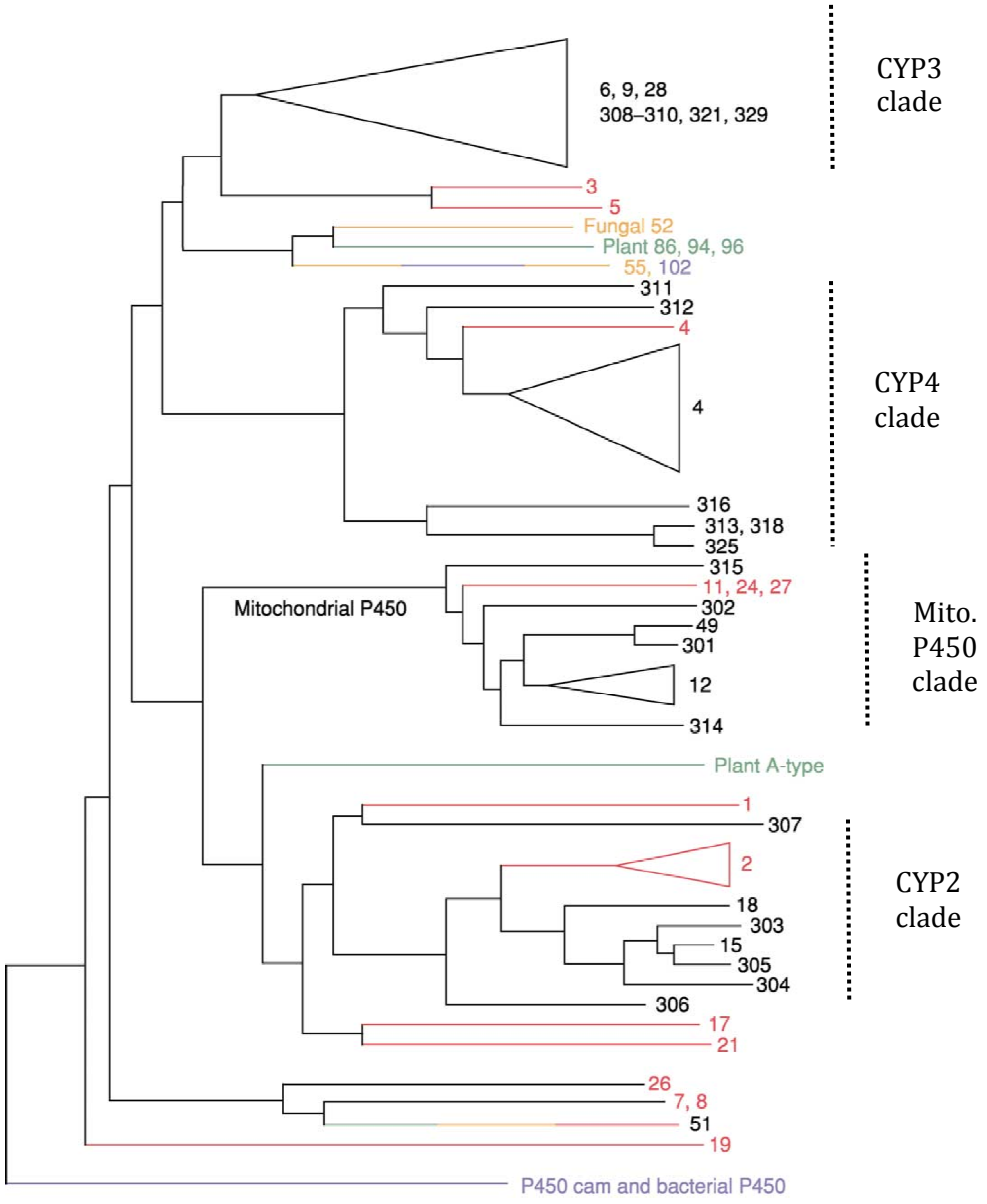


Figure 11: the four clades of insect P450 genes and relationships with major P450 families from other organisms. The color code is: black, insects; red, vertebrates; brown, fungi; green, plants; blue, bacteria. Triangles represent families with large numbers of genes. Plant A-type P450 genes are found only in plants. CYP55 and CYP102 are P450foxy and P450BM3, fatty acid hydroxylases fused with a P450 reductase domain. CYP51 is the sterol 14a-demethylase, the only P450 ortholog with CYP74 (not shown) found in different phyla. (From Feyereisen, 2006)

3.2 Substrate diversity

Functional identification of P450s can only be achieved through the biochemical characterization of its substrate selectivity. As the membrane-bound localisation of P450 make it difficult to study protein activity of individual P450 isolated from insect tissues, the expression of insect P450 cDNA in heterologous system has become a standard way of characterizing insect P450 proteins (Feyereisen, 2005). Insect P450s have therefore been expressed into different systems such as *Escherichia coli*, Baculovirus transfected lepidopteran cells, yeasts and Drosophila S2 cells. These techniques have allowed the identification of a large array of substrates that insect P450 can metabolize from endogenous compounds such as hormones to exogenous substrates such as plant allelochemicals and insecticides. For example CYP6A1 from the housefly expressed in *Escherichia coli* has been shown to metabolize the insecticide diazinon (Sabourault et al., 2001), some members of the CYP6AS family from the honeybee *Apis mellifera* expressed in baculovirus system were able to metabolize quercetin (Mao et al., 2009) or transfected S2 cells with CYP314A1 were reported to metabolize the insect hormone, ecdysone (Petryk et al., 2003).

3.3 Functions of insect P450s

From this ever increasing number of studies of functional characterization of insect P450s, we can divide their functional roles into two main categories: the metabolism of endogenous compounds, including signal molecules involved in growth, development and feeding, and the protection against xenobiotics, including resistance to pesticides and tolerance to plant toxins. Both of these aspects have been reviewed in Feyereisen (1999), Feyereisen (2005) and Schuler (1996).

3.3.1 Metabolism of endogenous substrates

Regarding the first aspect, it is now well known that P450s are involved in biosynthetic pathways of ecdysteroids and juvenile hormones, which are at the center stage of insect growth, development, and reproduction (Tijet et al., 2001) as it is depicted in figure 12 below. For example, it was found that Drosophila Halloween genes namely Phantom (Phm; CYP306A1), Disembodied (Dib; CYP302A1) and Shadow (Sad; CYP315A1) are involved into the biosynthesis pathway that transforms cholesterol into ecdysone. Shade (Shd; CYP314A1) catalyses the final hydroxylation step that transforms ecdysone into 20E (20-hydroxyecdysone) the principal insect molting hormone (Rewitz et al., 2006) (figure 13).

Another example of the role of insect P450s in the biosynthetic pathways of endogenous molecules is reported in the cockroach *Diploptera punctata*, where CYP4C7 and CYP15A1 have been shown to be involved in the biosynthesis and the regulation of the juvenile hormone III (JHIII) (Helvig et al., 2004; Sutherland et al., 1998).

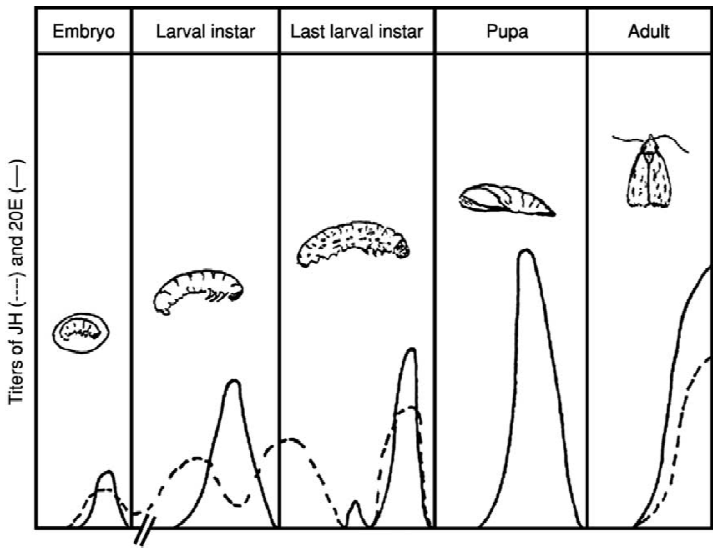


Figure 12: hormonal regulation of growth and development in insects. Metamorphosis of insect larvae into adults is under the control of two main hormones: the juvenile hormone (JH) and the moulting hormone, 20-hydroxyecdysone (2OE). (from Dhadialla et al., 2005)

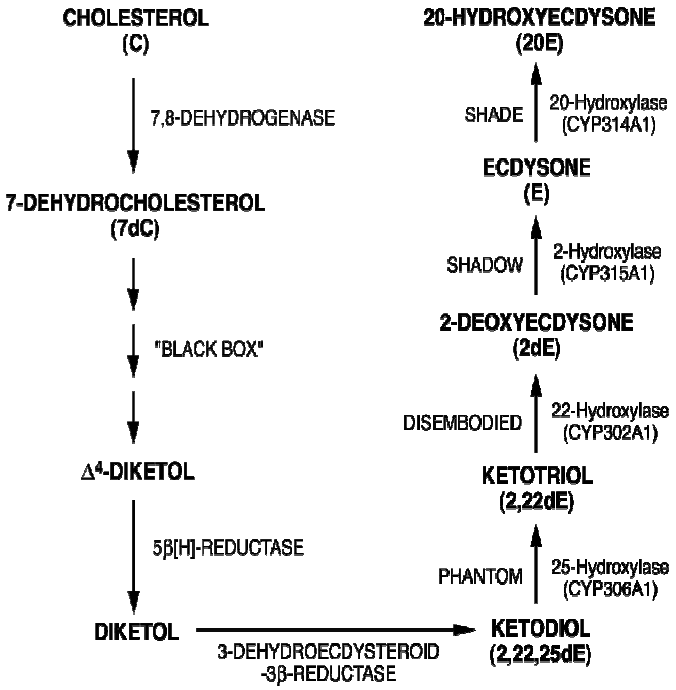


Figure 13: Scheme of 20-hydroxyecdysone (20E) biosynthesis in Drosophila. P450s involved in the last four hydroxylation steps leading to the fomrmation of the moulting hormone 20-hydroxyecdysone are indicated. (from Gilbert and Warren, 2005)

3.3.2 Metabolisms of xenobiotics

3.3.2.1 Plant allelochemicals

The metabolism of plant toxins by insects P450 has been extensively studied back in the BC era (Before Cloning, ©Feyereisen) as the measurement of the enzyme activity of the different reactions catalyzed by P450s, known as Mixed Function Oxidases (MFO) (reviewed in Brattsten, 1979), as well as the P450 protein content of insect microsomes (Yu, 1985) and NADPH reductase-dependent activities (Yu, 1986).

Based on the measurement of theses MFOs, many plant compounds have been shown to induce P450-based oxidases activities as well as microsomal P450 content. For example plant terpenoids in *Spodoptera eridania* (Brattsten et al., 1984), *Peridroma saucia* (Yu et al., 1979) and *Spodoptera frugiperda* (Gunderson et al., 1986; Yu, 1982);

glucosinolates/glucosinolate derivatives in *S. frugiperda* (Yu, 1983), flavonoids in *Heliothis zea* (Neal, 1987) and *S. frugiperda* (Wheeler et al., 1993) and nicotine in *Manduca sexta* (Snyder et al., 1993).

These indirect methods are a good first step in the characterization of P450-based plant allelochemicals metabolism in insects, however they do not give qualitative and quantitative information on which type of P450 is involved and at which extent. As it has been discussed in 3.2, the identification of individual P450 sequences has allowed the expression of their cDNAs in heterologous systems in order to investigate substrate selectivity but has also led to the correlation of mRNA induction levels with the previously identified protein levels and enzyme activities. For example in *Manduca sexta*, the plant allelochemical 2-tridecanone is an inducer of CYP4M1 and CYP4M3 (Snyder et al., 1995) and some P450s from the CYP9A family are induced by indole 3-carbinol, xanthotoxin and 2-tridecanone (Stevens et al., 2000) and in *Helicoverpa armigera*, the plant terpene gossypol is a potent inducer of CYP9A12 and CYP9A17 (Zhou et al., 2009).

3.3.2.2 insect P450s role in plant-insect interactions

As it was discussed in the first chapter of this manuscript, plants and insects arms race has led both parts to evolve sophisticated mechanisms to defend against each other, and insect P450s play an important role in this co-evolutionary process. In order to understand this, it is important to introduce the definition of host-plant specialization.

Herbivorous insects are classified into two groups: polyphagous species (generalists) which feed on a wide range of plant species, potentially encountering an array of toxic substances, and oligophagous species (specialists) feed on one or a small number of plant species and, thus, encounter a limited range of allelochemicals (Schuler, 1996). The majority of herbivorous insects belong to the second category and encounter large amounts of predictable chemistries, and have therefore characteristically high P450-based metabolism towards such chemistries (Stevens et al., 2000).

Probably the best story to illustrate this host plant specialization is the case of Papilio species detoxification of plant furanocoumarins through metabolism by P450s from the CYP6B family.

Furanocoumarins are plant allelochemicals found as two types in different plant species: the linear furanocoumarins (e.g. xanthotoxin and bergapten) and the angular type (e.g. angelicin). These substances are toxic to non-adapted herbivores (Berenbaum, 1990) by a photoreactivation mechanism which leads to the formation of DNA adducts. However, despite the toxicity of these substances, the black swallowtail *Papilio polyxenes* feeds almost exclusively on furanocoumarin containing plants. Early studies reported the ability of this species to detoxify xanthotoxin by a P450 monooxygenase-based mechanism (Bull et al., 1986; Cohen et al., 1989). In 1992, one specific P450 was cloned from xanthotoxin-treated larvae, the CYP6B1 (Cohen et al., 1992), and has been shown to be induced by and to metabolize linear but not angular furanocoumarins (Hung et al., 1995b; Ma et al., 1994; Prapaipong et al., 1994). However more recent studies using improved heterologous expression systems containing insect reductases reported that CYP6B1 was also able to metabolize angular furanocoumarins (angelicin) but at a lower level (Li et al., 2003; Wen et al., 2003).

A second CYP6B, CYP6B3, was isolated from *Papilio polyxenes* in 1995 and was shown to be inducible by linear as well as angular furanocoumarins (Hung et al., 1995a). Further CYP6B-like variants were then isolated from related Papilio species: *Papilio brevicauda* and *Papilio glaucus* (Cohen et al., 1992). The latter species is of particular interest as in contrast to *P. polyxenes*, it is a polyphagous species that encounter furanocoumarins only occasionally, but has however the ability to metabolize xanthotoxin through CYP6B metabolism. Moreover, six additional CYP6B have been isolated in *P. canadensis*, another generalist, and have shown to be induced by xanthotoxin (Li et al., 2002). Taken together, these results support the theory of Berenbaum et al. (1996) that high inducible activity toward a specific chemistry can serve as an adaptation of herbivores to a host containing this type of chemistry (Feyereisen, 1999).

3.3.2.3 Insecticide resistance

In addition to plant toxins, insect P450s can also metabolize a large array of insecticides compounds. Back in the early studies, MFO levels were found increased by several insecticides such as carbaryl (Gould and Hodgson, 1980), aldrin, heptachlor or biphenyl (Yu, 1985). P450-based metabolism of such substances has then been reported in some cases of heterologous expression of insect P450s. For example, Sabourault et al. found that over-expressed CYP6A1 from the house fly was able to metabolize diazinon (Sabourault et al., 2001) and CYP6B8 from *Helicoverpa zea* metabolises efficiently three insecticides, cypermethrin, diazinon and aldrin (Li et al., 2004).

This ability of P450 enzymes to metabolize insecticides plays an important role in insecticide resistance as it is now well established. P450 monooxygenase-mediated resistance is mainly due to increased detoxification than can result either from a change in the catalytic activity of the P450 involved and/or a change in the level of expression of the protein (Oppenoorth, 1984). Insecticide resistance associated with the overexpression of specific P450 enzymes have been largely reported in the literature. A good example to illustrate this phenomenon is the expression of Cyp6g1 in the DDT-resistant *Drosophila* strains. In 2001, Daborn and colleagues found that a single gene, Cyp6g1, was found overexpressed in DDT-resistant *Drosophila* strains and conferred cross-resistance to the neonicotinoid insecticide, imidacloprid (Daborn et al., 2001). Thanks to the availability of *Drosophila* complete genome, DNA microarrays were then constructed with all *Drosophila* P450 genes and hydridized with target cDNAs from susceptible and DDT-resistant strains of *Drosophila*. It was shown that Cyp6g1 was the one and only gene showing constitutive overexpression (Daborn et al., 2002). In addition, transgenic flies overexpressing CYP6g1 under the control of tubulin promotor in UAS/GAL4 expression system showed overexpression of only the CYP6g1 and cross-resistance to imidacloprid, acetamiprid and nitenpyram (Le Goff et al., 2003). Similar results were obtained along with 7 other P450s and showed that over-expression of CYP6g1 in transgenic flies increased survival to DDT, nitenpyram and dicyclanil (Daborn et al., 2007).

3.4 Regulation of insect P450s

Although less well studied than in vertebrates, insect P450 regulation has been explored in some cases and was found to be undergoing similar regulation mechanisms through transcription factors such as nuclear receptors, which will be discussed in the following chapter, as well as homologs to the AhR receptor as we will now see in the case of CYP6B family.

As it was discussed earlier, CYP6B inducibilty by furanocoumarins has been extensively studied in *Papilio* species, in particular in the case of CYP6B1 and CYP6B3 that are inducible by linear (such as xanthotoxin) and angular (such as angelicin) furanocoumarins (see section 3.3.2.2). Analyses of the CYP6B1v3 promotor in transient Sf9 cell expression assay have indicated that xanthotoxin-inducible transcription of this gene requires the presence of a Xenobiotic Response Element to xanthotoxin (XRE-Xan) (Petersen et al., 2003) and further sequence searches have also identified a putative xenobiotic response element to the AhR (XRE-AhR) (Denison et al., 1988). With the cloning of additional CYP6B genes, a number of additional regulatory sequences have been recorded, which are conserved among the different CYP6B members and have similarities to known regulatory sequences (Li et al., 2002; Petersen et al., 2001). For example, in the promotor region of CYP6B1 (Li et al., 2002) and CYP6B4 (McDonnell et al., 2004), an overlapping region of 3 different response elements was identified: a response element to the Ecdysone Receptor (EcRE), the receptor of the major moulting hormone in insects (see figure 12), a XRE-Xan as well as an Antioxidant Response Element (ARE), that has been identified as important for antioxidant-inducible expression of mammalian phase II detoxification genes. In addition to these elements, both of the CYP6B1 and CYP6B4 promoters contain XRE-AhR elements (xenobiotic response element to the aryl hydrocarbon receptor) similar to those found in mammalian P450 promoters that are activated by binding to the activated AhR–ARNT complexes. The fact that CYP6B genes contain response elements similar to those in mammalian genes that are the target of the AhR regulatory cascade suggest that this mechanisms might be conserved in insects.

The bHLH family of proteins has 56 members in Drosophila, and 13 are bHLH-PAS proteins related to AhR and ARNT (Hahn, 2002). The protein most closely related to mammalian AhR

is spineless-aristapedia (Ss), which has been characterised as a transcription factor present in the imaginal discs of developing Drosophila melanogaster larvae (Duncan et al., 1998). Ss interacts with different bHLH-PAS proteins (such as sima, sim, trh, dys and cyc) but most importantly to the ARNT ortholog called Tango (Tgo) that has been first identified as regulating antennal and tarsal development but shows also xenosensing functions by activating heterologous XRE-AhR elements (Emmons et al., 1999). In 2005, Brown and colleagues expressed Ss with and without Tgo proteins in Sf9 cells with wild-type and mutant CYP6B1:CAT constructs. They showed that Ss and Tgo increase basal expression of the wild-type CY6B1 promotor in an additive manner, suggesting that AhR/ARNT regulatory mechanisms of CYP1A1 in vertebrates might be conserved in insects (Brown et al., 2005).

bHLH-PAS proteins represent therefore one of the possible links between ingestion of the toxin and induction of detoxification genes. However, as signaling mechanisms of the detoxification machinery have been well studied in vertebrates, it was reported that other types of transcription factors were also involved in the xenobiotic transduction signal. These factors are nuclear hormone receptors (NRs) and will be discussed in the following chapter.

4. Nuclear Hormone Receptors

4.1 General properties

Nuclear hormone receptors (NRs) are one of the most abundant classes of transcriptional regulators in metazoans, in which they regulate functions as diverse as reproduction, differentiation, metabolism, metamorphosis and homeostasis (Escriva et al., 2000). This superfamily is present in all metazoans, and only in metazoans – no nuclear receptors have been found in the complete genome sequences currently available for plants, fungi, or unicellular eukaryotes (Laudet and Bonneton, 2005).

On the historical point of view, before the genes encoding nuclear receptors were cloned, the first NR was identified biochemically in the 1960s. Indeed, Elwood Jensen and his collaborators showed that estradiol was specifically retained in target cells of this hormone, leading to the discovery that its cellular activity is mediated by a specific high-affinity receptor. Subsequently, and only 20 years ago, the human glucocorticoid receptor (GR, NR3C1) was one of the first NRs to be cloned by Ron Evans and his colleagues together with the estrogen receptor (ER) cloned by the Pierre Chambon and Geoffrey Greene laboratories (Germain et al., 2006). Since then, NRs have become recognised as a superfamily of ligand-activated transcription factors, thus providing a direct link between signaling molecules that control these processes and transcriptional responses.

The NR research field has undergone very rapid development and covers areas ranging from structural and functional analyses to the molecular mechanisms of transcription regulation.

4.2 Structure

All NR proteins exhibit a characteristic modular structure that consists of five to six domains of homology (designated A to F, from the N-terminal to the C-terminal end) on the basis of regions of conserved sequence and function (Germain et al., 2006; Krust et al., 1986) (Figure 14). This common structural organization contains: a variable N-terminal region (A/B domain) containing the AF-1 transcription activator, a central well-conserved DNA binding domain (DBD, also termed C-domain), a non conserved hinge (D domain), a long and

moderately conserved ligand-binding domain (LBD, E domain) and eventually a C-terminal F domain (Escriva et al., 2000; Robinson-Rechavi and Laudet, 2003).

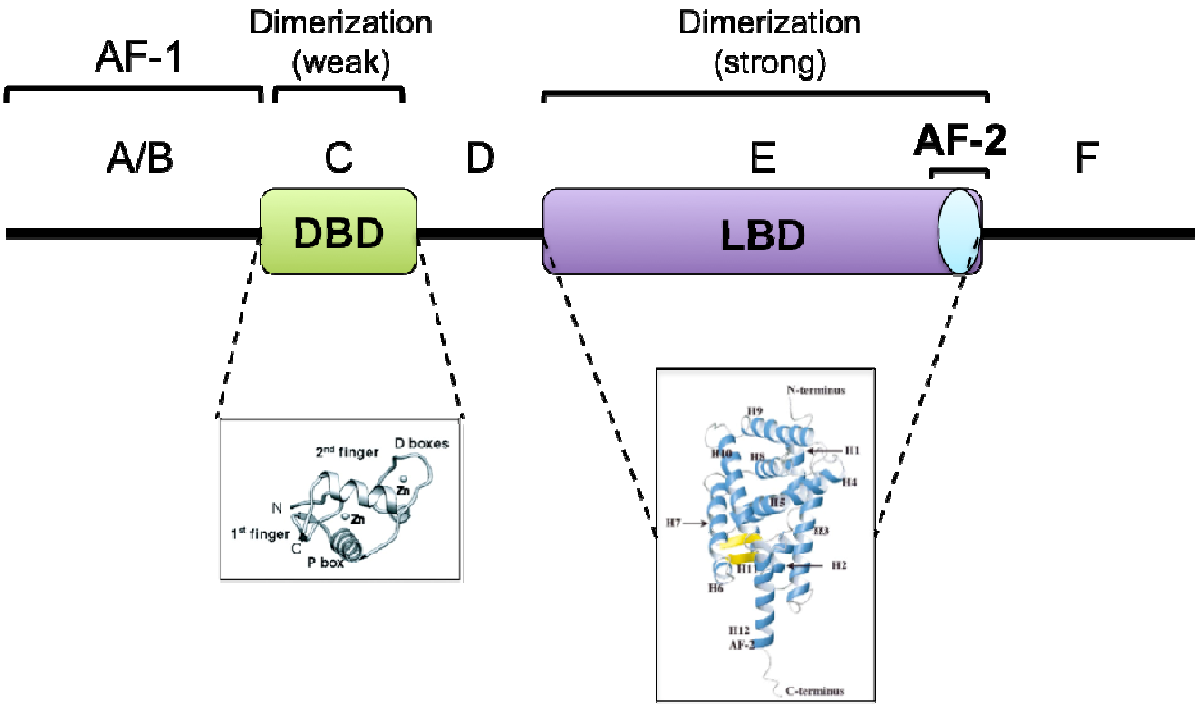


Figure 14: graphical representation of the nuclear receptor structure.

4.2.1 The A/B domain

The poorly defined N-terminal A/B region contains a transcriptional activation function, referred to as activation function 1 (AF-1), that can operate autonomously (Germain et al., 2006). Deletion of the LBD of steroid hormone receptors results indeed in a protein that is constitutively active in reporter gene assays (Lavery and McEwan, 2005). This led to the identification of the hormone-independent AF1 in the A/B domain. In contrast to the other activation function (AF-2) located in the LBD of liganded NRs, AF-1 can act in a ligand-independent manner when placed outside of the receptor.

The actual three-dimensional structure of the functionally folded AF-1 domain, ideally in the full-length receptor remains to be determined (Kumar and Litwack, 2009). The A/B domain and its AF-1 factor are variable in terms of amino acid sequence and length, showing little, if any, amino acid sequence homology, but has been shown to be important for steroid hormone receptor-dependent gene regulation, and is a major site for post-translational modifications such as phosphorylation and sumoylations (Lavery and McEwan, 2005; McEwan, 2004).

4.2.2 The DBD domain

The central C region of the NRs is the DBD that is the most conserved amino acid sequence among the members of the nuclear receptor superfamily. The DBD consists of two zinc-finger motifs, each containing four highly conserved cysteine molecules coordinating binding of a zinc atom. This results in the formation of a tertiary structure containing helices that interact specifically with DNA sequences that are organized appropriately in what are called response elements (RE) (Kumar and Thompson, 1999).

Residues in the first zinc-finger motif determine the specificity of the DNA recognition. It contains the P-box, which is the highly conserved part in the first zinc finger between the last two cysteines and determines the sequence specificity of the receptor-DNA binding RE (Germain et al., 2006).

The second zinc –finger motif is involved in dimerisation. Indeed, nuclear receptors bind to their REs as either homodimers or heterodimers.

Depending on the type of receptor, the C-terminal extension plays a role in sequence recognition, dimerisation, or both. The DBD is also the target of post-translational modifications and is involved furthermore in nuclear localisation and functions in interactions with transcription factors and coactivators (Claessens and Gewirth, 2004).

4.2.3 The D region

The D region is highly variable in both length and primary among nuclear receptors. As its name indicates, its main function is to serve a hinge between the DBD and the LBD. It allows the DBD and LBD to rotate and adopt different types of conformation without creating any steric hindrance troubles (Giguere, 1999). It also may contain some nuclear localisation signals (NLS) and contain residues whose mutation abolishes interaction with nuclear receptor corepressors (Aranda and Pascual, 2001).

4.2.4 The LBD domain

The LBD is a multifunctional domain that, in addition to the binding of ligand, mediates homo- and heterodimerisation, interaction with heat-shock proteins, ligand-dependent transcriptional activity, and in some cases, hormone reversible transcriptional repression (Aranda and Pascual, 2001). The C-terminal domain contains the ligand binding domain (LBD), whose overall architecture is well conserved between the various family members but which is nonetheless different enough to permit the high degree of ligand specificity characteristic of each member of the family. This domain harbours a ligand dependent activation function, called AF-2 that is crucially involved in transcriptional coregulator interaction (Laudet and Bonneton, 2005). The three-dimensional structure of the LBD has been determined for few unliganded (apo) and many liganded (holo) nuclear receptors, which has provided a good understanding of the mechanisms involved in ligand binding and transactivation (Gronemeyer et al., 2004).

Determination of the 3D structure of NRs shown that the LDBs of all nuclear receptors have a common overall three-dimensional structure that is structured as a three-layered α-helical antiparallel sandwich of 12 helices forming a hydrophobic pocket (see figure 15). This pocket is guarded by a twelfth helix (H12), which forms a movable lid over the pocket and contains residues that are crucial for the function of AF2. Many nuclear receptors are transcriptional silencers in the absence of ligand (apo-receptor) as a result of interaction with intermediary factors (i.e. corepressors). Upon ligand binding (holo-receptor), the ligand makes different contacts with the amino acids residues, promoting a conformational change that closes the

H12 “lid” on the pocket and the corepressor complex dissociates. Thus the activation domain within the H12 (AF2) is able to interact with co-activators and promotes transcription of target genes (Bourguet et al., 2000; Escriva et al., 2000; Laudet and Bonneton, 2005).

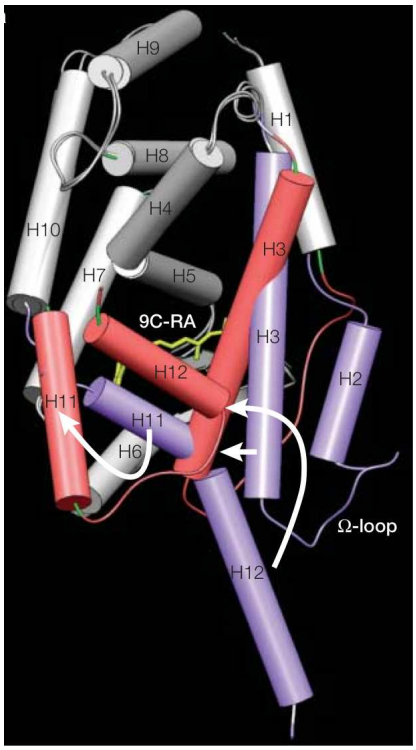


Figure 15: comparison of the crystal structures of the apo-retinoid X receptor-α (RXRα) ligand-binding domain (LBD) with the holo-RXRα LBD complexed with 9-*cis* retinoic acid. The coloured helices (purple in the apo-form; red in the holo form) are relocalised during the conformational change with the H12 helix that comes to close the « lid » of the ligand binding pocket. (from Gronemeyer et al., 2004)

4.2.5 The F region

Some of the nuclear receptors may have a C-terminal region called the F region, which is not conserved and is variable in length. For example the Retinoic Acid Receptor (RAR) and Hepatocyte Nuclear Factor 4 (HNF4) receptors have a region of respectively 36 and 102 aminoacids, when the Peroxysome Proliferator Activated Receptor (PPAR) or the Vitamin D Receptor (VDR) have no F region (Robinson-Rechavi and Laudet, 2003). To date, no exact function has been identified for this region.

4.3 Nomenclature

Given the plethora of names available for the same sequence, it has proved useful to construct a nomenclature based on the nuclear receptor sequences using molecular phylogeny.

The Nuclear Receptor Nomenclature Committee has elaborated a system to name NRs based on the nomenclature system that was developed for cytochrome P450 by Nebert et al. (1987). This system has proven to be convenient and flexible, allowing for the inclusion of an ever-increasing number of nuclear receptor genes (Committee, 1999).

Each receptor is described by the letters NR (for "nuclear receptor") and a three digit nomenclature: the subfamily to which a given receptor is indicated by Arabic numbers, groups by capital letters, and individual genes by Arabic numbers.

This system has proven to be flexible enough to integrate nuclear receptors from invertebrates as well as sequences generated from genome projects for which no pharmacological data are yet available.

This nomenclature allows the classification of nuclear receptors based on the sequence identity of the DBD and the LBD with other members of the same family. In mammals they are divided into 6 different sub-families (see table 3).

The first (Group I) family includes receptors of thyroid hormones (Thyroid Receptor Tr), RAR, VDR and PPAR as well as the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR).

The second sub-family (Group II) includes retinoid X receptor (RXR), HNF4, testis receptors (TR2) and receptors involved in eye development (TLX and PNR). It is important to point out the particularly important role of the RXR receptor which heterodimerize with some of the group I receptors.

The third sub-family (Group III) contains steroid receptors such as the androgen receptor (AR), the glucocorticoid receptor (GR) and the estrogen receptor (ER).

	Names	Nomenclature	Ligand
Group I	TRα	NR1A1	Thyroid hormones
	TRβ	NR1A2	Thyroid hormones
	RARα	NR1B1	Retinoic acid
	RARβ	NR1B2	Retinoic acid
	RARγ	NR1B3	Retinoic acid
	PPARα	NR1C1	Fatty acids, leukotriene B_4, fibrates
	PPARβ	NR1C2	Fatty acids
	PPARγ	NR1C3	Fatty acids, prostaglandin J_2, thiazolidinediones
	Rev-erbα	NR1D1	Orphan
	Rev-erbβ	NR1D2	Orphan
	RORα	NR1F1	Cholesterol, cholesteryl sulfate
	RORβ	NR1F2	Retinoic acid
	RORγ	NR1F3	Orphan
	LXRα	NR1H3	Oxysterols, T0901317, GW3965
	LXRβ	NR1H2	Oxysterols, T0901317, GW3965
	FXRα	NR1H4	Bile acids, fexaramine
	FXRβ[a]	NR1H5	Lanosterol
	VDR	NR1I1	Vitamin D, 1,25-dihydroxyvitamin D_3
	PXR	NR1I2	Xenobiotics, 16α-cyanopregnenolone
	CAR	NR1I3	Xenobiotics, phenobarbital
Group II	HNF4α	NR2A1	Orphan
	HNF4γ	NR2A2	Orphan
	RXRα	NR2B1	Retinoic acid
	RXRβ	NR2B2	Retinoic acid
	RXRγ	NR2B3	Retinoic acid
	TR2	NR2C1	Orphan
	TR4	NR2C2	Orphan
	TLL	NR2E2	Orphan
	PNR	NR2E3	Orphan
	COUP-TFI	NR2F1	Orphan
	COUP-TFII	NR2F2	Orphan
	EAR2	NR2F6	Orphan
Group III	ERα	NR3A1	Estradiol-17β, tamoxifen, raloxifene
	ERβ	NR3A2	Estradiol-17β, various synthetic compounds
	ERR α	NR3B1	Orphan
	ERRβ	NR3B2	DES, 4-OH tamoxifen
	ERRγ	NR3B3	DES, 4-OH tamoxifen
	GR	NR3C1	Cortisol, dexamethasone, RU486
	MR	NR3C2	Aldosterone, spirolactone
	PR	NR3C3	Progesterone, medroxyprogesterone acetate, RU486
	AR	NR3C4	Testosterone, flutamide
Group IV	NGFI-B	NR4A1	Orphan
	NURR1	NR4A2	Orphan
	NOR1	NR4A3	Orphan
Group V	SF1	NR5A1	Orphan
	LRH-1	NR5A2	Orphan
Group VI	GCNF	NR6A1	Orphan
Group 0	DAX-1	NR0B1	Orphan
	SHP	NR0B2	Orphan

Table 3: different sub-families of human nuclear receptors (adapted from Germain et al., 2006).

The receptors that contain only one of the two functional domains (such as DAX/SHP which lack LBD domain) are artificially clustered in subfamily 0.

The number of NRs identified in sequenced genomes varies considerably. To date 48 members have been identified in humans (Robinson-Rechavi et al., 2001), 21 in the genome of the fruit fly *Drosophila melanogaster* (Adams et al., 2000), over 270 are found in *Caenorhabditis elegans* (Sluder and Maina, 2001), 49 in the mouse and 47 in the rat genome (Zhang et al., 2004).

4.4 Evolution

The structural diversity of the ligands contrasts with the conservation and mode of action of their receptors. This and the large number of orphan receptors for which no ligand have been identified, have prompted much speculation on the origins of the signaling pathway.

Two main theories have been proposed: one says that orphan receptors have evolved as liganded molecules, which through gene duplication reached the present diversity. According to this model, orphan receptors would have lost their ability to bind ligands recently in evolution (Escriva et al., 2000).

The other theory supported by Escriva and colleagues suggest an alternative hypothesis in which ligand binding was acquired during NRs evolution (Escriva et al., 1997). As the presence of NRs for structurally different ligands within the same sub-family, together with the widespread distribution of orphan receptors in the phylogenetic tree, show no correlation between the evolutionary relationships of NRs and the nature of their ligands, this situation suggests independent gain of ligand binding capacity during nuclear receptor evolution (Laudet and Bonneton, 2005).

In their review of ligand binding evolution, Escriva and colleagues suggest that the first NR was an orphan receptor, whose descendants have secondarily, and several times, independently gained the ability to bind a ligand. Based on this theory and considering the known ligands and their respective affinities for their different NRs, they differentiate two types of ligand-binding acquisition: on the one hand, an ancestral orphan receptor recognizes a specific ligand with high affinity and keep that ligand specificity during evolution and on the other hand, an ancestral receptor that recognizes different types of ligands with low affinity and then either specialize for one ligand specificity or keep the ancestral status of low affinity for different related ligands (Escriva et al., 2000).

Taken together, their observations propose that nuclear receptors gained ligand-binding capacity during evolution from an original orphan receptor.

4.5 NR binding and response elements

Nuclear receptors regulate transcription by binding to specific DNA sequences in target genes known as hormone response elements (or HREs). These elements are located in regulatory sequences normally present in the 5-flanking region of the target gene. Although often the HREs are found relatively close to the core promoter, in some cases they are present in enhancer regions several kilobases upstream of the transcriptional initiation site (Aranda and Pascual, 2001). The analysis of a large number of HRE has revealed that 6bp sequence constitutes the core motif that is recognised by nuclear receptors: 6'Pu-GGTCA (Escriva et al., 2000). However there is a broad variety of different RE that have been generated throughout evolution and that are selective for a given class of receptors (Laudet and Bonneton, 2005).

Two consensus motifs have been identified: steroid hormone receptors typically bind to palindromes of the AGAACA sequence separated by three nucleotides, while the estrogen receptors recognize the consensus AGGTCA motif with the same configuration (Giguere, 1999).

These HREs directly reflect the mode of receptor binding, which can be as heterodimers, homodimers, or monomers (Mangelsdorf and Evans, 1995). Most receptors bind as homo- or heterodimers to HREs composed typically of two core hexameric motifs. For dimeric HREs, the half-sites can be configured as palindromes (Pal), inverted palindromes (IPs), everted or direct repeats (Ers or DRs). In contrast to steroid receptors that almost exclusively recognize palindromic elements, nonsteroidal receptors can bind to HREs with different configurations (Aranda and Pascual, 2001). For monomeric HREs, a single half-site is preceded by a 5-flanking A/T-rich sequence (Giguere, 1999). At present, only orphan receptors are known to bind DNA with high affinity as monomers (Escriva et al., 2000) (figure 16).

In the case of heterodimers, the RXR is the promiscuous partner for different receptors. A number of receptors including retinoic acid receptors (RARs), thyroid hormone receptors (TRs) and some orphan receptors heterodimerize with RXR and bind to direct repeats (DR) of the core motif (Aranda and Pascual, 2001). Heterodimerisation among nuclear receptor

superfamily members allows for the fine- tuning of nuclear receptor action by using combinatorial sets of ligands. In this way, RXR ligands can be used to enhance various signaling pathways as demonstrated by the ability of RXR agonists to synergize with RAR ligands, including some RAR antagonists (Bourguet et al., 2000).

Regarding the phylogenetic relationships between NRs, there is a good correlation of the nuclear receptors-HRE binding characteristics with the evolutionary history. For example, all members of the subfamily II are able to form homodimers on direct repeat sequences, whereas all members of subfamily III can form homodimers on palindromic sequences (Laudet and Bonneton, 2005).

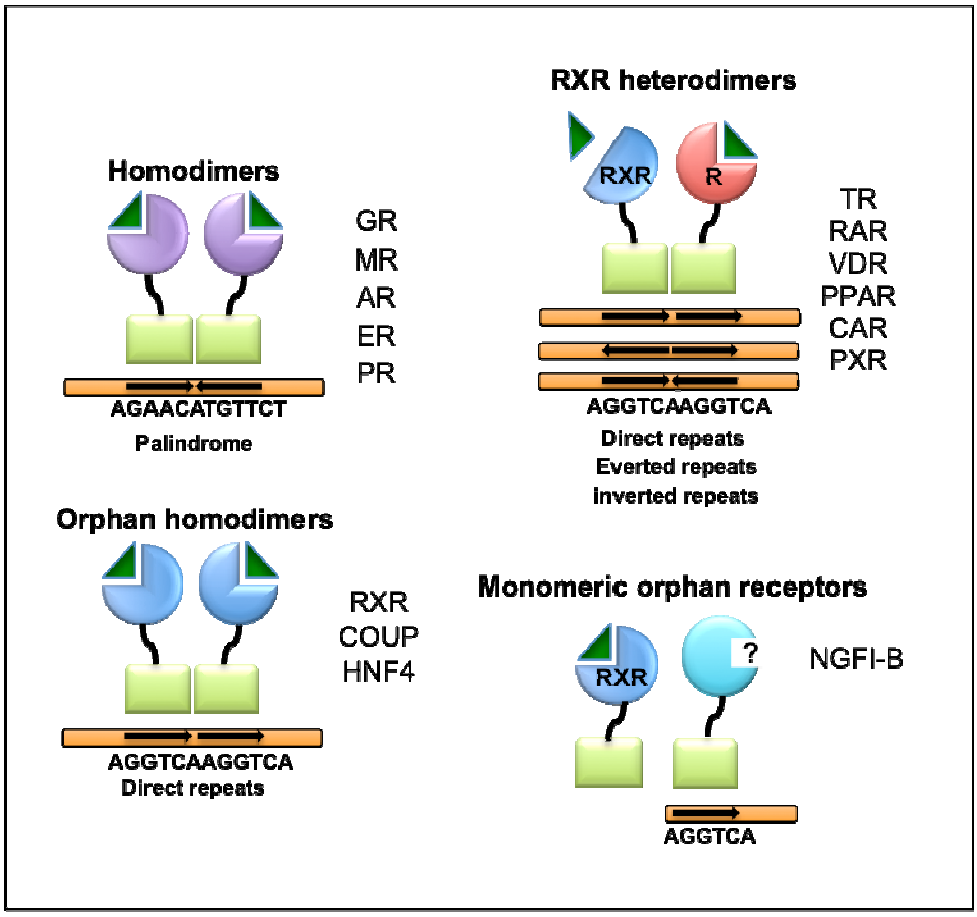


Figure 16: The different types of receptor binding to their response elements. Nuclear receptors can be grouped into 4 classes according to their ligand binding, DNA binding and dimerisation properties. Receptors can bind DNA as homodimers, such as steroid receptors, heterodimers with RXR partner, or monodimers. Homodimers recognize palindromic sequences or direct repeat sequences in the case of orphan homodimers. Heterodimers recognize different HRE arranged as direct, everted or inverted repeats. Monomers recognize their half-site sequence when it is preceded by a 5'-A/T rich sequence.

4.6 Nuclear receptors and P450s regulation: example of CAR and PXR

It is now well established that the regulation of P450s is mainly transcriptional and involves the activation of nuclear receptors. Indeed, several orphan receptors respond to xenobiotics in the environment that includes foreign chemicals such as environmental pollutants and prescription drugs. In response to xenobiotic compounds, these receptors mediate transcription of a variety of detoxifying enzymes that are members of the supergene family of cytochrome P450 molecules (Olefsky, 2001).

Progress in understanding the molecular mechanism of induction of P450 enzymes was made recently when the important roles of the pregnane X receptor (PXR) (Kliewer et al., 1998) and the constitutive androstane receptor (CAR) (Honkakoski et al., 1998) were discovered. Both of these receptors belong to the nuclear receptor superfamily and have been extensively studied for the last decade. They are known to bind an extremely diverse array of molecules (figure 17) including endogenous compounds such as steroids and bile acids. However, these two related receptors are most commonly considered to respond to a wide range of potentially toxic foreign compounds, or xenobiotics, and act as sensors for these lipophilic xenobiotics, hence their name as xenosensors (Moore et al., 2006).

PXR was first identified by the screening of an EST database derived from a liver cDNA library by Kliewer and his colleagues in 1998. Using a Gal4-PXR chimeric protein to perform an initial search for PXR activators, they found that synthetic pregnanes (C21 steroids) and both glucocorticoid agonists and antagonists were potent inducers of PXR activity. Based on the finding that PXR is best activated by pregnenolone and its derivatives, they proposed that the natural ligand for PXR is likely to be a pregnane; hence, the name pregnane X receptor (PXR). PXR was originally shown to regulate the expression of CYP3A isozymes by binding as a heterodimer with the 9-cis retinoic acid receptor RXR (NR2B1) to xenobiotic response elements located in the regulatory regions of these genes (Kliewer et al., 1998; Lehmann et al., 1998; Maglich et al., 2002).

CAR was originally identified through screening of a cDNA library with a degenerate oligonucleotide based on a conserved region of the nuclear receptor DBDs. The search for a ligand revealed that CAR is, in fact, a steroid receptor for androstenol and androstanol and

was therefore named Constitutive Androstane Receptor (CAR) (Giguere, 1999). Like PXR, CAR regulates the expression of target genes by binding to xenobiotic response elements as a heterodimer with 9-cis retinoic acid receptor and was originally demonstrated to regulate CYP2B gene expression (Honkakoski et al., 1998). The CYP2Bs are of special interest with regard to CAR due to the identification of phernobarbital-responsive elements (PBREM) in the 5′ regions of CYP2B genes followed by the elucidation of CAR as the receptor that is activated following exposure to phenobarbital (Sueyoshi et al., 1999).

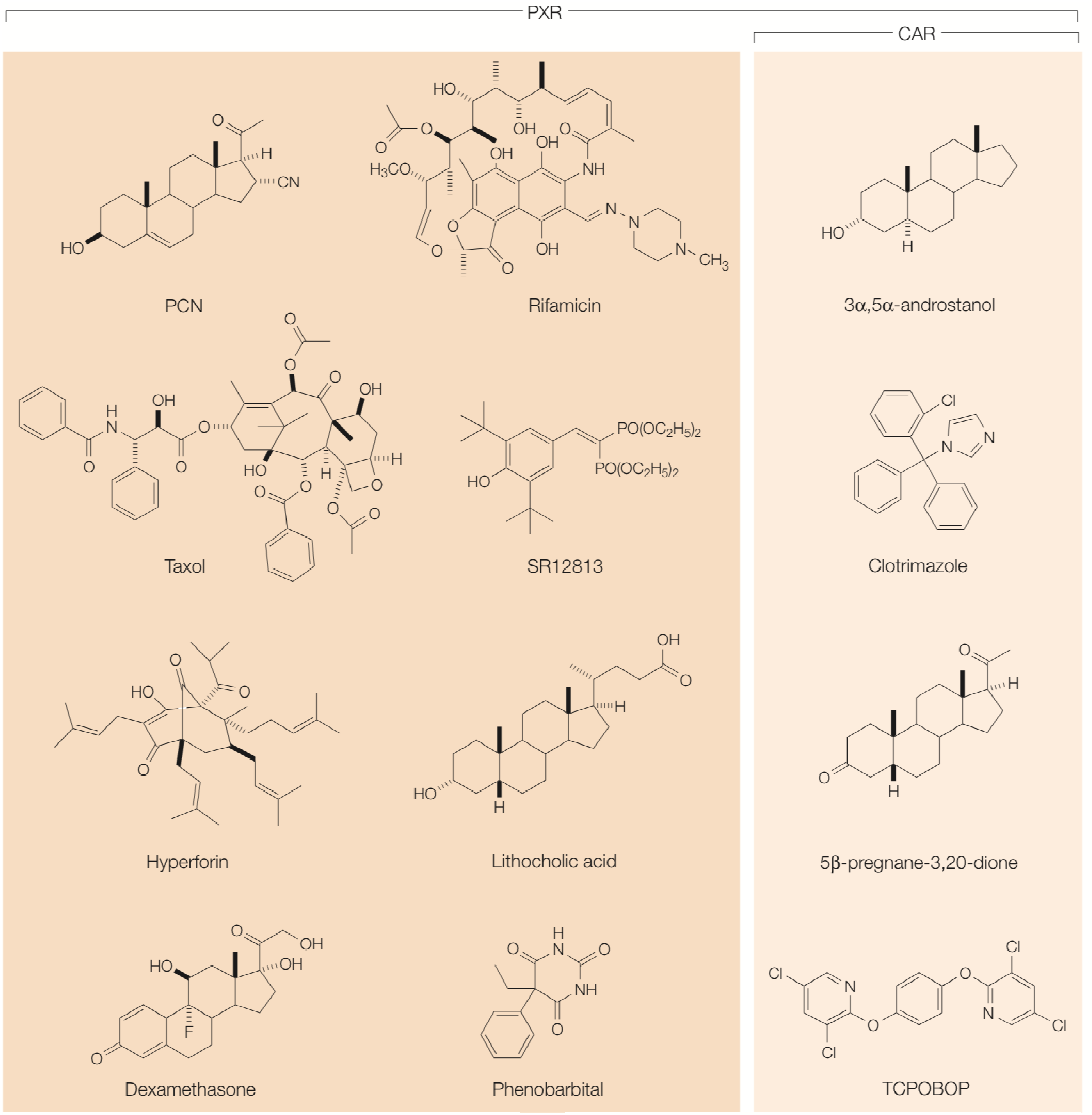


Figure 17 : PXR and CAR ligands**.** Examples of PXR ligands (dark and pale orange) and CAR ligands (pale orange only). Many CAR ligands are also PXR ligands (from Willson and Kliewer, 2002).

Since then, both receptors have been extensively studied and are shown to regulate different P450s mainly from the CYP3A and CYP2B families as well as other family members such as CYP1A1 and CYP1A2 for PXR (Maglich et al., 2002) and CYP24 for CAR (Moreau et al., 2007). In addition, they have been shown to regulate the expression of other genes involved in the detoxification machinery, such as GSTs or UDP-glucuronyltransferases (UGTs) (Maglich et al., 2002). A good review of the different genes regulated by CAR and PXR can be found in Honkakoski and Negishi (2000), Handschin and Meyer (2003) and Willson and Kliewer (2002).

PXR and CAR show overlapping but distinct control of the phase II and phase III detoxification pathways. Overall, since CAR and PXR are activated by some of the same ligands and induce specific but overlapping sets of genes, they provide the cell with two overlapping and semi- redundant mechanisms for recognizing and eliminating toxicants (Kretschmer and Baldwin, 2005).

CAR and PXR differ in their cellular localisation and transactivation mechanisms as PXR is exclusively nuclear, when CAR is found in the cytosol in its inactive state and one crucial step in its activation is its translocalisation into the nucleus upon the effect of activators such as phenobarbital (Kawamoto et al., 1999). There is however recent reports suggesting a cytosolic localisation of PXR as well, which then undergoes the same nuclear translocalisation as CAR when activated (Timsit and Negishi, 2007).

CAR and PXR bind as heterodimeric complexes with the RXR receptor to response elements in the regulatory regions of the induced genes (see figure 18 for an overview of CAR and PXR activation). These response elements have been identified as core motifs named Phenobarbital Response Element Modules (PBREM) for CAR and Xenobiotic Response Element Modules (XREM) for PXR (Timsit and Negishi, 2007), which contain direct or everted repeats of hexanucleotides. A review of these different response element motifs can be found in Sueyoshi and Negishi (2001).

Although CAR and PXR response elements or DNA motifs in the regulatory regions of these genes are distinct—it has now been established that CAR can activate CYP3A genes via PXR response elements and that PXR can regulate CYP2B genes via the CAR or phenobarbital response element (Nebert and Russell, 2002).

This crosstalk between CAR and PXR involving overlapping set of induced genes as well as reciprocal recognition of the same response elements has resulted from an adaptative advantage for living organisms to increase their ability to detect and respond to a wide variety of xenobiotics (Pascussi et al., 2008).

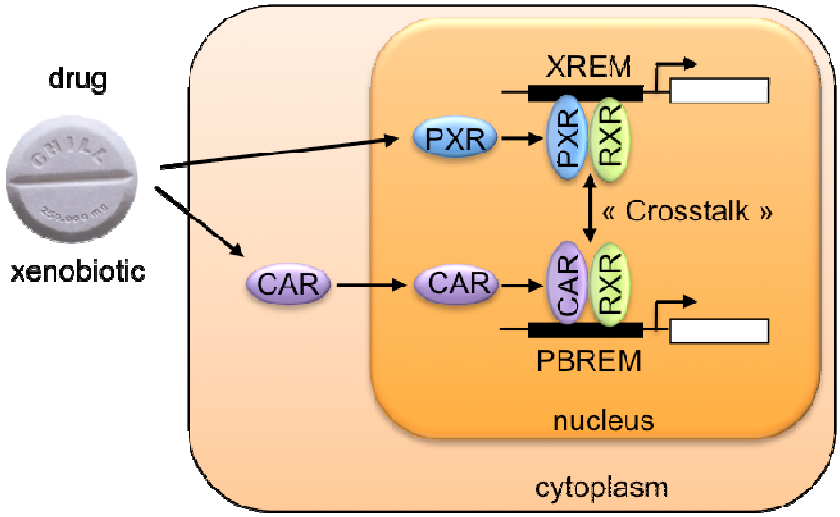


Figure 18 : Activation of the mammalian xenosensors PXR and CAR. After entering the cell, xenobiotics trigger cytoplasmic-nuclear translocation of CAR by and directly activate PXR in the nucleus. Subsequently, both PXR and CAR heterodimerize with RXR, bind to their respective response elements, and increase transcription of target genes. (from Handschin and Meyer, 2003)

The regulation of detoxification genes such as P450s in vertebrates is thus well established, involving a central role of CAR and PXR nuclear receptors. In insects however, the mechanisms that link the ingestion of the toxin and the expression of detoxification enzymes has received much less attention (Gatehouse, 2002). AhR-like mechanisms have been reported in some species as it was discussed in section 3.4, however other nuclear receptor-based mechanisms might be involved as it was recently suggested and will be discussed in the following chapter.

5. Nuclear receptors in insects

As it was discussed earlier, nuclear receptors have been identified in all metazoans, and regulate very diverse and essential functions such as reproduction, development and metabolism (Escriva et al., 2000). In insects, nuclear receptors are involved in similar important mechanisms as it will be discussed here, with an emphasis on metamorphosis regulated by 20-hydroxyecdysone (20E) and Juvenile Hormone (JH) and the detoxification of xenobiotics.

To date, 21 NRs have been identified in the fruit fly *Drosophila melanogaster* (Adams et al., 2000), 20 in the malaria mosquito *Anopheles gambiae* (Holt et al., 2002), 22 in the honey bee *Apis mellifera* (Velarde et al., 2006), 21 in the silkworm *Bombyx mori* genome (Cheng et al., 2008) 20 in the yellow fever mosquito *Aedes aegypti* (Cruz et al., 2009) and 21 in the red flour beetle *Tribolium castaneum* (Bonneton et al., 2008; Tan and Palli, 2008). Insect NRs have representatives in all 6 sub-families of NRs identified in vertebrates (see table 3) and are represented in figure 19.

The first sub-family of insect NRs (NR1) includes five receptors that are all directly involved into the ecdysteroid pathway of *Drosophila melanogaster*, such as EcR, which will be discussed later in this chapter. It also includes the only ortholog of mammalian CAR and PXR, DHR96. This receptor has been shown to be induced by 20E and is expressed during the onset of metamorphosis (Fisk and Thummel, 1995) and as its orthology with vertebrate xenosensors suggests, has also been shown to respond to xenobiotics (King-Jones et al., 2006). DHR96 functions will be reviewed later in this chapter.

The second family (NR2) contains the receptor Ultraspiracle (USP), which is the ortholog of vertebrate RXR and heterodimerize with EcR to form a fully functional ecdysone (20E) receptor (Yao et al., 1993; Yao et al., 1992). The requirement of heterodimerisation of EcR and USP has been found in all the species studied and it seems therefore that it forms the functional ecdysone receptor for all athropods (Laudet and Bonneton, 2005).

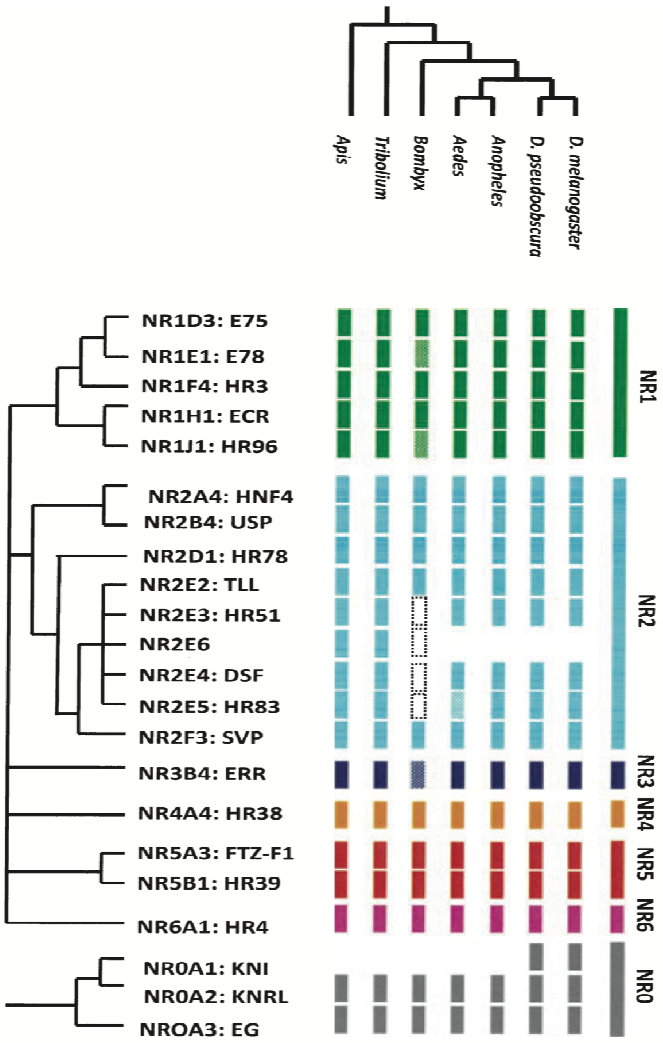


Figure 19: classification of identified insect nuclear receptors (from Bonneton et al., 2008; Nakagawa and Henrich, 2009)

The four remaining sub-families of insect NRs contain several nuclear receptros such as steroid receptors (e.g., ERR), an alternative binding partner for USP, DHR38, which plays a role in the ecdysone response (Sutherland et al., 1995), the best known and conserved FTZ-F1 receptor involved in embryogenesis and metamorphosis and atypical receptors from the NR0 sub-family, which lacks one of the two DBD or LBD.

5.1. The Ecdysone (20E) receptor: EcR/USP

Holometabolous insects have a typical development cycle that goes through distinct morphological changes from successive larval instars moults to pupation and metamorphosis into the adult form. The growth and development from one stage to another is regulated by two main hormones (see figure 12), the steroidal insect moulting hormone, 20-hydroxyecdysone (figure 20), and the sesquiterpenoid Juvenile Hormone (JH), of which there are at least six types (figure 21).

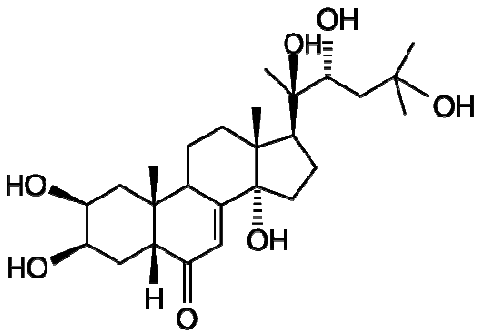


Figure 20: Chemical structure of the 20-hydroxyecdysone (from Dhadialla et al., 2005)

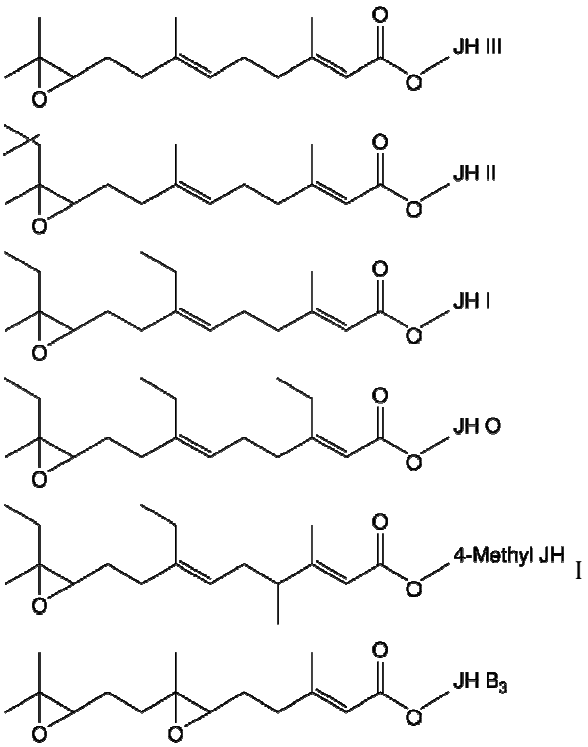


Figure 21: Chemical structures of naturally occurring JHs. JHIII is the major JH in most insects when JHI and JHII are the principal ones in Lepidoptera. (from Dhadialla et al., 2005)

Although the mode of action of JH is still not fully understood, 20E signal transduction is well understood (Riddiford et al., 2001; Thummel, 2002). Steroid hormones exert their effects on target tissues by activating their respective receptors, which are members of the nuclear receptor superfamily (Mangelsdorf et al., 1995). The Drosophila ecdysteroid receptor is a heterodimer of two such proteins: EcR (Ecdysone receptor, NR1H1) and the fly retinoid X receptor (RXR) homolog, USP (Ultraspiracle, NR2B4). EcR was first identified in Drosophila nearly 25 years after the structural identification of molting hormones (Koelle et al., 1991), along with USP (Oro et al., 1990). In *Drosophila melanogaster*, the EcR gene encodes three different isoforms, EcRA, EcRB1 and EcRB2 that contain unique amino termini but a common carboxy-terminal region including the characteristic DBD and LBD and the expression of these isoforms correlates with distinct cell- and tissue-specific responses to ecdysone during metamorphosis (Talbot et al., 1993). Although Drosophila has only one USP isoform, two different isoforms have been identified in several insect species that also show different patterns of induction related to the developmental stage. For example, *Manduca sexta* USP-1 is expressed during the intermoults periods whereas USP-2 is up-regulated at times of high ecdysteroid titer during the larval moult, when USP-1 dissapears (Jindra et al., 1997). In *Bombyx mori*, USP2 appeared also to be coordinate with the pulse of ecdysone during metamorphosis, and it was suggested that this isoform of USP might be the actual component of the EcR/USP complex (Cheng et al., 2008).

EcR/USP bind to various ecdysone response elements (EcREs) as a heterodimer to transactivate several target genes (Yao et al., 1993). The hormone-receptor complex directly induces a set of « early genes » including the Broad complex (BR-C) and E74 as well as early puff genes (King-Jones and Thummel, 2005; Sullivan and Thummel, 2003; Thummel, 2002). These genes encode transcription factors that transduce and amplify the ecdysone signal, regulating the expression of large batteries of downstream « late genes » leading to the appropriate stage- and tissue-specific biological responses such as tissue differentiation and cell death (Thummel, 2002).

The mode of action of 20E through the activation of EcR/USP complex has thus been well established. In addition, it has been found that USP could also heterodimerize with another nuclear receptor, DHR38, and interfering with the EcR pathway, suggesting that other receptor complex might be involved in the ecdysone response as well as the possibility of

USP to bind with alternative receptors (Sutherland et al., 1995). USP has also been suggested as the JH receptor for which the mode of action still remains to be elucidated.

The idea that USP could be the receptor of juvenile hormone (JH) or any of its derivatives is attractive because JH might directly modulate the activity of the EcR ⁄ USP complex (Nakagawa and Henrich, 2009). The docking model of JH to USP-LBD, proposed by Sasorith *et al*., suggested the plausibility of JH binding within the LBD of USP in *Heliothis* virescens (Sasorith et al., 2002). However, the percentage of occupancy of the LBP by these ligands was shown to lie in the bottom range of values for classical nuclear receptors. Even though JH can bind to the USP and stimulate oligomerization of the USP *in vitro* (Jones and Sharp, 1997), further experimentation will be required to establish the function of JH as a ligand for USP *in vivo*. In particular, the Kd for the binding of JH to the Drosophila USP is rather high (approximately 500 nm) (Jones and Sharp, 1997), whereas the typical Kd values for the binding between hormones and NRs are often very low (in the nanomolar range). Further studies conformed the micromolar range of affinity constant of JHIII with USP (Jones et al., 2006) and questions are still raised about the identification of JH receptor.

Recent work has suggested the role of Met (Methoprene tolerant) protein as a potential JH receptor. This protein was cloned and found to be an ARNT-like member of the bHLH-PAS protein family involved in the AhR pathway (see section 3.4) (Ashok et al., 1998) and was found to bind JH with high affinity (Miura et al., 2005). However, the major contention to the hypothesis that Met could be the JH receptor in that the Met mutants are viable in *Drosophila melanogaster* and recent reporter assay using two hybrid system failed in showing an increase of reporter gene activity when JH analog methoprene was added as a ligand for Met homodimers and Met:EcR and Met:USP heterodimers (Bitra and Palli, 2009). The identification of Met as the JH receptor needs therefore further research.

Insects are therefore able to achieve a fine-tuning of their development cycle through high specific signal transduction involving nuclear receptors. As it was discussed earlier, insects are also able to detoxify xenobiotic compounds that they may encounter in their environment thanks to the expression of detoxification enzymes such as P450s. However, little is known about the mechanisms linking the ingestion of the toxin and the expression of the detoxification enzymes. Recent work has nonetheless suggested similar nuclear receptor

pathways than those for vertebrate CAR and PXR with the identification of the Drosophila receptor, DHR96 as it will be discussed in the next chapter.

5.2 Insect nuclear receptor and xenobiotic metabolism: DHR96

In 1995, Fisk and Thummel have cloned and identify new members of the nuclear hormone receptors that are expressed during the onset of metamorphosis in *Drosophila melanogaster*. They isolated three new receptor genes, including one called DHR96. Sequence analysis showed that the DBD of DHR96 was 64% identical to the human VDR DBD and 52% to EcR (Fisk and Thummel, 1995).

They found that DHR96 encodes two transcripts of 2.8 and 0.6kb that are both expressed throughout third larval instars and prepupal development. As a subset of 20E regulated genes are coordinately induced at this time, they also checked for DHR96 induction by 20E and found that expression of DHR96 was indeed induced by the presence of 20E.

Finally, they found that DHR96 was able to recognize a unique response element, Hsp27-EcRE, which is also recognised by the EcR/USP receptor. Therefore they suggested that DHR96 might be involved in 20E response and compete for the binding site of ecdysone receptor but still no ligand was identified for this orphan receptor.

In 2000, Baker and colleagues studied the transactivation of EcR and the three previously identified receptors by 37 different insect and plant ecdysteroids and juvenoids. They used Gal4 chimeras in which the Gal4 DBD is fused to the LBD of the studied receptor, including DHR96. They found that the measured level of transcript did not exceed those that were treated with vehicle alone, failing to identify a potential ecdysteroid-derived ligand for DHR96 (Baker et al., 2000) .

No mutations have been yet reported in DHR96. However, its close phylogenetic relationship to other ligand-regulated members of the sub-family 1, such as EcR, VDR, CAR and PXR (see figure 22) indicates that this receptor might be regulated by a ligand (Escriva et al., 2000; Laudet and Bonneton, 2005).

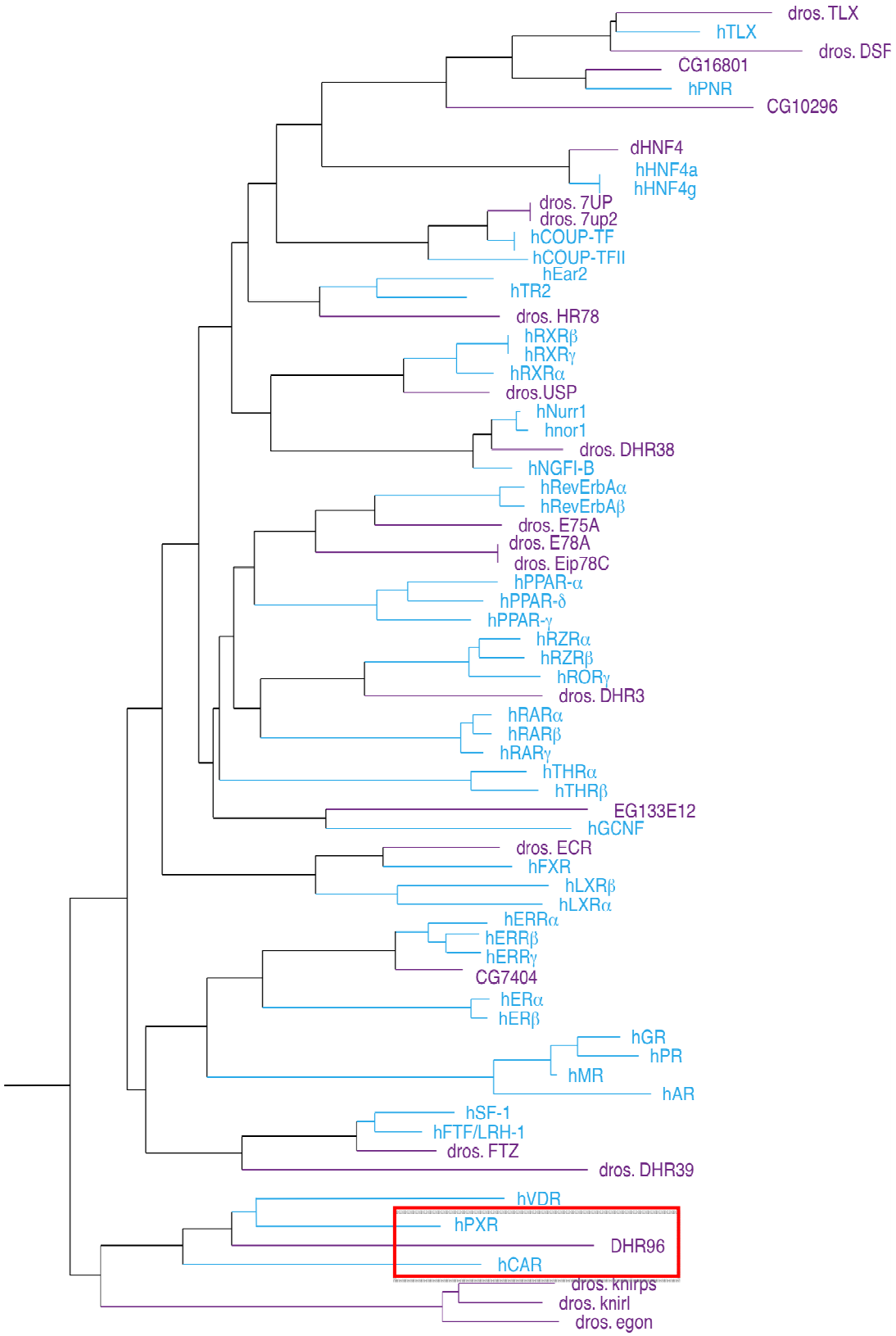


Figure 22: Phylogenetic tree representing human (blue) and drosophila (purple) nuclear receptor, with the particular cluster of genes coding for DHR96 and its closest human orthologs: CAR, PXR and VDR (from Enmark and Gustafsson, 2001)

DHR96 is represented by an orthologous group of 3 genes in *C. elegans*: nrh-8, nhr-48 and daf-12. Nhr-8 mutant are sensitive to chloroquine indicating a role in xenobiotic pathway (Lindblom et al., 2001), whereas daf-12 controls entry into diapause by monitoring dietary sterol levels (Antebi et al., 2000). The function of DHR96 therefore indicates two non-exclusive models: as an insect xenobiotic sensor that control detoxification pathways or as a sterol sensor (King-Jones and Thummel, 2005).

As it is shown in figure 23, DHR96 is found clustered with both human CAR and PXR, for which xenosensing functions have been well established (see section 4.6). Such a function can therefore be assumed for DHR96. Palanker *et al*. have tested this hypothesis in drosophila embryos expressing GAL4-DHR96 treated with the PXR-selective agonist PCN and the CAR- selective agonists TCPOBOP and CITCO. They found that only CITCO gave reproducible, strong activation of the DHR96 ligand sensor, indicating that the activation status of the DHR96 LBD can be regulated by xenobiotic compounds in a manner similar to that of its vertebrate orthologs CAR (Palanker et al., 2006) .

The same year, King-Jones and colleagues confirmed the xenosensing function of DHR96 and found that this receptor was involved in phenobarbital (PB)-regulated genes expression. They used DHR96 null mutant flies and found that although DHR96 mutant are viable and fertile, they show nonetheless an increased sensitivity to the sedative effect of PB and lower survival to chronic exposure to high doses of DDT. By using microarray analysis, they compared the pattern of gene expression in PB-treated wild type and DHR96 mutant flies and found that a large portion of genes were affected. However, some PB-responsive genes were superinduced in DHR96 mutant, including many detoxification genes such as P450s and the majority of PB-responsive genes were unaffected by the mutation. Overall, their study showed that DHR96 was involved into xenobiotic metabolism but also suggest that given the massive PB response in Drosophila, it is likely that additional nuclear receptors might feed into this pathway, such as bHLH-PAS proteins involved in the AhR pathway (King-Jones et al., 2006).

HR96 orthologs have been further identified in a small number of other insect species such as *Tribolium castaneum*, where RNA interference studies showed that injection of dsRNA targeted against TcHR96 in larvae had no effects on adult formation and female injected

with the same dsRNA produced viable offspring, suggesting that HR96 is not critical for molting and metamorphosis (Tan and Palli, 2008). Cheng *et al.* have also cloned a HR96 ortholog in *Bombyx mori* and found that it was co-ordinately expressed with USP-2 in this insect (Cheng et al., 2008). DHR96 was also found to be expressed through different developmental stages along with USP in Drosophila (Sullivan and Thummel, 2003), which in addition to the confirmation of Fisk's results showing the expression of DHR96 during the onset of metamorphosis, also points out an interesting feature of DHR96 and USP. Indeed, DHR96 belongs to the NR1 sub-family, which contains the ecdysone receptor EcR. This receptor is known to heterodimerize with USP, the insect ortholog of RXR (see section 5.1). In human, similar binding occurs between CAR and PXR and RXR (see section 4.6). We can therefore suggest that USP might also be a heterodimer partner for HR96. However, such a hypothesis has not yet been tested and molecular action of HR96 still remains to be elucidated.

The most recent work on DHR96 has revealed that this receptor could act as a sterol sensor as its ortholog LXR and as it was suggested in *C. elegans*. Horner *et al.* found that DHR96 is able to bind cholesterol and is required for the coordinate transcriptional response of genes that are regulated by cholesterol and involved in cholesterol up- take, trafficking, and Storage. However, they did not fully identify cholesterol as a ligand for DHR96 as they were unable to detect changes in DHR96 activity in response to exogenous cholesterol and dietary factors (Horner et al., 2009). In another study, they found that DHR96 is indeed indispensable for mediating the transcriptional response to dietary cholesterol and that it acts as a key regulator of genes involved in cholesterol metabolism (Bujold et al.).

HR96 seems therefore to exhibit two non-exclusive functions as a xenobiotic sensor and in the cholesterol metabolism. However, ligands for this receptor are still to be identified and potential heterodimer partners to be characterised. Moreover, the role of HR96 in the induction of detoxification genes such as P450s, as it has been demonstrated for CAR and PXR in mammals, has not yet been reported in insects. It would therefore be particularly interesting to characterise the role of HR96 as an insect xenosensors, which could bind plant allelochemicals and xenobiotic compounds and subsequently induce the expression of P450s. This is one of the objectives of this thesis, as it will be detailed below.

OBJECTIVES

The overall objective of this work is to study xenobiotic detoxification mechanisms in the polyphagous lepidopteran pest, *Spodoptera frugiperda* through the identification of the signaling mechanisms that link the ingestion of the toxin to the expression of detoxification enzymes as depicted in figure 23.

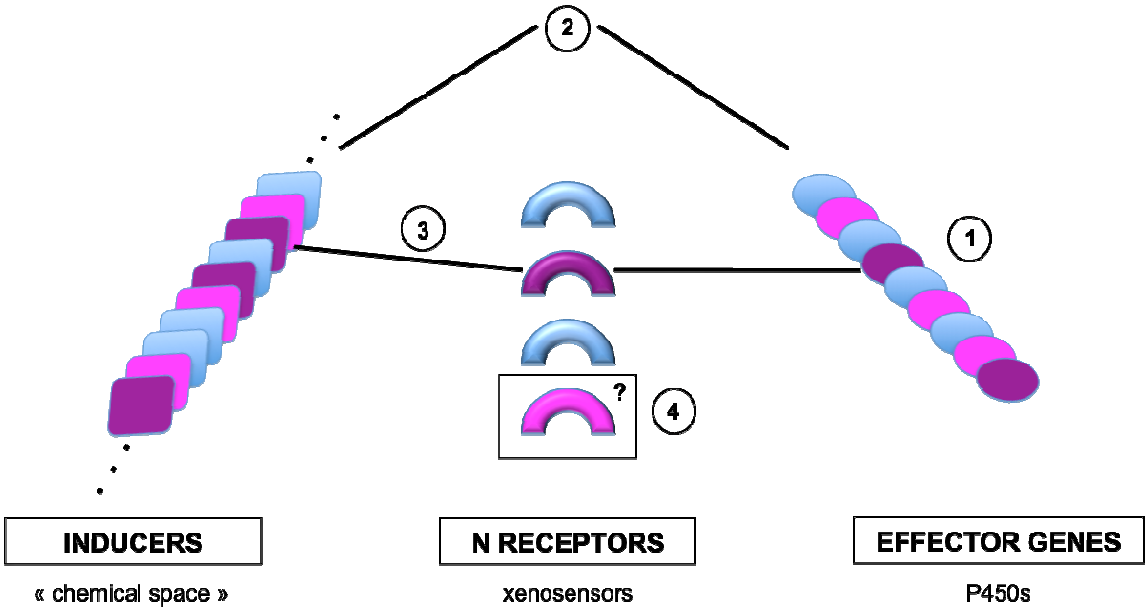


Figure 23: the combinatorial problem in insect detoxification mechanism. The chemical space represents a virtually infinite numbers of molecules, from which a small fraction such as insecticides and plant allelochemicals can bind to n nuclear receptors. The number of receptors is limited by the number of known receptors for fully sequenced and annotated insect genomes. These receptors after binding of the xenobiotic will induce typical effectors genes such as P450s for which the number is also limited by the number of their encoded genes present in the insect genome.

This will be done by 1) looking at the regulation of P450s expression in the model insect, *Drosophila melanogaster,* 2) extend this study to the patterns of gene expression and P450s in particular in *S. frugiperda* in response to xenobiotics, 3) studying one of the well characterised receptor-based signaling mechanisms involving EcR and USP in response to hormone agonists and 4) identify potential xenosensors in insects that are able to induce detoxification genes in response to a subset of inducing compounds.

1. In the first chapter, we will present our work on the model species *Drosophila melanogaster* where we have looked at the expression of the specific CYP6A2 cytochrome in response to various natural and synthetic compounds. We will compare this response to the CYP2 family of vertebrate and to the "CYPome" expression data that are available in the literature.

2. In the second chapter, we will present our results on the identification of patterns of gene induction in *S. frugiperda* midgut and in Sf9 cells exposed to different class of chemicals by a genome-wide analysis using a 10k genes microarray and quantitative analysis of P450 gene expression. By choosing a generalist herbivore as in insect model, we maximise our chance to cover many potential detoxification mechanisms. Indeed, in contrast to specialists, polyphagous, generalist herbivores face a more diverse array of chemistries that is directly proportional to the number of different host plants they feed on. Hence these insects have more complex P450-based detoxification mechanisms (Gatehouse, 2002). *S. frugiperda* is a major pest in agriculture in America and has been reported to feed on hosts from >25 plant families, making an excellent model organism to study the molecular mechanisms involved in the plant-insect interactions. Moreover, Sf9 cells derived from ovarian tissues of *S. frugiperda* represent a well-established cellular model that is used widely in thousands of studies, mainly as a heterologous expression system. Our approach will therefore allow a better understanding of *in vivo* mechanisms involved in insect response as well as the study of molecular mechanisms at a finer scale in an *in vitro* environment where we can control many factors. In order to cover as many potential detoxification mechanisms as possible, we will test the response of *S. frugiperda* to different classes of xenobiotic compounds that are presented in figure 24.

Methoprene and Methoxyfenozide are respectively a juvenile hormone analog and an ecdysone agonist. Deltamethrin is a commonly used pyrethrinoid and fipronil is an insecticide that targets GABA receptors. Quercetin is a flavonoid that is encountered in many Lepidoptera host plant species. Indole is a maize defensive compound and indole 3-carbinol a glucobracissin derivative. Xanthotoxin (or 8-methoxypsoralen) is a coumarin derivative that is a potent inducer in many insect species and 2-tridecanone is a secondary compound found in the trichome of wild tomatoes. Finally, the herbicide clofibrate and the barbiturate phenobarbital will be used as model inducers of P450s.

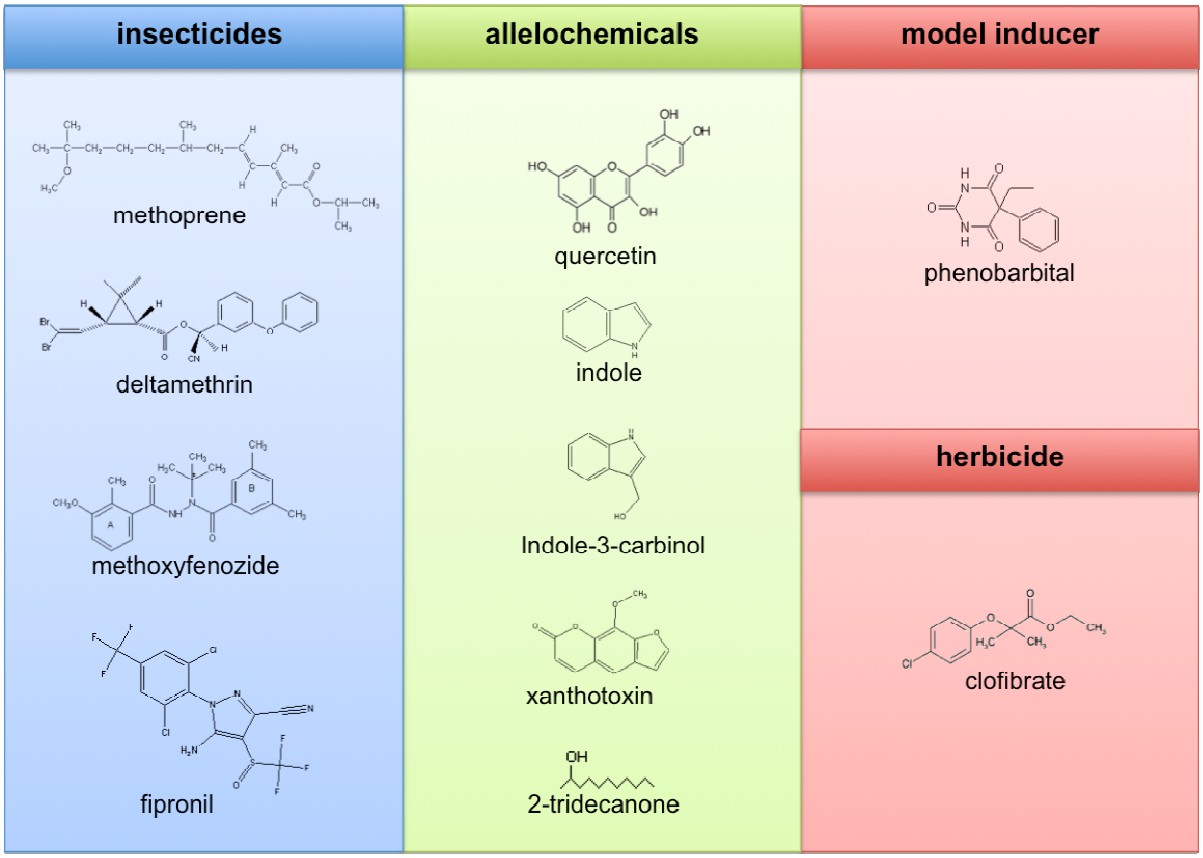


Figure 24: chemical structures of the 11 xenobiotic compounds used in *S. frugiperda* induction study.

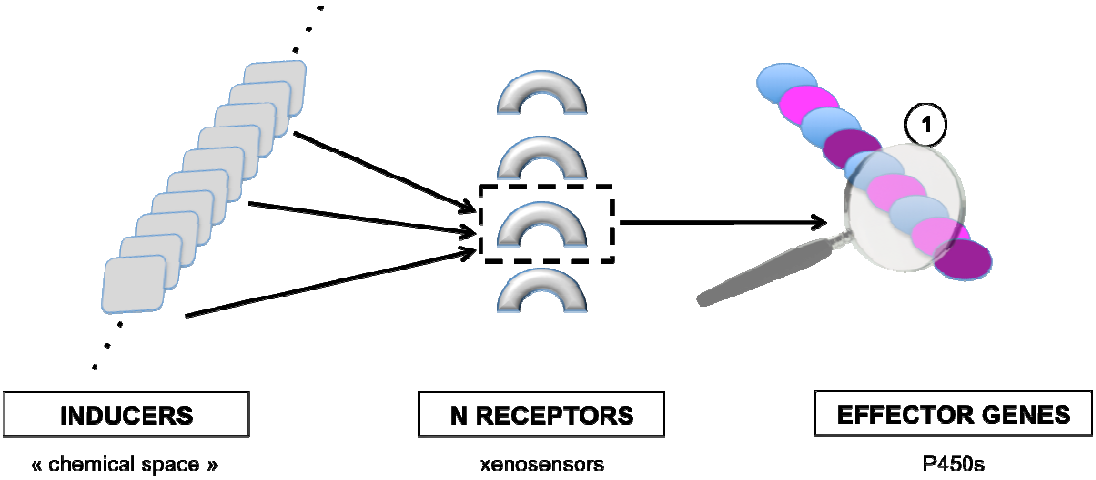
3. In the third chapter, we will present our work on the two most characterised nuclear hormone receptors that are essential for insect development and growth, EcR and USP. We will discuss our results on cellular and molecular effects of two hormone agonists, methoprene and methoxyfenozide, in *S. frugiperda* cells.

4. In the last chapter, we will present our results on the characterization of one specific nuclear receptor as a potential xenosensors in *S. frugiperda*, SfHR96 the ortholog of DHR96 in Drosophila.

CHAPTER 1

Article: Regulation of cytochrome P450 in Drosophila : genomic insights.

In press in *Pesticide Biochemistry and Physiology*



As a first step in the study of detoxification mechanisms in insects, we have chosen to identify gene expression patterns of detoxification enzymes in the model organism, *Drosophila melanogaster*.

Because of its historical importance, large research community, and powerful research tools, as well as its modest genome size (~180 Mb), *Drosophila* was chosen as a model organism in 1990 under the auspices of the Human Genome Project (Adams et al., 2000). The genomic tools available in *Drosophila* now allow both the detailed study of single genes and global approaches on the whole family of the 90 identified P450s in this insect (Tijet et al., 2001). The function of some of these P450s in xenobiotic response of insects has been identified. CYP6A2 for example was one of the first to be characterised as inducible by phenobarbital, over-expressed in resistant strains of *Drosophila* (Brun et al., 1996) and able to metabolize insecticide compounds such as aldrin, heptachlor and diazinon (Dunkov et al., 1997).

Here we focus on one effector (target) gene, *Drosophila* CYP6A2, and we analyze the literature for inducers of this gene, and show that its pattern of induction is similar to that of mammalian CYP2 genes that are induced by PXR/CAR nuclear receptors. Our results are presented below.

ARTICLE IN PRESS

Pesticide Biochemistry and Physiology xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Regulation of cytochrome P450 expression in *Drosophila*: Genomic insights

Maeva Giraudo [a], G. Chandran Unnithan [b], Gaëlle Le Goff [a], René Feyereisen [a,*]

[a] UMR INRA – CNRS – Université de Nice Sophia Antipolis, 06903 Sophia Antipolis, France
[b] Department of Entomology, University of Arizona, Tucson, AZ 85721, USA

ARTICLE INFO

Article history:
Received 14 October 2008
Accepted 24 June 2009
Available online xxxx

Keywords:
P450 induction
Phenobarbital
DNA microarrays
Cyp6a2
Resistance

ABSTRACT

Genomic tools such as the availability of the *Drosophila* genome sequence, the relative ease of stable transformation, and DNA microarrays have made the fruit fly a powerful model in insecticide toxicology research. We have used transgenic promoter-GFP constructs to document the detailed pattern of induced *Cyp6a2* gene expression in larval and adult *Drosophila* tissues. We also compared various insecticides and xenobiotics for their ability to induce this cytochrome P450 gene, and show that the pattern of *Cyp6a2* inducibility is comparable to that of vertebrate *CYP2B* genes, and different from that of vertebrate *CYP1A* genes, suggesting a degree of evolutionary conservation for the "phenobarbital-type" induction mechanism. Our results are compared to the increasingly diverse reports on P450 induction that can be gleaned from whole genome or from "detox" microarray experiments in *Drosophila*. These suggest that only a third of the genomic repertoire of *CYP* genes is inducible by xenobiotics, and that there are distinct subsets of inducers/induced genes, suggesting multiple xenobiotic transduction mechanisms. A relationship between induction and resistance is not supported by expression data from the literature. The relative abundance of expression data now available is in contrast to the paucity of studies on functional expression of P450 enzymes, and this remains a challenge for our understanding of the toxicokinetic aspects of insecticide action.
© 2009 Elsevier Inc. All rights reserved.

1. Introduction

Insecticidal action requires the presence of an active form of the insecticide at the target site at an effective concentration and for a sufficient time. The determinants of this bioavailability, transport, metabolism and sequestration, are therefore the toxicokinetic parameters [1] in insecticidal action. They have an equal importance to the molecular details of toxicodynamic, or mode of action, parameters. Efficacy, selective toxicity, and resistance can all be determined by either toxicokinetic or toxicodynamic differences between a sensitive species and a less sensitive species, a target or a non-target organism, and a susceptible or a resistant strain.

The increased use of *Drosophila* as a model insect in toxicology studies over the last decade is a logical consequence of the advantages it offers [2,3]. In the specific area of insecticide metabolism and resistance, the *Drosophila* genome sequence provided a first complete picture of cytochrome P450 diversity and abundance in insects with about 85 active CYP genes [4] while other insect genomes harbor more or fewer CYP genes [5]. The genomic tools available in *Drosophila* now allow both the detailed study of single genes and global approaches on the whole family of P450s in an insect.

Here, we provide examples of both approaches. First we focus on the *Cyp6a2* gene, a gene abundantly expressed in insecticide-resistant strains [6–9]. The CYP6A2 enzyme metabolizes organochlorine and organophosphorus insecticides [10] as well as dimethylbenzanthracene and aflatoxin B1 [11]. A mutant form of CYP6A2 has been reported to metabolize DDT as well [12]. The detoxification function of CYP6A2 is thus well established. *Cyp6a2* is also known to be inducible by barbiturates [7,9,10,13]. We report here the fine-scale mapping of the tissues in which the *Cyp6a2* gene is induced, and we report the pattern of *Cyp6a2* induction by various classes of chemicals using a screening approach with a transgenic GFP marker.

Secondly, we analyze the literature for induction of other CYP genes in *Drosophila* in order to place *Cyp6a2* in the context of the whole CYP family. The use of DNA microarrays allowed a genome-wide or "CYPome"-wide assessment of transcript abundance, and many important studies have been published since this field was reviewed [14]. Our analysis shows that only a third of the genomic repertoire of *CYP* genes is inducible by xenobiotics, and that there are distinct subsets of inducers/induced genes, suggesting multiple xenobiotic transduction mechanisms.

2. Materials and methods

2.1. Transgenic *Drosophila*

The 1.3 kb promoter fragment of the *Cyp6a2* gene [10] was cloned upstream of the Green Fluorescent Protein coding sequence in the

* Corresponding author.
E-mail address: rfeyer@sophia.inra.fr (R. Feyereisen).

0048-3575/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.pestbp.2009.06.009

Please cite this article in press as: M. Giraudo et al., Regulation of cytochrome P450 expression in *Drosophila*: Genomic insights, Pestic. Biochem. Physiol. (2009), doi:10.1016/j.pestbp.2009.06.009

ARTICLE IN PRESS

pCasper P-element vector and this was used to transform the w^{1119} line of *Drosophila melanogaster*. The P[w+-1.3-*Cyp6a2*-GFP]-transformed lines were screened for high inducibility of GFP fluorescence following phenobarbital treatment. A double X-chromosome transformant line 1E; 11B; Sco/SM6b was used for further study.

2.2. CYP6A2-GFP induction

Induction of *Cyp6a2*-GFP by phenobarbital and by the polychlorobiphenyl mixture Aroclor 1254 (PCB) in different tissues/organs in larva, pupa and adult of the transgenic stock 1E; 11B; Sco/SM6b was determined using confocal microscopy. Late first instar or early second instar larvae, and 1-day-old adults were fed for 3–5 days on Ward's *Drosophila* instant diet mixed with phenobarbital (1 g dry instant diet + 2.5 ml 0.4% phenobarbital sodium in distilled water). One-day-old flies were also treated with PCB, by contact for 4–5 days; for this, 15–20 flies were kept in 20 ml glass scintillation vials coated with 0.1 mg (3 μg/cm^2) PCB dissolved in acetone. PCB-treated flies were also fed wet instant diet placed at the bottom of the vial. For controls, larvae adults were treated similarly but without phenobarbital sodium or PCB. Whole larvae after molting into second or third instar, adults, and freshly dissected organs from larvae and adults were mounted on microscope slide in 80% glycerin in PBS and were examined for GFP fluorescence using a laser scanning confocal microscope (BioRad 1024 confocal scanning head attached to a Nikon Optiphot 2 microscope with PlanApo objectives). Confocal images were processed, using Lasersharp software (BioRad, Hercules, CA) and Adobe Photoshop. Treatment of flies with other chemicals was either by contact (DDD: 0.1 mg/vial; DDE: 0.01 mg/vial; dicofol: 0.1 mg/vial; aldrin: 1 μg/vial; clofibrate: 10 μg/vial) or by incorporation in the media, i.e. ingestion (*trans*-stilbene oxide: 0.3%; limonene: 0.1%).

Table 1
PCB/phenobarbital-induced GFP in various tissues/organs in larvae and adults.

Organs/tissues	Larva[a]	Adult
Cuticle	–	–
Tormogen cells of bristles	–	+
Epidermis	+	+
Imaginal disk	–	na
Skeletal muscles	+	+
Flight muscles	na	+
Fat body	+	+
Heart	–[b]	–[b]
Pericardial cells and nephrocytes	–	+[c]
Hemolymph (hemocytes)	–	+
Pharynx, esophagus, proventriculus/cardia	+	+
Midgut, hindgut, rectum	+	+
Caecum	–	–
Crop muscles	na	+
Salivary gland	+[d]	+
Malpighian tubules	+[e]	+[e]
Tracheal cells/lining	+	
Spiracular glands	+	na
Brain, thoracic ganglion, peripheral nerves	+	+
Ring gland	–	–
Sense cells/organs	+	+
Compound eye and ocellus	na	+
Ovary/eggs	na	–
Uterus	na	+
Accessory gland	na	–[b]
Testis, vas deferens, ejaculatory duct, sperm pump	na	+

The results are expressed as presence or absence of green fluorescence and are not meant to be interpreted quantitatively. na, not applicable.
[a] Third instar larva.
[b] Only very weak green fluorescence (GF).
[c] Strong yellow/orange auto-fluorescence masked GF.
[d] GF only in the anterior region of the salivary gland in the larva.
[e] Weak GF in the distal region.

3. Results

3.1. Tissue specificity of *Cyp6a2*-GFP induction

Cyp6a2-GFP fluorescence was induced in many but not all the tissues/organs in treated larvae and adults (Table 1). In addition to this non-ubiquitous expression, there were also apparent quantitative differences among different tissues/organs. Yellow/orange auto-fluorescence of chitinous cuticle and cuticular lining of internal structures (trachea, crop, foregut, proventriculus/cardia, hindgut, rectum and salivary gland duct), food content (digestive system), or other inclusions (distal part of the Malpighian tubules, nephrocytes and pericardial cells) sometimes masked or modified the green fluorescence (GF). All muscles/muscle fibers in both larvae and adults showed GF, but in the flight muscles GF was less intense than in other muscular components. Heart also showed only weak GF. In adults, regions of the body covered with only the membranous cuticle showed uniform green fluorescence because of the GFP in hemolymph (hemocytes) and epidermis. The fat body and the entire intestinal wall were intensely green fluorescent, but not the gastric caecum. GFP appeared to be induced throughout the central and peripheral nervous system, and sensory cells and their axons; but GF was weak in the larval brain and ganglion. In adult brain and ganglion, GF was more intense in the neuropile and lamina of the optic lobes than in the neurons. The ring gland and ovary did not show detectable GF, however, accessory glands showed faint GF. In the newly formed pupa, produced from phenobarbital-fed larva, fat body and digestive system were green (Fig. 1L); other internal structures were not distinguishable in whole-mounted pupae examined. None of the tissues/organs in control larvae, pupae and adult flies showed green fluorescence (Fig. 1J, K and M, Fig. 2L–N).

3.2. *Cyp6a2* induction by xenobiotics

The clear difference in green fluorescence between induced and non-induced insects allowed us to screen for the ability of various chemicals to induce *Cyp6a2*. Table 2 shows the response of the *Cyp6a2*-GFP transgenic line to treatment by various chemicals, compared to the induction pattern of vertebrate *CYP1A* and *CYP2B* genes [15–17]. Phenobarbital, pentobarbital, organochlorines (DDT and aldrin), *trans*-stilbene oxide and limonene were all inducers of *Cyp6a2* and these are known inducers of the *CYP2B* genes, but not of the *CYP1A* genes. In contrast, *Cyp6a2* was not inducible by β-naphthoflavone or 3-methylcholanthrene, known inducers of the *CYP1A* genes. The case of polychlorobiphenyls is particularly interesting, as a mixture of coplanar and non-coplanar PCBs (Aroclor 1254) induces all three types of genes, but pure isomers show a distinguishable pattern: 2,4,5,2′,4′,5′-HCB (non-coplanar) induces *Cyp6a2* and the *CYP2B* genes, but 3,4,5,3′,4′,5′-HCB (coplanar) only induces the *CYP1A* genes [18,19]. Ethanol and clofibrate, typical inducers of the vertebrate *CYP2E* and *CYP4A* genes, respectively, did not induce *Cyp6a2*.

3.3. From *Cyp6a2* induction to induction of the CYPome

Cyp6a2 was initially reported to be phenobarbital (or barbital) inducible, and it is also regulated by ecdysone [20]. It has recently been shown to be also induced by caffeine [21]. Moreover, a number of DNA microarray studies have now identified *Cyp6a2* as being responsive to chemical treatments. We have therefore compared and evaluated the results of ten published studies from 17 datasets on xenobiotic treatment of *D. melanogaster* that used either custom DNA arrays ("detox chips" [22,23]) or whole [24–28] (and partial [29–31]) genome arrays (Table 3). These studies

Please cite this article in press as: M. Giraudo et al., Regulation of cytochrome P450 expression in *Drosophila*: Genomic insights, Pestic. Biochem. Physiol. (2009), doi:10.1016/j.pestbp.2009.06.009

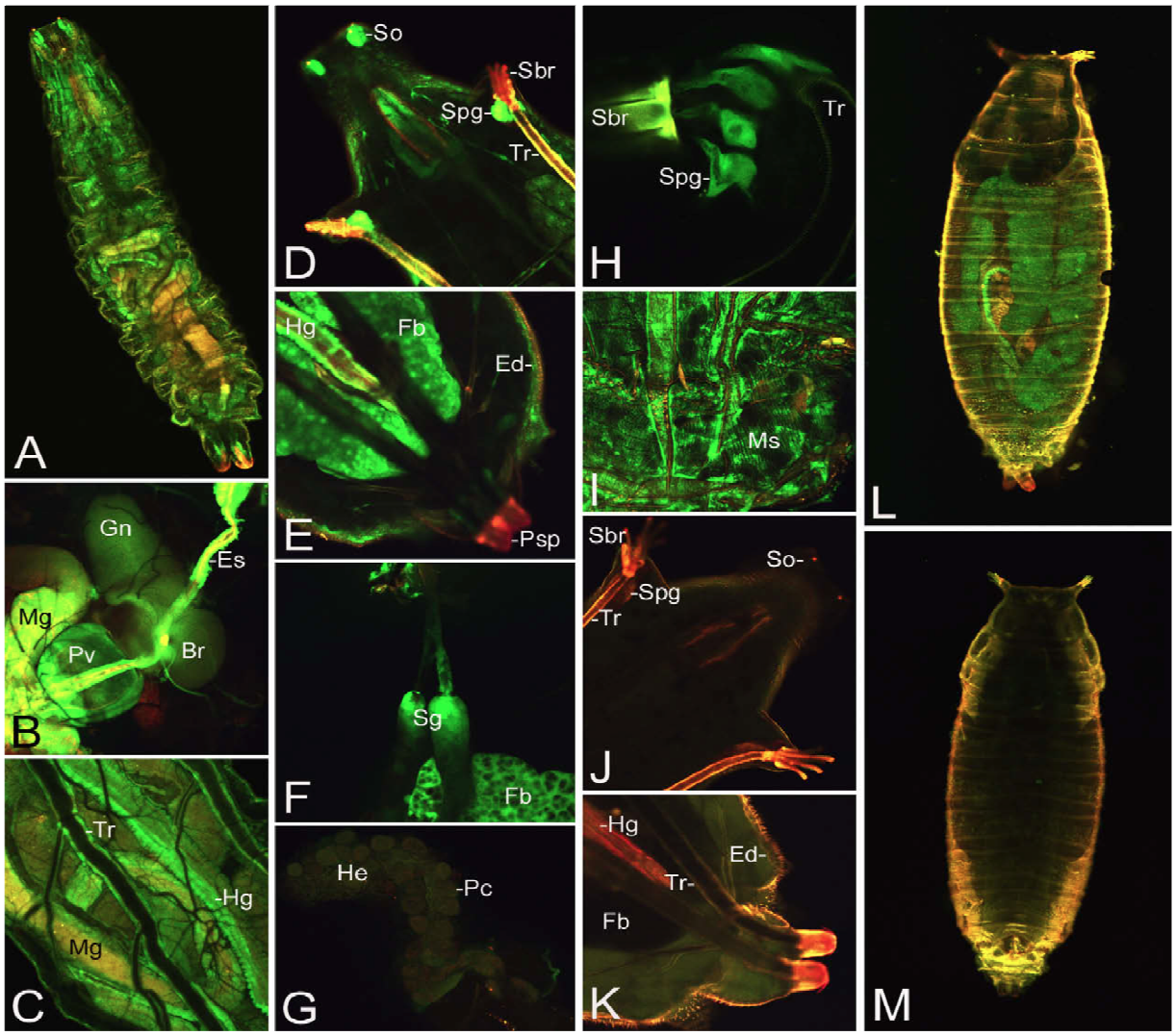


Fig. 1. Phenobarbital-induced GFP in third instar larva and pupa. (A) Early third instar larva. (B) Brain-ganglion complex and anterior part of the digestive system. (C) Abdominal region showing body wall and internal organs. (D and E) anterior and posterior ends of the larva. (F) Anterior part of the salivary gland. (G) Heart and pericardial cells. (H) Anterior spiracle and spiracular glands. (I) Body wall showing muscles. (J and K) Anterior and posterior ends of control larva (without Phenobarbital). (L) Newly formed pupa, treated with Phenobarbital at larval stage. (M) Control pupa. Br, brain; Ed, epidermis; Es, esophagus; Fb, fat body; Gn, ganglion; He, heart; Hg, hindgut; Mg, midgut; Ms, muscles; Pv, proventriculus; Sbr, spiracular branches; So, sense organ; Sp, spiracle; Spg, spiracular glands; Tr, trachea.

covered ten chemicals, with different treatment regimes (concentrations and exposure times), as well as different developmental stages (third instar larvae and adults). Despite the differences in techniques and statistical treatments, we feel that the consensus outcome of this comparison provides an interesting first glimpse of the patterns of xenobiotic response of the fruit fly. Negative results, i.e. chemical treatments that did not induce P450 genes were not considered, although these are also informative [22]. We included the studies on exposure to *Piper nigrum* extracts [30] because the main component piperamides are well known. We did not include the study on dietary shift in larvae (standard cornmeal to bananas) [32] that showed a slight induction of *Cyp9b2* and a moderate decrease in *Cyp6g1* and *Cyp4d2* expression. It is known that P450 genes are involved in the adaptation to diet in other *Drosophila* species, for instance *Cyp28a1* and *Cyp4d10* in *Drosophila mettleri* [33], and that the transcriptome is affected by starvation. Every diet should be considered as a collection of inducers and inhibitors of various potencies and concentrations, but here we focus on identified xenobiotics.

The results of this survey show that between one and twelve CYP genes are affected by each chemical treatment, and that no more than a third (27 P450s) of the CYPome is responsive to xenobiotics. Different classes of compounds induce different CYP genes, and there is considerable overlap between some chemicals while others stand out (Fig. 3). For instance, paraquat (11 genes) and tunicamycin (7 genes) seem to induce a set of CYP genes with little overlap with the set induced by the other chemicals. Tunicamycin causes a stress at the level of the endoplasmic reticulum and paraquat causes stress through the generation of reactive oxygen species [26]. The CYP genes seen induced in those treatments are perhaps best described as stress-responsive. *Cyp6d4*, *Cyp28a5* and *Cyp4p1* are the common genes in that category. A broader overlap is seen in the response to the other chemicals, with 11 genes retained in the consensus for phenobarbital induction (from six

Please cite this article in press as: M. Giraudo et al., Regulation of cytochrome P450 expression in *Drosophila*: Genomic insights, Pestic. Biochem. Physiol. (2009), doi:10.1016/j.pestbp.2009.06.009

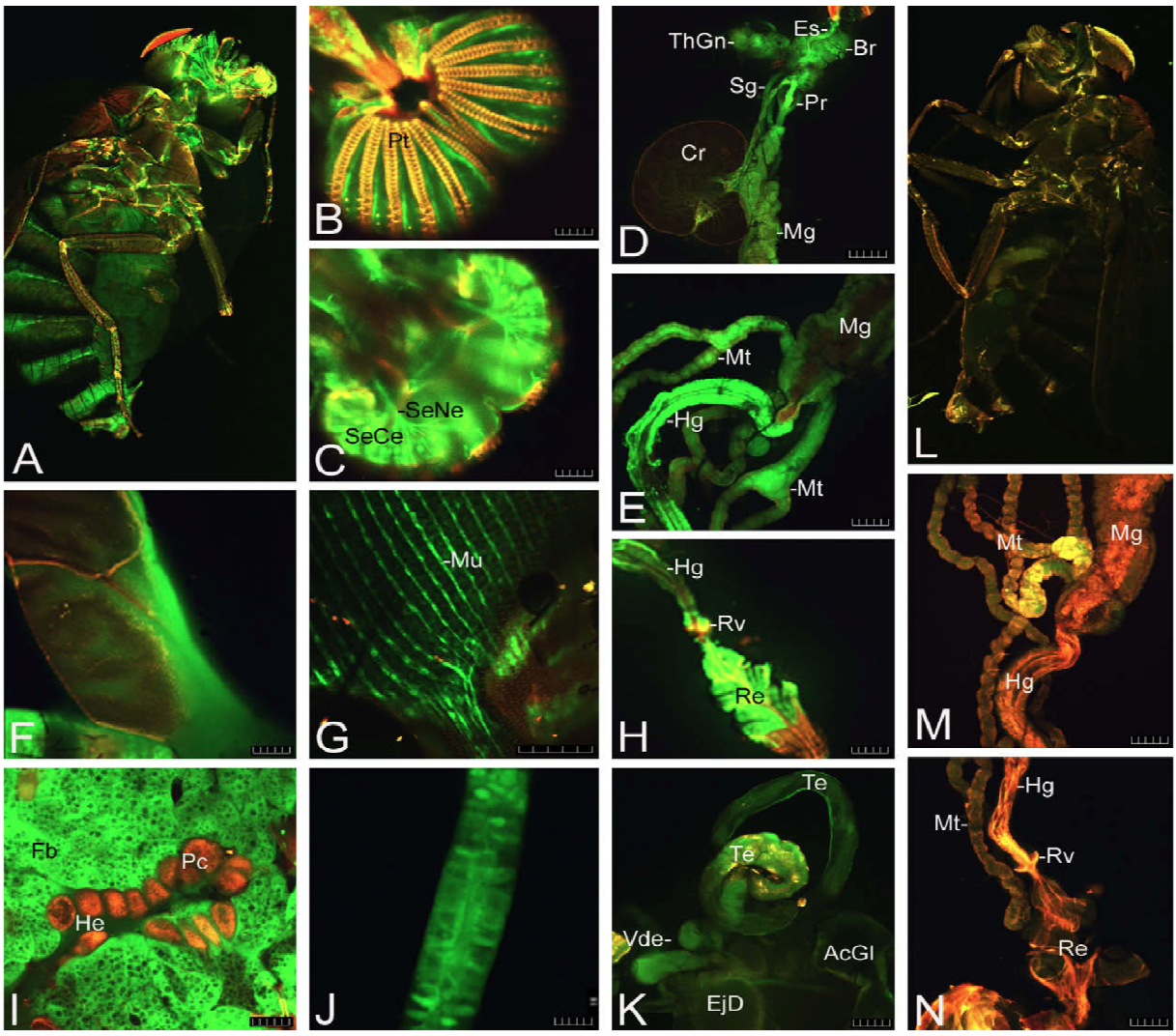


Fig. 2. Phenobarbital (Pheno)- or PCB-treated adults. (A) Female fly (Pheno). (B and C) Apex of proboscis (frontal view), surface and interior (PCB). (D) Brain, thoracic ganglion and anterior region of the digestive system (Pheno). (E) Part of midgut and hindgut and proximal region of Malpighian tubules (PCB). (F) Wing (PCB). (G) Abdominal wall showing tergosternal muscles and epidermis (PCB). (H) Hindgut and rectum (Pheno). (I) Abdominal fat body, heart and pericardial cells (Pheno). (J) Part of the salivary gland (Pheno). (K) Part of male reproductive system (PCB). (L–N) Control female, and parts of the digestive system. AcGl, Accessory gland; Br, brain; Cr, crop; Ejd, ejaculatory duct; Fb, fatbody; He, heart; Hg, hindgut; Mg, midgut; Mt, malpighian tubule; Mu, muscles; Pc, pericardial cells; Pr, proventriculus; Pt, pseudotrachea; Re, rectum; Rv, rectal valve; SeCe, sensory cells; SeNe, sensory nerve; Sg, salivary gland Te, testis; Vde, vas deferens. Scale bar:D, 40 μm, E,F, H, K, M and N, 100 μm; B, C, G and I, 50 μm; J, 10 μm.

experimental conditions), 12 genes for piperonyl butoxide and 11 genes for caffeine. *P. nigrum* extracts, atrazine and pyrethrum induced fewer genes, whereas DDT and ethanol had only one major target in the CYPome (*Cyp12d1* and *Cyp6a8*, respectively). Those common genes responding to a chemical challenge are *Cyp6a2*, *Cyp12d1*, *Cyp6a8*, *Cyp6d5* and *Cyp6w1*. The wide chemical responsiveness of *Cyp6a2* that we observed using the transgenic fly model is thus confirmed by the various microarray studies.

3.4. Induction and insecticide resistance

Several reports in the literature have noted that CYP genes that are constitutively overexpressed in insecticide-resistant strains are also inducible by xenobiotics in susceptible strains, in *Drosophila* and in other insect species. We thus compared the data on CYP gene induction by xenobiotics that were collected in DNA microarray experiments with the list of genes that are currently known to be constitutively overexpressed in resistant strains or otherwise related to resistance (Fig. 4). Of the 27 genes known to be inducible by one or the other xenobiotics, and of the 12 genes known to be associated with resistance, eight are overlapping. Of these eight only three (*Cyp6a2*, *Cyp6g1* and *Cyp12d1*) have been shown to be causally related to resistance. Two (*Cyp12a4* and *Cyp6g2*) have been experimentally linked to resistance but have not been shown to be inducible by any of the nine compounds tested to date.

4. Discussion and conclusions

4.1. Tissue-specific expression

The midgut, Malphighian tubules and fat body are the tissues recognized as the major sites of P450-mediated detoxification in

Please cite this article in press as: M. Giraudo et al., Regulation of cytochrome P450 expression in *Drosophila*: Genomic insights, Pestic. Biochem. Physiol. (2009), doi:10.1016/j.pestbp.2009.06.009

Table 2
Inducers of mammalian *CYP1A* and *CYP2B* genes compared with inducers of fruit fly *Cyp6a2*.

Inducer	Mammalian P450		*Drosophila*
	CYP1A	*CYP2B*	*Cyp6a2*
Phenobarbital	–	+	+
Pentobarbital	–	+	+
OCs			
DDT/DDD/DDE/dicofol	–	+	+
Aldrin	–	+	+
trans-Stilbene oxide	–	+	+
PCBs			
Aroclor 1254	+	+	+
2,4,5,2′,4′,5′-HCB	–	+	+
3,4,5,3′,4′,5′-HCB	+	–	–
Limonene	–	+	+
β-Naphthoflavone	+	–	–
3-Methylcholanthrene	+	–	–
Ethanol	–	–	–
Clofibrate	–	–	–

Induction of *Cyp6a2* was estimated *in vivo* by the green fluorescence of treated transgenic *6a2*-GFP flies, see Section 2 for details. Induction patterns of *CYP1A* and *CYP2B* genes were compiled from the literature.

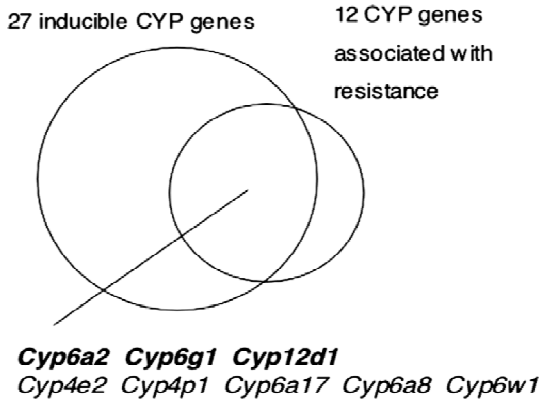


Fig. 4. Venn diagram showing the overlap between *CYP* genes inducible by chemicals and *CYP* genes associated to resistance in field or laboratory studies. The overlapping genes are identified, and of those, the three genes that have been causally linked to resistance in field-derived strains or experimental studies are in bold.

Table 3
Induction of the CYPome.

Phenobarbital	Atrazine	Caffeine	*Piper nigrum* extract	Piperonyl butoxide	Pyrethrum	Ethanol or DDT	Paraquat	Tunicamycin
6 datasets [22–24,27] Ad-, L- F-, M-	2 datasets [23] Ad- F-, M-	1 dataset [22] Ad- M-	1 dataset [30] Ad- F-	2 datasets [25] Ad- M-	1 dataset [31] Ad- F-	1 dataset [28] Ad- M-	2 datasets [26,29] Ad- M-	1 dataset [26] Ad- M-
Cyp4d4	***Cyp6a2***	*Cyp4ae1*	***Cyp6a8***	*Cyp4ae1*	*Cyp9f2*	***Cyp6a8*** (ethanol)	*Cyp4ac3*	*Cyp4e1*
Cyp4e2	***Cyp6d5***	*Cyp4d14*	*Cyp6d4*	*Cyp4e2*	***Cyp12d1***	***Cyp12d1*** (DDT)	***Cyp4p1***	***Cyp4p1***
Cyp6a2	*Cyp6g1*	*Cyp4e2*	***Cyp6d5***	*Cyp4p1*			*Cyp6a13*	*Cyp6a23*
Cyp6a8	***Cyp6w1***	***Cyp6a2***	***Cyp6w1***	***Cyp6a2***			*Cyp6a22*	***Cyp6d4***
Cyp6a17	*Cyp12d1*	***Cyp6a8***	*Cyp9b2*	***Cyp6a8***			*Cyp6a23*	*Cyp9b2*
Cyp6a23	*Cyp304a1*	*Cyp6a21*	***Cyp12d1***	*Cyp6a21*			***Cyp6d4***	***Cyp28a5***
Cyp6d5		*Cyp6d4*		*Cyp6a23*			*Cyp6d5*	*Cyp313a1*
Cyp6g1		***Cyp6d5***		***Cyp6d5***			***Cyp28a5***	
Cyp6w1		*Cyp6g1*		***Cyp6w1***			*Cyp309a1*	
Cyp9b2		***Cyp6w1***		*Cyp12b2*				
Cyp12d1		*Cyp12d1*		*Cyp12c1*				
				Cyp12d1				

Genes listed in bold are the genes most representative of inducibility following chemical challenge or stress response (see Fig. 3).
L-, Ad-: third instar larvae or adults.
M-, F-: male or female.

insects [34–36]. DNA microarray analyses in *Drosophila* have confirmed that P450 transcripts are enriched in these tissues. In Malpighian tubules of adult flies, the *Cyp6a18* transcript was enriched >25-fold and *Cyp6a2* enriched 8-fold [37]. The data of that study reveal that while 1,457 genes (10% of all genes) are expressed more than 2-fold in the Malphighian tubules, 29 P450 genes (or 28% of all P450 genes) are expressed over 2.8-fold. Similarly, 36 P450 genes (40%), including *Cyp6a2*, are represented in the larval midgut transcriptome [38]. A distillation of FlyAtlas data on tissue expression of P450 genes [39] shows that *Cyp6a2* is not expressed at high levels in normal flies, with Malpighian tubules, hindgut and head being the major sites of expression. Our results also show that *Cyp6a2* basal expression is very low, although our technique does not measure the *Cyp6a2* transcript levels but the accumulation of GFP protein driven by the *Cyp6a2* promoter, and is therefore qualitatively different and perhaps less sensitive. Following induction, however, the gene is highly expressed both in larvae and in adults, in the midgut, Malpighian tubules and fat body. These results confirm and expand in detail the early results obtained by in situ hybridization in adult flies [7]. They also clearly show that *Cyp6a2*, after induction, is expressed in many other tissues as well as in the recognized sites of detoxification. The expression in the nervous system, in both larvae and adults is of significance, as the majority of insecticides have nervous system targets, such as the

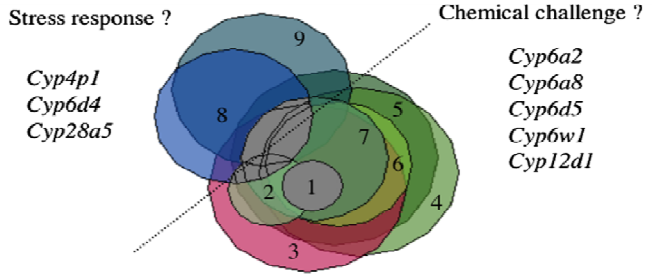


Fig. 3. Venn diagram summarizing the inducibility of *Drosophila CYP* genes by various chemicals. 1: ethanol; 2: pyrethrum; 3: phenobarbital; 4: caffeine; 5: piperonyl butoxide; 6: *Piper nigrum* extract; 7: atrazine; 8: tunicamycin; 9: paraquat. The limited overlap between the *CYP* genes induced by paraquat and tunicamycin and those induced by the other chemicals led us to draw an arbitrary line to separate the genes that we designate as representing the response to stress from those representing the response to chemical challenge.

Please cite this article in press as: M. Giraudo et al., Regulation of cytochrome P450 expression in *Drosophila*: Genomic insights, Pestic. Biochem. Physiol. (2009), doi:10.1016/j.pestbp.2009.06.009

organophosphorus insecticides that are metabolized by CYP6A2 [10]. Expression of *CYP6D1* in the thoracic ganglia of adult house flies has also been reported to contribute to cypermethrin resistance in the Lpr strain [40]. The metabolism of xenobiotics by P450 enzymes is highly "uncoupled", costing much NADPH and generating considerable amounts of reactive oxygen species, as shown recently in a detailed study of house fly CYP6A1 [53]. The uninduced, low basal production of P450 enzymes is therefore probably an optimal state in the absence of chemical challenge. The observation that induced *Cyp6a2* should be found in a wide variety of tissues, beyond those in the front line of defense against xenobiotics such as the midgut and Malpighian tubules, may be an evolved response of insects who lack a closed circulatory system and therefore have all their tissues exposed to xenobiotics, once these have crossed the midgut or integument barriers. *Cyp6a2* has a pattern of expression that is similar to that of *Cyp6g1*. Chung et al. [50] have used in situ hybridization as well as GFP reporter constructs to study the spatial expression of the *Cyp6g1* gene. They showed that the wild-type *Cyp6g1* is expressed in parts of the midgut and in the Malpighian tubules. In wandering larvae, expression is also detected in the fat body. In resistant strains carrying a fragment of the Accord transposable element, the expression is higher in those tissues, the whole midgut, and also includes the gastric caeca. Manipulation (RNAi or overexpression) of *Cyp6g1* transcript levels in transgenic flies under the control of a Malpighian tubule promoter is sufficient to affect DDT resistance, whereas RNAi suppression of *Cyp6g1* expression in fat body or brain is without effect [39]. Thus, the tissue expression pattern is critical in determining the toxicodynamics of insecticides (or more generally xenobiotics) that are metabolized by P450 enzymes.

4.2. Specificity of induction

The *Cyp6a2* gene appears to be responding to multiple chemical challenges, but not to all chemical treatments. Other genes in the CYPome also appear to have a specific range of response. It is obvious that the detailed study of one gene or the cursory analysis of the whole CYPome as seen through various microarray experiments does not allow definitive conclusions to be drawn, and that more detailed analyses should focus on the time course of induction and its dose response (see [22] for time courses). Nonetheless, it seems that about a third of the CYPome is inducible, while the majority of P450 genes is not regulated by xenobiotic exposure. The non-inducible genes probably include the Halloween genes encoding enzymes of ecdysteroid biosynthesis [41] but also include many genes of unknown function, that are expressed at low levels in the whole insect and may in fact have a very restricted physiological function and pattern of expression. Among the inducible genes, there are obvious subsets, evidenced in our survey as stress-responsive genes and genes responding to chemical challenge. This suggests that the chemical diversity of xenobiotics is perceived by different, probably overlapping transduction mechanisms, and this leads to an adapted response. In vertebrates, this is achieved by two types of receptors, the members of the nuclear receptor family including the xenosensors PXR and CAR, and the bHLH-PAS proteins such as the Ah receptor. Cross-talk between these receptors and with receptors for endogenous hormones probably allows a fine-tuning of the response [42].

The inducibility of certain P450 genes in bacteria, plants, and insects by phenobarbital remains somewhat mysterious. However, the remarkable parallel between *Cyp6a2* induction and the known induction pattern of mammalian *CYP2B* genes strongly suggests that the "phenobarbital-type" induction in insects and mammals may share some characteristic that has been conserved during evolution. The nuclear receptor CAR is an essential component of the mouse response to the drug [43,44]. Analysis of the evolutionary relationships between *Drosophila*, *Caenorhabditis elegans* and vertebrate nuclear receptors revealed that the orphan receptor DHR96 was most closely related to the nematode NHR-8 and DAF-12 receptors and to vertebrate CAR, vitamin D receptor (VDR) and Pregnane X receptor (PXR) [45,46]. The nematode NHR-8 receptor is essential for wild-type resistance to colchicine and chloroquine [45]. Recently, King-Jones et al. [24] presented evidence for the role of DHR96 in the response of *Drosophila* to phenobarbital. They obtained DHR96 null mutants and showed that the expression of some but not all phenobarbital-inducible genes was affected in the mutants.

We attempted to silence the DHR96 gene by RNAi, using the UAS–GAL4 system wherein a transgenic line with a promoter-driven GAL4 expression was crossed to a transgenic line with UAS-driven expression of DHR96 dsRNA (line 11783R-2 kindly provided by Dr. Ueno, NIG-Fly, Japan). The heat shock promoter (hsp70-GAL4) or the promoter with moderate expression from the *Cyp6g1* gene (*Cyp6g1*-GAL4; line kindly provided by Dr. Ffrench-Constant, Univ. Exeter) were insufficient as drivers to obtain significant downregulation of DHR96, whereas a stronger, ubiquitous promoter (tubulin-GAL4) led to high mortality in both male and female crosses (results not shown). These negative results nonetheless indicate a complex role for DHR96 that may be involved in essential physiological or developmental process in addition to its reported implication in the response to xenobiotics.

4.3. Resistance and induction

Cyp6a2 and *Cyp12d1* are both inducible by at least four compounds and their constitutive overexpression has been causally linked to resistance. CYP6A2 metabolizes organophosphorus insecticides [10] and CYP12D1, when overexpressed in transgenic flies, confers resistance to dicyclanil and to DDT [47]. We note here that *Cyp12d1* is a recently duplicated gene (i.e. *Cyp12d1* and *Cyp12d2* that differ by only three non-neutral nucleotide changes (http://p450.sophia.inra.fr), *Cyp12d2* having been masked by a gap in the initial release of the *Drosophila* genome sequence). A third gene, *Cyp6g1*, is inducible by phenobarbital and by caffeine, and its overexpression in natural populations of *Drosophila* [48,49] as well as in transgenic flies [48,50,47,39] causes resistance to DDT and to neonicotinoids. Five other genes, *Cyp4e2*, *Cyp4p1*, *Cyp6a17*, *Cyp6a8* and *Cyp6w1* are both inducible and overexpressed in some resistant strains, but there is no evidence for their causal relationship to resistance. The case of *Cyp6a8* is noteworthy, as the recombinant enzyme metabolizes aldrin to a modest degree but not heptachlor or DDT [51], and its overexpression in transgenic flies does not confer resistance to DDT, nitenpyram, dicyclanil or diazinon [47]. Two genes are known to be linked to resistance but have not been shown to be inducible in any of the studies that we analyzed. *Cyp12a4* confers a selective resistance to lufenuron in field strains and in laboratory, transgenic strains [52]. *Cyp6g2* is a gene expressed at very low levels in the head [39] but its transgenic overexpression in gut and Malpighian tubules confers diazinon and dicyclanil resistance [47]. These discrepancies highlight the danger of associating resistance with constitutive overexpression, and resistance with induction. We have argued [23] that inducibility of a P450 gene implies the presence of *cis*-regulatory sequences in addition to the sequences controlling the correct tissue and developmental expression. The broader target (*cis*-sequences of the induced gene and genes for the *trans*-factors that are necessary for its induction) for mutational events is therefore a risk factor for resistance when the gene encodes a xenobiotic-metabolizing enzyme. It is well known that inducibility is under genetic control with different insect strains showing different induction patterns (see e.g. [54–56]). Hällström [57] has compared a resistant strain in which the resistance mutation has caused increased constitutive

Please cite this article in press as: M. Giraudo et al., Regulation of cytochrome P450 expression in *Drosophila*: Genomic insights, Pestic. Biochem. Physiol. (2009), doi:10.1016/j.pestbp.2009.06.009

expression and has abolished further inducibility to a "constitutive mutant". Any other link between induction and resistance is probably coincidental rather than causal, because the time needed for induction and its dose–response would not sufficiently improve the toxicokinetics of most fast-acting neurotoxins used as insecticides to explain resistance.

While this paper was under review, Chung et al. [58] reported on the constitutive expression of CYP genes in *Drosophila* larvae. They obtained expression patterns for about two thirds of the CYPome, of which half were expressed in the fat body, midgut and Malpighian tubules, including all the genes that we highlighted in Fig. 3. Many other genes had a more discrete expression pattern, sometimes specific to small organs or cell types. Their results and our survey lead to a similar conclusion, that only a restricted subset of P450 enzymes are likely to be involved in detoxification. Many endogenous functions remain to be discovered for the other P450 enzymes.

Acknowledgments

Work on *Cyp6a2*-GFP was supported by National Institutes of Health Grants GM39014 and ES06694. We thank Dr. Ueno and Dr. Ffrench-Constant for the UAS or GAL4 transgenic lines. We thank B. Dunkov for help with the GFP transgenic lines and the Synthetic Core Facility, Southwest Environmental Health Sciences Center, University of Arizona, for the hexachlorobiphenyls.

References

[1] W. Welling, G.D. Paterson, Toxicodynamics of Insecticides, In: G.A. Kerkut, L.I. Gilbert (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Pergamon Press, Oxford, 1985, pp. 603–645.
[2] T.G. Wilson, Resistance of *Drosophila* to toxins, Annu. Rev. Entomol. 46 (2001) 545–571.
[3] T.G. Wilson, *Drosophila*: sentinels of environmental toxicants, Integr. Comp. Biol. 45 (2005) 127–136.
[4] N. Tijet, C. Helvig, R. Feyereisen, The cytochrome P450 gene superfamily in *Drosophila melanogaster*: annotation, intron–exon organization and phylogeny, Gene 262 (2001) 189–198.
[5] R. Feyereisen, Evolution of insect P450, Biochem. Soc. Trans. 34 (2006) 1252–1255.
[6] L.C. Waters, A.C. Zelhof, B.J. Shaw, L.Y. Ch'ang, Possible involvement of the long terminal repeat of transposable element 17.6 in regulating expression of an insecticide resistance-associated P450 gene in *Drosophila*, Proc. Natl. Acad. Sci. USA 89 (1992) 4855–4859 (published erratum appears in Proc. Natl. Acad. Sci. USA 89 (24) (1992) 12209).
[7] A. Brun, A. Cuany, T. Le Mouel, J. Berge, M. Amichot, Inducibility of the *Drosophila melanogaster* cytochrome P450 gene, CYP6A2, by phenobarbital in insecticide susceptible or resistant strains, Insect Biochem. Mol. Biol. 26 (1996) 697–703.
[8] J.M. Bride, A. Cuany, M. Amichot, A. Brun, M. Babault, T.L. Mouel, G. De Sousa, R. Rahmani, J.B. Berge, Cytochrome P-450 field insecticide tolerance and development of laboratory resistance in grape vine populations of *Drosophila melanogaster* (Diptera: Drosophilidae), J. Econ. Entomol. 90 (1997) 1514–1520.
[9] S. Maitra, S.M. Dombrowski, L.C. Waters, R. Ganguly, Three second chromosome-linked clustered Cyp6 genes show differential constitutive and barbital-induced expression in DDT-resistant and susceptible strains of *Drosophila melanogaster*, Gene 180 (1996) 165–171.
[10] B.C. Dunkov, V.M. Guzov, G. Mocelin, F. Shotkoski, A. Brun, M. Amichot, R.H. Ffrench-Constant, R. Feyereisen, The *Drosophila* cytochrome P450 gene Cyp6a2: structure, localization, heterologous expression, and induction by phenobarbital, DNA Cell Biol. 16 (1997) 1345–1356.
[11] C. Saner, B. Weibel, F.E. Wurgler, C. Sengstag, Metabolism of promutagens catalyzed by *Drosophila melanogaster* CYP6A2 enzyme in *Saccharomyces cerevisiae*, Environ. Mol. Mutagen. 27 (1996) 46–58.
[12] M. Amichot, S. Tares, A. Brun-Barale, L. Arthaud, J.M. Bride, J.B. Berge, Point mutations associated with insecticide resistance in the *Drosophila* cytochrome P450 Cyp6a2 enable DDT metabolism, Eur. J. Biochem. 271 (2004) 1250–1257.
[13] S.M. Dombrowski, R. Krishnan, M. Witte, S. Maitra, C. Diesing, L.C. Waters, R. Ganguly, Constitutive and barbital-induced expression of the Cyp6a2 allele of a high producer strain of CYP6A2 in the genetic background of a low producer strain, Gene 221 (1998) 69–77.
[14] R. Feyereisen, Insect cytochrome P450, in: L.I. Gilbert, K. Iatrou, S.S. Gill (Eds.), Comprehensive Molecular Insect Science, Elsevier, Oxford, 2005, pp. 1–77.
[15] P. Honkakoski, R. Moore, K.A. Washburn, M. Negishi, Activation by diverse xenochemicals of the 51-base pair phenobarbital-responsive enhancer module in the CYP2B10 gene, Mol. Pharmacol. 53 (1998) 597–601.
[16] H. Yamada, Y. Ishii, M. Yamamoto, K. Oguri, Induction of the hepatic cytochrome P450 2B subfamily by xenobiotics: research history, evolutionary aspect, relation to tumorigenesis, and mechanism, Curr. Drug Metab. 7 (2006) 397–409.
[17] M.S. Denison, S.D. Seidel, W.J. Rogers, M. Ziccardi, G.M. Winter, S. Heath-Pagliuso, Natural and synthetic ligands for the Ah receptor, in: A. Puga, K.B. Wallace (Eds.), Molecular Biology of the Toxic Response, Taylor and Francis, 1999, pp. 393–410.
[18] A. Parkinson, S.H. Safe, L.W. Robertson, P.E. Thomas, D.E. Ryan, L.M. Reik, W. Levin, Immunochemical quantitation of cytochrome P-450 isozymes and epoxide hydrolase in liver microsomes from polychlorinated or polybrominated biphenyl-treated rats. A study of structure–activity relationships, J. Biol. Chem. 258 (1983) 5967–5976.
[19] J.A. Goldstein, P. Hickman, H. Bergman, J.D. McKinney, M.P. Walker, Separation of pure polychlorinated biphenyl isomers into two types of inducers on the basis of induction of cytochrome P-450 or P-448, Chem. Biol. Interact. 17 (1977) 69–87.
[20] V.S. Spiegelman, S.Y. Fuchs, G.A. Belitsky, The expression of insecticide resistance-related cytochrome P450 forms is regulated by molting hormone in *Drosophila melanogaster*, Biochem. Biophys. Res. Commun. 232 (1997) 304–307.
[21] S. Bhaskara, E.D. Dean, V. Lam, R. Ganguly, Induction of two cytochrome P450 genes, Cyp6a2 and Cyp6a8, of *Drosophila melanogaster* by caffeine in adult flies and in cell culture, Gene 377 (2006) 56–64.
[22] L. Willoughby, H. Chung, C. Lumb, C. Robin, P. Batterham, P.J. Daborn, A comparison of *Drosophila melanogaster* detoxification gene induction responses for six insecticides, caffeine and phenobarbital, Insect Biochem. Mol. Biol. 36 (2006) 934–942.
[23] G. Le Goff, F. Hilliou, B.D. Siegfried, S. Boundy, E. Wajnberg, L. Sofer, P. Audant, R.H. Ffrench-Constant, R. Feyereisen, Xenobiotic response in *Drosophila melanogaster*: sex dependence of P450 and GST gene induction, Insect Biochem. Mol. Biol. 36 (2006) 674–682.
[24] K. King-Jones, M.A. Horner, G. Lam, C.S. Thummel, The DHR96 nuclear receptor regulates xenobiotic responses in *Drosophila*, Cell Metab. 4 (2006) 37–48.
[25] L. Willoughby, H. Chung, C. Lumb, C. Robin, P. Batterham, P.J. Daborn, A comparison of *Drosophila melanogaster* detoxification gene induction responses for six insecticides, caffeine and phenobarbital, Insect Biochem. Mol. Biol. 36 (2006) 934–942.
[26] F. Girardot, V. Monnier, H. Tricoire, Genome wide analysis of common and specific stress responses in adult *Drosophila melanogaster*, BMC Genomics 5 (2004) 74.
[27] W. Sun, V.M. Margam, L. Sun, G. Buczkowski, G.W. Bennett, B. Schemerhorn, W.M. Muir, B.R. Pittendrigh, Genome-wide analysis of phenobarbital-inducible genes in *Drosophila melanogaster*, Insect Mol. Biol. 15 (2006) 455–464.
[28] T.V. Morozova, R.R. Anholt, T.F. Mackay, Transcriptional response to alcohol exposure in *Drosophila melanogaster*, Genome Biol. 7 (2006) R95.
[29] S. Zou, S. Meadows, L. Sharp, L.Y. Yan, Y.N. Jan, Genome-wide study of aging and oxidative stress response in *Drosophila melanogaster*, Proc. Natl. Acad. Sci. USA 97 (2000) 13726–13731.
[30] H.R. Jensen, I.M. Scott, S. Sims, V.L. Trudeau, J.T. Arnason, Gene expression profiles of *Drosophila melanogaster* exposed to an insecticidal extract of *Piper nigrum*, J. Agric. Food Chem. 54 (2006) 1289–1295.
[31] H.R. Jensen, I.M. Scott, S.R. Sims, V.L. Trudeau, J.T. Arnason, The effect of a synergistic concentration of a *Piper nigrum* extract used in conjunction with pyrethrum upon gene expression in *Drosophila melanogaster*, Insect Mol. Biol. 15 (2006) 329–339.
[32] L.D. Carsten, T. Watts, T.A. Markow, Gene expression patterns accompanying a dietary shift in *Drosophila melanogaster*, Mol. Ecol. 14 (2005) 3203–3208.
[33] J.M. Bono, L.M. Matzkin, S. Castrezana, T.A. Markow, Molecular evolution and population genetics of two *Drosophila mettleri* cytochrome P450 genes involved in host plant utilization, Mol. Ecol. 17 (2008) 3211–3221.
[34] F. Cariño, J.F. Koener, F.W. Plapp Jr., R. Feyereisen, Expression of the cytochrome P450 gene CYP6A1 in the housefly, *Musca domestica*, in: C.A. Mullin, J.G. Scott (Eds.), Molecular Mechanisms of Insecticide Resistance, American Chemical Society, Washington, DC, 1992, pp. 31–40.
[35] J.G. Scott, S.S. Lee, Tissue distribution of microsomal cytochrome P-450 monooxygenases and their inducibility by phenobarbital in the insecticide resistant LPR strain of house fly, *Musca domestica* L., Insect Biochem. Mol. Biol. 23 (1993) 729–738.
[36] E. Hodgson, Microsomal monooxygenases, in: G.A. Kerkut, L.I. Gilbert (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Pergamon, Oxford, 1985, pp. 225–331.
[37] J. Wang, L. Kean, J. Yang, A.K. Allan, S.A. Davies, P. Herzyk, J.A. Dow, Function-informed transcriptome analysis of *Drosophila* renal tubule, Genome Biol. 5 (2004) R69.
[38] H.M. Li, G. Buczkowski, O. Mittapalli, J. Xie, J. Wu, R. Westerman, B.J. Schemerhorn, L.L. Murdock, B.R. Pittendrigh, Transcriptomic profiles of *Drosophila melanogaster* third instar larval midgut and responses to oxidative stress, Insect Mol. Biol. 17 (2008) 325–339.
[39] J. Yang, C. McCart, D.J. Woods, S. Terhzaz, K.G. Greenwood, R.H. Ffrench-Constant, J.A. Dow, A *Drosophila* systems approach to xenobiotic metabolism, Physiol. Genomics 30 (2007) 223–231.
[40] P.J. Korytko, J.G. Scott, CYP6D1 protects thoracic ganglia of houseflies from the neurotoxic insecticide cypermethrin, Arch. Insect Biochem. Physiol. 37 (1998) 57–63.

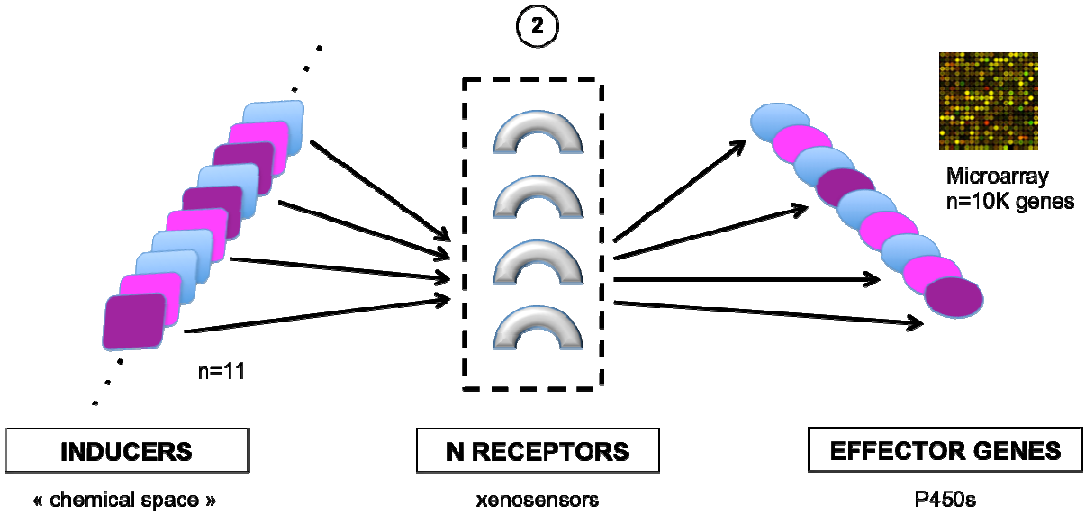
Please cite this article in press as: M. Giraudo et al., Regulation of cytochrome P450 expression in *Drosophila*: Genomic insights, Pestic. Biochem. Physiol. (2009), doi:10.1016/j.pestbp.2009.06.009

[41] L.I. Gilbert, Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*, Mol. Cell. Endocrinol. 215 (2004) 1–10.
[42] J.M. Pascussi, S. Gerbal-Chaloin, C. Duret, M. Daujat-Chavanieu, M.J. Vilarem, P. Maurel, The tangle of nuclear receptors that controls xenobiotic metabolism and transport: crosstalk and consequences, Annu. Rev. Pharmacol. Toxicol. 48 (2008) 1–32.
[43] P. Wei, J. Zhang, M. Egan-Hafley, S. Liang, D.D. Moore, The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism, Nature 407 (2000) 920–923.
[44] K. Swales, M. Negishi, CAR, driving into the future, Mol. Endocrinol. 18 (2004) 1589–1598.
[45] T.H. Lindblom, G.J. Pierce, A.E. Sluder, A *C. elegans* orphan nuclear receptor contributes to xenobiotic resistance, Curr. Biol. 11 (2001) 864–868.
[46] K. King-Jones, C.S. Thummel, Nuclear receptors – a perspective from *Drosophila*, Nat. Rev. Genet. 6 (2005) 311–323.
[47] P.J. Daborn, C. Lumb, A. Boey, W. Wong, R.H. Ffrench-Constant, P. Batterham, Evaluating the insecticide resistance potential of eight *Drosophila melanogaster* cytochrome P450 genes by transgenic over-expression, Insect Biochem. Mol. Biol. 37 (2007) 512–519.
[48] P.J. Daborn, J.L. Yen, M.R. Bogwitz, G. Le Goff, E. Feil, S. Jeffers, N. Tijet, T. Perry, D. Heckel, P. Batterham, R. Feyereisen, T.G. Wilson, R.H. Ffrench-Constant, A single P450 allele associated with insecticide resistance in *Drosophila*, Science 297 (2002) 2253–2256.
[49] F. Catania, M.O. Kauer, P.J. Daborn, J.L. Yen, R.H. Ffrench-Constant, C. Schlotterer, World-wide survey of an Accord insertion and its association with DDT resistance in *Drosophila melanogaster*, Mol. Ecol. 13 (2004) 2491–2504.
[50] H. Chung, M.R. Bogwitz, C. McCart, A. Andrianopoulos, R.H. Ffrench-Constant, P. Batterham, P.J. Daborn, Cis-regulatory elements in the Accord retrotransposon result in tissue-specific expression of the *Drosophila melanogaster* insecticide resistance gene Cyp6g1, Genetics 175 (2007) 1071–1077.
[51] C. Helvig, N. Tijet, R. Feyereisen, F.A. Walker, L.L. Restifo, *Drosophila melanogaster* CYP6A8, an insect P450 that catalyzes lauric acid (omega-1)-hydroxylation, Biochem. Biophys. Res. Commun. 325 (2004) 1495–1502.
[52] F. Catania, M.O. Kauer, P.J. Daborn, J.L. Yen, R.H. Ffrench-Constant, C. Schlotterer, World-wide survey of an Accord insertion and its association with DDT resistance in *Drosophila melanogaster*, Mol. Ecol. 13 (2004) 2491–2504.
[53] M.B. Murataliev, V.M. Guzov, F.A. Walker, R. Feyereisen, P450 reductase and cytochrome b5 interactions with cytochrome P450: effects on house fly CYP6A1 catalysis, Insect Biochem. Mol. Biol. 38 (2008) 1008–1015.
[54] L.C. Terriere, S.J. Yu, The induction of detoxifying enzymes in insects, J. Agric. Food Chem. 22 (1974) 366–373.
[55] I. Hallstrom, J. Magnusson, C. Ramel, Relation between the somatic toxicity of dimethylnitrosamine and a genetically determined variation in the level and induction of cytochrome P450 in *Drosophila melanogaster*, Mutat. Res. 92 (1982) 161–168.
[56] R. Feyereisen, Polysubstrate monooxygenases (cytochrome P-450) in larvae of susceptible and resistant strains of house flies, Pestic. Biochem. Physiol. 19 (1983) 262–269.
[57] I. Hallstrom, Genetic regulation of the cytochrome P-450 system in *Drosophila melanogaster*. II. Localization of some genes regulating cytochrome P-450 activity, Chem. Biol. Interact. 56 (1985) 173–184.
[58] H. Chung, T. Sztal, S. Pasricha, M. Sridhar, P. Batterham, P.J. Daborn, Characterization of *Drosophila melanogaster* cytochrome P450 genes, Proc. Natl. Acad. Sci. USA 106 (2009) 5731–5736.

Please cite this article in press as: M. Giraudo et al., Regulation of cytochrome P450 expression in *Drosophila*: Genomic insights, Pestic. Biochem. Physiol. (2009), doi:10.1016/j.pestbp.2009.06.009

CHAPTER 2

Manuscript: Xenobiotic gene induction response in *Spodoptera frugiperda* midgut and Sf9 cells.



Now that we have identified patterns of gene induction in the model species *Drosophila melanogaster*, we would like in this second chapter to extend our study to a polyphagous lepidopteran pest, *Spodoptera frugiperda*.

As it was discussed earlier (see section 3.3.2.2 of the introduction), because the majority of phytophagous insects have a restricted host range (three or fewer families of plants; (Dicke, 2000)), they encounter large amounts of predictable chemistries, and have characteristically high P450-based metabolism towards such chemistries. This is clearly the case for CYP6B1, a P450 from the black swallowtail, *Papilio polyxenes*, that is induced by and metabolizes toxic host plant furanocoumarins (review in Berenbaum, 2002). Polyphagous, generalist herbivores on the other hand face a more diverse array of chemistries. It is well known that plant chemicals, pesticides and other xenobiotics can induce the activity of insect P450 enzymes and the expression of some specific genes (review in Feyereisen, 2005). It is not yet known which types of chemicals induce which type of P450 genes. It is not known how relevant concentrations of plant-derived inducers affect the transcriptome in the cells exposed to, and responding to the inducer - principally the midgut in the case of Lepidoptera.

Thanks to the Génoscope BAC sequencing project "comparative genomics of Lepidoptera" and the access to ESTs from 8 different *S. frugiperda* libraries built from different tissues and development stages as well as from treated individuals, we were able to print long oligonucleotide microarrays containing 9773 60-mers oligonucleotides from *S. frugiperda*. In

addition, we were able to identify 33 sequences of P450 genes in *S. frugiperda*, out of which 9 could be efficiently tested in qRT-PCR reactions in Sf9 cells and 18 in *S. frugiperda* midgut. The remaining P450s were either not detectable in the midgut or in Sf9 cells or did not show acceptable efficiency in qRT-PCR and were thus not tested.

We have used both approaches, microarray and qRT-PCR on specific P450 genes to study the xenobiotic response in the polyphagous pest *S. frugiperda* both *in vivo* in the larvae midgut and *in vitro* in Sf9 cells exposed to a battery of xenobiotic compounds containing plant allelochemicals, insecticides and potent inducers, as described earlier in the objectives. By studying the effect of different types of xenobiotics, we wish to span different paths of the chemical space that insects have to navigate through. As we said in the first chapter of this manuscript, chemical space encompasses every possible existing molecules, with only a small part being biologically relevant, meaning synthesised and/or used by living organisms. These molecules can be characterised by different descriptors from their molecular weight to the partition coefficient in octanol relative to water. We have summarised in table 4 the main descriptors of the xenobiotic compounds that we have used in this study. They range from 117 to 505 in MW and from somewhat polar to liphophilic.

We will now present our results where we have identified patterns of gene induction in response to xenobiotics by microarray analysis and specific induction of P450 genes by qRT-PCR.

Table 4 : chemical properties of the xenobiotic compounds used as inducers in S. frugiperda larvae and Sf9 cells

compound	logP octanol/ water	MW	source	Molar volume (cm^3)	H bond donors	H bond acceptors	Molar refractivity (cm^3)	Polar surface area (angström)
Deltamethrin	6.20	505.2	Synthetic	316.7	0	4	116.0	59.32
Quercetin	1.82	304.2	Many	178.6	5	7	73.7	72.45
Methoxyfenozide	3.72	368.5	Synthetic	335.3	1	5	108.2	49.85
Xanthotoxin	1.93	216.2	Apiaceae	158.0	0	4	56.6	48.67
Indole	2.14	117.1	Many	101.8	1	1	38.5	4.93
Indole 3-carbinol	0.96	147.2	Brassicacae	115.3	2	3	45.0	14.16
Clofibrate	3.32	242.7	Synthetic	211.4	0	3	62.7	35.53
2-tridecanone	5.16	198.3	Solanaceae	240.2	0	1	62.3	17.07
Fipronil	3.21	437.1	Synthetic	233.6	2	5	81.8	81.13
Methoprene	5.63	310.5	synthetic	339.8	0	3	93.4	35.53

Xenobiotic gene induction response in *Spodoptera frugiperda* midgut and Sf9 cells.

Maeva Giraudo[a], René Feyereisen[a]*, Frédérique Hilliou[a], Trang Tran[b] and Gaëlle Le Goff[a].

[a] UMR 1301 IBSV, INRA-CNRS-Université de Nice Sophia Antipolis, 400 Route des Chappes, 06903 Sophia-Antipolis, France

[b] Lanaud Gestion-Pôle de Lanaud, 87220 Boisseuil, France

*Corresponding author: René Feyereisen, UMR 1301 IBSV, INRA-CNRS-Université de Nice Sophia-Antipolis, 400 Route des Chappes, 06903 Sophia-Antipolis, FRANCE; phone : 33 492386450, fax : 33 492386401, e-mail : rfeyer@sophia.inra.fr

short title: *Spodoptera frugiperda* xenobiotic response

Abstract

Phytophagous insects have developed sophisticated mechanisms to metabolicaaly inactivate the potentially toxic plant allelochemicals and insecticides that they ingest. These mechanisms are mainly based on the induction of detoxification genes such as cytochrome P450s. We have studied the xenobiotic response in the polyphagous lepidopteran pest*, Spodoptera frugiperda* and in the derived Sf9 cells in response to different chemical compounds. Microarray analysis showed that the xenobiotic response was different *in vitro* and *in vivo*, with a common pattern involving the regulation of detoxification genes. qRT-PCR analysis showed that the plant allelochemicals indole and xanthotoxin were the strongest inducers of a small subset of P450s in the midgut (CYP321A7, CYP321A9, CYP9A32) whereas insecticides induced P450 expression mostly *in vitro* (CYP9A30, CYP9A31). Our results suggest that the xenobiotic response in a polyphagous pest rely on detoxification gene regulation involving the induction of different subset of P450s.

Keywords: P450, Spodoptera frugiperda, microarray, xenobiotics.

1. Introduction

The chemical space, which encompasses all possible existing small molecules (>10^{60}), comprises only a small fraction of biological relevant molecules that are synthesized and/or used by living organisms (Dobson, 2004). Plant metabolome represents a large part of that space as plants synthesize a broad range of secondary metabolites with some 200 000 compounds well known to pharmacologists (Hartmann, 2004). These plant allelochemicals are essential for their growth and development as well as defensive compounds against their natural enemies, such as herbivorous insects. Indeed, plants and insects have co-evolved for more than 350 millions years, entering an evolutionary arms-race in which plants evolved allelochemicals-based mechanisms to fend off their natural enemies and herbivorous insects in turn have developed sophisticated mechanisms to counteract these plant defences (Gatehouse, 2002; Rausher, 2001).

Some of the most important mechanisms that have allowed herbivorous insects to persist in a modern agriculture are their ability to metabolize and detoxify xenobiotics that they encounter as constituents in their host plants (Ferry et al., 2004; Li et al., 2007). This ability has been shown to grant phytophagous insects with the capacity of developing cross-resistance to insecticide compounds used in pest management. This is the case for example in the Lepidoptera *Helicoverpa zea,* which shows increased tolerance to the insecticide carbaryl after being exposed to 2-tridecanone, a toxic allelochemicals from trichomes of wild tomatoes (Riskallah et al., 1986). Lepidoptera represent a diverse and important group of agricultural insect pests that cause widespread economic damage on food and fiber crop plants, fruit trees, forests, and stored grains. Understanding detoxification machinery in these species has therefore become critically important in order to develop efficient pest control strategies.

This detoxification machinery includes different classes of enzymes, the most abundant and the best known being the cytochrome P450 enzymes. Insects have about 100 P450 genes (Ranson et al., 2002; Tijet et al., 2001) that play numerous functional roles, including growth, development, feeding, resistance to pesticide and tolerance to plant toxins (Feyereisen, 1999). Given the importance of insect P450 in metabolism, this versatile metabolic system has been a main topic in insect research for many decades. It is now well known that plant chemicals, pesticides and other xenobiotics can induce the activity of insect P450 enzymes and the expression of some specific genes as it has been reviewed in (Feyereisen, 2005; Yu, 1986). For example, members of the CYP4 family in the tobacco hornworm, *Manduca sexta* (Snyder et al., 1995) of the CYP9 family in *Helicoverpa armigera* (Zhou et al., 2009) and the CYP6 family in *Helicoverpa zea* (Li et al., 2002) have all been reported to be inducible by host plant chemicals and xenobiotics. However, the pattern of response within the whole multigene families or the selectivity of the inducer has yet to be documented.

With the recent advent of complete, annotated genomes of different insect species such as Drosophila (Adams et al., 2000) and Tribolium (Richards et al., 2008), the study of xenobiotic response in insects has been taken to the whole transcriptome level thanks to the development of post-genomic tools such as DNA microarrays and more recently to the pyrosequencing. DNA microarray hybridization has rapidly become a method of choice to investigate genome wide response to xenobiotics. For example, this technique has been used to study the molecular response of *Drosophila* to chemical stressors such as H_2O_2, paraquat and tunicamycin (Girardot et al., 2004). Moreover, the design of specific "detox" chips that represent all members of the detoxification gene families have provided an excellent tool to identify inducible genes. For example, detox chips have been used in *Drosophila* to study P450 gene induction in response to phenobarbital and atrazine (Le Goff

et al., 2006) or to measure the induction of detoxification gene families such as P450s, GST sans esterases in response to insecticides, caffeine and phenobarbital (Willoughby et al., 2006). Similar approaches have also been widely used in mosquitoes such as *Anopheles gambiae* (Vontas et al., 2005) and *Aedes aegypti* (Poupardin et al., 2008) to identify detoxification response to insecticide exposure.

In Lepidoptera, a relatively lower number of studies have used post-genomic tools to investigate patterns of gene expression in these insects. For example, cDNA libraries were constructed from pheromone glands of *Heliothis virescens* and compared to other available moths cDNA databases to identify genes involved in the biosynthesis of pheromones (Vogel et al., 2010) and the recently developed pyrosequencing technique was applied to *Manduca sexta* to define larval midgut transcriptome and revealed the identification of new genes, among which 36 new P450s (Pauchet et al., 2010). With the sequence of *Helicoverpa armigera* on its way, there is no doubt that lepidopteran transcriptomic studies will grow, using recent advances in high-throughput genomic analysis such as pyrosequencing.

Among Lepidoptera, the fall armyworm *Spodoptera frugiperda* (JE Smith) is a serious polyphagous pest present mostly in the American continent that causes severe damages on several economically important crops such as corn, soybeans, peanuts, sorghum, Bermuda grass and cowpeas. This pest has developed resistance to major classes of insecticides in many areas as a result of chemical control (Negre et al., 2006; Yu and McCord, 2007). In addition to being important agricultural pests these noctuids are biological models studied for several purposes. For example, *S. frugiperda* is well known through its famous Sf21 cell line and its Sf9 subclone (Vaughn et al., 1977) which is used for numerous heterologous protein productions (Negre et al., 2006).

We have constructed a *Spodoptera frugiperda* microarray consisting of 9773 60-mers oligonucleotides designed from ESTs databases obtained from eight different tissues, developmental stages and treated insects. Here we report the use of this microarray to study the detoxification response in this polyphagous lepidopteran pest exposed to different plant allelochemicals and insecticides. By choosing a generalist species, we have increased our chance to cover many different detoxification pathways as in contrast to specialist insects, they encounter large amounts of predictable chemistries, and have therefore characteristically high P450-based metabolism towards such chemistries (Stevens et al., 2000). We have therefore also monitored P450 gene induction in response to the different compounds tested to identify their role in the xenobiotic response of *S. frugiperda*.

2. Material and Methods

2.1 Reagents

The analytical grade plant allelochemicals xanthotoxin, quercetin, 2-tridecanone, indole, indole 3-carbinol and the herbicide clofibrate used in this study, as well as the drug phenobarbital were purchased from Sigma-Aldrich (St Quentin Fallavier, France). The insecticides deltamethrin, methoxyfenozide and fipronil were purchased from Cluzeau (CIL, France). Methoprene was a gift from the former Zoecon Corp., Palo Alto, California.

2.2 Insect rearing and treatments

Larvae of *Spodoptera frugiperda* were fed *ad libitum* on artificial medium adapted from Poitout and Bues (Poitout and Bues, 1974) and were reared at 25,5°C ±1°C, and 70%

relative humidity under a L 14: D 10 photoperiod. Under these conditions, the 5th (penultimate) and 6th (ultimate) larval stage last about 2 to 3 days each, with additional two pre-pupal days characterized by the arrest of wandering and beginning of weight lost towards pupal formation.

For xenobiotic induction of gene expression, newly molted 6th instar larvae were fed for 24h on artificial medium containing doses of different xenobiotic compounds chosen according to the literature for plant allelochemicals and/or to toxicity testing for insecticide compounds (table 2). Briefly, newly moulted 6th instar larvae were fed for 24h with increasing concentrations of each of the xenobiotic compound and mortality was recorded for 10 larvae for each of the concentrations. Induction doses were chosen the highest dose that causes no mortality in 10 larvae.

Newly molted 6th instar larvae were then fed either 0.5% phenobarbital, 0.05% xanthotoxin, 0.25% 2-tridecanone, indole 3-carbinol, indole, clofibrate or quercetin, 20nM methoxyfenozide, 2µM methoprene, 10nM deltamethrin, 2µM fipronil or the equal amount of DMSO for control experiments.

2.3 cell culture and treatments

Sf9 cell line derived from the pupal ovarian tissue of *Spodoptera frugiperda* was purchased from Invitrogen. The cells were cultured and maintained in insect-Xpress serum free medium (Lonza) at 27°C in suspension spinner flasks with an agitation rate of 100 rpm and passaged routinely every third day. Cell density was determined by Malassez hemocytometer counts and cell viability was evaluated by methylene blue (1mg/mL, v/v)

staining. Prior to experiments, cells were sowed in 6 well plates (TPP) at 5.10^5 cells/ml and left at 27°C for adhesion. Attached cells were treated for 24h with doses of different xenobiotics chosen after 24h MTT cytotoxicity testing. Briefly, Sf9 cells were seeded in 96-well culture plates and treated in triplicates for 24 hours with increased concentrations of each of the different compounds. Cells in culture were then loaded with MTT (5mg/ml) and incubated at 27°C for 2 hours. Cell homogenates were used to measure absorbance at 570nm using a microplate reader (SpectraMax, Molecular Devices). Induction doses were chosen at the DL10, which represents the dose lethal to 10% of the cells (table 3). Sf9 cells were treated for 24h with 500µM 2-tridecanone, 250µM phenobarbital, indole and indole 3-carbinol, 100µM xanthotoxin, clofibrate, and deltamethrin, 50µM quercetin, 25µM methoprene, 1µM fipronil 0.1µM methoxyfenozide or the same volume of DMSO.

2.4 RNA extraction

For xenobiotic induction of gene expression studies in *S. frugiperda* larvae, midguts were dissected from larvae fed for 24h on artifical medium containing each of the tested compounds at the concentration stated above.

Total RNA was extracted from the midgut using TRIzol Reagent (Invitrogen Life technologies) according to the manufacturer's protocol. After quantification of RNA concentration at 260nm and verification of RNA quality on 2% agarose gels, RNAs were stored at -80°C until use. RNA extractions were performed on 3 independent biological replicates of 5-pooled midguts.

Total RNA was extracted from cells of a well of the 6 wells plate also using Trizol Reagent and RNA were analysed and stored the same way than for tissue samples. Extractions were performed on three independent biological replicates.

2.6 Quantitative real-time PCR

Total RNA (1µg) was reverse transcribed using the iScript cDNA Synthesis kit (Biorad). qRT-PCR reactions were carried out on an Opticon monitor 2 (Biorad) using the qPCR Mastermix plus for SYBR Green I no ROX (Eurogentec). PCR primer pairs for *S. frugiperda* P450s and the three control genes (G6PD, L18 and Rpl4) are described in table 1. Final concentration of primers was 20nM. Efficiency of each primer pair was determined by absolute standard curves for the different gene transcripts and their controls by serial (5x) dilutions of 6th instar midgut and Sf9 cells cDNA. The PCR conditions were as follows: 50°C for 2 min, 95°C for 10min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Each reaction was performed in duplicate and the mean of the three independent biological replicates was calculated for midgut and Sf9 cells. All results were normalized using mRNA level of three control genes (RpL4, L18 and G6PD) and relative expression values were calculated in R using the RqPCRBase package developed in our laboratory (Hilliou and Tran, manuscript in preparation).

2.6. Microarray experimental design

Our oligonucleotides were designed from 79148 ESTs sequences of eight different cDNA libraries of *Spodoptera frugiperda* representing many tissues including midgut and Sf9 cells (see http://bioweb.ensam.inra.fr/spodobase/). Using the assembly analysis (programme CAP3), we obtained 10092 contigs and singletons from these ESTs. Our *Spodoptera frugiperda* microarray consist of 9773 60-mers oligonucleotides synthesised by Sigma-Aldrich that were designed to match unique contigs or singletons and to suit our hybridization conditions (GC content average 46% and average Tm of 46°C). Each

comparison consisted in six microarrays, three biological replicates hybridized with dye swap (fully balance dye swap design) and duplicate spots. cDNA were synthesized from 7µg of total RNA and labelled with the dyes Cy3-dCTP and Cy5-dCTP (Amersham) using the ChipShot direct labelling system Promega according to the manufacturer's instructions. The microarrays were hybridized with cDNA prepared as described in Le Goff *et al* (2006) and scanned using GenePixPro scanner (Axon,version 3.01). Experimental data and associated microarray designs have been deposited in the NBCI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under serie X and platform record X using Mediante database for data transfer (Le Brigand and Barbry, 2007).

2.7. Microarray data analysis

We used the Bioconductor suite of statistical packages (Gentleman et al., 2004): *limma* (Wettenhall and Smyth, 2004) for our data analysis. The expression intensities were obtained by subtracting the background intensity from the foreground intensity for each non-flag spot (all flagged spots were eliminated). The expression data were normalised by the use of the within-array normalization with the "loess method" and the between-array normalization using the "quantile method" (Yang and Thorne, 2003). The linear model for series of arrays and empirical Bayes method were then applied for assessing differential expression (Smyth, 2004). The false discovery rate of the p-value for multiple tests was controlled by using Benjamini-Hochberg method. Differentially expressed genes were selected if the absolute value of log2-fold-change greater than 1 and adjusted p-value below 0.01 and if the average intensity is greater than two time of average background. In order to provide an overall measure of evidence of differential expression, we used the Fisher's

method for combining adjusted p-values from independent tests of significance of duplicate spots (Hess and Iyer, 2007).

3. Results

3.1 Choice of compounds and doses used

In order to study the xenobiotic response in *S. frugiperda*, last instar larvae and Sf9 cells were exposed for 24h to different plant allelochemicals: indole-3-carbinol, a glucobracissin derivative that is a known inducer in *S. frugiperda* and an Ah receptor activator in vertebrates; xanthotoxin (8-methoxypsoralen), a coumarin derivative that is a potent inducer in many insect species; 2-tridecanone, a secondary compound found in the trichomes of the wild tomato; indole as maize defensive compound and quercetin, a flavonoid that is encountered in many Lepidoptera host plant species. Some commonly used insecticides have also been tested: methoprene and methoxyfenozide, respectively juvenile hormone analog and ecdysone agonist; deltamethrin, a commonly used pyrethrinoid and fipronil, an insecticide that targets GABA receptors. In addition, we used phenobarbital as archetypal inducer and the PPAR-type inducer clofibrate.

The different xenobiotics were used at doses similar to the ones reported in the literature and known to induce detoxification genes when it was possible (table 2), as this was the case for plant allelochemicals in *in vivo* experiments. However, induction doses for insecticides in *S. frugiperda* larvae and for all xenobiotic tested in Sf9 cells (table 3) were chosen from mortality monitoring assay and cytotoxicity testing respectively. That way we could chose doses that would be high enough to potentially induce molecular xenobiotic response in

both systems without causing toxic-related effects that could interfere with the identification of detoxification machinery response. Our results show that insecticides had quite different toxic effects in *S. frugiperda* larvae as some compounds such as fipronil and methoprene that start to cause mortality at doses within the micromolar range, when methoxyfenozide and deltamethrin seem to exert toxic effects at a much lower nanomolar range (table 2).

In Sf9 cells, some compounds showed no cytotoxicity at the highest doses tested such as deltamethrin and 2-tridecanone, when compounds such as methoxyfenozide were toxic for the cells at concentrations as low as 0.1 µM (table 3). Interestingly, methoprene was the only compound that showed a more important toxic effect in larvae than in the cells.

3.2 Microarray results

These doses were then used in 24h exposure of *S. frugiperda* larvae and Sf9 cells to monitor gene expression patterns in both systems by microarray analysis. The number of genes for which levels of expression were significantly affected by each of the tested compounds in both systems is depicted in figure 1. Our results indicate that *S*. *frugiperda* larvae and Sf9 cells show a striking different gene expression response to xenobiotics. Plant allelochemicals have the most important effect *in vivo*, such as indole, quercetin and xanthotoxin that regulate the expression of 227, 277 and 117 genes respectively, but have a moderate effect *in vitro* compared to clofibrate, which affected 173 genes in Sf9 cells. In addition, the number of commonly affected genes in both systems is extremely low, not exceeding 6 common genes in response to indole 3-carbinol and none in response to deltamethrin. Table 4 summarizes the genes that are affected both in larvae and cells. Expression values are represented as the log of the fold change for each of the genes and its associated *p* value.

Sequences of each gene were submitted to blastx to retrieve gene functions. The sequences for which no homology was found by blastx were submitted to a blastn in Butterflybase and classified in "hypothetical protein" category if homology was found with the sequence of another lepidopteran transcript, while the sequences restricted to *Spodoptera frugiperda* were put in a "hypothetical transcripts" category. As we can see, some genes are found up-regulated in both cells and larvae for most of the compounds such as carboxyl/choline esterase, which is known to be involved in detoxification metabolism of xenobiotic in insects and insecticide resistance (Yu et al., 2009). However, some genes show opposite regulation patterns in larvae and cells as it is the case in response to clofibrate and methoprene, where all commonly affected genes are found up-regulated in Sf9 cells and down-regulated in larvae. *In vivo* and *in vitro* systems show therefore a different response to xenobiotics, which do not seem to be correlated as shown in figure 2. The number of gene affected by each of the treatments shows indeed no correlation between the larvae and the cell line.

When looking at the number of genes found up- and down-regulated in *S. frugiperda* larvae and Sf9 cells in response to xenobiotics, our results show once again that both systems display different regulation patterns of gene expression as it can be seen in figure 3. We found that the plant allelochemicals xanthotoxin, quercetin and indole were the stronger inducers of gene expression in *S. frugiperda* larvae with 46, 100 and 88 genes up-regulated by these compounds respectively. In Sf9 cells, it seems that the herbicide clofibrate was the stronger inducer with 84 genes up-regulated by this xenobiotic when only 35 were induced in the larvae.

Among the genes that respond significantly to the tested xenobiotic, we have found that a number of them were involved in the detoxification response as it is shown in table 5 for *in*

vivo experiments and in table 6 for Sf9 cells. Genes such as carboxylesterases and glutathione S-transferases (GSTs) were up-regulated in response to indole, indole 3-carbinol, methoxyfenozide, phenobarbital and xanthotoxin in larvae, in addition we also found a glucosyltransferase induced by fipronil and phenobarbital and two P450s, CYP6B40 and CYP4M18 that were induced by methoxyfenozide and quercetin respectively. Genes involved in oxidative stress response were also found up-regulated in larvae such as a peroxiredoxin in response to clofibrate, a catalase and a peroxidase were up-regulated by indole and methoxyfenozide induced the expression of a superoxide dismutase. Interestingly, a number of the detoxification genes were also down-regulated in response to the different xenobiotics. This is the case for several GSTs in response to indole, indole 3-carbinol, quercetin and xanthotoxin and for two P450s: CYP6B40 in significantly down-regulated by indole and CYP9A25 by xanthotoxin. In addition, one CYP9A9 related gene and CYP6B6 was down-regulated by quercetin and a P450 similar to CYP321A1 from *Helicoverpa armigera* also showed a reduced expression in response to xanthotoxin. We found similar detoxification gene response in Sf9 cells with the induction of carboxylesterases in several treatments, including indole, indole 3-carbinol, methoprene, phenobarbital and xanthotoxin as it was found in larvae. Oxidative stress related genes such as peroxiredoxin and peroxidase were also found up-reglated in response to clofibrate and indole respectively, in concordance to the larvae response. We also found that CYP4M18 was induced by deltamethrin, as well as the *Helicoverpa* related CYP321A1 and CYP4M15 showed an increased expression in response to 2-tridecanone.

3.3 P450 expression monitored by qRT-PCR

We have further explored specific P450 gene expression in *S. frugiperda* larvae and Sf9 cells by quantitative-real time PCR analysis and results are presented in table 7 and table 8. We found 33 P450 genes in *S. frugiperda* databases built from different tissues, developmental stages and treated individuals. Out of these 33 genes, we were able to measure with acceptable qRT-PCR efficiency 19 of them in *S. frugiperda* midgut and 9 in Sf9 cells. The remaining P450s were either not detectable in one or the other system or did not show qRT-PCR efficiency in the 90-110% range. Except for CYP321A7 and CYP333B2, which are both induced in larvae and cells in response to indole 3-carbinol and fipronil respectively, none of the compounds showed the same patterns of gene induction in both systems. Plant allelochemicals were the stronger P450 inducers in larvae, with CYP321A7 being induced by 162 fold in larvae treated with xanthotoxin and by 135 fold in response to indole. Insecticides such as methoprene, deltamethrin and the herbicide clofibrate did not induce significantly P450 gene expression. Different results were observed in Sf9 cells, with these latter 3 compounds showed an increase expression of several P450s and xanthotoxin being unable to induce any of them. We were not able to validate microarray results that showed specific induction or repression of P450 genes as for example, CYP6B40 that was found down-regulated by indole in microarray anaylsis but up-regulated in qRT-PCR experiments.

Taken together, our results show that Sf9 cell response to xenobiotics does not match *in vivo* response and that both system show different patterns of gene induction. However different these patterns are, they still involve detoxification gene expression and P450s in particular.

Discussion

We have studied gene expression patterns in the midgut of *S. frugiperda* larvae exposed to various plant allelochemicals and insecticides and used the same battery of chemicals on the Sf9 cell line derived from this species. Insect cell culture have been used in numerous studies as research tools in virology, in studies of signaling mechanisms to study insect immunity, hemocyte migration, and to test hypotheses about gene expression, and in screening programs designed to discover new insecticide chemistries (for a review see Smagghe et al., 2009). Sf9 cells have however mainly been used as a heterologous expression system through baculovirus mediated expression. We report here the first evidence of the use of Sf9 cells as a model system to study xenobiotic response of insects. Our results show that *S. frugiperda* larvae and Sf9 cells display gene expression response to xenobiotic exposure involving several detoxification genes. We found that plant allelochemicals such as xanthotoxin, indole and indole 3-carbinol were able to induce carboxylesterases in both systems, an enzyme class that has been shown to be involved in the detoxification of xenobiotics and insecticide resistance such as carbamate and pyrethroids (Yu et al., 2009). We also found that GSTs were found up-regulated in response to many different xenobiotics, which is consistent with the literature, for example GSTs induction was reported in Drosophila in response to atrazine and phenobarbital (Le Goff et al., 2006; Willoughby et al., 2006) and to indole and xanthotoxin (Li et al., 2007). We have found one GST induced in the midgut exposed to phenobarbital and to xanthotoxin and two GSTs over-expressed in response to indole. We also showed that some GSTs were down-regulated by some compounds such as the plant allelochemicals quercetin. This is not surprising as quercetin has been shown to inhibit the expression of some GSTs in *Trichoplusia ni* (Ahmad and Pardini, 1990). Glucosyltransferases (UGTs) were found mostly down-regulated in the

midgut by compounds such as clofibrate, fipronil and quercetin and similarly in response to clofibrate, indole 3-carbinol and 2-tridecanone in Sf9 cells. UGTs have been identified in many insect species and although their function is not yet clearly established, that have been involved in detoxifcation of xenobiotic and insecticide resistance (Luque et al., 2002). The fact that these genes were found mostly down-regulated in both systems could account for the toxic effect of the corresponding compounds. In addition, we found a number of antioxidant enzymes coding genes that were affected by the different xenobiotic treatments in both larvae and cells. Oxidative stress is a major deleterious mechanisms that occurs in every aerobic living organisms by the generation of reactive oxygen species (ROS), and occurs in insects through respiration and the ingestion of ROS generating compounds such as phenols (Barbehenn, 2002). We found that clofibrate was able to induce one peroxiredoxin *in vivo* and in Sf9 cells. This enzyme was also found to respond to H_2O_2 and temperature stress in *Bombyx mori* (Wang et al., 2008) and the bumble bee (Hu et al., 2010). We also found that indole and methoxyfenozide were inducers of a catalase and a superoxide dismutase respectively in the midgut, both being active antioxidant enzymes found in many insect species, such as in the grasshopper in reponse to tanic acid diets (Barbehenn, 2002). Our results show therefore a common response in *S. frugipera* larvae and Sf9 cells involving detoxification genes. However, the regulation of these genes showed different patterns in both systems, suggesting different sensitivity of midgut and cells to xenobiotics. This is confirmed by the number of genes found up- and down-regulated that differs remarkably in both systems in response to the same xenobiotic compounds. Cell lines have always been a model system is toxicological studies in vertebrate and have been successfully used in insect to understand endogenous regulatory pathways involved in ecdysteroid signalling mechanisms and the effects of hormone agonists such as methoprene

and methoxyfenozide (Dhadialla et al., 1998). Our results indicate that although this system might be useful to study detoxification response in insects, it does not represent what is happening in insect tissues such as insect midguts. We have indeed found that P450 gene induction was quite different in larvae and in Sf9 cells, with compounds such as xanthotoxin and indole being strong P450 inducers in midguts and inversely having no or moderately effects in the cells. Similarly, insecticides such as methoprene and deltamethrin were only able to induce P450s in the cells. Sf9 cells have been shown to be a reliable system to study the induction of an heterologous CYP6B1 by xanthotoxin (Brown et al., 2005). However, we were unable to show a significant induction of any of the 9 P450s tested in Sf9 cells by xanthotoxin, thus indicating that additional P450 not yet identified in the cells might be involved. P450 gene induction was also found different in microarray analysis and qRT-PCR studies, with CYP6B40 being induced by methoxyfenozide in midgut by specific qRT-PCT measurement but the corresponding gene in the microarray analysis was found down-regulated. Discrepancies between microarray and real-time quantitative PCR data can result from biological variation or technical issues during microarray statistical analysis, which might have eliminated genes that did not reach normalization and statistical reproducibility requirements, as it was also the case in P450 gene expression studies in *Aedes aegypti* (Poupardin et al., 2008). Our results confirm that microarray experiments require cross-validation with other gene-expression profiling techniques. We have therefore explored the available catalogue of P450 gene expression in both system in response to plant allelochemicals and xenobiotics. P450s are the best known enzymes involved in the detoxification machinery of xenobiotics in insects (Feyereisen, 2005). We found that the CYP9A and CYP321A families were the most induced by the plant toxins xanthotoxin and indole. Our results confirm the inducibility of theses P450 that is found in the literature.

Indeed, CYP321A1 from *Helicoverpa zea* is induced by indole and xanthotoxin (Zeng et al., 2007), and CYP9A members are actively induced in *Manduca sexta* by xanthotoxin, phenobarbital, indole 3-carbinol, 2-tridecanone and clofibrate (Stevens et al., 2000). CYP9A30, 31 and 32 were inducible in Sf9 cells by deltamethrin as it was found for CYP9A12 and CYP9A17 in *Helicoverpa armigera* (Zhou et al., 2009), however we could not detect any induction of these genes in the midgut nor in response to phenobarbital as it is the case for *H. armigera*. In addition, we found that CYP4M14 was induced by 2-tridecanone and methoprene in Sf9 cells and CYP4M members have already been shown to respond to 2-tridecanone, clofibrate and phenobarbital in *Manduca sexta* (Snyder et al., 1995).

Our results according to the literature indicate that detoxification mechanisms in insects are complicated, hence in a polyphagous species such as *S. frugiperda*, which encounters a large array of xenobiotic compounds, and is mainly based on P450 gene induction. We have identified numerous P450s induced by the different xenobiotics, with genes from the CYP9A and CYP321A families that respond particularly to plant allelochemicals.

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F.*, et al.* (2000). The genome sequence of Drosophila melanogaster. Science *287*, 2185-2195.

Ahmad, S., and Pardini, S. (1990). Antioxidant defense of the cabbage looper, Trichoplusia ni: enzymatic responses to the superoxide-generating flavonoid, quercetin and photodynamic furanocoumarin, xantotoxin. Photochem Photobiol *51*, 305-312.

Barbehenn, R.V. (2002). Gut-based antioxidant enzymes in a polyphagous and a graminivorous grasshopper. J Chem Ecol *28*, 1329-1347.

Brown, R.P., McDonnell, C.M., Berenbaum, M.R., and Schuler, M.A. (2005). Regulation of an insect cytochrome P450 monooxygenase gene (CYP6B1) by aryl hydrocarbon and xanthotoxin response cascades. Gene *358*, 39-52.

Dhadialla, T.S., Carlson, G.R., and Le, D.P. (1998). New insecticides with ecdysteroidal and juvenile hormone activity. Annu Rev Entomol *43*, 545-569.

Dobson, C.M. (2004). Chemical space and biology. Nature *432*, 824-828.

Ferry, N., Edwards, M.G., Gatehouse, J.A., and Gatehouse, A.M. (2004). Plant-insect interactions: molecular approaches to insect resistance. Curr Opin Biotechnol *15*, 155-161.

Feyereisen, R. (1999). Insect P450 enzymes. Annu Rev Entomol *44*, 507-533.

Feyereisen, R. (2005). Insect cytochrome P450. In Comprehensive molecular insect science (Elsevier), pp. 1-77.

Gatehouse, J.A. (2002). Plant resistance towards insect hrebivores: a dynamic interaction. New Phytol *156*, 145-169.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J.*, et al.* (2004). Bioconductor: open software development for computational biology and bioinformatics. Genome Biol *5*, R80.

Girardot, F., Monnier, V., and Tricoire, H. (2004). Genome wide analysis of common and specific stress responses in adult drosophila melanogaster. BMC Genomics *5*, 74.

Hartmann, T. (2004). Plant-derived secondary metabolites as defensive chemicals in herbivorous insects: a case study in chemical ecology. Planta *219*, 1-4.

Hess, A., and Iyer, H. (2007). Fisher's combined p-value for detecting differentially expressed genes using Affymetrix expression arrays. BMC Genomics *8*, 96.

Hu, Z., Lee, K.S., Choo, Y.M., Yoon, H.J., Lee, S.M., Lee, J.H., Kim, D.H., Sohn, H.D., and Jin, B.R. (2010). Molecular cloning and characterization of 1-Cys and 2-Cys peroxiredoxins from the bumblebee Bombus ignitus. Comp Biochem Physiol B Biochem Mol Biol *155*, 272-280.

Le Brigand, K., and Barbry, P. (2007). Mediante: a web-based microarray data manager. Bioinformatics *23*, 1304-1306.

Le Goff, G., Hilliou, F., Siegfried, B.D., Boundy, S., Wajnberg, E., Sofer, L., Audant, P., ffrench-Constant, R.H., and Feyereisen, R. (2006). Xenobiotic response in Drosophila melanogaster: sex dependence of P450 and GST gene induction. Insect Biochem Mol Biol *36*, 674-682.

Li, X., Berenbaum, M.R., and Schuler, M.A. (2002). Plant allelochemicals differentially regulate Helicoverpa zea cytochrome P450 genes. Insect Mol Biol *11*, 343-351.

Li, X., Schuler, M.A., and Berenbaum, M.R. (2007). Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu Rev Entomol *52*, 231-253.

Luque, T., Okano, K., and O'Reilly, D.R. (2002). Characterization of a novel silkworm (Bombyx mori) phenol UDP-glucosyltransferase. Eur J Biochem *269*, 819-825.

Negre, V., Hotelier, T., Volkoff, A.N., Gimenez, S., Cousserans, F., Mita, K., Sabau, X., Rocher, J., Lopez-Ferber, M., d'Alencon, E.*, et al.* (2006). SPODOBASE: an EST database for the lepidopteran crop pest Spodoptera. BMC Bioinformatics *7*, 322.

Pauchet, Y., Wilkinson, P., Vogel, H., Nelson, D.R., Reynolds, S.E., Heckel, D.G., and ffrench-Constant, R.H. (2010). Pyrosequencing the Manduca sexta larval midgut transcriptome: messages for digestion, detoxification and defence. Insect Mol Biol *19*, 61-75.

Poitout, S., and Bues, R. (1974). [Linolenic acid requirements of lepidoptera Noctuidae Quadrifinae Plusiinae: Chrysodeixis chalcites Esp., Autographa gamma L.' Macdunnoughia confusa Stph., Trichoplusia ni Hbn. reared on artificial diets]. Ann Nutr Aliment *28*, 173-187.

Poupardin, R., Reynaud, S., Strode, C., Ranson, H., Vontas, J., and David, J.P. (2008). Cross-induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito Aedes aegypti: impact on larval tolerance to chemical insecticides. Insect Biochem Mol Biol *38*, 540-551.

Ranson, H., Claudianos, C., Ortelli, F., Abgrall, C., Hemingway, J., Sharakhova, M.V., Unger, M.F., Collins, F.H., and Feyereisen, R. (2002). Evolution of supergene families associated with insecticide resistance. Science *298*, 179-181.

Rausher, M.D. (2001). Co-evolution and plant resistance to natural enemies. Nature *411*, 857-864.

Richards, S., Gibbs, R.A., Weinstock, G.M., Brown, S.J., Denell, R., Beeman, R.W., Gibbs, R., Bucher, G., Friedrich, M., Grimmelikhuijzen, C.J.*, et al.* (2008). The genome of the model beetle and pest Tribolium castaneum. Nature *452*, 949-955.

Riskallah, M.R., Dauterman, W.C., and Hodgson, E. (1986). Nutritional effetcs of the induction of cytochrome P-450 and glutathione transferase in larvae of the tobacco budworm, Heliothis virescens (F.). Insect Biochem *16*, 491-499.

Smagghe, G., Goodman, C.L., and Stanley, D. (2009). Insect cell culture and applications to research and pest management. In Vitro Cell Dev Biol Anim *45*, 93-105.

Smyth, G.K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol *3*, Article3.

Snyder, M.J., Stevens, J.L., Andersen, J.F., and Feyereisen, R. (1995). Expression of cytochrome P450 genes of the CYP4 family in midgut and fat body of the tobacco hornworm, Manduca sexta. Arch Biochem Biophys *321*, 13-20.

Stevens, J.L., Snyder, M.J., Koener, J.F., and Feyereisen, R. (2000). Inducible P450s of the CYP9 family from larval Manduca sexta midgut. Insect Biochem Mol Biol *30*, 559-568.

Tijet, N., Helvig, C., and Feyereisen, R. (2001). The cytochrome P450 gene superfamily in Drosophila melanogaster: annotation, intron-exon organization and phylogeny. Gene *262*, 189-198.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J., and McCawley, P. (1977). The establishment of two cell lines from the insect Spodoptera frugiperda (Lepidoptera; Noctuidae). In Vitro *13*, 213-217.

Vogel, H., Heidel, A.J., Heckel, D.G., and Groot, A.T. (2010). Transcriptome analysis of the sex pheromone gland of the noctuid moth Heliothis virescens. BMC Genomics *11*, 29.

Vontas, J., Blass, C., Koutsos, A.C., David, J.P., Kafatos, F.C., Louis, C., Hemingway, J., Christophides, G.K., and Ranson, H. (2005). Gene expression in insecticide resistant and susceptible Anopheles gambiae strains constitutively or after insecticide exposure. Insect Mol Biol *14*, 509-521.

Wang, Q., Chen, K., Yao, Q., Zhao, Y., Li, Y., Shen, H., and Mu, R. (2008). Identification and characterization of a novel 1-Cys peroxiredoxin from silkworm, Bombyx mori. Comp Biochem Physiol B Biochem Mol Biol *149*, 176-182.

Wettenhall, J.M., and Smyth, G.K. (2004). limmaGUI: a graphical user interface for linear modeling of microarray data. Bioinformatics *20*, 3705-3706.

Willoughby, L., Chung, H., Lumb, C., Robin, C., Batterham, P., and Daborn, P.J. (2006). A comparison of Drosophila melanogaster detoxification gene induction responses for six insecticides, caffeine and phenobarbital. Insect Biochem Mol Biol *36*, 934-942.

Yang, Y.H., and Thorne, N.P. (2003). Normalization for two-color cDNA microarray data. . In Science and Statistics: A Festschrift for Terry Speed, IMS Lecture Notes - Monograph Series, D.R. Goldstein., ed., pp. 403-418.

Yu, Q.Y., Lu, C., Li, W.L., Xiang, Z.H., and Zhang, Z. (2009). Annotation and expression of carboxylesterases in the silkworm, Bombyx mori. BMC Genomics *10*, 553.

Yu, S.J. (1983). Induction of detoxifying enzymes by allelochemicals and host plants in the fall armyworm. Pest Biochem Physiol *19*, 330-336.

Yu, S.J. (1986). Consequences of induction of foreign compound-metabolizing enzymes in insects. 153-175.

Yu, S.J., and McCord, E., Jr. (2007). Lack of cross-resistance to indoxacarb in insecticide-resistant Spodoptera frugiperda (Lepidoptera: Noctuidae) and Plutella xylostella (Lepidoptera: Yponomeutidae). Pest Manag Sci *63*, 63-67.

Zeng, R.S., Wen, Z., Niu, G., Schuler, M.A., and Berenbaum, M.R. (2007). Allelochemical induction of cytochrome P450 monooxygenases and amelioration of xenobiotic toxicity in Helicoverpa zea. J Chem Ecol *33*, 449-461.

Zhou, X., Ma, C., Li, M., Sheng, C., Liu, H., and Qiu, X. (2009). CYP9A12 and CYP9A17 in the cotton bollworm, Helicoverpa armigera: sequence similarity, expression profile and xenobiotic response. Pest Manag Sci.

Table 1 : primers used in qRT-PCR reactions

	Forward primer	Reverse primer
CYP9A24	GGCACTAAACAACAGAGTGTGG	ACCAAGCGTTCCTGTACGTC
CYP9A25	AATGCAAAGGCTGAGAAGGA	GAAAAACGATCAGGGTCGAA
CYP9A26	TGAAAGGCCAAGAATGGAAG	GTCATCTGACGCCTCGATTT
CYP9A27	CATCAAGTATCGCACGCCTA	ATTCAAATCTGCCGACGAAC
CYP9A30	GTCCTGGTGGCTGTGGTATT	GTGCGAAAAATGATCGTGTG
CYP9A31	ATGCTCGTCTTGGTCTGGTT	CTGCCCATGTTACCGAAGAT
CYP9A32	ATCATTCGTAAGGGCCAGTG	AAGTGAACGGGACGATTTTG
CYP6Bp2	CAATCCAGCACGATGAGAAA	GTGCGAATTTTGACCAAGG
CYP6B40	CTGTATCGGTATGCGGTTTG	TTCCACCTTTAGGTCCGATG
CYP4L9	TCGGTGATGACATGGAAAGA	AGAACGACAGACGTGCCTTTT
CYP4L13	ACGAACGTGAGTCTGCCTATGTGA	ACGACGTCCGGACCAAAAATC
CYP4M14	TGATCTCGGACTTGCACTTG	GTCCAGCGCTGAAAGGAATA
CYP4M15	TGATCTTGGACGTGCATTAT	GCCCAGCACTGAAGGGAATG
CYP4M17	AGAGTCGCTGCGCATATACC	GGGTTCGGGAATAAATCCTC
CYP4M18	TCCTACCCGAGAACAGCATC	ACTTCTGCCACAGCCATCTT
CYP321A9	GCGTGGTGTAGCCTTCTACG	CGGGTCAATGACAAACAGTG
CYP321A7	TCCAGACCCAGAAGTTTTCG	CGGCCTGGACTTGTAATTTG
CYP333B2	GAATTATGCCGGTGGTGTCT	TAGCGACATGTCTCGGTGAG
CYP337B1	CCGTTTGGTGAAGGAAACA	GACCGAAGGGACTTCTTTCA
G6PD	GGCCCTGTGGCTAACAGAAT	CATCGTCTCTACCAAAAGGCTTC
L18	CGTATCAACCGACCTCCACT	AGGCACCTTGTAGAGCCTCA
Rpl4	CAACAAGAGGGGTTCACGAT	GCACGATCAGTTCGGGTATC

Table 2: induction doses used in *S. frugiperda* larvae *in vivo* exposition for each of the tested compounds. References found in the literature are stated when possible for plant allelochemicals. Doses for insecticide compounds were chosen from 24h toxicity testing with the higher concentrations causing no larvae mortality (<DL05).

Compounds	doses	references
xanthotoxin	0.05 %	(Zeng et al., 2007)
Indole	0.25 %	Chosen similar to other allelochemicals
Indole 3-carbinol	0.25 %	(Yu, 1986)
Quercetin	0.25 %	(Yu, 1983)
2-tridecanone	0.25 %	(Riskallah et al., 1986)
Clofibrate	0.25 %	(Stevens et al., 2000)
Phenobarbital	0.50 %	(Snyder et al., 1995)
Methoxyfenozide	20 nM	<DL05 from toxicity testing
Methoprene	2 µM	<DL05 from toxicity testing
Deltamethrin	10 nM	<DL05 from toxicity testing
Fipronil	2 µM	<DL05 from toxicity testing

Table 3: induction doses used in Sf9 cells exposition studies for each of the tested compounds. Doses were chosen from 24h MTT cytotoxicity testing at DL10, unless no toxicity was recorded in which case the higher dose that remains soluble in culture medium was chosen.

Compounds	DL10	Dose chosen
Xanthotoxin	>100 µM	100 µM (limit of solubility)
Indole	250 µM	250 µM
Indole 3-carbinol	250 µM	250 µM
Quercetin	50 µM	50 µM
2-tridecanone	> 500µM	500 µM (limit of solubility)
Clofibrate	100 µM	100 µM
Phenobarbital	250 µM	250 µM
Methoxyfenozide	0.1 µM	0.1 µM
Methoprene	25 µM	25 µM
Deltamethrin	>100µM	100 µM (limit of solubility)
Fipronil	1 µM	1 µM

Table 4: microarray analysis of genes up- and down- regulated in both *S. frugiperda* larvae and Sf9 cells after exposure for 24h to different xenobiotics. Larvae and Sf9 cells were exposed for 24h to sublethal and >DL10 doses of xenobiotic respectively. Only genes found significantly up- or down-regulated in both systems as depicted in figure 1 are shown.

			Midgut		Sf9	
compound	Probe number	Function	Log FC	p value	Log FC	p value
Fipronil	34868	Pyruvate deshydrogenase	0.98	3.6E-2	-0.81	3.1E-2
Xanthotoxin	34868	Pyruvate deshydrogenase	1.75	6.9E-3	0.62	1.3E-2
	40592	Hypothetical transcript	1.84	4.0E-4	1.24	9.1E-8
	40600	Carboxyl cholinesterase	1.66	8.0E-4	1.09	3.7E-7
Indole	27227	Mitochondrial ribosomal protein	2.85	3.8E-9	1.72	4.2E-7
	33976	Oxidase peroxidase	0.71	2.9E-2	2.12	3.3E-2
	38033	Ribosomal protein	-0.81	5.0E-4	0.88	3.3E-2
	40600	Carboxyl cholinesterase	0.97	4.0E-4	2.05	4.2E-8
2-tridecanone	40999	Myosin regulatory light chain	-4.45	1.0E-2	2.93	2.8E-2
Clofibrate	39073	Small nuclear ribonucleoprotein	-0.82	5.4E-3	1.16	1.0E-4
	39351	ATP synthase b	-0.71	3.4E-2	0.80	6.5E-5
	40139	Hypothetical transcript	-0.63	4.3E-2	0.97	4.1E-2
Indole 3-carbinol	27227	Mitochondrial ribosomal protein	2.70	5.0E-4	1.94	4.8E-10
	36136	Hypothetical protein	-1.33	4.6E-3	-0.80	4.0E-2
	36395	Kinesin-like protein	-1.04	5.0E-3	0.87	7.1E-3
	40592	Hypothetical transcript	1.79	1.7E-6	2.41	2.6E-3
	40600	Carboxyl cholinesterase	1.65	4.5E-5	2.10	2.8E-8
	40870	Hsp 70 hsp 90 organizing protein	3.30	4.9E-2	2.93	1.9E-2
Methoprene	40592	Hypothetical transcript	-0.77	7.0E-3	2.04	1.5E-8
	40600	Carboxyl cholinesterase	-0.71	2.0E-4	1.97	3.7E-7
Phenobarbital	27227	Mitochondrial ribosomal protein	1.34	3.7E-8	0.82	8.8E-7
	40600	Carboxyl cholinesterase	1.25	3.7E-3	1.15	5.2E-7
Methoxyfenozide	25793	Vitellogenin	0.88	1.6E-2	0.77	1.4E-2
	40151	Gelsolin	0.72	1.9E-5	0.67	4.0E-2
quercetin	25385	Peptidyl prolyl isomerase	0.88	2.0E-2	0.68	2.7E-2

Table 5: microarray analysis of selected detoxification genes up- and down-regulated in *S. frugiperda* midgut after exposure for 24h to different xenobiotics.

compound	Gene description	Probe number	Log FC	*p* value
xanthotoxin	glutathione S-transferase 9	adhoc-41019	-1.03	5.84E-03
	CYP9A25	adhoc-38527	-0.80	3.02E-03
	cytochrome P450 monooxygenase CYP321A1	adhoc-34609	-0.67	4.86E-03
	carboxylesterase	adhoc-40600	1.66	8.41E-04
	glutathione S-transferase 14	adhoc-27466	1.53	1.90E-04
indole	glutathione S-transferase 9	adhoc-41019	-1.58	1.57E-04
	glutathione S-transferase 7	adhoc-39161	-0.68	1.29E-02
	CYP6B40	adhoc-40444	-0.67	1.47E-04
	catalase	adhoc-40325	1.36	1.20E-03
	glutathione S-transferase	adhoc-34379	1.31	4.40E-03
	glutathione S-transferase 14	adhoc-27466	1.22	5.45E-07
	carboxylesterase	adhoc-40600	0.97	4.02E-04
	epoxide hydrolase	adhoc-36011	0.74	4.41E-02
	peroxidase	adhoc-33976	0.71	2.89E-02
Indole 3-carbinol	S-formylglutathione hydrolase	adhoc-35696	-2.43	3.01E-02
	glutathione transferase zeta	adhoc-24957	-2.27	2.31E-04
	glutathione S-transferase S4	adhoc-40718	-0.68	3.70E-02
	carboxylesterase	adhoc-40600	1.65	4.50E-05
2-tridecanone	glutathione S-transferase 14	adhoc-39621	-0.60	3.96E-02
quercetin	carboxylesterase-6	adhoc-40352	-1.09	1.30E-02
	glutathione S-transferase 14	adhoc-39621	-0.88	4.09E-04
	phenol UDP-glucosyltransferase	adhoc-35493	-0.86	5.32E-03
	carboxylesterase-6	adhoc-40797	-0.83	2.42E-02
	P450 9A9 [Spodoptera exigua], CYP9A19 [Bombyx mori]	adhoc-27215	-0.81	2.27E-02
	Multidrug-Resistance like Protein 1 CG6214-PE, isoform E	adhoc-34231	-0.79	5.62E-03
	cytochrome p450 6B6 [Helicoverpa armigera]	adhoc-26019	-0.75	2.32E-03
	phenol UDP-glucosyltransferase	adhoc-41144	-0.66	1.60E-03
	CYP4M18	adhoc-24805	1.32	1.24E-02
deltamethrin	Reactive mitochondrial oxygen species modulator 1	adhoc-26149	0.76	2.90E-02
	multi drug resistance-associated protein (MRP)	adhoc-34231	0.62	3.77E-02
fipronil	phenol UDP-glucosyltransferase	adhoc-41144	-0.74	4.45E-02
	phenol UDP-glucosyltransferase	adhoc-36536	2.20	3.66E-02
methoxyfenozide	cytochrome c oxidase, subunit VB	adhoc-36011	2.10	2.22E-02
	glutathione transferase zeta	adhoc-44426	1.79	6.63E-03
	Mn superoxide dismutase	adhoc-40571	1.38	2.55E-02
	carboxylesterase-6	adhoc-36431	0.98	4.99E-02
	glutathione S-transferase 9	adhoc-40352	0.73	1.09E-02
	CYP6B40	adhoc-41019	0.59	1.45E-02
methoprene	carboxylesterase	adhoc-40600	-0.72	2.54E-04
phenobarbital	glutathione S-transferase 2	adhoc-26178	2.97	1.47E-02
	carboxylesterase	adhoc-40600	1.25	3.71E-03
	phenol UDP-glucosyltransferase	adhoc-41144	0.60	2.75E-02
clofibrate	oxidative stress protein	adhoc-38734	-2.63	2.87E-02
	phenol UDP-glucosyltransferase	adhoc-38067	-0.63	1.05E-03
	1-Cys peroxiredoxin	adhoc-38799	1.32	5.26E-03

Table 6: microarray analysis of selected detoxification genes up- and down-regulated in Sf9 cells midgut after exposure for 24h to different xenobiotics.

compound	Gene description	adhoc	Log FC	*p* value
xanthotoxin	glutathione S-transferase	adhoc-24868	-4.22	2.88E-03
	glutathione S-transferase	adhoc-26347	-0.77	3.99E-02
	peroxidase	adhoc-33976	-0.73	1.32E-02
	Carboxylesterase	adhoc-40600	1.09	3.78E-07
indole	thioredoxin peroxidase	adhoc-26099	-0.79	3.51E-02
	uridine diphosphate glucosyltransferase	adhoc-35495	-0.68	1.69E-02
	peroxidase	adhoc-33976	2.12	3.40E-02
	carboxylesterase	adhoc-40600	2.05	4.27E-08
Indole 3-carbinol	catalase	adhoc-41112	-0.61	2.77E-02
	carboxylesterase	adhoc-40600	2.10	2.84E-08
2-tridecanone	carboxylesterase-6	adhoc-34509	-1.26	1.31E-02
	phenol UDP-glucosyltransferase	adhoc-38443	-1.13	2.39E-03
	glutathione transferase zeta	adhoc-24957	2.20	4.44E-02
	CYP4M15	adhoc-24793	1.45	2.24E-02
quercetin	Carboxylesterase	adhoc-40600	-0.59	3.97E-02
	similar to oxidative stress protein	adhoc-38734	4.05	7.84E-03
deltamethrin	cytochrome P450	adhoc-35655	-0.94	3.14E-02
	carboxylesterase	adhoc-40600	2.40	4.28E-10
	CYP4M18	adhoc-34767	0.84	3.96E-03
	cytochrome P450 monooxygenase CYP321A1	adhoc-34609	0.72	3.33E-02
fipronil	gst1	adhoc-26914	-0.95	2.15E-04
	carboxylesterase	adhoc-34965	-0.61	4.81E-02
	carboxylesterase	adhoc-40600	0.81	8.79E-03
methoxyfenozide				
methoprene	uridine diphosphate glucosyltransferase	adhoc-35495	-0.87	4.22E-02
	carboxylesterase	adhoc-40600	1.97	3.75E-07
phenobarbital	sulfotransferase	adhoc-25711	-1.71	4.91E-02
	similar to copper-zinc superoxide dismutase	adhoc-38960	1.99	3.78E-02
	Carboxylesterase	adhoc-40600	1.15	5.22E-05
clofibrate	phenol UDP-glucosyltransferase	adhoc-38443	-0.99	2.63E-05
	CYP6B39	adhoc-27179	-0.71	1.85E-05
	carboxylesterase	adhoc-40600	2.00	4.16E-05
	1-Cys peroxiredoxin	adhoc-26528	0.95	1.96E-03

Table 7: real-time quantitative PCR analysis of the differential expression of 19 P450 genes in *S. frugiperda* larvae exposed for 24h to sub-lethal doses of 11 different xenobiotic compounds. Gene expression values are indicated as fold expression in larvae exposed to each xenobiotic comparatively to unexposed larvae (controls). The three reference genes Rpl4, G6PD and L18 were used as internal controls for normalization. Gene expression values in bold are significantly different from the corresponding control as measured by pair-wise t-student test ($p<0.05$).

	xanthotoxin	2-tridecanone	indole 3-carbinol	indole	quercetin	methoxyfenozide	methoprene	deltamethrin	fipronil	clofibrate	phenobarbital
CYP4L9	0.32	0.79	0.44	0.44	0.46	0.53	1.12	0.86	1.11	1.02	1.25
CYP4L13	0.69	**5.09**	0.70	0.54	0.29	0.71	0.96	0.91	1.18	1.22	2.90
CYP4M14	**0.14**	0.56	0.31	0.46	0.47	0.58	0.80	0.60	0.61	0.76	0.71
CYP4M15	0.19	0.78	0.61	0.48	0.45	1.12	1.03	0.98	0.93	1.18	1.08
CYP4M17	0.17	1.01	0.33	0.36	0.41	0.86	1.10	0.86	0.78	0.75	1.25
CYP4M18	**0.38**	1.04	0.61	0.58	0.69	1.05	1.04	0.92	1.05	0.98	1.08
CYP6B40	**2.89**	0.72	2.50	**3.26**	0.42	1.75	0.90	1.14	1.42	0.63	**3.26**
CYP6Bp2	0.26	0.53	0.71	0.53	0.92	0.74	0.59	0.89	0.77	1.04	1.23
CYP9A24	0.99	0.63	0.53	0.53	0.42	0.92	1.07	1.36	1.48	1.00	0.94
CYP9A25	1.85	0.77	0.55	0.45	0.52	**3.80**	1.14	1.76	2.89	1.16	0.83
CYP9A26	1.41	0.70	0.46	1.43	0.23	1.45	1.29	0.91	1.89	0.56	0.95
CYP9A27	**2.92**	0.74	0.65	0.77	0.63	2.46	1.56	1.94	1.41	1.16	0.60
CYP9A30	1.53	0.75	1.45	2.10	0.35	1.18	1.11	1.20	0.91	0.86	1.03
CYP9A31	**21.04**	0.72	2.96	**8.13**	0.38	1.43	0.94	1.55	2.12	1.01	**2.93**
CYP9A32	**28.10**	0.73	1.15	3.28	0.48	3.02	1.70	3.05	2.34	1.42	0.67
CYP321A7	**162.11**	0.88	**64.31**	**135.95**	0.34	1.09	1.13	1.21	1.41	3.62	3.86
CYP321A9	**20.97**	0.92	**11.70**	**28.59**	0.61	1.58	1.49	1.52	1.12	1.22	3.42
CYP333B2	**2.66**	0.98	0.93	1.64	0.66	1.83	1.31	2.18	**2.49**	1.33	1.19
CYP337B1	1.23	1.12	0.61	0.74	1.01	1.33	1.08	1.27	1.65	1.04	1.28

Table 8: real-time quantitative PCR analysis of the differential expression of 9 P450 genes in Sf9 cells exposed for 24h to >DL10 doses of 11 different xenobiotic compounds. Gene expression values are indicated as fold expression in cells exposed to each xenobiotic comparatively to unexposed cells (controls). The three reference genes Rpl4, G6PD and L18 were used as internal controls for normalization. Gene expression values in bold are significantly different from the corresponding control as measured by pair-wise t-student test ($p<0.05$).

	xanthotoxin	2-tridecanone	indole 3-carbinol	indole	quercetin	methoxyfenozide	methoprene	deltamethrin	fipronil	clofibrate	phenobarbital
CYP4M14	1.11	**2.76**	0.53	1.64	1.57	1.04	**3.18**	1.39	1.01	1.52	1.77
CYP4M15	0.54	1.10	0.28	0.93	0.73	1.22	2.02	0.88	0.64	0.67	1.14
CYP9A24	0.65	**4.17**	0.96	1.17	1.56	**3.06**	1.08	1.57	0.87	**5.11**	1.23
CYP9A26	1.17	**2.87**	0.76	2.22	1.18	1.32	**3.59**	2.05	1.32	2.51	2.34
CYP9A30	6.85	**9.25**	5.12	5.79	1.24	0.47	**8.21**	**14.05**	2.60	**22.39**	2.63
CYP9A31	2.05	**12.40**	6.46	6.46	0.71	0.86	**9.27**	**7.19**	1.77	**13.75**	3.77
CYP9A32	1.91	**7.56**	2.07	2.32	1.49	1.20	**3.70**	**4.31**	1.52	**6.23**	1.07
CYP321A9	1.42	**3.86**	**4.03**	1.90	0.85	0.92	**5.74**	3.08	1.00	1.92	1.00
CYP333B2	2.10	1.97	**2.71**	**5.11**	**3.04**	0.17	**4.36**	**5.39**	**3.91**	**4.17**	1.48

Figures legends

Figure 1: Venn diagrams representing the number of regulated genes in *S. frugiperda* midgut and Sf9 cells exposed to 11 different xenobiotic compounds for 24h measured by microarray analysis. The number of genes regulated by each of the xenobiotic compound tested is indicated in the upper dark grey circle for the midgut and in the lower light grey circle for Sf9 cells. Numbers in bold represented at the intersection of both circles are genes found regulated in both systems.

Figure 2: Correlation between numbers of genes found up- or down-regulated by microarray analysis in the midgut of *S. frugiperda* and Sf9 cells after exposure to 11 different xenobiotics for 24h.

Figure 3: Numbers of genes up- and down-regulated in *S. frugiperda* midgut (A) and Sf9 cells (B) after exposure to sub-lethal doses of 11 xenobiotic compounds for 24h measured by microarray analysis. The total number of genes presents on the DNA microarray (9773) is represented in yellow and the respective number of genes up- and down-regulated found after exposure to each of the compound are represented in red and yellow, respectively.

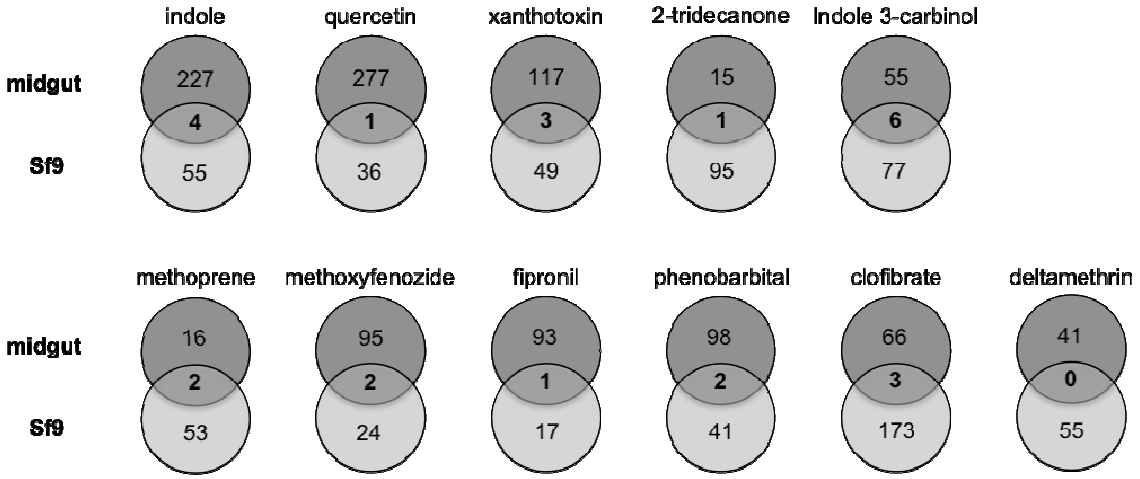

indole
quercetin
xanthotoxin
2-tridecanone
Indole 3-carbinol
midgut
Sf9
227
4
55
277
1
36
117
3
49
15
1
95
55
6
77
methoprene
methoxyfenozide
fipronil
phenobarbital
clofibrate
deltamethrin
midgut
Sf9
16
2
53
95
2
24
93
1
17
98
2
41
66
3
173
41
0
55

figure 1

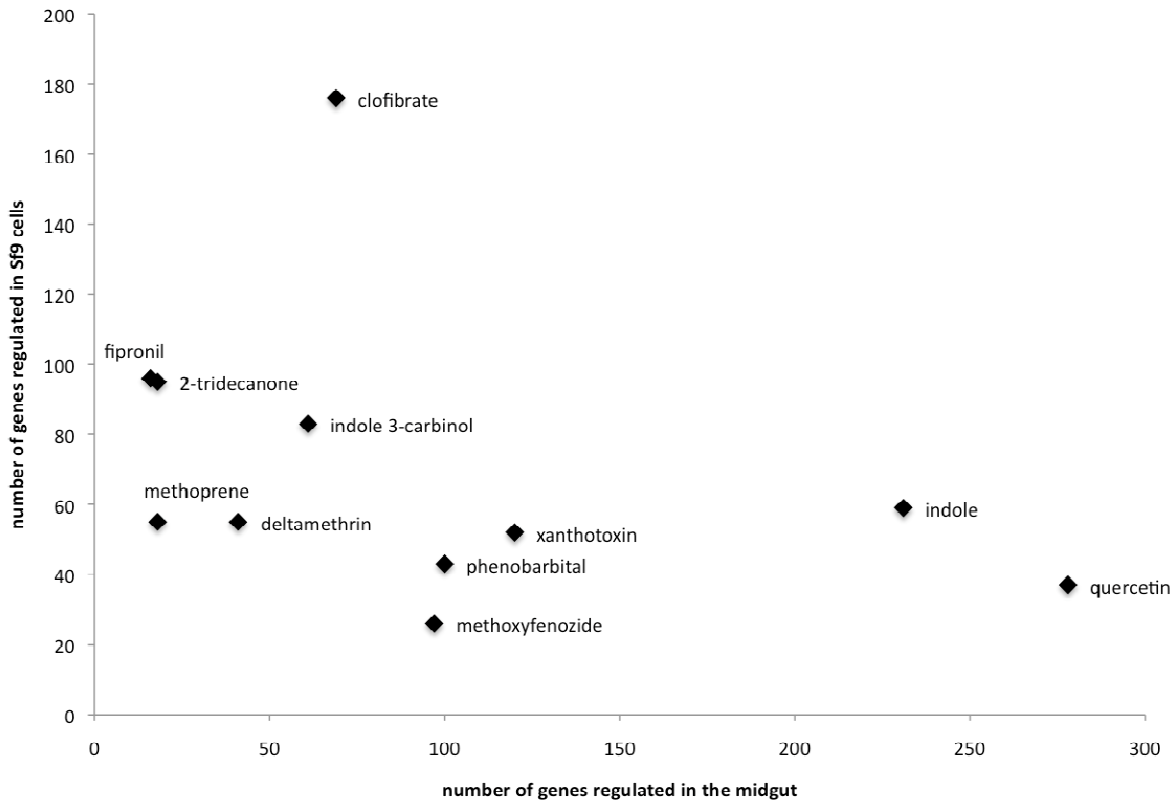
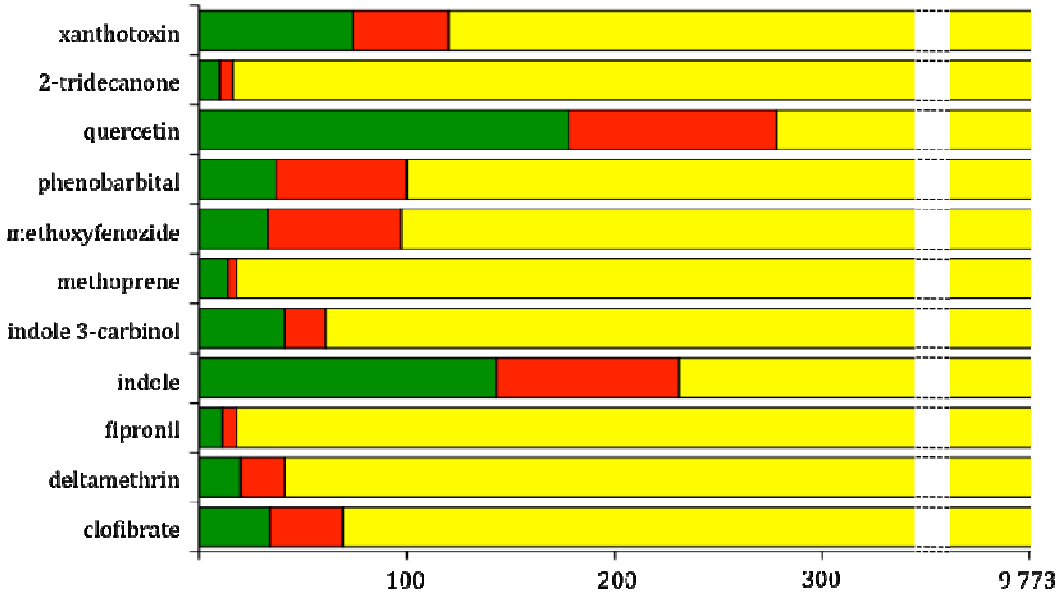

number of genes regulated in Sf9 cells
number of genes regulated in the midgut
clofibrate
fipronil
2-tridecanone
indole 3-carbinol
methoprene
deltamethrin
xanthotoxin
phenobarbital
methoxyfenozide
indole
quercetin
0
20
40
60
80
100
120
140
160
180
200
0
50
100
150
200
250
300

Figure 2

A midgut



B Sf9 cells

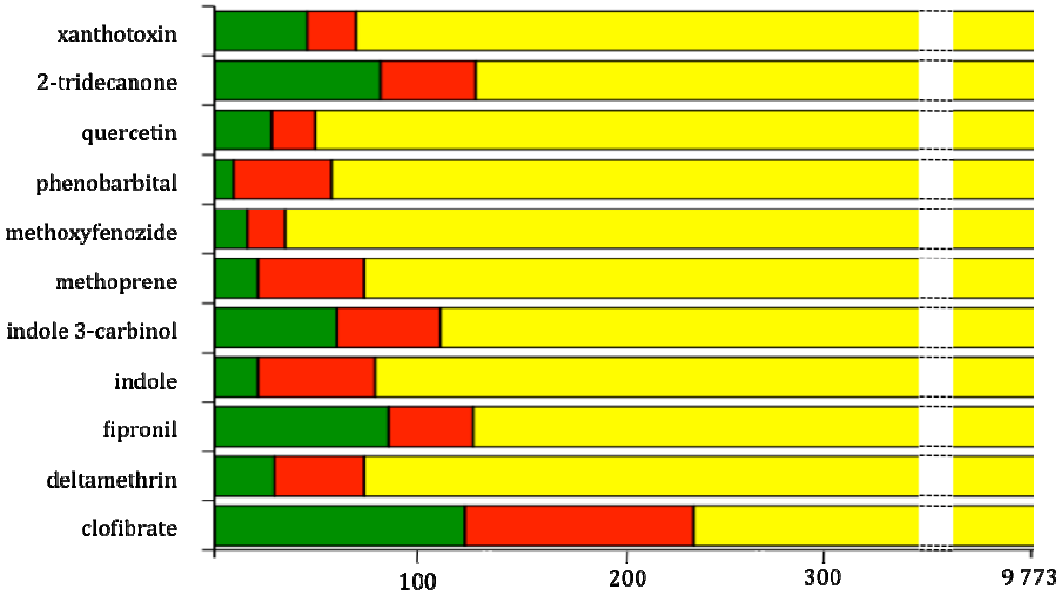
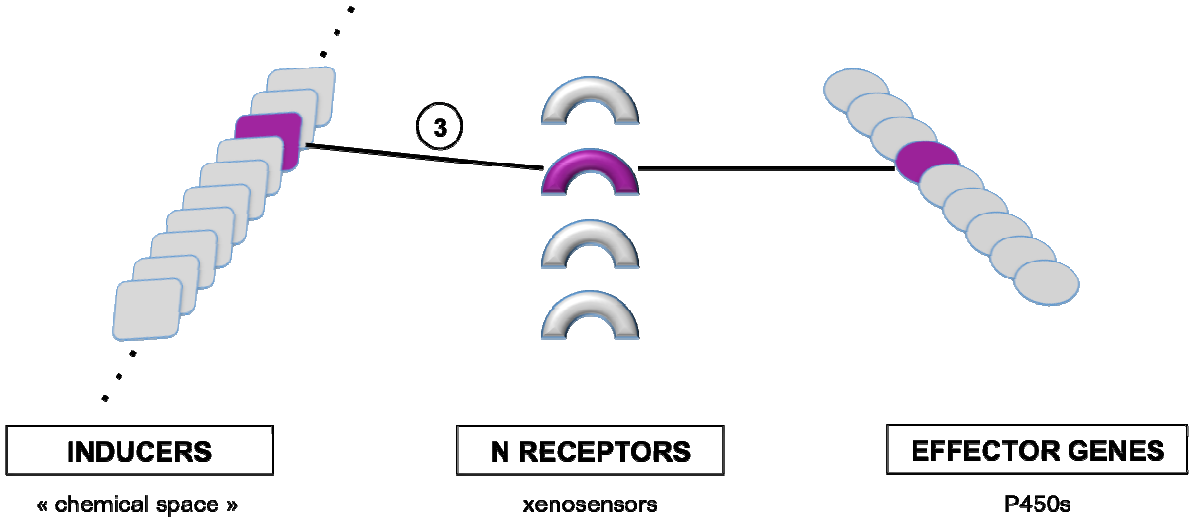


Figure 3

CHAPTER 3

Manuscript: Effects of hormone agonists on the Sf9 cells, cellular proliferation and cell cycle arrest.



We have identified which type of xenobiotic induces which P450 in *S. frugiperda* larvae and Sf9 cells. We will now focus our attention on two specific insecticide inducers: the 20-hydroxyecdysone (20E) agonist, methoxyfenozide and the Juvenile Hormone (JH) analog, methoprene.

20-E and JH are two major hormones that control insect development and metamorphosis. They have therefore been attractive target for the elaboration of effective insecitides that would disrupt insect development. Methoxyfenozide and methoprene belong to these hormone mimic insecticides and present the advantage of being potent to insect pests but less toxic to non-target organisms (Dhadialla et al., 1998).

The mode of action of these insecticides is regulated through nuclear receptors. 20-E agonists exert their activity by binding to the ecdysone receptor complex that comprises the two nuclear receptors EcR and USP. JH analog mode of action however remains to be elucidated as they are several receptor candidates for JH among which MET (methoprene tolerant) (Ashok et al., 1998) and USP (ultraspiracle) (Jones and Sharp, 1997).

We will present here the results of our study on the effects of methoprene and methoxyfenozide in Sf9 cells.

Effects of hormone agonists on the Sf9 cells, cellular proliferation and cell cycle arrest

Maeva Giraudo[a], Jérôme Califano[a,b], Nathalie Taquet[a,c], Frédérique Hilliou[a], Trang Tran[a,d], René Feyereisen[a] and Gaëlle Le Goff[a,e]

[a] UMR 1301 IBSV, INRA-CNRS-Université de Nice Sophia Antipolis, 400 Route des Chappes, 06903 Sophia-Antipolis, France

[b] Département des affaires réglementaires (parfum, arômes, matières premières), Robertet SA 37 avenue Sidi-Brahim 06130 Grasse, France

[c] Institute of Developmental Biology and Cancer, Polarized growth in yeast, CNRS UMR6543 Université de Nice – Sophia Antipolis, Faculté des Sciences - Parc Valrose, 06108 Nice Cedex 2, France

[d] Lanaud Gestion-Pôle de Lanaud, 87220 Boisseuil, France

[e] Corresponding author: Gaëlle Le Goff, UMR 1301 IBSV, INRA-CNRS-Université de Nice Sophia-Antipolis, 400 Route des Chappes, 06903 Sophia-Antipolis, FRANCE; phone : 33 492386578, fax : 33 492386401, e-mail : legoff@sophia.inra.fr

Short title : hormone agonist insecticides on Sf9

In preparation for Journal of Insect Physiology

Abstract:

Methoxyfenozide and methoprene are two insecticides, which mimic the action of the main hormones involved in the control of insect growth and development, the 20-hydroxyecdysone and the juvenile hormone. We used the *Spodoptera frugiperda* cell line Sf9 to investigate their mode of action. Toxicological tests showed that methoxyfenozide was more potent than methoprene in cell viability tests and in the inhibition of cellular proliferation. Cell growth arrest occured in G1 phase after a methoxyfenozide treatment and in G2/M for methoprene. Microarray experiments and qPCR to follow the expression of receptors were performed to understand the molecular action of these hormone agonists. Twenty-six genes were differentially expressed after methoxyfenozide treatment and 55 genes after methoprene treatment but no gene was shared between the two treatments. Our results suggest two different signalling pathways in Sf9 cells.

Keywords: methoxyfenozide, methoprene, Sf9 cells, cell cycle arrest

1. Introduction

Growth and development are controlled by two major hormones, the steroid 20-hydroxyecdysone (20E) and the sesquiterpenoid juvenile hormone (JH) insects (Truman and Riddiford, 1999). The cross-talk between these two hormones regulates all stages between egg-larva-pupa to adult. High level of 20E is required to initiate all developmental transitions and JH determines the nature of the molt (Dubrovsky, 2005). JH is necessary for larval molting and growth but metamorphosis occurs in it absence (Riddiford, 1996). The signalling action of these hormones involves nuclear receptors. If the mode of action of 20E is well-known, it remains more enigmatic for JH due to the lack of knowledge of its receptor. 20E exerts it action through the binding to a nuclear receptor heterodimer consisting of an ecdysone receptor (EcR) and ultraspiracle (USP), which is the insect ortholog of retinoid-X-receptor from vertebrate (Yao et al., 1992). The complex will regulate expression of target genes by binding to promoting region. In Drosophila, it was shown that 20E linked to its receptor will activate early genes among which are transcription factor regulators Broad complex (BR-C), E74 and E75 (Karim and Thummel, 1992; Thummel, 2001). It is those transcription factors that in turn will regulate late genes that have a direct role in metamorphosis mechanism (like cell death, cellular proliferation, differentiation, cuticle production…). Several receptor candidates for JH exist among which MET (Methoprene tolerant) a member of the bHLH-PAS transcription factor family (Ashok et al., 1998) and USP (Jones and Sharp, 1997). MET can bind JH at physiological concentration (Miura et al., 2005) whereas USP was shown to bind JH with low affinity, at least 100 times lower than expected for a nuclear receptor (Jones et al., 2001). However the situation is complex and it is difficult

to generalize finding about Met and USP from one insect group to another. Indeed, Met has a close paralog in Drosophila, germ cell expressed (gce) (Moore et al., 2000) and it is one of the explanation why Met-null mutants are fully viable (Wilson and Ashok, 1998). The Met/gce duplication is recent and the two paralogs are found in the Drosophila genus but is not found in mosquitoes (Baumann et al., 2010). In the other insects, Met has therefore only one ortholog and in *Tribolium castaneum* its depletion by RNAi causes premature pupal morphogenesis (Konopova and Jindra, 2007). A phylogenetic study of USP receptors shows that there are two types of receptor in arthropods, one having lost the ability to bind the ligand in *Bemisia tabaci* (Hemiptera) and *Tribolium castaneum* (Coleoptera) and another still able to bind the ligand in Diptera and Lepidoptera (Iwema et al., 2007). Moreover the molecular signalling mechanism downstream of JH binding to its putative receptor remains limited. Two transcription factors, Broad complex (BR-C) and Krüppel homolog 1 (Kr-h1) seem to play an important role (Konopova and Jindra, 2008; Minakuchi et al., 2008; Parthasarathy et al., 2008; Suzuki et al., 2008). Minakuchi et al. (2009) have proposed a model in the red flour beetle *Tribolium castaneum* where Kr-h1 works downstream of Met in the larval stage and downstream of Met but upstream BR-C in the pupa, allowing metamorphosis inhibition in one case or its initiation in the other (Minakuchi et al., 2009).

These hormonal receptors (EcR, USP and Met) are also the target for insecticides, which act by disrupting insect development. Agonist hormone insecticides are of growing interest because some are shown to have selective toxicity, they are potent against pest insects and less or non toxic for the beneficial insects, mammals, fish and birds (Dhadialla et al., 1998). Among 20E agonists are diacylhydrazines, a non-steroidal agonist family, having insecticide activity by binding to the EcR-USP receptors. This family of compounds provokes a premature molt that leads to the death of the insect; they are only acting on larvae. The

activity spectrum of these compounds varies within insect orders and it is directly correlated to receptor affinity for the insecticide (Dhadialla et al., 1998; Smagghe et al., 2002). As an example, methoxyfenozide which we are using in our study is more effective against Lepidoptera (Carlson et al., 2001). The other insecticides that mimic hormone action are juvenile agonists (JHA), initially designed to be metabolically stable JH analogs. Their precise molecular target is less well-known due to the still controversial mode of action of JH. JHA block insects at an intermediate stage during development making them unable to emerge normal adults. They also disrupt reproduction in insects where JH is gonadotropic. Methoprene was the first successfully used JHA (Henrick et al., 1973) and it is more effective against dipteran insects compared to Lepidoptera.

Cell lines could be a useful tool to understand insecticide mode of action. Several members of the biacylhydrazines were used on cell lines, showing an inhibition of cellular proliferation. This is the case for Drosophila Kc cell treated by RH-5849 and tebufenozide (Mikitani, 1996; Wing, 1988). Similar effects on arrest of cell growth have also been observed with these compounds in a lepidopteran cell line IAL-PID2 from the imaginal wing disks of *Plodia interpunctella* (Silhacek et al., 1990), as well as in the epithelial cell line from *Chironomus tentans* (Quack et al., 1995). Further studies on IAL-PID2 with tebufenozide have shown a G2/M arrest with an induction of mRNA transcripts for EcR and USP associated with a decreased in the expression of cyclin B, one of the protein involved in the cell cycle control (Auzoux-Bordenave et al., 2005). Effects on cell proliferation were also reported for JHA like on IAL-PID2 (Oberlander et al., 2000) but the molecular mechanism leading to this arrest was not clarified.

In our study we were interested in the effects of insecticides that mimic hormone action on *Spodoptera frugiperda* Sf9 cell line. The toxicity of methoxyfenozide and methoprene was evaluated. As expected, both insecticides inhibit cellular proliferation. Flow cytometry analysis showed a distinct action between these compounds with a G2/M arrest after methoprene treatment whereas methoxyfenozide induced a G1 arrest. To investigate the differential molecular mode of action of these hormone agonists we have performed microarray experiments and the expression of receptors was followed by qRT-PCR. Our results suggest two different signalling pathways.

2. Materials and Methods

2.1. Cell culture

Sf9 cell line was derived from the pupal ovarian tissue of *Spodoptera frugiperda*. Cells were cultured at 27°C in monolayer with the insect-Xpress protein free medium (Lonza). For experiments cells were cultured in 6 well plates, sowed at 5.10^5 cells/ml. Cell proliferation was estimated by scraping off cells from a well and a cell aliquot added volume/volume of 1mg/ml of methylene blue was counting in a Malassez haemocytometer under the microscope.

2.2. Cell viability by MTT assay

Sf9 cells were seeded in 96-well culture plates and treated for 24, 48 and 72 hours with increased concentrations of methoprene and methoxyfenozide. Methoxyfenozide was purchased from Cluzeau and MTT from Sigma. Methoprene was a gift from the former Zoecon Corp., Palo Alto, California. Cells in culture were then loaded with MTT (5mg/ml) and

incubated at 27°C for 2 hours. Cell homogenates were used to measure absorbance at 570nm using a microplate reader (SpectraMax, Molecular Devices).

2.3. Cell cycle analysis

Cellular DNA content was determined by staining cells with propidium iodide and measuring fluorescence (FACSCalibur, Becton Dickinson). The Sf9 cells were incubated during 48 hours with Methoxyfenozide or Methoprene and resuspended and fixed in ice during 30 minutes with 70% ethanol/PBS (10 mM Na2HPO4, 138 mM NaCl, 2.7 mM KCl, pH 7.4). The fixed cells were incubated in a solution containing 50 mg/ml RNAse and 50 mg/ml propidium iodide for 20 min at 37°C. For each cell population, 10,000 cells were analysed by FACS and the percentage of cells in a specific phase of the cell cycle was determined with the propidium iodide DNA staining technique. Cells were classified in G0/G1, S and G2/M phases depending on the intensity of the fluorescence peaks.

2.4. RNA extraction

Total RNA was extracted from cells of a well of the 6 wells plate using Trizol Reagent (Invitrogen Life technologies). Extractions were performed on the three independent biological replicates.

2.5. Microarray experimental design

Our oligonucleotides were designed from 79148 ESTs sequences of eight different tissues of *Spodoptera frugiperda* (fat body, hemocytes, midgut…). Using the assembly analysis (programme CAP3), we obtained 10092 contigs and singleton from these ESTs. Our *Spodoptera frugiperda* microarray consists of 9773 60-mers oligonucleotides synthesised by

Sygma-Aldrich that were designed to match unique contigs or singleton and to suit our hybridization conditions (GC content average 46% and average Tm of 46°C). Each comparison consisted in six microarrays, three biological replicates hybridised with dye swap (fully balance dye swap design) and duplicate spots. cDNA were synthesized from 7µg of total RNA and labelled with the dyes Cy3-dCTP and Cy5-dCTP (Amersham) using the ChipShot direct labeling system Promega according to the manufacturer's instructions. The microarray were hybridised with cDNA prepared as described in (Le Goff et al., 2006)and scanned using GenePixPro scanner (Axon,version 3.01). Experimental data and associated microarray designs have been deposited in the NBCI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under serie X and platform record X using Mediante database for data transfer (Le Brigand and Barbry, 2007).

2.6. Data analysis

We used the Bioconductor suite of statistical packages (Gentleman et al., 2004): *limma* (Wettenhall and Smyth, 2004) for our data analysis. The expression intensity was obtained by subtracting the background intensity from the foreground intensity for each non-flag spot (all flagged spots were eliminated). The expression data were normalised by the use of the within-array normalization with the "loess method" and the between-array normalization using the "quantile method" (Yang and Thorne, 2003). The linear model for series of arrays and empirical Bayes method were then applied for assessing differential expression (Smyth, 2004). The false discovery rate of the p-value for multiple tests was controlled by using Benjamini-Hochberg method. Differentially expressed genes were

selected if the absolute value of log2-fold-change greater than 1 and adjusted p-value below 0.01 and if the average intensity is greater than two time of average background. In order to provide an overall measure of evidence of differential expression, we used the Fisher's method for combining adjusted p-values from independent tests of significance of duplicate spots (Hess and Iyer, 2007).

2.7. Quantitative real-time PCR

Total RNA (1µg) was reverse transcribed using the iScript cDNA Synthesis kit (Biorad). QPCR reactions were carried out on an Opticon monitor 2 (Biorad) using the qPCR Mastermix plus for SYBR Green I no ROX (Eurogentec). The PCR conditions were as follows: 50°C for 2 min, 95°C for 10min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Each reaction was performed in triplicate and the mean of the three independent biological replicates was calculated. All results were normalized using mRNA level of three control genes (RpL4, L18 and G6PD) and relative expression values were calculated in R using the RqPCRBase package developed in our laboratory (Hilliou and Tran, manuscript in preparation).

3. Results

3.1. Toxicological effects of methoxyfenozide and methoprene on the Sf9 cells

Cell viability was determined by MTT test after an insecticide exposure of 24, 48 and 72 hours. Methoxyfenozide had already a marked effect at 100nM, the lowest concentration tested (Fig.1A). Surprisingly, increased insecticide concentrations did not sensibly modify the

cell viability. The IC_{50} is below 100nM at 72 h and can not be calculated for the time 24 and 72h. Figure 1B shows the results for methoprene. Almost no effects were observed up to 25µM, whatever the time of treatment (90% of cells are viable). For a concentration range between 50 and 75µM, toxicity was low (after 72h, still 70% of viable cells). The calculated IC_{50} at 48 and 72h was around 200µM and below 100µM respectively.

Thus the two hormone agonists induced cell death of the Sf9 cell line and methoxyfenozide was more potent than methoprene by a toxicological factor of about 1,000.

3.2. Cellular proliferation inhibition

The potential effect of these insecticides on the cellular proliferation was monitored. Cells were sowed at 5.10^5 cells/ml in 6 well plates, then agonist hormone treatment was started 24h after sowing. Treated cells were counted daily during 3 days. Cells in the DMSO control grew to reach to 15 to 17.10^5 cells/ml at 72h (Fig.2). Cell density at 24h and 72h in DMSO was significantly different, indicating that cells proliferated. A normal and significant growth of the cells treated by methoxyfenozide was observed at the lower concentration used 10nM, however all the other tested concentrations had an antiproliferative effect (Fig.2A). At 50µM of methoxyfenozide, the number of cells remained stable for 3 days. Methoprene had no effect at 1µM with a cell density correlated to that of the DMSO control. An inhibition of cell proliferation was observed at the concentration range between 50 and 100µM, with no significant difference between numbers of cells counted at 24h or 72h (Fig.2B).

Methoprene and methoxyfenozide therefore both caused an arrest of cell proliferation in a dose-dependent manner.

3.3. Distinct phases of arrest in the cell cycle

In order to evaluate in which phase of the cell cycle the cells are arrested after insecticide treatment, we turned to flow cytometry. First, the distribution of untreated cells in the different phases was evaluated (Fig. 3A). 1.7% of the cells were found in apoptosis, 26.19% in G1, 15.85% in S and 56.25% in G2/M. No significant difference was observed for cells treated only with the solvent (DMSO) used to solubilise the insecticides (Fig. 3B). The insecticide-treated cells were then analyzed. In cells treated with methoxyfenozide, a shift occurred between the percentage of cells in G2/M compared to the percentage of cells in G1 (Fig.3C). 42.42% of cells are in the G1 phase related to 26.19% of the control. In cells treated with methoprene, cells were arrested in the G2/M phase with 75.28% of cells in this stage (Fig.3D).

3.4. Molecular pathways involved in the cell response to hormone agonist treatments

Although the phenotypic effects were identical between Sf9 cells treated by methoprene or methoxyfenozide (arrest of the cell growth but cell morphology identical to control cells, data not shown), nevertheless the insecticides had different molecular effects with an arrest of the cell cycle in G1 for methoxyfenozide and G2/M for methoprene. The molecular genetic regulation leading to this arrest still needs to be elucidated. We compared the transcriptional effect mediated by the signalling transduction pathway of each hormone agonist by using a specifically designed oligonucleotide microarray. We chose the first concentration of insecticide having a significant effect on cell proliferation, i.e 100nM for

methoxyfenozide and 25µM for methoprene, in order to focus on physiological effects and avoid toxicological effects.

Genes were considered as differentially regulated by the insecticide if their expression ratio was >1.5 or <0.66 and P value <0.05. Sequences of these genes were analysed in Blast2Go to assign them Gene Ontology (GO) terms and then classified in biological process level 3. The sequences for which no homology was found by blastx were submitted to a blastn in Butterflybase and classified in "hypothetical protein" category if homology was found with the sequence of another lepidopteran transcript, while the sequences restricted to *Spodoptera frugiperda* were put in a "hypothetical transcripts" category. Table 1 represents these results. After methoprene treatment, 55 genes were differentially expressed with 39 overexpressed and 16 down-regulated. In the case of methoxyfenozide, 26 genes were differentially regulated with 14 over-expressed and 12 down-regulated. We first noted that there was no overlap between the genes regulated by methoprene and methoxyfenozide. The main category of genes up-regulated by methoprene is the hypothetical transcripts category (15 genes). The next categories are the genes involved in cellular metabolic process (9 genes) and transcription/translation (7 genes). These categories are also the most populated for cells treated by methoxyfenozide. Five upregulated genes by methoprene belonged to the response to stress category. One gene could be present in different biological process.

Looking in more detail to the list of differentially regulated gene may help to identify the potential function of genes involved in the molecular and cellular effects of methoxyfenozide (Table2). In the transport proteins, two vacuolar ATPases are regulated, subunit B that is upregulated and subunit C that is downregulated. Insect vacuolar proteins

consist in two functional parts, the peripheral catalytic V1 complex composed of eight different subunits (from A to H) that hydrolyzes ATP and the integral membrane V0 complex consisting of four different subunits (a,c,d,e) that transports protons across the membrane (Beyenbach and Wieczorek, 2006). These two parts can disassemble and reassemble depending on conditions which regulate V-ATPase activity (Sumner et al., 1995; Wieczorek et al., 2000). Both subunits can bind actin filament (Holliday et al., 2000; Vitavska et al., 2003), but subunit C is the only subunit that can be phosphorylated (Voss et al., 2007). Another specificity of subunit C is its release in the cytosol upon dissociation of the two complexes (Kane, 2000; Merzendorfer et al., 2000). Clearly, subunit C has its own properties and can be a good candidate to mediate signalling pathways (Wieczorek et al., 2009). So it may not be surprising to see opposite regulation of subunit B and C. Moreover promoter studies have revealed different regulatory elements between the two subunits genes in *Manduca sexta* (Wieczorek et al., 2000). Down regulation of V-ATPase in apical globelet cell of *Manduca sexta* during moulting and starvation was also suggested (Graf et al., 1996; Sumner et al., 1995). These data may indicate a possible involvement of hormone on the regulation of V-ATPase expression. Calcium may play a role in the signalling because at least two proteins whose function is calcium-dependent, cadherin and calreticulin are up-regulated. Cadherin is an essential protein for cell adhesion and calreticulin is a quality-control chaperone. Futhermore, it was shown on mammalian cell line that over-expression of calreticulin correlates with increased cell adhesiveness and increased of cadherin expression (Fadel et al., 2001). These proteins are also known in mammals to be regulated by steroid hormones. Crossin et al. have shown that inhibition of rat astrocytes proliferation is mediated through the glucocorticoid receptor pathway and they also observed a level of mRNA for calreticulin increased (Crossin et al., 1997). Among other genes over-expressed

three are implicated in the translation/transcription process. PolyA binding protein 2 has a function in translation initiation of polyadenylated mRNA. P27BBP/eIF6 known as eukaryotic translation initiation factor 6 is a ribosomal anti-association factor (Gartmann et al., 2010). Dead box RNA helicase catalyzes the ATP-dependent unwinding of double-stranded RNA. In the spruce budworm (*Choristoneura fumiferana*) tebufenozide another 20E agonist was shown to enhance expression of a dead box RNA helicase (Zhang et al., 2004). In human, it has been suggested that this type of proteins could be involved in cell cycle control and the regulation of apoptosis (Schroder, 2009). A chymotrypsin gene is up-regulated, in *Tribolium castaneum* RNAi of several genes coding for chymotrypsin lead to severe molting defects (Broehan et al., 2010). Certain genes in the list are known to be regulated by the 20E like vitellogenin. An over-expression of this gene after treatment by an agonist of the hormone is consistent. Vitellogenin transcription is activated by 20E in mosquito via direct binding of the heterodimer receptor EcR-USP (Martin et al., 2001).

Genes differentially regulated by methoprene are listed in the table 3. The two main category processes are transcription/translation and response to stress with 7 genes (5 up-regulated and 2 down-regulated) and 8 genes (7 up-regulated and one down) respectively. In translation several ribosomal proteins were over-expressed, these data can be related to study on the human HeLa cells where authors have identified that translation-related genes seem essential for G2/M progression (Mukherji et al., 2006), siRNA of ribosomal protein reduced cell proliferation. In response to stress different enzyme types are overexpressed, carboxylesterases already well known in insecticide resistance (Ffrench-Constant, 2007), apolipoprotein D precursor, in Drosophila correlation exists between overexpression of

apolipoprotein D and increased stress resistance (Walker et al., 2006). In a general way, these enzymes are over-expressed which is understandable after an insecticide treatment, nevertheless uridine diphosphate glucosyltransferase and DNAJ-1 are down-regulated. Overexpression is found for genes implicated in spindle assembly. Microtubule associated protein RP/EB family 3 may be involved in microtubule polymerization and stabilization. Kinesins are class of motor protein and beta-tubulin cofactor E is involved in the formation of the tubulin dimer. All of three can regulate microtubule dynamics and stabilization, in mammals an increased in microtubule stabilization leads to a mitotic G2/M arrest (Gallagher, 2007) which is in agreement with our results. Other gene categories are overexpressed like S-adenosylmethionine decarboxylase, it is an enzyme implicated in the synthesis of polyamines (Larsson and Rasmuson-Lestander, 1997). Polyamines affect the cell cycle progression, alterations in their relative amount can cause an arrest in cell cycle (Ackermann et al., 2003). Among this gene list, we can also cited gene down-regulated such as 14-3-3 epsilon protein. This protein family is involved in a variety of molecular and cellular functions (Darling et al., 2005). However, in Drosophila loss of 14-3-3 epsilon protein induces growth repression (Nielsen et al., 2008).

3.5. Expression of hormone receptors

Microarrays were not sensitive enough to detect EcR or USP, and we did not have probes for Met. Indeed we were unable to find the Met sequence on Spodobase (http://www.spodobase.univ-montp2.fr/Spodobase). Therefore, we used RT-qPCR approaches to study the expression of EcR and USP. qPCR primers were designed and expression of EcR and USP was followed in Sf9 cells treated by methoxyfenozide or methoprene and this was compared to cells treated by DMSO. As our results shown in figure

4, the two insecticides significantly induced the expression of both receptors. EcR was induced by a 1.92 fold in methoxyfenozide treated cells when USP expression was increased by 1.82 fold in methoprene treated cells.

4. Discussion

Cell lines provide a useful tool to investigate the molecular mode of action of insecticides. The *Spodoptera frugiperda* Sf9 cell line is probably the most widely used for high level expression of recombinant protein (Atmann et al., 1999). However, *Spodoptera frugiperda* from which the Sf9 cell line is derived is a major crop pest, a polyphagous insect able to feed on more than 25 different host plants. We decided to use this cell line in order to determine the effects of two insecticides, which are agonists of major hormones controlling insect development, the 20E and JH.

Methoxyfenozide acts as mimics of 20E and by binding to the EcR/USP receptor complex. Its affinity for the receptor in Lepidoptera is 10000 times higher than for the insect moulting hormone. However the affinity varies within insect orders, more potent on the lepidopteran cell line *Plodia interpunctella* than on the Drosophila Kc cells (Dhadialla et al., 1998). We have shown that methoxyfenozide was able to induce the expression of EcR in Sf9 cells, which is consistent with the affinity of this compound for the receptor. Methoprene has a different spectrum of activity, being very effective against dipteran insects but less so against Lepidoptera (Staal, 1975). In our results, methoprene induced the expression of USP, suggesting that USP might be responding to JH as it was already suggested.

In a second step, we have shown that these two insecticides could inhibit cellular proliferation. Treatment by tebufenozide, another bisacylhydrazine, leads to the same effects in two other lepidoptera cell lines, IAL-PID2 from *Plodia interpunctella* (Auzoux-Bordenave et al., 2005) and Se4 from *Spodoptera exigua* (Decombel et al., 2005). Methoprene and another analog of JH, fenoxycarb, significantly inhibit cell proliferation of the IAL-PID2 cell line (Oberlander et al., 2000). In this same cell line Auzoux-Bordenave et al. reported that tebufenozide arrested the cell cycle in G2/M (Auzoux-Bordenave et al., 2005). However, most studies to date have focused on hormone action. 20E causes an arrest in G2 in Kc cell (Stevens et al., 1980) and in IAL-PID2 cells (Mottier et al., 2004) whereas arrest occurs in the G1 phase in mosquito C7-10 cells (Gerenday and Fallon, 2004). To understand in which phase of the cell cycle the cells are accumulated after our insecticide treatments, flow cytometry experiments were performed. Inhibition of cellular proliferation associated with methoxyfenozide treatment induces an arrest of the cell cycle in G1 phase. In contrast, methoprene blocks the cells in G2/M.

Then finally, we have investigated insecticide mode of action at the molecular level. Which pathway leads to this arrest? Is there any element in common between the two treatments? We used a custom microarray consisting of 9773 probes of *Spodoptera frugiperda*. Very few genes are differentially expressed following a methoxyfenozide or a methoprene treatment, 26 in one case and 55 in the other. The cell cycle consists of four distinct phases: G1 phase where cells grow and cyclin D is expressed, S phase during which DNA replication occurs and cyclins E and A predominate, then the G2 phase, with degradation of the cyclin E and accumulation of the cyclin B. This phase is followed by the mitosis which leads to the cell division (Fallon and Gerenday, 2010). The progression through the cell cycle is controlled by cyclins and cyclin dependent kinases (CDK). The cyclins form

complexes with CDK, CDK4 for cyclin D, CDK2 for cyclin E. Our array only has one probe for cyclin A, but not for other cyclins. No change in cyclin A levels was detected although Mottier et al. reported a significant decrease in the expression of cyclin A and B after a 20E treatment of IAL-PID2 and the level of both cyclins remain very low between 12 and 36h post-treatment (Mottier et al., 2004). On the other hand, some results are consistent with an arrest of the cell cycle as we observed overexpression of different genes involved in the spindle assembly after the methoprene treatment. Indeed a biosynthetic step occurs during the G2 phase, mainly involving the production of microtubules, which are required during the process of mitosis. Genes such as cadherin are overexpressed in our methoxyfenozide experiments and these have been shown to be overexpressed in cells that had stopped to proliferate (Crossin et al., 1997). Genes and pathways involved in the various stages of the cell cycle progression were identified in a study on human HeLa cells (Mukherji et al., 2006). Several concordant observations can be made between genes shown to be essential in that study and genes differentially regulated in our study. For example, several ribosomal proteins, kinesin, DNA-J have been shown to be essential for G2/M progression and are differentially regulated after a methoprene treatment. Similarly, eIF, ATPase and dead box RNA helicase are essential in G1 phase and are differentially regulated after the methoxyfenozide treatment. Although an evolutionary conservation of core cell-cycle regulatory transcripts among species would be expected, there are in fact only few genes (16) conserved between human and yeast cell-cycle regulatory networks (Mukherji et al., 2006)

References

Ackermann, J.M., Pegg, A.E., and McCloskey, D.E. (2003). Drugs affecting the cell cycle via actions on the polyamine metabolic pathway. Prog Cell Cycle Res *5*, 461-468.

Ashok, M., Turner, C., and Wilson, T.G. (1998). Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. Proc Natl Acad Sci U S A *95*, 2761-2766.

Atmann, F., Staudacher, E., and Wilson, I.B. (1999). Insect cells as hosts for the expression of recombinant glycoproteins. Glycoconj J *2*, 109-123.

Auzoux-Bordenave, S., Solvar, M., Queguiner, I., Bozzolan, F., Mottier, V., Siaussat, D., Porcheron, P., and Debernard, S. (2005). Comparative effects of a non-steroidal ecdysone agonist RH-5992 and 20-hydroxyecdysone in a lepidopteran cell line (IAL-PID2). Insect Biochem Mol Biol *35*, 1033-1042.

Baumann, A., Fujiwara, Y., and Wilson, T.G. (2010). Evolutionary divergence of the paralogs Methoprene tolerant (Met) and germ cell expressed (gce) within the genus Drosophila. J Insect Physiol.

Beyenbach, K.W., and Wieczorek, H. (2006). The V-type H+ ATPase: molecular structure and function, physiological roles and regulation. J Exp Biol *209*, 577-589.

Broehan, G., Arakane, Y., Beeman, R.W., Kramer, K.J., Muthukrishnan, S., and Merzendorfer, H. (2010). Chymotrypsin-like peptidases from Tribolium castaneum: a role in molting revealed by RNA interference. Insect Biochem Mol Biol *40*, 274-283.

Carlson, G.R., Dhadialla, T.S., Hunter, R., Jansson, R.K., Jany, C.S., Lidert, Z., and Slawecki, R.A. (2001). The chemical and biological properties of methoxyfenozide, a new insecticidal ecdysteroid agonist. Pest Manag Sci *57*, 115-119.

Crossin, K.L., Tai, M.H., Krushel, L.A., Mauro, V.P., and Edelman, G.M. (1997). Glucocorticoid receptor pathways are involved in the inhibition of astrocyte proliferation. Proc Natl Acad Sci U S A *94*, 2687-2692.

Darling, D.L., Yingling, J., and Wynshaw-Boris, A. (2005). Role of 14-3-3 proteins in eukaryotic signaling and development. Curr Top Dev Biol *68*, 281-315.

Decombel, L., Tirry, L., and Smagghe, G. (2005). Action of 24-epibrassinolide on a cell line of the beet armyworm, Spodoptera exigua. Arch Insect Biochem Physiol *58*, 145-156.

Dhadialla, T.S., Carlson, G.R., and Le, D.P. (1998). New insecticides with ecdysteroidal and juvenile hormone activity. Annu Rev Entomol *43*, 545-569.

Dubrovsky, E.B. (2005). Hormonal cross talk in insect development. Trends Endocrinol Metab *16*, 6-11.

Fadel, M.P., Szewczenko-Pawlikowski, M., Leclerc, P., Dziak, E., Symonds, J.M., Blaschuk, O., Michalak, M., and Opas, M. (2001). Calreticulin affects beta-catenin-associated pathways. J Biol Chem *276*, 27083-27089.

Fallon, A.M., and Gerenday, A. (2010). Ecdysone and the cell cycle: Investigations in a mosquito cell line. J Insect Physiol.

Ffrench-Constant, R.H. (2007). Which came first: insecticides or resistance? Trends Genet *23*, 1-4.

Gallagher, B.M., Jr. (2007). Microtubule-stabilizing natural products as promising cancer therapeutics. Curr Med Chem *14*, 2959-2967.

Gartmann, M., Blau, M., Armache, J.P., Mielke, T., Topf, M., and Beckmann, R. (2010). Mechanism of eIF6-mediated inhibition of ribosomal subunit joining. J Biol Chem *285*, 14848-14851.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B. *et al.* (2004). Bioconductor: open software development for computational biology and bioinformatics. Genome Biol *5*, R80.

Gerenday, A., and Fallon, A.M. (2004). Ecdysone-induced accumulation of mosquito cells in the G1 phase of the cell cycle. J Insect Physiol *50*, 831-838.

Graf, R., Harvey, W.R., and Wieczorek, H. (1996). Purification and properties of a cytosolic V1-ATPase. J Biol Chem *271*, 20908-20913.

Henrick, C.A., Staal, G.B., and Siddall, J.B. (1973). Alkyl 3,7,11-trimethyl-2,4-dodecadienoates, a new class of potent insect growth regulators with juvenile hormone activity. J Agri Food Chem *21*, 354-359.

Hess, A., and Iyer, H. (2007). Fisher's combined p-value for detecting differentially expressed genes using Affymetrix expression arrays. BMC Genomics *8*, 96.

Holliday, L.S., Lu, M., Lee, B.S., Nelson, R.D., Solivan, S., Zhang, L., and Gluck, S.L. (2000). The amino-terminal domain of the B subunit of vacuolar H+-ATPase contains a filamentous actin binding site. J Biol Chem *275*, 32331-32337.

Iwema, T., Billas, I.M., Beck, Y., Bonneton, F., Nierengarten, H., Chaumot, A., Richards, G. *et al* (2007). Structural and functional characterization of a novel type of ligand-independent RXR-USP receptor. Embo J *26*, 3770-3782.

Jones, G., and Sharp, P.A. (1997). Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. Proc Natl Acad Sci U S A *94*, 13499-13503.

Jones, G., Wozniak, M., Chu, Y., Dhar, S., and Jones, D. (2001). Juvenile hormone III-dependent conformational changes of the nuclear receptor ultraspiracle. Insect Biochem Mol Biol *32*, 33-49.

Kane, P.M. (2000). Regulation of V-ATPases by reversible disassembly. FEBS Lett *469*, 137-141.

Karim, F.D., and Thummel, C.S. (1992). Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. EMBO J *11*, 4083-4093.

Konopova, B., and Jindra, M. (2007). Juvenile hormone resistance gene Methoprene-tolerant controls entry into metamorphosis in the beetle Tribolium castaneum. Proc Natl Acad Sci U S A *104*, 10488-10493.

Konopova, B., and Jindra, M. (2008). Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolan metamorphosis. Development *135*, 559-568.

Larsson, J., and Rasmuson-Lestander, A. (1997). Cloning, mapping and mutational analysis of the S-adenosylmethionine decarboxylase gene in Drosophila melanogaster. Mol Gen Genet *256*, 652-660.

Le Brigand, K., and Barbry, P. (2007). Mediante: a web-based microarray data manager. Bioinformatics *23*, 1304-1306.

Le Goff, G., Hilliou, F., Siegfried, B.D., Boundy, S., Wajnberg, E., Sofer, L., Audant, P., ffrench-Constant, R.H., and Feyereisen, R. (2006). Xenobiotic response in Drosophila melanogaster: sex dependence of P450 and GST gene induction. Insect Biochem Mol Biol *36*, 674-682.

Martin, D., Wang, S.F., and Raikhel, A.S. (2001). The vitellogenin gene of the mosquito Aedes aegypti is a direct target of ecdysteroid receptor. Mol Cell Endocrinol *173*, 75-86.

Merzendorfer, H., Reineke, S., Zhao, X.F., Jacobmeier, B., Harvey, W.R., and Wieczorek, H. (2000). The multigene family of the tobacco hornworm V-ATPase: novel subunits a, C, D, H, and putative isoforms. Biochim Biophys Acta *1467*, 369-379.

Mikitani, K. (1996). A new nonsteroidal chemical class of ligand for the ecdysteroid receptor 3, 5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide shows apparent insect molting hormone activities at molecular and cellular levels. Biochem Biophys Res Commun *227*, 427-432.

Minakuchi, C., Namiki, T., and Shinoda, T. (2009). Kruppel homolog 1, an early juvenile hormone-response gene downstream of Methoprene-tolerant, mediates its anti-metamorphic action in the red flour beetle Tribolium castaneum. Dev Biol *325*, 341-350.

Minakuchi, C., Zhou, X., and Riddiford, L.M. (2008). Kruppel homolog 1 (Kr-h1) mediates juvenile hormone action during metamorphosis of Drosophila melanogaster. Mech Dev *125*, 91-105.

Miura, K., Oda, M., Makita, S., and Chinzei, Y. (2005). Characterization of the Drosophila Methoprene -tolerant gene product. Juvenile hormone binding and ligand-dependent gene regulation. FEBS J *272*, 1169-1178.

Moore, A.W., Barbel, S., Jan, L.Y., and Jan, Y.N. (2000). A genomewide survey of basic helix-loop-helix factors in Drosophila. Proc Natl Acad Sci U S A *97*, 10436-10441.

Mottier, V., Siaussat, D., Bozzolan, F., Auzoux-Bordenave, S., Porcheron, P., and Debernard, S. (2004). The 20-hydroxyecdysone-induced cellular arrest in G2 phase is preceded by an inhibition of cyclin expression. Insect Biochem Mol Biol *34*, 51-60.

Mukherji, M., Bell, R., Supekova, L., Wang, Y., Orth, A.P., Batalov, S., Miraglia, L., Huesken, D., Lange, J., Martin, C.*, et al.* (2006). Genome-wide functional analysis of human cell-cycle regulators. Proc Natl Acad Sci U S A *103*, 14819-14824.

Nielsen, M.D., Luo, X., Biteau, B., Syverson, K., and Jasper, H. (2008). 14-3-3 Epsilon antagonizes FoxO to control growth, apoptosis and longevity in Drosophila. Aging Cell *7*, 688-699.

Oberlander, H., Leach, C.E., and Shaaya, E. (2000). Juvenile hormone and juvenile hormone mimics inhibit proliferation in a lepidopteran imaginal disc cell line. J Insect Physiol *46*, 259-265.

Parthasarathy, R., Tan, A., Bai, H., and Palli, S.R. (2008). Transcription factor broad suppresses precocious development of adult structures during larval-pupal metamorphosis in the red flour beetle, Tribolium castaneum. Mech Dev *125*, 299-313.

Quack, S., Fretz, A., Spindler-Barth, M., and Spindler, K.D. (1995). Receptor affinities and biological responses of non-steroidal ecdysteroid agonists on the epithelial cell line from *Chironomus tentans* (Diptera: Chironomidae). Eur J Entomol *92*, 341-347.

Riddiford, L.M. (1996). Juvenile hormone: the status of its "status quo" action. Arch Insect Biochem Physiol *32*, 271-286.

Schroder, M. (2009). Human DEAD-box protein 3 has multiple functions in gene regulation and cell cycle control and is a prime target for viral manipulation. Biochem Pharmacol *79*, 297-306.

Silhacek, D.L., Oberlander, H., and Porcheron, P. (1990). Action of RH 5849, a non-steroidal acdysteroid mimic, on Plodia interpunctella (Hubner) in vivo and in vitro. Arch Insect Biochem Physiol *15*, 201-212.

Smagghe, G., Dhadialla, T.S., and Lezzi, M. (2002). Comparative toxicity and ecdysone receptor affinity of non-steroidal ecdysone agonists and 20-hydroxyecdysone in Chironomus tentans. Insect Biochem Mol Biol *32*, 187-192.

Smyth, G.K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol *3*, Article3.

Staal, G.B. (1975). Insect growth regulators with juvenile hormone activity. Annu Rev Entomol *20*, 417-460.

Stevens, B., Alvarez, C.M., Bohman, R., and O'Connor, J.D. (1980). An ecdysteroid-induced alteration in the cell cycle of cultured Drosophila cells. Cell *22*, 675-682.

Sumner, J.P., Dow, J.A., Earley, F.G., Klein, U., Jager, D., and Wieczorek, H. (1995). Regulation of plasma membrane V-ATPase activity by dissociation of peripheral subunits. J Biol Chem *270*, 5649-5653.

Suzuki, Y., Truman, J.W., and Riddiford, L.M. (2008). The role of Broad in the development of Tribolium castaneum: implications for the evolution of the holometabolous insect pupa. Development *135*, 569-577.

Thummel, C.S. (2001). Molecular mechanisms of developmental timing in C. elegans and Drosophila. Dev Cell *1*, 453-465.

Truman, J.W., and Riddiford, L.M. (1999). The origins of insect metamorphosis. Nature *401*, 447-452.

Vitavska, O., Wieczorek, H., and Merzendorfer, H. (2003). A novel role for subunit C in mediating binding of the H+-V-ATPase to the actin cytoskeleton. J Biol Chem *278*, 18499-18505.

Voss, M., Vitavska, O., Walz, B., Wieczorek, H., and Baumann, O. (2007). Stimulus-induced phosphorylation of vacuolar H(+)-ATPase by protein kinase A. J Biol Chem *282*, 33735-33742.

Walker, D.W., Muffat, J., Rundel, C., and Benzer, S. (2006). Overexpression of a Drosophila homolog of apolipoprotein D leads to increased stress resistance and extended lifespan. Curr Biol *16*, 674-679.

Wettenhall, J.M., and Smyth, G.K. (2004). limmaGUI: a graphical user interface for linear modeling of microarray data. Bioinformatics *20*, 3705-3706.

Wieczorek, H., Beyenbach, K.W., Huss, M., and Vitavska, O. (2009). Vacuolar-type proton pumps in insect epithelia. J Exp Biol *212*, 1611-1619.

Wieczorek, H., Grber, G., Harvey, W.R., Huss, M., Merzendorfer, H., and Zeiske, W. (2000). Structure and regulation of insect plasma membrane H(+)V-ATPase. J Exp Biol *203*, 127-135.

Wilson, T.G., and Ashok, M. (1998). Insecticide resistance resulting from an absence of target-site gene product. Proc Natl Acad Sci U S A *95*, 14040-14044.

Wing, K.D. (1988). RH 5849, a nonsteroidal ecdysone agonist: effects on a Drosophila cell line. Science *241*, 467-469.

Yang, Y.H., and Thorne, N.P. (2003). Normalization for two-color cDNA microarray data. . In Science and Statistics: A Festschrift for Terry Speed, IMS Lecture Notes - Monograph Series, D.R. Goldstein., ed., pp. 403-418.

Yao, T.P., Segraves, W.A., Oro, A.E., McKeown, M., and Evans, R.M. (1992). Drosophila ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell *71*, 63-72.

Zhang, D.Y., Zheng, S.C., Zheng, Y.P., Ladd, T.R., Pang, A.S., Davey, K.G., Krell, P.J., Arif, B.M., Retnakaran, A., and Feng, Q.L. (2004). An ecdysone-inducible putative "DEAD box" RNA

helicase in the spruce budworm (Choristoneura fumiferana). Insect Biochem Mol Biol *34*, 273-281.

Table 1. Classification of genes regulated in Sf9 cells after treatment by methoxyfenozide or methoprene according to GO terms, blast2Go annotation level 3

	methoxyfenozide		**methoprene**	
Biological process	up-regulated	down-regulated	up-regulated	down-regulated
biosynthetic process	2	1	4	3
cellular component organisation	3	0	1	3
cell cycle	0	0	1	1
cellular metabolic process	8	4	9	4
establisment of localization	2	1	3	1
multicellular organismal development	1	1	2	2
regulation of biological process	2	1	2	3
reproductive process	2	0	1	2
response to stress	0	2	5	2
transcription/translation	4	1	7	1
transport	2	1	2	1
hypothetical protein	0	2	4	0
hypothetical transcript	2	3	15	7
unknown function	0	1	0	0

Table 2. Microarray data for selected genes after Sf9 treatment by methoxyfenozide

Gene description and putative function	**adhoc**	**ratio**	**P value**
Actin cytoskeleton			
gelsolin	adhoc-40151	1.59	0.0406
Carbohydrate metabolism			
6-phosphogluconolactonase	adhoc-27223	2.39	0.0102
Catabolism process			
3-hydroxyisobutyryl Coenzyme A hydrolase	adhoc-25654	0.64	0.0083
hydroxyphenylpyruvate dioxygenase	adhoc-39567	1.64	0.0486
Cell adhesion			
cadherin	adhoc-26462	6.32	0.0098
Chaperone proteins			
calreticulin	adhoc-40650	4.47	0.0168
Proteolysis			
von Hippel-Lindau tumour suppressor protein	adhoc-24976	0.18	0.0014
chymotrypsin	adhoc-25557	2.9	0.0386
Reproductive protein			
vitellogenin	adhoc-25793	1.69	0.0149
Response to stress/Detoxification			
heat shock protein 90	adhoc-39008	0.57	0.0440
aldehyde oxidase	adhoc-34664	0.14	0.0436
Sugar synthesis			
chondroitin sulfate synthase	adhoc-44313	3.55	0.0254
Translation/Transcription			
polyadenylate binding protein 2	adhoc-36158	6.19	0.0102
p27BBP/eIF6	adhoc-33876	3.58	0.0252
dead box RNA helicase	adhoc-25789	3.11	0.0422
Transport			
vacuolar ATPase subunit B	adhoc-34648	9.44	0.0059
vacuolar ATPase subunit C	adhoc-40849	0.64	0.0131

Table 3 . Microarray data for selected genes after Sf9 treatment by methoprene

Gene description and putative function	**adhoc**	**ratio**	**P value**
Amino acids biosynthesic process			
phosphoserine phosphatase	adhoc-25880	0.62	0.0022
Extracellular matrix protein			
hemicetin like protein 1	adhoc-25674	1.7	0.0355
Immune protein			
scolexin B like	adhoc-34835	1.77	0.0116
Mitotic cell cycle checkpoint			
14-3-3 epsilon protein	adhoc-41120	0.62	0.0022
Phospholipid biosynthetic process			
choline/ethanolamine kinase	adhoc-36601	0.54	0.0273
Polyamine synthesis			
S-adenosylmethionine decarboxylase	adhoc-44296	1.71	0.0151
Regulation of Rab GTPase activity			
Tbc1 domain family	adhoc-39160	0.57	0.0026
Response to stress/Detoxification			
carboxylesterase	adhoc-40600	3.89	3.75E-07
prophenoloxidase activating factor	adhoc-26333	2.55	0.0086
aldehyde dehydrogenase 7 family member A1	adhoc-25971	1.89	0.0019
pheromone degrading enzyme 2	adhoc-34999	1.59	0.0172
apolipoprotein D precursor	adhoc-35472	1.55	0.0119
DNAJ-1	adhoc-38331	0.65	0.0456
uridine diphosphate glucosyltransferase	adhoc-35495	0.55	0.0422
Spindle assembly			
microtubule associated protein RP/EB family 3	adhoc-44445	2.86	0.0441
kinesin like protein	adhoc-36395	2.75	0.0110
beta-tubulin cofactor E	adhoc-36883	2.07	0.0152
Structural constituent of cuticule			
cuticle protein 1 like	adhoc-38620	0.61	0.0462
Translation/Transcription			
mitonchondrial ribosomal protein L49	adhoc-27227	4.62	3.83E-06
60S ribosomal protein L31	adhoc-25976	1.77	0.0019
coiled-ciol-helix-coiled-coil helix domain containing 8	adhoc-34522	1.67	7.20E-05
ribosomal protein L10	adhoc-38355	1.55	0.0139
spt3 associated factor 42	adhoc-41154	1.52	0.0044
mitochondrial translational release factor 1 like	adhoc-40890	0.43	0.0035
tRNA splicing endonuclease 2	adhoc-35120	1.9	1.29E-05
bip2 like	adhoc-41062	1.5	0.0050
Transport			
phosphate transport protein	adhoc-38062	1.85	0.0017
translocase of inner mitochondrial membrane	adhoc-40155	0.61	0.0079
Vesicle trafficking			
exocyst complex component 6	adhoc-36460	1.5	0.0340

Figure Legends

Figure 1. Methoxyfenozide and methoprene toxicity on Sf9 cells.

Cell viability is followed by MTT test at 24, 48 and 72h post-treatment of Sf9 cells by methoxyfenozide (A) or methoprene (B). Data calculated are a mean of three independent experiments.

Figure 2. Effects of methoxyfenozide and methoprene on Sf9 cellular proliferation.

Cells were numbered 24, 48 and 72h post-insecticide treatment, (A) methoxyfenozide, (B) methoprene. Data calculated are a mean of three independent experiments. A t-test was performed to determine result significance.

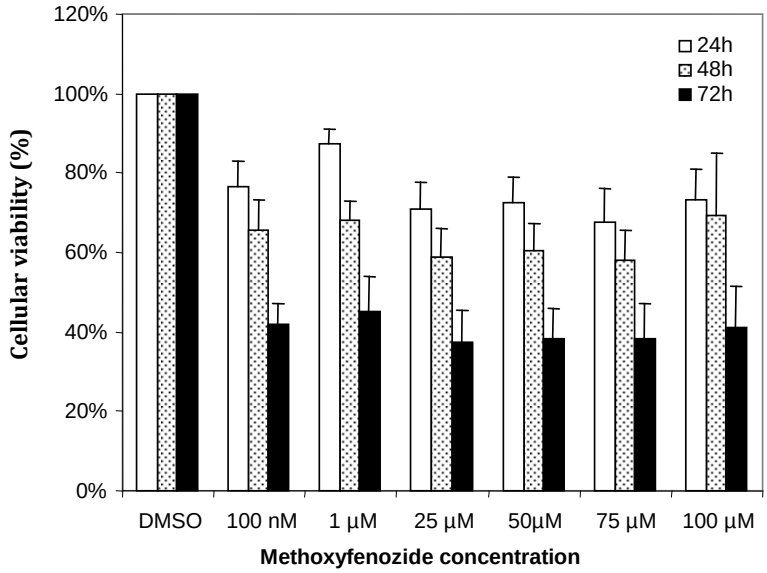
Figure 3. Distribution of Sf9 cells in the different phases of the cell cycle.

Cellular DNA content was determined by staining cells with propidium iodide and measuring fluorescence.

(A) Control, cells untreated.
(B) DMSO control, cells were treated during 48h by the solvent used for insecticide.
(C) Methoxyfenozide treatment, cells were treated during 48h at 10nM methoxyfenozide.
(D) Methoprene treatment, cells were treated during 48h at 10µM methoprene.

Figure 4. Gene expression levels of hormone nuclear receptors after insecticide treatment. Expression levels are normalized with the three reference genes G6PD, Rpl4 and L18

A


120%
100%
80%
60%
40%
20%
0%
Cellular viability (%)
24h
48h
72h
DMSO
100 nM
1 µM
25 µM
50µM
75 µM
100 µM
Methoxyfenozide concentration

B

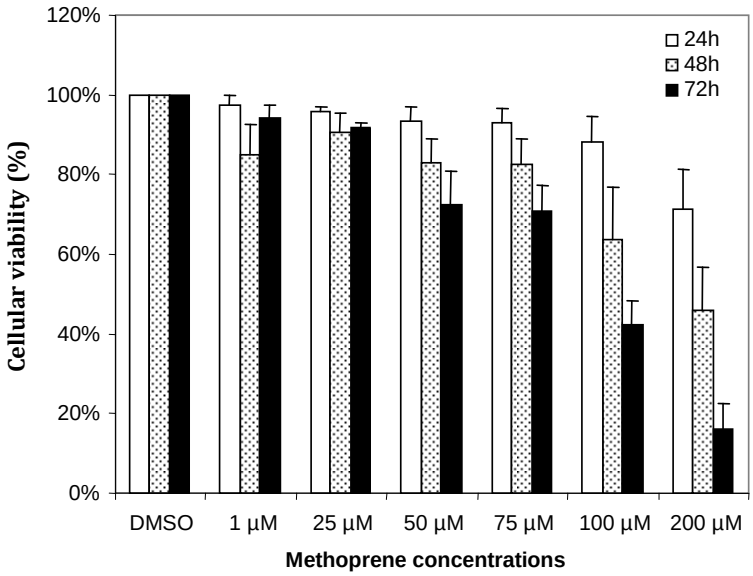
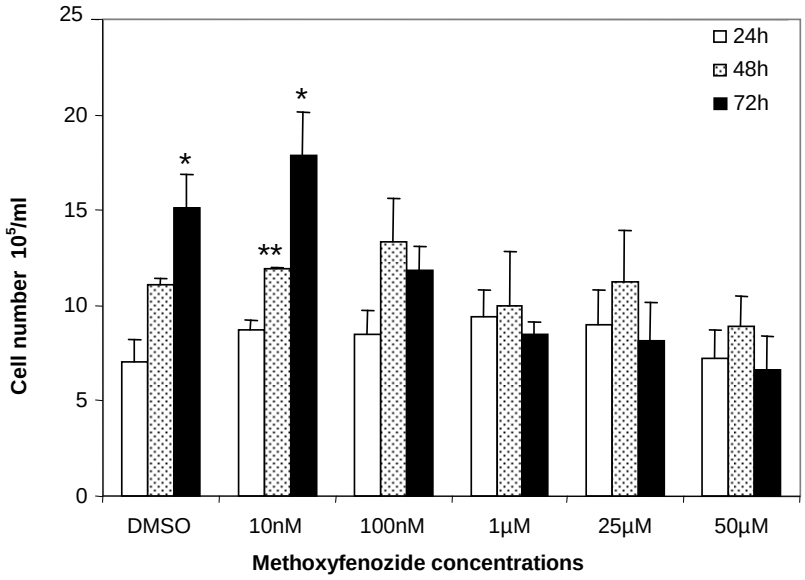

120%
100%
80%
60%
40%
20%
0%
Cellular viability (%)
24h
48h
72h
DMSO
1 µM
25 µM
50 µM
75 µM
100 µM
200 µM
Methoprene concentrations

Figure 1

A


25
20
15
10
5
0
24h
48h
72h
*
**
*
Cell number 10⁵/ml
DMSO
10nM
100nM
1µM
25µM
50µM
Methoxyfenozide concentrations

B

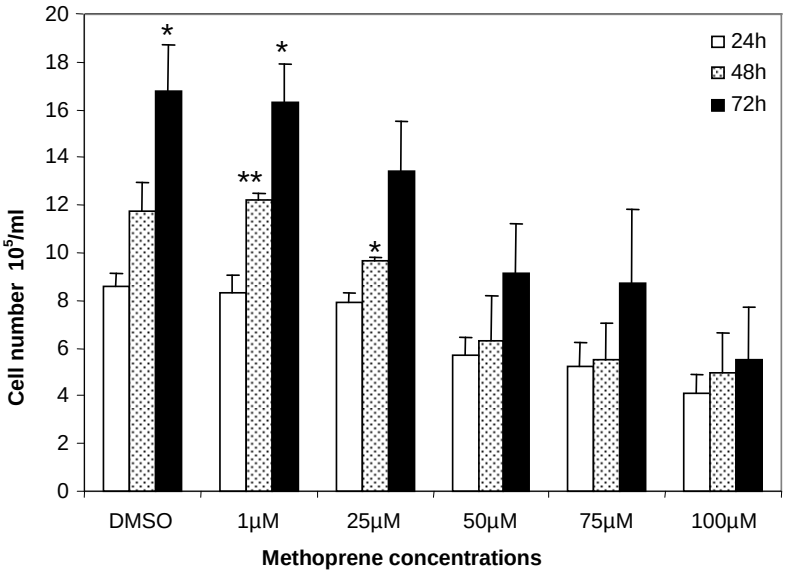

20
18
16
14
12
10
8
6
4
2
0
24h
48h
72h
*
**
*
Cell number 10⁵/ml
DMSO
1µM
25µM
50µM
75µM
100µM
Methoprene concentrations

Figure 2

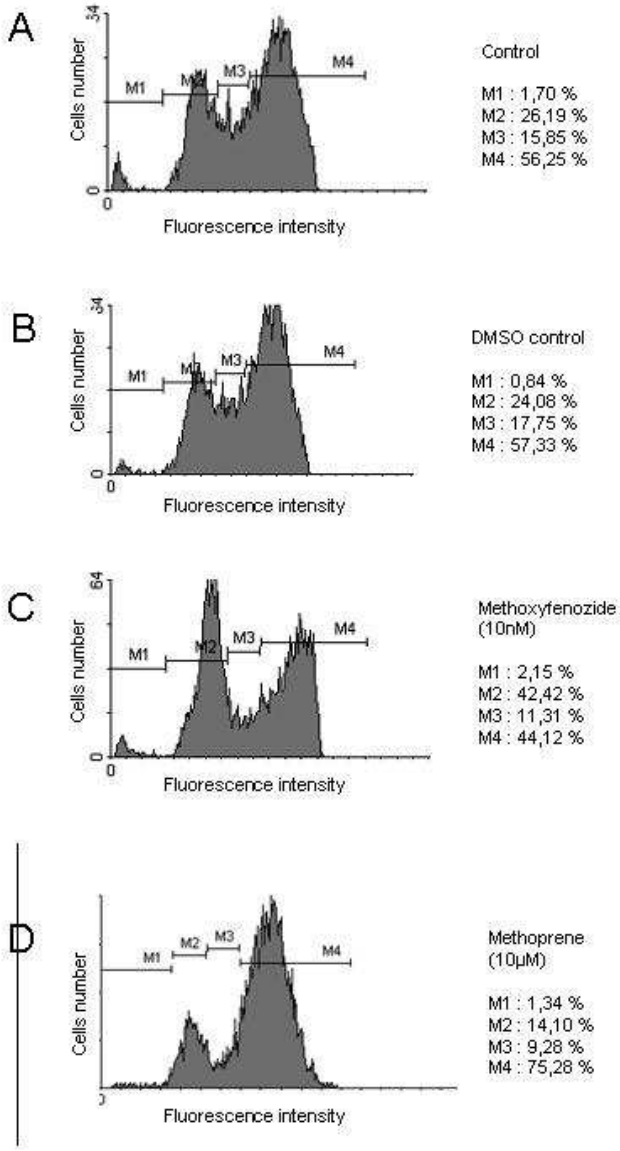

A
Cells number
34
0
0
M1
M2
M3
M4
Fluorescence intensity
Control
M1 : 1,70 %
M2 : 26,19 %
M3 : 15,85 %
M4 : 56,25 %
B
Cells number
34
0
0
M1
M2
M3
M4
Fluorescence intensity
DMSO control
M1 : 0,84 %
M2 : 24,08 %
M3 : 17,75 %
M4 : 57,33 %
C
Cells number
64
0
0
M1
M2
M3
M4
Fluorescence intensity
Methoxyfenozide
(10nM)
M1 : 2,15 %
M2 : 42,42 %
M3 : 11,31 %
M4 : 44,12 %
D
Cells number
M1
M2
M3
M4
Fluorescence intensity
Methoprene
(10µM)
M1 : 1,34 %
M2 : 14,10 %
M3 : 9,28 %
M4 : 75,28 %

Figure 3

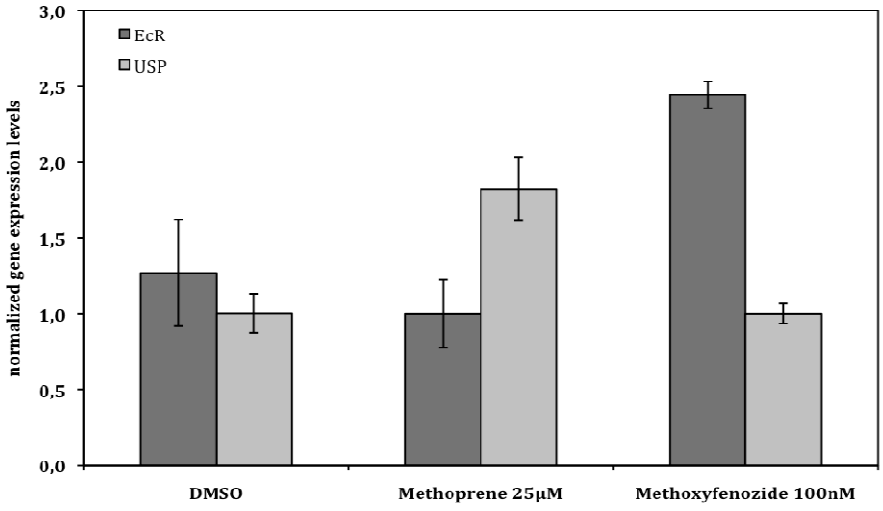
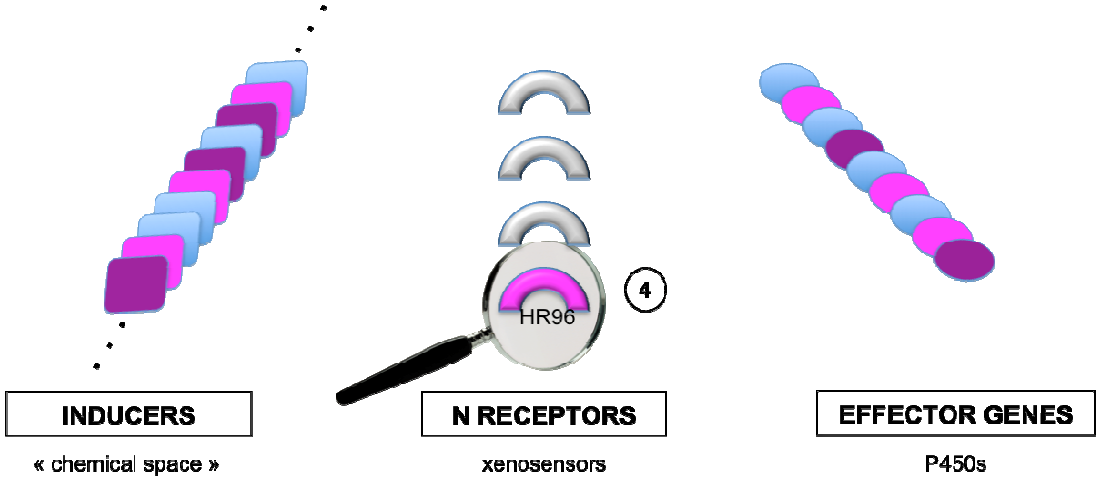

3,0
2,5
2,0
1,5
1,0
0,5
0,0
normalized gene expression levels
EcR
USP
DMSO
Methoprene 25µM
Methoxyfenozide 100nM

Figure 4

CHAPTER 4

Manuscript: Molecular cloning and expression of nuclear receptors in *Spodoptera frugiperda* midgut ans Sf9 cells.



In order to elucidate detoxification mechanisms involved in insects, we have identified patterns of gene induction in the model organism *Drosophila* and the polyphagous pest *Spodoptera frugiperda* in response to different types of xenobiotic compounds. By focusing gene expression studies on P450 induction, we have now a good overview of the "catalogue" of effector genes induced in response to xenobiotics in a polyphagous species.

As we have discussed in the previous chapters, we found that only a small subset of P450s were induced in *Drosophila* and *Spodoptera* in response to xenobiotics and that they are different subsets of inducers/induced genes suggesting multiple xenobiotic transduction mechanisms (see chapter 1 and 2 in results). We have therefore studied as a first step in the understanding of molecular mechanims allowing signal transduction between inducers and effector genes, the response of two well characterised nuclear receptors in insect, EcR and USP, to respective agonists and analog of the two major hormones edcysone and juvenile hormone (chapter 3).

As a final step, we have chosen to identify potential xenosensors in *S. frugiperda* by a candidate gene approach focusing on the only known ortholog of CAR and PXR in *Drosophila*, DHR96. We have discussed earlier that CAR and PXR were two members of the nuclear hormone superfamily that play a central role in the detoxification of xenobiotics by notably inducing the expression of P450s (see section 4.6 of the introduction). Moreover, DHR96 was shown to be involved in xenobiotic response in Drosophila by increasing phenobarbital-

sedative effects and decreasing DDT survival in DHR96 mutants (King-Jones et al., 2006). We therefore expect the same regulation mechanisms of HR96 in *Spodoptera*.

We will now present our results on the cloning of the nuclear receptors SfHR96 and USP in *S. frugiperda*, with the study of temporal and tissue specific expression of these receptors and discuss the role of SfHR96 in xenobiotic response of insects.

Molecular cloning and expression of nuclear receptors in *Spodoptera frugiperda* midgut and Sf9 cells.

Maeva Giraudo[a], René Feyereisen[a]*, Frédérique Hilliou[a], Trang Tran[b] and Gaëlle Le Goff[a].

[a] UMR 1301 IBSV, INRA-CNRS-Université de Nice Sophia Antipolis, 400 Route des Chappes, 06903 Sophia-Antipolis, France

[b] Lanaud Gestion-Pôle de Lanaud, 87220 Boisseuil, France

*Corresponding author: René Feyereisen, UMR 1301 IBSV, INRA-CNRS-Université de Nice Sophia-Antipolis, 400 Route des Chappes, 06903 Sophia-Antipolis, FRANCE; phone : 33 492386450, fax : 33 492386401, e-mail : rfeyer@sophia.inra.fr

Short title: cloning of Spodoptera frugiperda HR96 and USP.

Abstract

Full-length sequence of HR96 and USP were cloned from *Spodoptera frugiperda* using a PCR-based approach employing degenerate primers designed on the basis of conserved regions of nuclear receptors, together with 5'- and 3'-RACE. The sequence of SfHR96 showed 70% and 66% identity in the DNA and ligand binding domain respectively, with its ortholog in Drosophila, DHR96. Cloning of USP revealed the presence of two isoforms, SfUSP-1 and SfUSP-2 in this species, that differ in their N-terminal region. SfHR96 was constitutively expressed in Sf9 cells and in larval midgut, fat body and Malpighian tubules throughout the last two instars and pupation. However, SfHR96 expression was lower than the other nuclear receptors EcR (Ecdysone receptor) and USP (ultraspiracle). EcR and SfUSP-2 showed peaks of expression before larval moults and during metamorphosis, whereas SfUSP-1 was mainly expressed in the pre-pupal stage. Exposure of cells and larvae to different xenobiotics did not induce the expression of SfHR96. EcR was significantly induced by the 20-hydroxyecdysone agonist, methoxyfenozide, and SfUSP showed an increase expression when exposed to the juvenile hormone analog, methoprene.

Keywords: *Spodoptera frugiperda*, Sf9, nuclear receptors, HR96, USP.

1. Introduction

The Nuclear receptor (NR) superfamily comprises transcription factors in metazoans in which they regulate functions as diverse as reproduction, differentiation, metabolism, metamorphosis and homeostasis (Escriva et al., 2000; Mangelsdorf et al., 1995; Robinson-Rechavi et al., 2001). These proteins are characterized by a common structure among family members containing two functional domains, the highly-conserved DNA Binding Domain (DBD) consisting of two C4 type zinc-fingers, and the less conserved Ligand Binding Domain (LBD), which contains a ligand binding pocket, dimerization domain and activation domain (Germain et al., 2006; Robinson-Rechavi and Laudet, 2003). The number of NRs identified in sequenced genomes varies considerably. To date 48 members have been identified in humans (Robinson-Rechavi et al., 2001), 21 in the genome of the fruit fly *Drosophila melanogaster* (Adams et al., 2000) and over 270 are found in *Caenorhabditis elegans* (Sluder and Maina, 2001). Recently sequenced insect genomes of *Anopheles gambiae* revealed 20 NRs (Holt et al., 2002), 22 in the honey bee *Apis mellifera* (Velarde et al., 2006), 21 in the silkworm *Bombyx mori* genome (Cheng et al., 2008), 20 in the yellow fever mosquito *Aedes aegypti* (Cruz et al., 2009) and 21 in the red flour beetle *Tribolium castaneum* (Bonneton et al., 2008; Tan and Palli, 2008). In vertebrates, NRs are able to bind endogenous compounds such as steroids and thyroid hormones and play therefore a key role in a variety of biological pathways from embryonic development to metabolic processes. Similar regulation mechanisms occur in insects, where 20-hydroxyecdysone (20E), the major moulting hormone, binds to an ecdysteroid receptor that regulates the expression of target genes involved in development and metamorphosis (for review, see Thummel, 2002 and Nakagawa and Henrich, 2009). The functional ecdysteroid receptor in insects is a heterodimer of EcR (Ecdysone receptor) and USP (ultraspiracle), two members of the NR family that are

structurally conserved among different species (Mangelsdorf et al., 1995). The identification of both of these receptors in many insect species has revealed the presence of three different isoforms for EcR, EcRA, EcRB1 and EcRB2 (Talbot et al., 1993) and in some cases the presence of two distinct isoforms for USP, USP-1 and USP-2, with USP-2 being 50 amino acids shorter than the other isoform.

The ability of nuclear receptors to bind small lipophilic ligands has triggered the study of NR functions in xenobiotic metabolism and the identification of NRs as drug targets. Detailed studies have defined a central role for two vertebrate NRs in xenobiotic metabolism, the human steroid and xenobiotic receptor (SXR, PXR in mice) and the constitutive androstane receptor (CAR) (Willson and Kliewer, 2002). PXR and CAR are able to bind a wide range of xenobiotics and regulate the expression of overlapping set of genes, including genes encoding Phase I detoxification enzymes such as cytochrome P450s (Maglich et al., 2002; Moore et al., 2006; Pascussi et al., 2005; Willson and Kliewer, 2002). In contrast to these studies in human and mice, relatively little data are available on the regulation of insect xenobiotic responses. Recently, a single ortholog of PXR and CAR has been identified in *Drosophila melanogaster*, DHR96, and has been shown to be involved in xenobiotic response of this insect by playing a role in the regulation of many phenobarbital-regulated genes, including some P450s (King-Jones et al., 2006). Moreover, further studies have reported that DHR96 could be activated by a CAR-selective agonist, suggesting that it may be regulated in a manner similar to that of the vertebrate xenobiotic receptors (Palanker et al., 2006). However, evidence of the ability of DHR96 to bind xenobiotics and transactivate the expression of detoxification enzymes such as P450s still remains to be clearly established.

It is now well know that insect P450s are involved in the detoxification of xenobiotics that insect encounter in their diet, such as plant allelochemicals and insecticides (for a review, see (Feyereisen, 2005). We have recently identified patterns of gene expression induced by different types of xenobiotics in the polyphagous lepidopteran pest, *Spodoptera frugiperda*, with a focus on cytochrome P450 induction (Giraudo et al., unpublished). As a first step in understanding how expression of these P450s is regulated, we have cloned HR96 in *S. frugiperda* (SfHR96) using a PCR-based cloning method employing degenerate primers based on the highly conserved domains of the insect nuclear receptors. In order to compare expression patterns of SfHR96 with other nuclear receptors, we have also cloned two isoforms of SfUSP, for which only a partial sequence was available in public databases. Here we report the results of this cloning and the patterns of expression of SfHR96 in comparison with EcR and SfUSP in *S. frugiperda* larval tissues and Sf9 cells.

2. Material and Methods

2.1 Reagents

The analytical grade plant allelochemicals xanthotoxin, quercetin, 2-tridecanone, indole, indole 3-carbinol and the herbicide clofibrate used in this study, as well as the drug phenobarbital were purchased from Sigma-Aldrich (St Quentin Fallavier, France). The insecticides deltamethrin, methoxyfenozide and fipronil were purchased from Cluzeau (CIL, France). Methoprene was a gift from the former Zoecon Corp., Palo Alto, California.

2.2 Insect rearing and treatments

Larvae of *Spodoptera frugiperda* were fed *ad libitum* on artificial medium (Poitout and Bues, 1974) and were reared at 25,5°C ±1°C, and 70% relative humidity under a L 14: D 10 photoperiod. Under these conditions, the 5th (penultimate) and 6th (ultimate) larval stages last about 2 to 3 days each, with additional two pre-pupal days characterized by the arrest of wandering and beginning of weight lost towards pupal formation.

For xenobiotic induction of nuclear receptor gene expression, newly molted 6^{th} instar larvae were fed for 24h on artificial medium containing either 0.5% phenobarbital, 0.05% xanthotoxin, 0.25% 2-tridecanone, indole 3-carbinol, indole, clofibrate or quercetin, 20nM methoxyfenozide, 2µM methoprene, 10nM deltamethrin, 2µM fipronil or the corresponding concentration of DMSO.

2.3 cell culture and treatments

Sf9 cells derived from the pupal ovarian tissue of *Spodoptera frugiperda* were cultured and maintained in insect-Xpress serum free medium (Lonza) at 27°C in suspension spinner flasks with an agitation rate of 100 rpm and passaged routinely every third day. Cell density was determined by Malassez hemocytometer counts and cell viability was evaluated by methylene blue (1mg/mL, v/v) staining. Prior to experiments, cells were sowed in 6 well plates (TPP) at 5.10^5 cells/ml and left at 27°C for adhesion. For induction of gene expression studies, attached cells were treated for 24h with 500µM 2-tridecanone, 250µM phenobarbital, indole and indole 3-carbinol, 100µM xanthotoxin, clofibrate, and deltamethrin, 50µM quercetin, 25µM methoprene, 1µM fipronil 0.1µM methoxyfenozide or the same volume of DMSO.

2.4 RNA extraction

For tissue and stage specific gene expression studies, midguts, fat bodies and Malpighian tubules were dissected in Phosphate buffer (100mM potassium phosphate buffer, pH7.2, 1mM EDTA) from last day 5th instars larvae and at different time points during the last larval stage: three times a day for the two first days of the 6th instar, and then once per day for the 2 pre-pupal larval stages. Early (~2 days) and Late (~10 days) pupae were ground to powder in liquid nitrogen.

For xenobiotic induction of gene expression studies, midguts were dissected from larvae fed for 24h on artifical medium containing each of the tested compounds at the concentration stated earlier.

Total RNA was extracted from the different tissues using TRIzol Reagent (Invitrogen Life technologies) according to the manufacturer's protocol. RNAs were stored at -80°C until use. RNA extractions were performed on 4 independent biological replicates for tissue and stage specific gene expression measurements and on 3 biological replicates of 5-pooled midguts for the induction of gene expression studies.

Total RNA was extracted from cells of a well of the 6 wells plate also using Trizol Reagent and RNA were analysed and stored the same way than for tissue samples. Extractions were performed on three independent biological replicates.

2.5 PCR amplification and sequencing

Degenerate primers were designed based on the sequences of DHR96 and putative HR96 sequences found in *Bombyx mori*, *Anopheles gambiae*, *Apis mellifera* and *Tribolium castaneum*. These sequences were first aligned in the web-based program Block-Maker (http://bioinformatics.weizmann.ac.il/blocks/blockmkr/www/make_blocks.html), which

finds conserved blocks in a group of two or more aligned protein sequences. The conserved blocks obtained were further analyzed by CODEHOP (http://bioinformatics.weizmann.ac.il/blocks/codehop.html), which designs degenerate primers with a clamp region specific to the conserved blocks. SfHR96 degenerate primers were found in the LBD region and were as follows: (5'-GACCAAGTCGCTCTGCTGAARGGNGGNTG-3') for SfHR96-F1 forward primer and (5'-CAGGTAGTAGTAGGAGTTTTGTTCCAGTYKDATNACRT-3') for SfHR96-R1 reverse primer. The conditions for the initial PCR amplification were as follows: 1 cycle of 94°C for 1 min, followed by 35 cycles of 94°C for 15 sec, 60°C for 30 sec and 68°C for 30 sec, using the Accuprime Taq polymerase (invitrogen). The 320pb fragment obtained was purified using the Minelute PCR purification kit (Qiagen) and subsequently cloned into pcR2.1 vector (TA cloning kit Invitrogen) and sequenced.

2.6 Rapid Amplification of cDNA 5-' and 3'- ends (5'/3' RACE)

5' and 3' RACE were carried out to obtain the 5' and 3' ends of the cDNA for SfHR96 and SfUSP from *S. frugiperda* midgut using the Marathon cDNA amplification kit (Clontech). Briefly, first and second strand cDNA are synthesized from polyA+ mRNA isolated from *S. frugiperda* midgut with the Illustra Quickprep micromRNA purification kit (GE Healthcare). Specific adaptors are then ligated to the obtained ds cDNA generating a library of adaptor-ligated ds cDNA from which 5'- and 3'- end are amplified using the following gene specific primers SfHR96-F2 (5'-TGGACAGAGGAAGCAGTGGAAGATCC-3') and SfHR96-R2 (5'-ATTACATCGGGATGCACGACCTTGG-3') for SFHR96 designed from the 320bp fragment obtained during the initial PCR amplification (figure 1) and SfUSP-F1 (5'-ATGTCTCGCGTGCGGGATGAAGAGG-3') and SfUSP-R1 (5'-CCCACACCACCAACGCGGCTATCTG-

3') for SfUSP designed from the partial *Spodoptera frugiperda* USP sequence available in Genbank aligned with the complete sequence of USP in *S. littoralis*. Gene specific primers are used together with the adaptor specific primer AP1 supplied with the kit to obtain 5'- and 3'- RACE fragments. A second round of PCR amplification is then realized on the resulting 5'- and 3'- fragments using the following nested gene specific primers: SfHR96-F3 (5'-AAGCTGGCCAAGGGCAACATCTACC-3') and SfHR96-R3 (5'-GGTAGATGTTGCCCTTGGCCAGCTT-3') for SfHR96 and SfUSP-F2 (5'-AGGAGCGACAAAGGGCAGCCAGAGG-3') and SfUSP-R2 (5'-GGGTCAGCCACCAGCGACTCCATCT-3') for SfUSP. These nested gene specific primers are used together with adaptor specific primer AP2 supplied with the kit to generated 5' and 3'- end specific fragments. The 5'- and 3'- RACE products are then cloned in pcR2.1 vector (TA cloning kit, Invitrogen) and sequenced. Cloning of the full-length cDNA/ORF based on the 5'- and 3'-RACE sequenced were realised to confirm by sequencing that the sequence obtained is not a chimera.

2.6 Quantitative real-time PCR

Total RNA (1µg) was reverse transcribed using the iScript cDNA Synthesis kit (Biorad) and resulting cDNA were diluted 10 times for quantitative real time PCR reactions. qRT-PCR reactions were carried out on an Opticon monitor 2 (Biorad) using the qPCR Mastermix plus for SYBR Green I no ROX (Eurogentec). PCR primer pairs for *S. frugiperda* HR96 (SfHR96), USP-1, USP-2 and the common part of USP isoforms (SfUSP-1, SfUSP-2, SfUSPcom), EcR and the three control genes (G6PD, L18 and Rpl4) were as follows: (5'-ACAAGGCGGAAAAGAGACGGAAAT-3'/5'-TGCAGCGGTATGAGCCCAGTAT-3') for SfHR96, (5'-TGACGGCACTTATCAACTGG-3'/5'-CCAGTGAACAGTCAACAGTCG-3') for SfUSP-1, (5'-GGAGCCCTCGAGAGATTCAG-3'/5'-GGGGGTAGTTCTTGAATGCAG-3') for SfUSP-2 and (5'-

ATAGCGAGGCTGGTCTGGTA-3'/5'-GGCATGTCCGACTCTTCTTC-3') for SfEcR. Final concentration of primers was 20nM. qRT-PCR efficiency of each primer pair was determined by absolute standard curves for the different gene transcripts and their controls by serial (5x) dilutions of 6th instar midgut and Sf9 cells cDNA. The PCR conditions were as follows: 50°C for 2 min, 95°C for 10min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Each reaction was performed in duplicate and the mean of the four (tissue-stage-specific induction) or the three (xenobiotic induction of gene expression and Sf9 cells) independent biological replicates was calculated. All results were normalized using mRNA level of three control genes (RpL4, L18 and G6PD) and relative expression values were calculated in R using the RqPCRBase package developed in our laboratory (Hilliou and Tran, unpublished)

2.7 Phylogenetic analysis

Twenty two Nuclear Receptors were compared including NR1IJK sequences: eight vertebrate PXR/CAR/VDR sequences, six insect HR96 sequences and NR1H sequences: three arthropod EcR sequences and four chordate LXR/FXR sequences (rat, mouse and the tunicate *Ciona intestinalis*). Drosophila DHR3 (NR1F) was used as outgroup.

The analysis was performed on the Phylogeny.lirmm.fr platform and comprised the following steps. Sequences were aligned with MUSCLE (v3.7) configured for highest accuracy (MUSCLE with default settings). Two methods of tree reconstruction were used. (1) Using the neighbor joining method implemented in the BioNJ program (settings gamma 1, 1000 bootstraps) and (2) using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT). Reliability of the internal branches was assessed using the aLRT test.

Graphical representation and edition of the phylogenetic trees were performed with TreeDyn (v198.3).

3. Results

3.2. Cloning of SfHR96 and SfUSP

We have designed degenerate primers based from the amino acid sequence of DHR96 and other putative HR96 sequences that allowed us to amplify a 320bp fragment from *S. frugiperda* midgut cDNA. This fragment shares 63% identity with DHR96 LBD as shown in figure 1. This LBD fragment allowed us to design specific primers for the amplification of 5'- and 3-' end by RACE PCR reactions. Both 5' and 3' RACE reactions did not give specific results from the first round of amplification as many non specific bands were obtained for both reactions. A second round of PCR amplification using nested primer pairs designed in the inner sequence of the first round PCR reaction was needed to obtain both 3' and 5' fragments of SfHR96 of approximately 1700bp for 5'-end and 600bp for the 3'-end. The sequence of both fragments shared a long enough overlapping region that allowed the reconstruction of a putative whole sequence of 1722bp for SfHR96. From this reconstituted sequence, we have cloned the full-length cDNA/ORF of SfHR96 and confirmed by sequencing that the sequence obtained was not a chimera. Alignment of the deduced amino-acid sequence of SfHR96 with DHR96 showed that both sequences share >50% of identity in their whole sequence, with respectively 79% and 66% identity within the DNA and ligand binding domain of the receptor as shown in figure 2.

For SfUSP, the design of specific primers based on the sequence of USP in *S. littoralis* allowed us to obtain two fragments of approximately 900bp for the 5' end and 450bp for the 3' end.

The sequencing of these fragments showed actually some consistent differences in the 5' region of different clones. Indeed, the deduced amino acid sequence of some clones showed a 50 residues shorter 5' sequence. The alignment of our SfUSP with other insect USP amino acid sequences showed the presence of two isoforms that differ in their 5' end in *S. frugiperda* as it is shown in figure 3, consistently with the presence of two isoforms of this receptor in some insect species.

3.3 Phylogenetic analysis of SfHR96

The deduced amino-acid sequence of SfHR96 that we obtained by RACE-PCR cloning was compared to other insect HR96 ortholog and related nuclear receptors as shown in figure 4. Two phylogenetic trees were built using neighbour joining and maximum likelihood methods.

The two trees are relatively consistent showing that our *S. frugiperda* sequence is most closely related to its Bombyx ortholog obtained from the complete genome sequence. In both trees, the VDR/PXR/CAR sequences were monophyletic and most related to the HR96 sequences. The trees differed in the relative position of LXR, FXR and EcR sequences, were the neighbor-joining tree has more support than the maximum likelihood tree.

3.3 Tissue- and stage- specific expression of nuclear receptors *in S. frugiperda*

Using qRT-PCR method, we examined gene expression of the *S. frugiperda* nuclear receptors SfHR96, USP-1, USP-2 and EcR during development from the last fifth and 6^{th} instars to the pupae. Although EcR presents three different isoforms in other insect species, we have measured the level of expression using primers designed on the common part of these three isoforms, without differentiating respective contribution of each of the isoforms. Our results

show that EcR is expressed in midgut, fat bodies and Malpighian tubules with a peak of expression in the late 5th instar, just before the 6th instar moult and in the second day of the pre-pupal stage, with consistent high levels of expression in pupal stages (figure 5). SfUSP-1 and SfUSP-2 are also expressed in the three tissues examined, with SfUSP-1 showing a higher level of expression in Malpighian tubules during the 6th instar (figure 7) than SfUSP-2. SfUSP-1 also shows a peak of expression in the last pre-pupal stage, consistently with SfUSP-2 expression. However, levels of gene expression of SfUSP-1 are much lower in pupae than the second isoform (figure 8). SfHR96 is expressed in all tissues examined with higher levels in midguts and Malpighian tubules compare to fat bodies (figure 6). This receptor did not show the same peak of expression in the pre-pupal and pupae stages as it was seen for EcR and USP and seems to be constitutively expressed throughout the development stages, with a slight increase in the midgut during the late 5th instar and in Malpighian tubules during the first day of the 6th instar.

3.4 Induction of nuclear receptors expression by xenobiotics

Specific induction of the nuclear receptors SfHR96, EcR, USP-1 and USP-2 were monitored in *S. frugiperda* midgut fed for 24h with sub-lethal doses of different xenobiotic compounds. Our results show that out of the four receptors studied, only EcR and USP-2 were significantly induced respectively by the ecdysone agonist methoxyfenozide (*p*=7E-03) and the juvenile hormone analog methoprene (*p*=5E-02), with USP-2 also showing an induction when larvae were exposed to the insecticide fipronil (table 1A). None of the tested compounds were able to affect the expression of USP-1, as it was also the case for SfHR96 except for two xenobiotics, phenobarbital and xanthotoxin. Interestingly, these two compounds reduced significantly the expression of SfHR96 in *S. frugiperda* midguts.

We also measured the induction level of the three receptors in Sf9 cells exposed to the same battery of chemicals (table 1B). We were not able to use specfic qRT-PCR primers to differentiate the two USP isoforms with acceptable efficiency in Sf9 cells and we therefore used primer designed on the common sequence of the two isoforms. Similarly to midgut, SfHR96 was not induced by any of the compounds tested. EcR and USP showed however the same induction patterns in response to methoxyfenozide and methoprene as it was seen in the midgut, with additional compounds found to induce the expression of these receptors. 2-tridecanone and clofibrate were able to induce both EcR and USP expression, and USP expression was also higher in response to indole.

Discussion

The structural features of nuclear receptors, notably the highly conserved domains (DNA and ligand binding domains), enabled us to design degenerate primers, in conjunction with PCR, for cloning SfHR96 and two isoforms of SfUSP, SfUSP-1 and SfUSP-2 from the polyphagous pest *Spodoptera frugiperda*. Our result show that USP isoforms shares high identity with the known USP isoforms identified in *Manduca sexta* (Jindra et al., 1997) and *Bomby mori* (Cheng et al., 2008) and we have found that the partial sequence of SfUSP available in Genbank corresponds in fact to our SfUSP-2. We show that both isoforms are expressed throughout developmental stages and tissues in *S. frugiperda*, with a peak of expression in fat bodies for SfUSP-2 in the final days of 5^{th} and 6^{th} instars just before the moult, as it has been shown in the epidermis for *Manduca sexta* USP-2 (Jindra et al., 1997). In this study, it was found that MsUSP-1 disappears during larval and pupal moults when ecdysteroid titers are high, and Ms-USP2 increases, suggesting that MsUSP-2 could be the isoform that

interacts with EcR for new cuticle production during the insect moult. Similar results were observed in *Bombyx mori* were USP-2 expression appeared to be coordinate with the pulse of ecdysone during metamorphosis, suggesting that this isoform of USP may be the actual component of the EcR/USP receptor complex (Cheng et al., 2008). We found similar increase of expression of SfUSP-2 before larval moult, concomitant with the first peak of expression of EcR. However, SfUSP-1 was the predominantly expressed isoform in the prepupal stage, when EcR expression reaches its second peak. Insects have different isoforms of EcR. EcRA is mainly expressed in the prepupal and pupal stage in Drosophila, when EcRB appears just before larval and pupal moult (King-Jones and Thummel, 2005). In our results, we only studied the common isoform of EcR and we cannot differenciate which isoform is associated with respective peaks of expression. It is therefore possible that SfUSP-1 and 2 interact with one or another EcR isoform depending on the developmental stage, which could explain the difference in SfUSP expression.

We obtained the sequence of HR96 in *Spodoptera frugiperda*. It shares high identity with its Drosophila ortholog and phylogenetic analysis revealed that it was indeed clustered with other identified HR96 sequences in insects, being most closely related to the *Bombyx mori* HR96. The first identification of HR96 in Drosophila showed that its DBD shared high identity with the human vitamin D receptor and EcR (Fisk and Thummel, 1995) and that this receptor was closely related to the vertebrate receptors CAR and PXR (Escriva et al., 2000; Laudet and Bonneton, 2005), representing the single ortholog of these receptors (King-Jones et al., 2006). Our result confirm these data as SfHR96 in both phylogenetic trees showed that HR96 cluster was most related to the monophyletic group containing VDR/PXR/CAR sequences.

We found that this receptor is expressed constitutively at low levels in all developmental stages and tissues examined with a light increase before and after the last instar moult. These results are consistent with the expression of DHR96 in all digestive tissues of Drosophila (Palanker et al., 2006). However, temporal expression studies in *B. mori* showed that BmHR96 was only expressed at specific time points co-ordinately with ecdysone peaks and along with the expression of BmUSP-2 (Cheng et al., 2008). Our results support these observations with the increase of expression of SfHR96 before the last larval moult, concomitant with the first peak of ecdsyone and the expression of SfUSP-2 but we did not find an increase of expression during the second ecdysone peak before pupation.

HR96 belong to the large family of orphan receptors for which no ligand has been identified, nor potential hetero-dimerization partner. NRs indeed function mainly as homo or heterodimer, for example the functional ecdysone receptor consists of a heterodimer of EcR and USP (Yao et al., 1992). USP has also been shown to form a heterodimer with DHR38 (Sutherland et al., 1995), suggesting that it can bind to other nuclear receptors. In vertebrate, USP ortholog RXR binds to the nuclear receptors CAR and PXR, which are the ortholog of HR96 in insects. We can therefore suggest that USP could be a good dimerization candidate for HR96. This hypothesis is particularly attractive as the recruitment of USP by HR96 would make it less available for EcR and thus interfere with ecdysteroid signaling mechanisms. Our results support this hypothesis with a concomitant expression of SfHR96 and SfUSP-2 before the larval moult. However, interactions between SfHR96 and USP still remain to be elucidated.

In vertebrate, CAR and PXR regulate the expression of detoxification genes, such as P450s, when they are activated by xenobiotics (Willson and Kliewer, 2002). In Drosophila, DHR96

has been identified as a potential xenosensors involved in the regulation of many phenobarbital-regulated genes (King-Jones et al., 2006) and was shown to be activated by CAR agonists suggesting similar regulation mechanisms than its vertebrate ortholog (Palanker et al., 2006). Regulation of CAR and PXR involves transcriptional expression, with compounds such as dexamethasone inducing the expression of both receptors (Pascussi et al., 2000a; Pascussi et al., 2000b). We have therefore tested the ability of different plant allelochemicals and xenobiotics to induce the expression of SfHR96 in *S. frugiperda* larvae and Sf9 cells. Our results show that none of the compounds were inducers, with xanthotoxin showing a repression of the expression of this receptor. This may suggest that SfHR96 might regulate the expression of detoxification genes after activation by xenobiotics without increasing its own expression.

As its vertebrate orthologs CAR and PXR regulate the expression of a number of detoxification genes, including P450s (Willson and Kliewer, 2002), we could suggest that SfHR96 act as a xenosensor by regulating P450s in *S. frugiperda* in a similar manner. We have recently identified patterns of P450 induction in response to different plant chemicals and xenobiotics in *S. frugiperda* larvae and Sf9 cells (Giraudo et al., manuscript in preparation). The next logical step will therefore be the study of the effect of SfHR96 silencing on the expression of P450s in these systems. Extinction of the expression of SfHR96 by RNAi will be tried in order to validate its function as a xenosensors and make the link between the ingestion of the toxic compound and the induction of detoxificaton genes in *S. frugiperda*..

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F.*, et al.* (2000). The genome sequence of Drosophila melanogaster. Science *287*, 2185-2195.

Bonneton, F., Chaumot, A., and Laudet, V. (2008). Annotation of Tribolium nuclear receptors reveals an increase in evolutionary rate of a network controlling the ecdysone cascade. Insect Biochem Mol Biol *38*, 416-429.

Bujold, M., Gopalakrishnan, A., Nally, E., and King-Jones, K. Nuclear receptor DHR96 acts as a sentinel for low cholesterol concentrations in Drosophila melanogaster. Mol Cell Biol *30*, 793-805.

Cheng, D., Xia, Q., Duan, J., Wei, L., Huang, C., Li, Z., Wang, G., and Xiang, Z. (2008). Nuclear receptors in Bombyx mori: insights into genomic structure and developmental expression. Insect Biochem Mol Biol *38*, 1130-1137.

Cruz, J., Sieglaff, D.H., Arensburger, P., Atkinson, P.W., and Raikhel, A.S. (2009). Nuclear receptors in the mosquito Aedes aegypti: annotation, hormonal regulation and expression profiling. FEBS J *276*, 1233-1254.

Escriva, H., Delaunay, F., and Laudet, V. (2000). Ligand binding and nuclear receptor evolution. Bioessays *22*, 717-727.

Feyereisen, R. (2005). Insect cytochrome P450. In Comprehensive molecular insect science (Elsevier), pp. 1-77.

Fisk, G.J., and Thummel, C.S. (1995). Isolation, regulation, and DNA-binding properties of three Drosophila nuclear hormone receptor superfamily members. Proc Natl Acad Sci U S A *92*, 10604-10608.

Germain, P., Staels, B., Dacquet, C., Spedding, M., and Laudet, V. (2006). Overview of nomenclature of nuclear receptors. Pharmacol Rev *58*, 685-704.

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., Nusskern, D.R., Wincker, P., Clark, A.G., Ribeiro, J.M., Wides, R.*, et al.* (2002). The genome sequence of the malaria mosquito Anopheles gambiae. Science *298*, 129-149.

Horner, M.A., Pardee, K., Liu, S., King-Jones, K., Lajoie, G., Edwards, A., Krause, H.M., and Thummel, C.S. (2009). The Drosophila DHR96 nuclear receptor binds cholesterol and regulates cholesterol homeostasis. Genes Dev *23*, 2711-2716.

Jindra, M., Huang, J.Y., Malone, F., Asahina, M., and Riddiford, L.M. (1997). Identification and mRNA developmental profiles of two ultraspiracle isoforms in the epidermis and wings of Manduca sexta. Insect Mol Biol *6*, 41-53.

King-Jones, K., Horner, M.A., Lam, G., and Thummel, C.S. (2006). The DHR96 nuclear receptor regulates xenobiotic responses in Drosophila. Cell Metab *4*, 37-48.

King-Jones, K., and Thummel, C.S. (2005). Nuclear receptors--a perspective from Drosophila. Nat Rev Genet *6*, 311-323.

Laudet, V., and Bonneton, F. (2005). evolution of nuclear hormone receptors in insects. In Comprehensive molecular insect science (Elsevier), pp. 287-318.

Maglich, J.M., Stoltz, C.M., Goodwin, B., Hawkins-Brown, D., Moore, J.T., and Kliewer, S.A. (2002). Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. Mol Pharmacol *62*, 638-646.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P.*, et al.* (1995). The nuclear receptor superfamily: the second decade. Cell *83*, 835-839.

Moore, D.D., Kato, S., Xie, W., Mangelsdorf, D.J., Schmidt, D.R., Xiao, R., and Kliewer, S.A. (2006). International Union of Pharmacology. LXII. The NR1H and NR1I receptors: constitutive androstane receptor, pregnene X receptor, farnesoid X receptor alpha, farnesoid X receptor beta, liver X receptor alpha, liver X receptor beta, and vitamin D receptor. Pharmacol Rev *58*, 742-759.

Nakagawa, Y., and Henrich, V.C. (2009). Arthropod nuclear receptors and their role in molting. FEBS J *276*, 6128-6157.

Palanker, L., Necakov, A.S., Sampson, H.M., Ni, R., Hu, C., Thummel, C.S., and Krause, H.M. (2006). Dynamic regulation of Drosophila nuclear receptor activity in vivo. Development *133*, 3549-3562.

Pascussi, J.M., Drocourt, L., Fabre, J.M., Maurel, P., and Vilarem, M.J. (2000a). Dexamethasone induces pregnane X receptor and retinoid X receptor-alpha expression in human hepatocytes: synergistic increase of CYP3A4 induction by pregnane X receptor activators. Mol Pharmacol *58*, 361-372.

Pascussi, J.M., Gerbal-Chaloin, S., Fabre, J.M., Maurel, P., and Vilarem, M.J. (2000b). Dexamethasone enhances constitutive androstane receptor expression in human hepatocytes: consequences on cytochrome P450 gene regulation. Mol Pharmacol *58*, 1441-1450.

Pascussi, J.M., Robert, A., Nguyen, M., Walrant-Debray, O., Garabedian, M., Martin, P., Pineau, T., Saric, J., Navarro, F., Maurel, P.*, et al.* (2005). Possible involvement of pregnane X receptor-enhanced CYP24 expression in drug-induced osteomalacia. J Clin Invest *115*, 177-186.

Poitout, S., and Bues, R. (1974). [Linolenic acid requirements of lepidoptera Noctuidae Quadrifinae Plusiinae: Chrysodeixis chalcites Esp., Autographa gamma L.' Macdunnoughia confusa Stph., Trichoplusia ni Hbn. reared on artificial diets]. Ann Nutr Aliment *28*, 173-187.

Robinson-Rechavi, M., Carpentier, A.S., Duffraisse, M., and Laudet, V. (2001). How many nuclear hormone receptors are there in the human genome? Trends Genet *17*, 554-556.

Robinson-Rechavi, M., and Laudet, V. (2003). Bioinformatics of nuclear receptors. In Methods in enzymology, Elsevier, ed., pp. 95-118.

Sluder, A.E., and Maina, C.V. (2001). Nuclear receptors in nematodes: themes and variations. Trends Genet *17*, 206-213.

Sutherland, J.D., Kozlova, T., Tzertzinis, G., and Kafatos, F.C. (1995). Drosophila hormone receptor 38: a second partner for Drosophila USP suggests an unexpected role for nuclear receptors of the nerve growth factor-induced protein B type. Proc Natl Acad Sci U S A *92*, 7966-7970.

Talbot, W.S., Swyryd, E.A., and Hogness, D.S. (1993). Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell *73*, 1323-1337.

Tan, A., and Palli, S.R. (2008). Identification and characterization of nuclear receptors from the red flour beetle, Tribolium castaneum. Insect Biochem Mol Biol *38*, 430-439.

Thummel, C.S. (2002). Ecdysone-regulated puff genes 2000. Insect Biochem Mol Biol *32*, 113-120.

Velarde, R.A., Robinson, G.E., and Fahrbach, S.E. (2006). Nuclear receptors of the honey bee: annotation and expression in the adult brain. Insect Mol Biol *15*, 583-595.

Willson, T.M., and Kliewer, S.A. (2002). PXR, CAR and drug metabolism. Nat Rev Drug Discov *1*, 259-266.

Yao, T.P., Segraves, W.A., Oro, A.E., McKeown, M., and Evans, R.M. (1992). Drosophila ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell *71*, 63-72

Table 1: qRT-PCR analysis of nuclear receptors gene induction in *S.frugiperda midgut* (A) and Sf9 cells (B) by xenobiotics and insecticides. Larvae and cells were exposed for 24h to sublethal concentrations of the different compounds as described in material and methods. Gene expression values are indicated as fold expression in larvae and cells exposed to each xenobiotic comparatively to unexposed larvae and cells (controls). The three reference genes Rpl4, G6PD and L18 were used as internal controls for normalization. Gene expression values in bold are significantly different from the corresponding control as measured by pair-wise t-student test ($p<0.05$) .

A	SfHR96		EcR		USP-1		USP-2	
xenobiotics	ratio	P value	ratio	P value	ratio	P value	ratio	P value
Phenobarbital	0.58	2.0E-02	0.45	6.0E-02	0.84	5.5E-01	0.68	1.4E-01
Xanthotoxin	**0.39**	**4.0E-03**	0.60	2.2E-01	0.73	3.7E-01	0.63	1.8E-01
2-tridecanone	0.78	2.5E-01	0.57	1.8E-01	0.70	3.2E-01	0.75	3.5E-01
Indole 3-carbinol	0.81	3.2E-01	0.64	2.7E-01	1.07	8.0E-01	0.91	7.4E-01
Clofibrate	0.92	6.9E-01	0.84	6.2E-01	0.90	7.5E-01	0.95	8.6E-01
Quercetin	0.81	3.3E-01	0.71	3.7E-01	0.77	4.4E-01	1.31	2.5E-01
Indole	0.80	3.0E-01	0.67	3.0E-01	1.05	8.6E-01	0.97	7.3E-01
Methoxyfenozide	0.99	9.6E-01	**1.92**	**7.0E-03**	1.10	7.4E-01	1.44	1.1E-01
Methoprene	0.91	6.4E-01	0.92	7.9E-01	0.73	3.8E-01	**1.54**	**1.1E-02**
Deltamethrin	0.92	6.6E-01	0.88	7.1E-01	1.00	8.2E-01	1.22	4.2E-01
Fipronil	0.85	7.3E-01	1.09	7.7E-01	1.07	8.1E-01	1.69	5.0E-02

B	SfHR96		EcR		USP-com	
xenobiotics	ratio	P value	ratio	P value	ratio	P value
Phenobarbital	1.05	8.6E-01	1.22	8.5E-01	1.48	1.6E-01
Xanthotoxin	2.29	4.9E-01	1.35	1.4E-02	1.42	1.8E-01
2-tridecanone	1.78	1.6E-01	**2.92**	**3.5E-03**	**1.90**	**6.1E-03**
Indole 3-carbinol	2.36	1.9E-02	1.06	4.6E-03	1.45	1.5E-01
Clofibrate	1.90	1.1E-01	**3.43**	**4.1E-04**	**2.27**	**2.7E-04**
Quercetin	1.80	1.9E-01	1.28	2.1E-03	1.78	2.7E-02
Indole	2.05	7.9E-01	1.56	3.5E-01	**1.87**	**7.6E-03**
Methoxyfenozide	0.75	6.7E-01	**2.55**	**1.0E-02**	1.60	5.7E-02
Methoprene	2.15	7.0E-01	1.98	1.1E-01	**2.74**	**4.3E-06**
Deltamethrin	2.32	2.2E-02	1.61	3.1E-01	1.63	4.7E-02
Fipronil	1.36	1.1E-01	1.36	5.5E-01	1.36	2.1E-01

Figure legends

Figure 1: alignment of the deduced amino-acid sequence of SfHR96 LBD obtained in the initial PCR amplification with the protein sequence of DHR96 using CLUTALW2. DHR96 LBD is indicated by the frame.

Figure 2: alignment of SfHR96 and DHR96. The amino-acid sequence deduced from the full length SfHR96 that we cloned by RACE-PCR was aligned with the protein sequence of DHR96 using CLUSTALW2. Highly conserved DBD and LBD are indicated in frames. Poorly conserved hinge region between the LBD and the DBD showed weak alignment and was therefore not shown (indicated by double slashes).

Figure 3: alignment of the deduced amino-acid sequences obtained from the cloning of SfUSP-1 **(A)** and SfUSP-2 **(B)** with USP-1 and -2 orthologs in insects. Ms: *Manduca sexta*, Bm: *Bombyx mori*, SfUSP-G: partial sequence of *S. frugiperda* USP (Genbank acc. Number AF411255.

Figure 4: Phylogenetic analysis of SfHR96. Trees were built with the neighbour joininig method **(A)** of maximum likelihood method **(B)**. Boostrap values are indicated for each branch. Dmel : *Drosophila melanogaster* ; Agam : *Anopheles gambiae* ; Bmor *Bombyx mori* ; Sfru : *Spodoptera frugiperda* ; Amel : *Apis mellifera* ; Tcas : *Tribolium castaneum*.

Figures 4-5-6-7: qRT-PCR analysis of nuclear receptors gene expression in different tissues and developmental stages of *S. frugiperda* larvae. Expression was monitored in last day 5th instar larvae (L5), at different time points of the first and second days of 6th instars (L6-1 to 3 and L6-4 to 6 respectively), in first and second pre-pupal stages (PP1 and PP2) and in early (~2days) and late (~10 days) pupae. EcR levels of expression are shown in figure 2, SfHR96 in

figure 3, SfUSP-1 in figure 4 and SfUSP-2 in figure 5. Expression levels are normalized with the three reference genes G6PD, Rpl4 and L18.

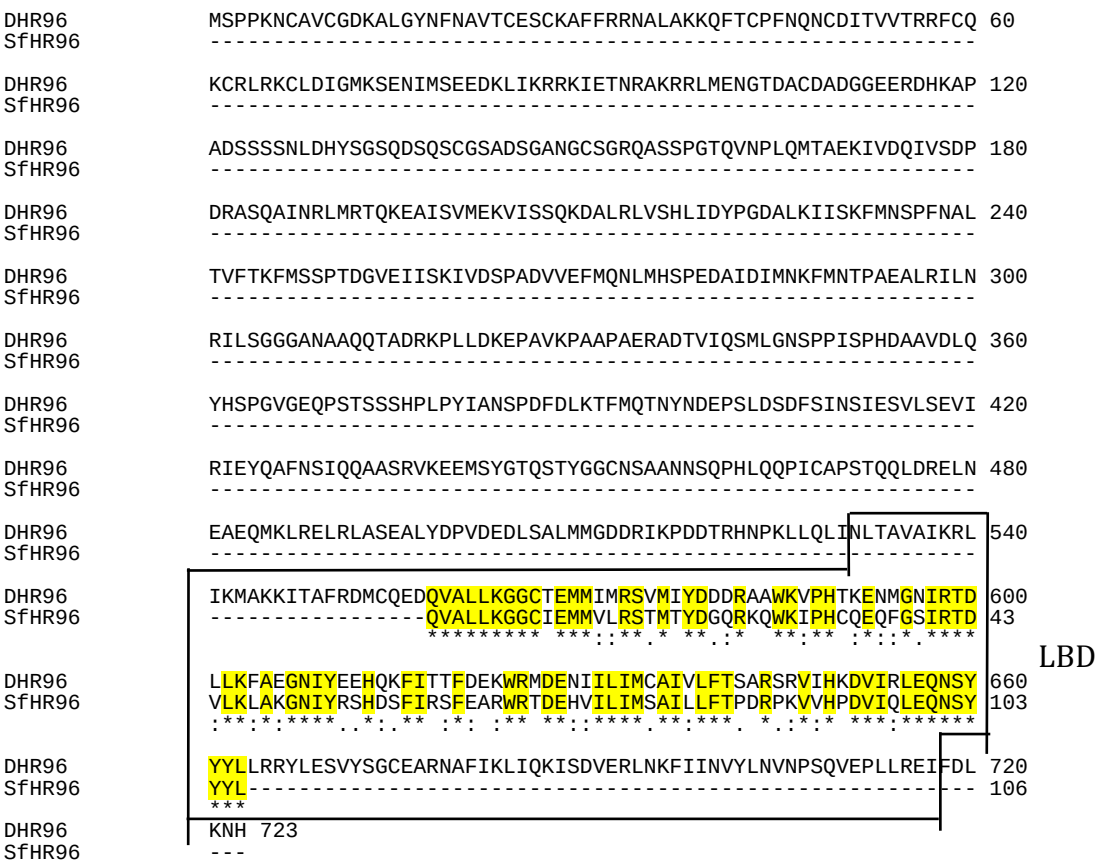

DHR96 MSPPKNCAVCGDKALGYNFNAVTCESCKAFFRRNALAKKQFTCPFNQNCDITVVTRRFCQ 60
SfHR96 ----------
DHR96 KCRLRKCLDIGMKSENIMSEEDKLIKRRKIETNRAKRRLMENGTDACDADGGEERDHKAP 120
SfHR96 ----------
DHR96 ADSSSSNLDHYSGSQDSQSCGSADSGANGCSGRQASSPGTQVNPLQMTAEKIVDQIVSDP 180
SfHR96 ----------
DHR96 DRASQAINRLMRTQKEAISVMEKVISSQKDALRLVSHLIDYPGDALKIISKFMNSPFNAL 240
SfHR96 ----------
DHR96 TVFTKFMSSPTDGVEIISKIVDSPADVVEFMQNLMHSPEDAIDIMNKFMNTPAEALRILN 300
SfHR96 ----------
DHR96 RILSGGGANAAQQTADRKPLLDKEPAVKPAAPAERADTVIQSMLGNSPPISPHDAAVDLQ 360
SfHR96 ----------
DHR96 YHSPGVGEQPSTSSSHPLPYIANSPDFDLKTFMQTNYNDEPSLDSDFSINSIESVLSEVI 420
SfHR96 ----------
DHR96 RIEYQAFNSIQQAASRVKEEMSYGTQSTYGGCNSAANNSQPHLQQPICAPSTQQLDRELN 480
SfHR96 ----------
DHR96 EAEQMKLRELRLASEALYDPVDEDLSALMMGDDRIKPDDTRHNPKLLQLINLTAVAIKRL 540
SfHR96 ----------
DHR96 IKMAKKITAFRDMCQEDQVALLKGGCTEMMIMRSVMIYDDDRAAWKVPHTKENMGNIRTD 600
SfHR96 ----------QVALLKGGCIEMMVLRSTMTYDGQRKQWKIPHCQEQFGSIRTD 43
LBD
DHR96 LLKFAEGNIYEEHQKFITTFDEKWRMDENIILIMCAIVLFTSARSRVIHKDVIRLEQNSY 660
SfHR96 VLKLAKGNIYRSHDSFIRSFEARWRTDEHVILIMSAILLFTPDRPKVVHPDVIQLEQNSY 103
DHR96 YYLLRRYLESVYSGCEARNAFIKLIQKISDVERLNKFIINVYLNVNPSQVEPLLREIFDL 720
SfHR96 YYL---------- 106
DHR96 KNH 723
SfHR96 ---

Figure 1

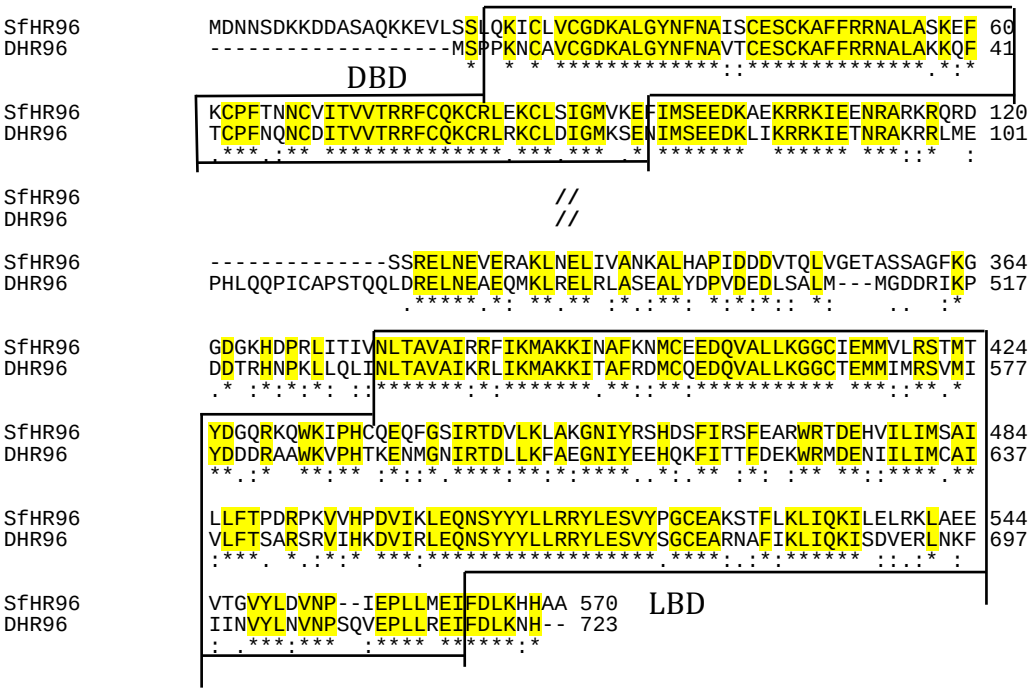

SfHR96 MDNNSDKKDDASAQKKEVLSSLQKICLVCGDKALGYNFNAISCESCKAFFRRNALASKEF 60
DHR96 ----------MSPPKNCAVCGDKALGYNFNAVTCESCKAFFRRNALAKKQF 41
DBD
SfHR96 KCPFTNNCVITVVTRRFCQKCRLEKCLSIGMVKEFIMSEEDKAEKRRKIEENRARKRQRD 120
DHR96 TCPFNQNCDITVVTRRFCQKCRLRKCLDIGMKSENIMSEEDKLIKRRKIETNRAKRRLME 101
SfHR96 //
DHR96 //
SfHR96 ----------SSRELNEVERAKLNELIVANKALHAPIDDDVTQLVGETASSAGFKG 364
DHR96 PHLQQPICAPSTQQLDRELNEAEQMKLRELRLASEALYDPVDEDLSALM---MGDDRIKP 517
SfHR96 GDGKHDPRLITIVNLTAVAIRRFIKMAKKINAFKNMCEEDQVALLKGGCIEMMVLRSTMT 424
DHR96 DDTRHNPKLLQLINLTAVAIKRLIKMAKKITAFRDMCQEDQVALLKGGCTEMMIMRSVMI 577
SfHR96 YDGQRKQWKIPHCQEQFGSIRTDVLKLAKGNIYRSHDSFIRSFEARWRTDEHVILIMSAI 484
DHR96 YDDDRAAWKVPHTKENMGNIRTDLLKFAEGNIYEEHQKFITTFDEKWRMDENIILIMCAI 637
SfHR96 LLFTPDRPKVVHPDVIKLEQNSYYYLLRRYLESVYPGCEAKSTFLKLIQKILELRKLAEE 544
DHR96 VLFTSARSRVIHKDVIRLEQNSYYYLLRRYLESVYSGCEARNAFIKLIQKISDVERLNKF 697
LBD
SfHR96 VTGVYLDVNP--IEPLLMEIFDLKHHAA 570
DHR96 IINVYLNVNPSQVEPLLREIFDLKNH-- 723

Figure 2

A

```
MsUSP-1          MSSVAKKDKRTMSVTALINRAWPLTPAPHQQQ-SMPSSQPSNFLQPLATPSTTPSVE--L 57
BmUSP-1          MSSVAKKDKRTMSVTALINRAWPMTPSPQQQQQMVPSTQHSNFLHAMATPSTTPNVE--L 58
SfUSP-1          -MSVAKKDKPTMSVTALINWARPLPPG-QQQQPMTPTS-PGSMLQPMATPSNIPTVDCSL 57

MsUSP-1          DIQWLNIEPGFMSPMSPPEMKPDTAMLDGLRDDSTPPPAFKNYPPNHPLSGSKHLCSICG 117
BmUSP-1          DIQWLNIESGFMSPMSPPEMKPDTAMLDGFRDDSTPPPPFKNYPPNHPLSGSKHLCSICG 118
SfUSP-1          DIQWLNLESGFMSPMSPPEMKPDTAMLDGLRDDSTPPPAFKNYPPNHPLSGSKHLCSICG 117

MsUSP-1          DRASGKHYGVYSCEGCKGFFKRTVRKDLTYACREDRNCIIDKRQRNRCQYCRYQKCLACG 177
BmUSP-1          DRASGKHYGVYSCEGCKGFFKRTVRKDLTYACREDKNCIIDKRQRNRCQYCRYQKCLACG 178
SfUSP-1          DRASGKHYGVYSCEGCKGFFKRTVRKDLTYACREERNCIIDKRQRNRCQYCRYQKCLACG 177

MsUSP-1          MKREAVQEERQRAARGTEDAHPSS--SVQELSIERLLEIESLVADPPEEFQFLRVGPESG 235
BmUSP-1          MKREAVQEERQRAARRTEDAHPSS--SVQELSIERLLELEALVADSAEELQILRVGPESG 236
SfUSP-1          MKREAVQEERQRAARGTEDAHPSSSVQVQELSIERLLEMESLVADPTEEYQFLRVGPDSN 237

MsUSP-1          VPAKYRAPVSSLCQIGNKQIAALVVWARDIPHFGQLELEDQILLIKNSWNELLLFAIAWR 295
BmUSP-1          VPAKYRAPVSSLCQIGNKQIAALIVWARDIPHFGQLEIDDQILLIKGSWNELLLFAIAWR 296
SfUSP-1          VPPKFRAPVSSLCQIGNKQIAALVVWARDIPHFSSLELEDQMLLIKGSWNELLLFAIAWR 297

MsUSP-1          SMEYLTDERENVDS---RSTAPPQLMCLMPGMTLHRNSALQAGVGQIFDRVLSELSLKMR 352
BmUSP-1          SMEFLNDERENVDS---RNTAPPQLICLMPGMTLHRNSALQAGVGQIFDRVLSELSLKMR 353
SfUSP-1          SMEYLTEEREVVDSSGNRTTSPPQLMCLMPGMTLHRNSALQAGVGQIFDRVLSELSLKMR 357

MsUSP-1          TLRMDQAEYVALKAIILLNPDVKGLKNKPEVVVLREKMFSCLDEYVRRSRCAEEGRFAAL 412
BmUSP-1          SLRMDQAECVALKAIILLNPDVKGLKNKQEVDVLREKMFLCLDEYCRRSRGGEEGRFAAL 413
SfUSP-1          ALRVDQAEYVALKAIILLNPDVKGLKNRPEVEILREKMFSCLDEYVRRSRGGEEGRFAAL 417

MsUSP-1          LLRLPALRSISLKCFEHLYFFHLVADTSIASYIHDALRNHAPSIDTSIL 461
BmUSP-1          LLRLPALRSISLKSFEHLYLFHLVAEGSVSSYIRDALCNHAPPIDTNIM 462
SfUSP-1          LLRLPALRSISLKSFEHLFFFHLVADTSIASYIREALRSHAPPIDANLM 466
```

B

```
SfUSP-G           MMEPSRDSGLNLESGFMSPMSPPEMKPDTAMLDGLRDDSTPPPAFKNYPPNHPLSGSKHL 60
SfUSP-2          MMEPSRDSGLNLESGFMSPMSPPEMKPDTAMLDGLRDDSTPPPAFKNYPPNHPLSGSKHL 60
BmUSP-2          -MEPSRESGLNIESGFMSPMSPPEMKPDTAMLDGFRDDSTPPPPFKNYPPNHPLSGSKHL 59

SfUSP-G          CSICGDRASGKHYGVYSCEGCKGFFKRTVRKDLTYACREERNCIIDKRQRNRCQYCRYQK 120
SfUSP-2          CSICGDRASGKHYGVYSCEGCKGFFKRTVRKDLTYACREERNCIIDKRQRNRCQYCRYQK 120
BmUSP-2          CSICGDRASGKHYGVYSCEGCKGFFKRTVRKDLTYACREDKNCIIDKRQRNRCQYCRYQK 119

SfUSP-G          CLACGMKREAVQEERQRAARGTEDAHPSSSVQVQELSIERLLEMESLVADPTEEYQFLRV 180
SfUSP-2          CLACGMKREAVQEERQRAARGTEDAHPSSSVQVQELSIERLLEMESLVADPTEEYQFLRV 180
BmUSP-2          CLACGM------------------------------------------------------ 125

SfUSP-G          GPDSNVPPKFRAPVSSLCQIGNKQIAALVVWARDIPHFSSLELEDQMLLIKGSWNELLLF 240
SfUSP-2          GPDSNVPPKFRAPVSSLCQIGNKQIAALVVWARDIPHFSSLELEDQMLLIKGSWNELLLF 240
BmUSP-2          ------------------------------------------------------------

SfUSP-G          AIAWRSMEYLTEEREVVDSFGNRTTSPPQLMCLMPG------------------------ 276
SfUSP-2          AIAWRSMEYLTEEREVVDSSGNRTTSPPQLMCLMPGMTLHRNSALQAGVGQIFDRVLSEL 300
BmUSP-2          ------------------------------------------------------------

SfUSP-G          ------------------------------------------------------------
SfUSP-2          SLKMRALRVDQAEYVALKAIILLNPDVKGLKNRPEVEILREKMFSCLDEYVRRSRGGEEG 360
BmUSP-2          ------------------------------------------------------------

SfUSP-G          ----------------------------------------------------
SfUSP-2          RFAALLLRLPALRSISLKSFEHLFFFHLVADTSIASYIREALRSHAPPIDANLM 414
BmUSP-2          ----------------------------------------------------
```

Figure 3

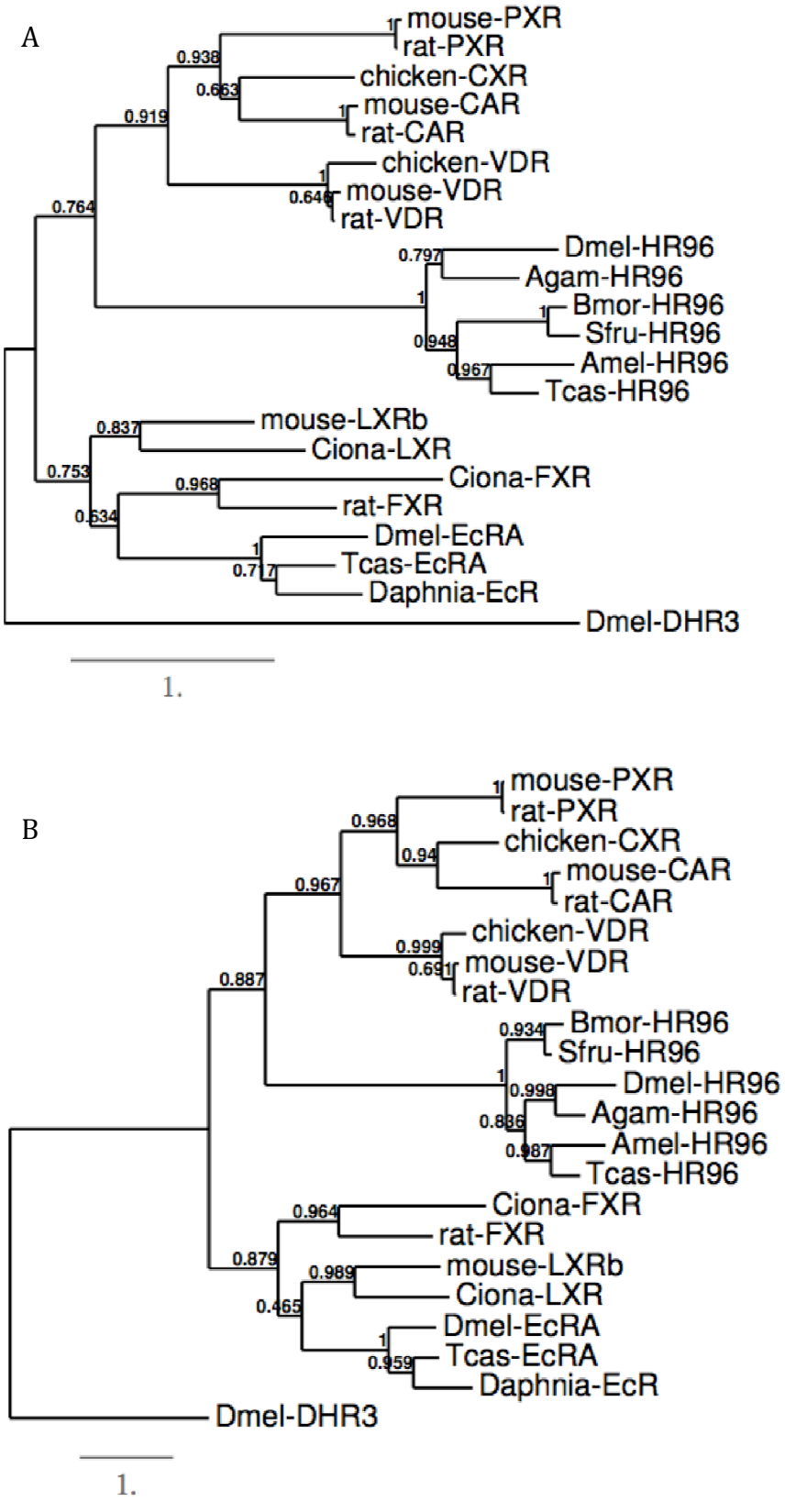

A
mouse-PXR
rat-PXR
chicken-CXR
mouse-CAR
rat-CAR
chicken-VDR
mouse-VDR
rat-VDR
Dmel-HR96
Agam-HR96
Bmor-HR96
Sfru-HR96
Amel-HR96
Tcas-HR96
mouse-LXRb
Ciona-LXR
Ciona-FXR
rat-FXR
Dmel-EcRA
Tcas-EcRA
Daphnia-EcR
Dmel-DHR3
1.
B
mouse-PXR
rat-PXR
chicken-CXR
mouse-CAR
rat-CAR
chicken-VDR
mouse-VDR
rat-VDR
Bmor-HR96
Sfru-HR96
Dmel-HR96
Agam-HR96
Amel-HR96
Tcas-HR96
Ciona-FXR
rat-FXR
mouse-LXRb
Ciona-LXR
Dmel-EcRA
Tcas-EcRA
Daphnia-EcR
Dmel-DHR3
1.

Figure 4

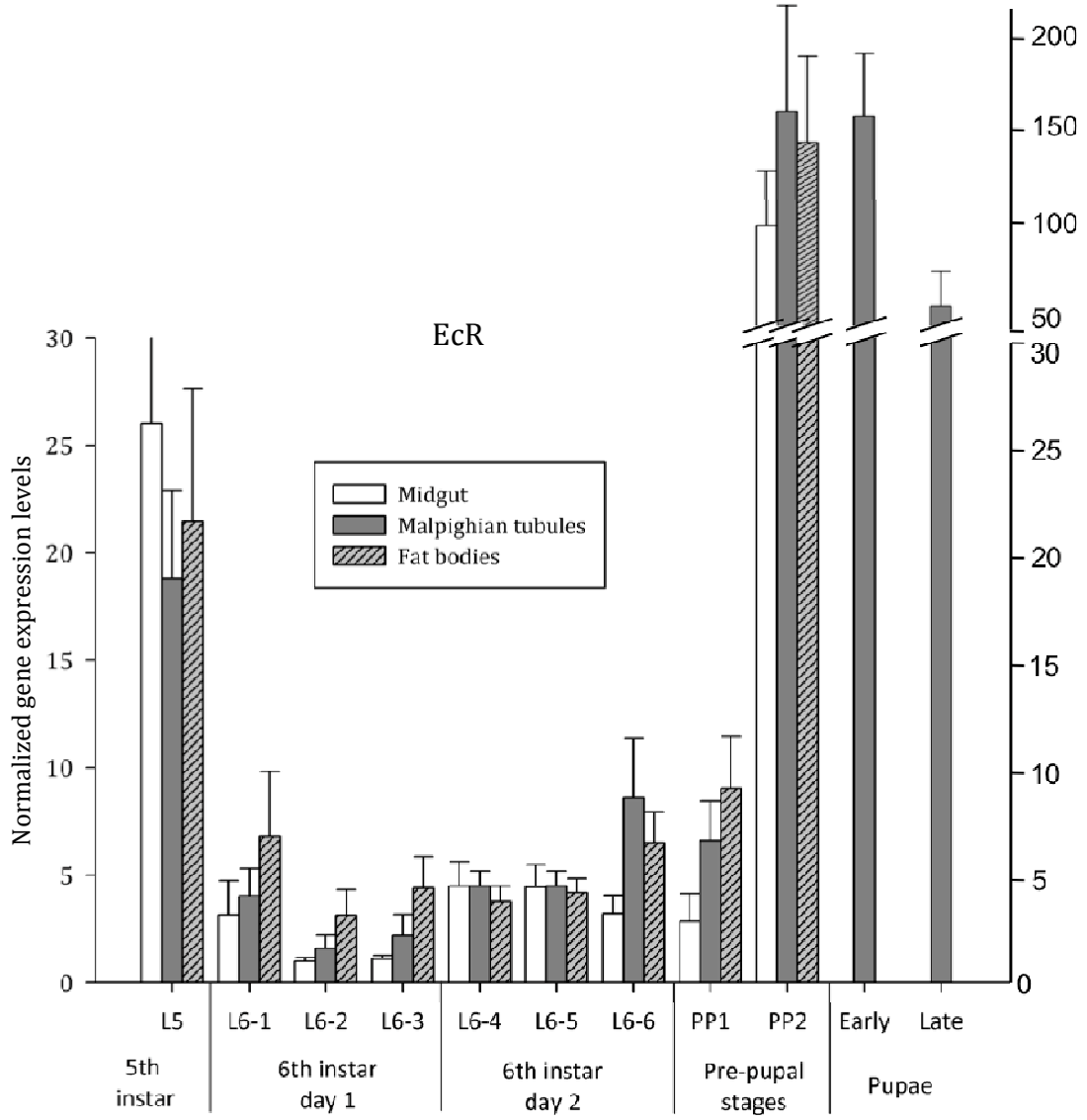

EcR
Normalized gene expression levels
Midgut
Malpighian tubules
Fat bodies
0
5
10
15
20
25
30
50
100
150
200
L5
L6-1
L6-2
L6-3
L6-4
L6-5
L6-6
PP1
PP2
Early
Late
5th instar
6th instar day 1
6th instar day 2
Pre-pupal stages
Pupae

Figure 5

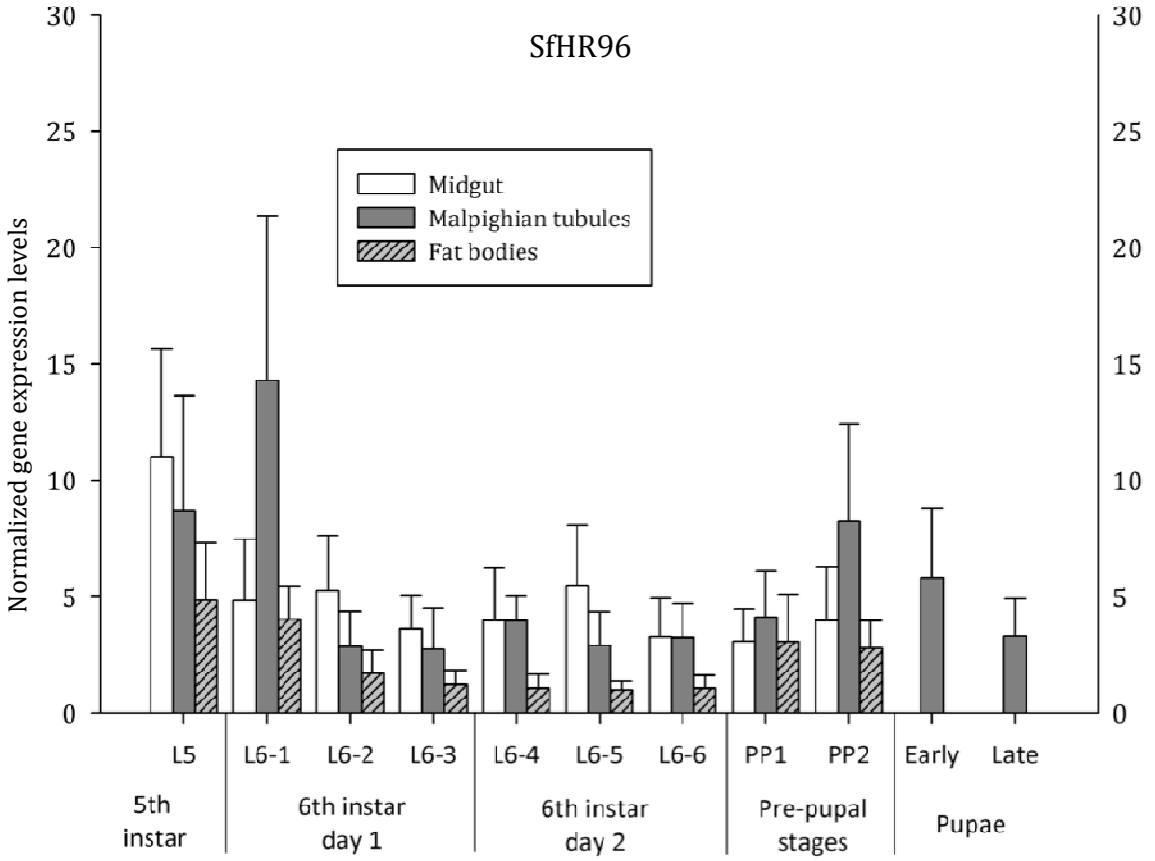

SfHR96
Normalized gene expression levels
30
25
20
15
10
5
0
Midgut
Malpighian tubules
Fat bodies
L5
L6-1
L6-2
L6-3
L6-4
L6-5
L6-6
PP1
PP2
Early
Late
5th instar
6th instar day 1
6th instar day 2
Pre-pupal stages
Pupae

Figure 6

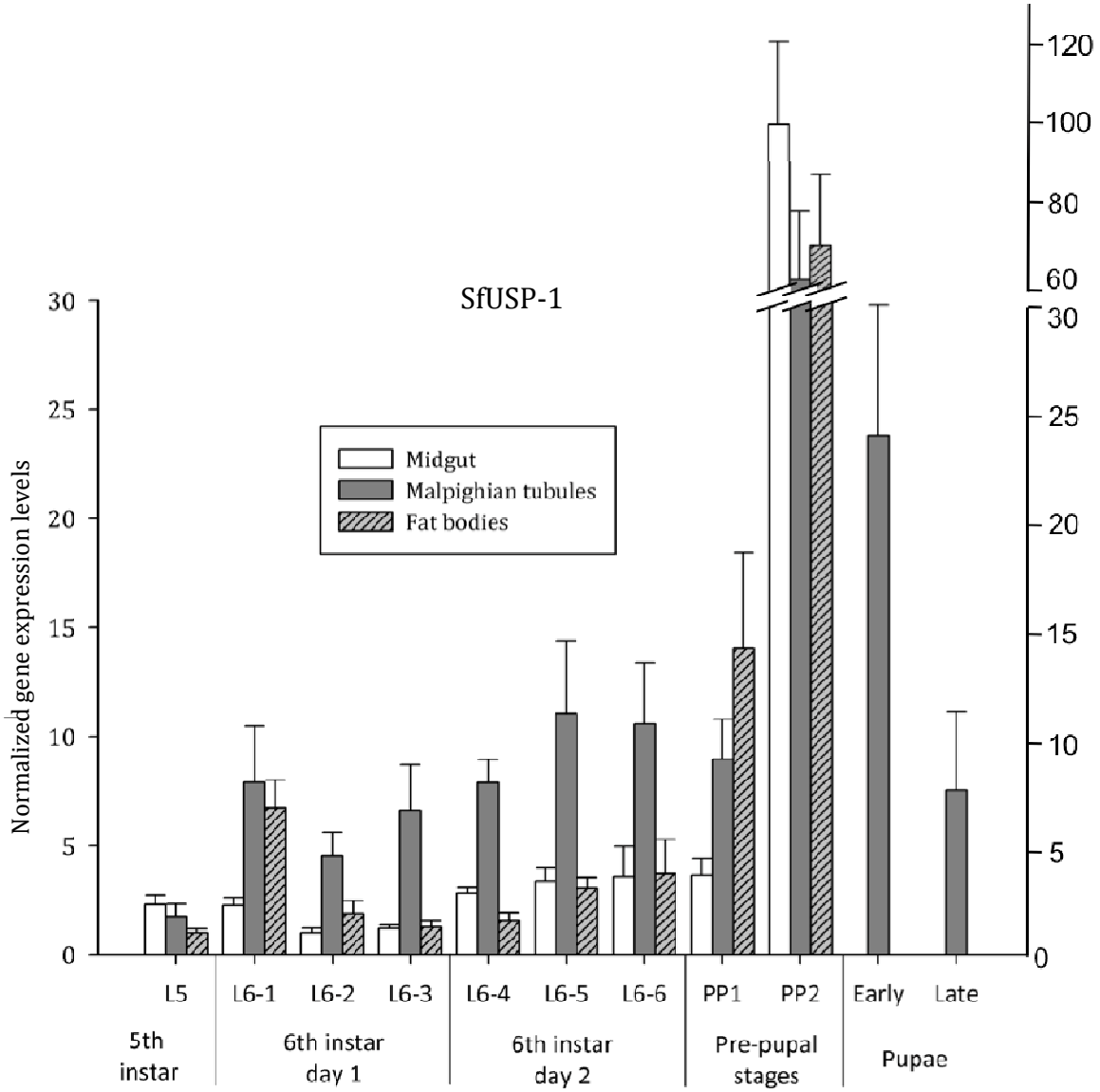

SfUSP-1
Normalized gene expression levels
Midgut
Malpighian tubules
Fat bodies
0
5
10
15
20
25
30
60
80
100
120
L5
L6-1
L6-2
L6-3
L6-4
L6-5
L6-6
PP1
PP2
Early
Late
5th instar
6th instar day 1
6th instar day 2
Pre-pupal stages
Pupae

Figure 7

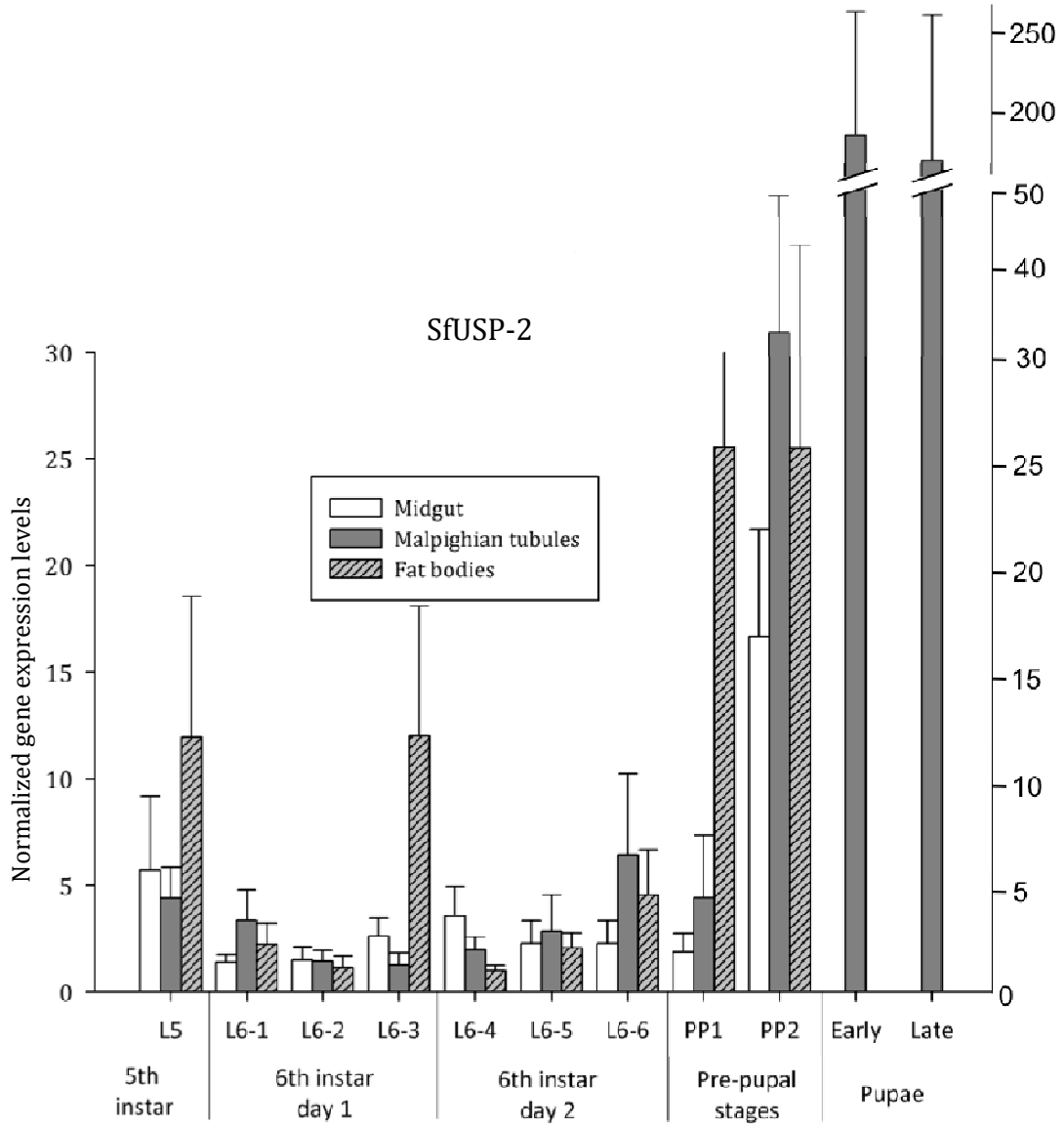

SfUSP-2
Normalized gene expression levels
Midgut
Malpighian tubules
Fat bodies
0
5
10
15
20
25
30
40
50
200
250
L5
L6-1
L6-2
L6-3
L6-4
L6-5
L6-6
PP1
PP2
Early
Late
5th instar
6th instar day 1
6th instar day 2
Pre-pupal stages
Pupae

Figure 8

Additional results:

1. RNAi impairment of SfHR96

We have successfully cloned SfRH96, the ortholog of DHR96 in *Spodoptera frugiperda* and shown that it was expressed in the three major tissues involved in detoxification, the midgut fat body and Malpighian tubules. Of relevance to the use of Sf9 cells as tools to study induction, we found that SfHR96 was expressed in those cells. This receptor seemed to be constitutively expressed independently of xenobiotic exposure. In an attempt to identify the function of SfHR96 in the detoxification response of this insect, we have tried to knock-down the expression of the gene encoding the receptor by RNAi experiments *in vivo* in *S. frugiperda* larvae and *in vitro* in Sf9 cells and to study the effect of this impairment on P450 gene induction.

1.1 *in vivo* experiments

dsRNA were synthesized using MEGAscript RNAi kit (Ambion) from *S. frugiperda* midgut cDNA. Specific primers were designed based on the sequence of SfHR96 that we have cloned and a fragment of 334bp was amplified by PCR using these primers and was used as a template to synthesize the dsRNA. 3µg of dsRNA were then injected to newly molted 5^{th} instar larvae and newly molted dsRNA 6^{th} instar larvae were then fed for 48h with artificial medium containing 0.05% of xanthotoxin or the equivalent amount of DMSO. In parallel, we have also injected larvae with dsRNA prepared from the Phenylalanine ammonia lyase (PAL) gene from *Arabidopsis thaliana* as a control. RNA was then extracted from the midguts of larvae and the expression of specific P450s was measured by qRT-PCR analysis in dsHR96 xanthotoxin treated larvae, dsPAL xanthotoxin treated larvae and uninjected xanthotoxin treated larvae. Primers for P450 genes and SfHR96, qRT-PCR conditions, and data analysis were the same as described in the material and methods of chapter 2. We have chosen xanthotoxin as inducer as it was identified as the stronger inducer of P450 genes in *S. frugiperda* midgut, with induction levels of 162.1 for CYP321A7 and 20.97 for CYP321A9.

Tabel 5 presents our results of CYP321A7 and CYP321A9 expression levels in dsRNA and control larvae measured in 5 independent biological replicates

Table 5 : levels of P450 expression[a] measured by qRT-PCR analysis in *S. frugiperda* larvae injected with dsRNA targeted against SfHR96 and exposed to xanthotoxin. Larvae injected with dsPAL from *A. thaliana* served as control.

	CYP321A9	CYP321A7	SfHR96
Replicat 1			
dsPAL - DMSO	4.2	1.9	1.2
dsPAL - Xantho 0.05%	33.2	572.1	1.0
dsHR96 - DMSO	1.0	1.0	2.1
dsHR96 - Xantho 0.05%	70.0	1289.1	1.3
Replicat 2			
dsPAL - DMSO	1.7	1.0	6.7
dsPAL - Xantho 0.05%	13.0	777.5	1.0
dsHR96 - DMSO	1.0	3.0	9.1
dsHR96 - Xantho 0.05%	6.3	334.0	1.2
Replicat 3			
dsPAL - DMSO	13.1	16.5	3.5
dsPAL - Xantho 0.05%	6.9	79.2	1.7
dsHR96 - DMSO	1.0	1.0	1.0
dsHR96 - Xantho 0.05%	69.81	3612.7	1.6
Replicat 4			
dsPAL - DMSO	1.0	1.0	1.6
dsPAL - Xantho 0.05%	25.3	363.8	1.0
dsHR96 - DMSO	4.0	23.0	1.1
dsHR96 - Xantho 0.05%	5.8	59.4	1.3
Replicat 5			
dsPAL - DMSO	3.3	1.2	2.1
dsPAL - Xantho 0.05%	19.5	253.0	1.0
dsHR96 - DMSO	1.0	1.0	1.2
dsHR96 - Xantho 0.05%	3.1	78.6	1.2

[a] for each replicate and each P450, relative expression values were calculated by qBase, which rescale the lowest relative quantity to 1 for each gene in every sample. Expression values in red show genes for which the level of induction is decreased in dsHR96 treated cells exposed to xanthotoxin, in comparison to dsPAL transfected cells.

Our results show that we could observe a significant gene expression decrease in 3 replicates, with levels of CYP321A7 being between 2 and 6 times less induced in dsRNA treated larvae exposed to xanthotoxin, compared to uninjected larvae. However, the exact same pattern was also observed in 2 replicates where the expression of CYP321A7 was induced by a 2 and 45 fold in dsRNA treated larvae exposed to xanthotoxin.

We have tried to improve our results by injecting dsRNA at different instars or with different quantity of dsRNA but could not get a sufficient reproducibility showing a consistent decrease in P450 gene expression in dsRNA larvae. Expression levels of SfHR96 did not show

a significant decrease in dsRNA treated larvae either but as the basal level of expression of this gene is very low, it might be difficult to measure accurately a decrease in expression, which however do not mean that RNAi did not work.

1.2 *in vitro* experiments

We have therefore tried to knock-down SfHR96 in Sf9 cells by transfection of eihter dsRNA or siRNA. dsRNA were the same used for *in vivo* experiments and siRNA were designed and synthesized by Eurogentec based on our cloned sequence of SfHR96.

2µg of dsRNA were transfected into Sf9 cells with three different transfection reagents according to the manufacturer's protocol: Insect GeneJuice (Novagen), polyethylenimin and TransIT-siQuest (Mirus Bio). Cells were then treated for 24h with xanthotoxin, 24h after transfection. Expression levels of CYP9A30, 9A31 and 9A32 were followed in dsRNA xanthotoxin induced cells and untransfected xanthotoxin treated cells, with dsPAL transfected cells used as control. We have chosen to follow the expression of the CYP9A members as they were identified as the most induced P450s in Sf9 cells by xanthotoxin in preliminary studies (data not shown). However, we have observed in chapter 2 that actually xanthotoxin did not induce significantly the expression of any of these P450s. Our preliminary results indicating the opposite could therefore have been false positive resulting from experimental biases. This observation could therefore explain why we did not observe any significant decrease in P450 induction nor SfHR96 expression in the cells treated with dsHR96 (data not shown).

We have therefore tried to silence the expression of SfHR96 by transfecting Sf9 cells with siRNA at different concentrations, with TranIT-siQuest (Mirus Bio) transfection reagents. Our first results are depicted in figure 26.

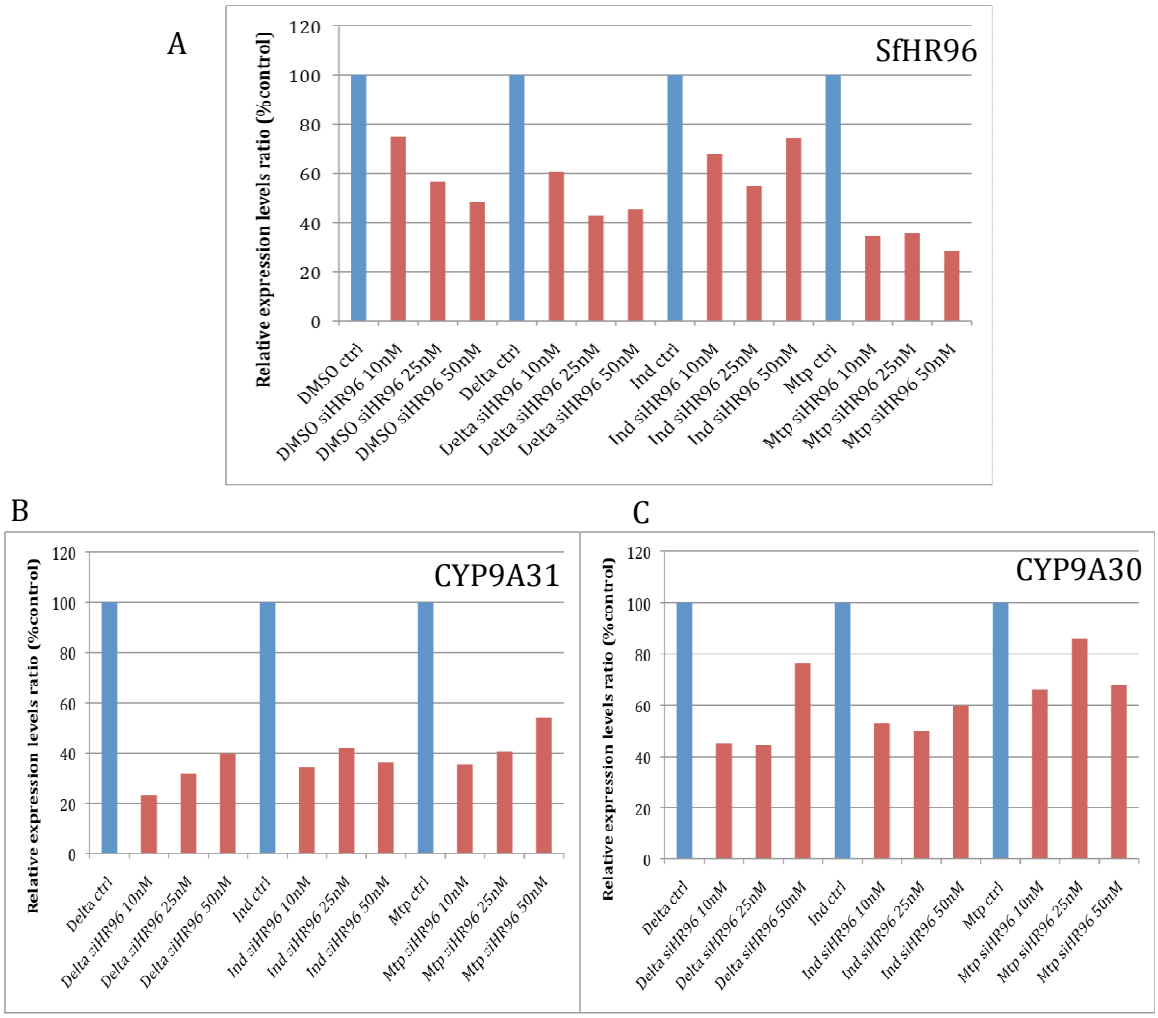


Figure 26 : Preliminary results of gene expression levels of SfHR96 (A), CYP9A31 (B) and CYP9A30 (C) in Sf9 cells transfected with 3 concentrations (10, 25 and 50 nM) of siRNA targeted against SfHR96 (siHR96) and untransfected cells (ctrl) exposed to 50µM deltamethrin (delta), 250µM indole (Ind) or 25µM methoprene (Mtp). Gene expression values are represented as the ratio between xenobiotic treated cells and untreated cells, in percentage of control (untransfected exposed cells)

Our results show that we could efficiently silence the expression of SfHR96 in Sf9 cells with an extinction ratio between 20 and 60% (figure 26A). A concomitant decrease of P450 gene expression was observed for CYP9A31 (figure 26B) and CYP9A30 (figure 26C), the latter showing the strongest decrease with only 20% expression remaining in siHR96 transfected cells exposed to deltamethrin.

These preliminary results suggest that SfHR96 is necessary for the induction of CYP9A30 and CYP9A31 in Sf9 cells, in contrary to the results we obtained with dsRNA transfections.

However, like the situation in *S. frugiperda* larvae injected with dsRNA, we were unable to reproduce these results. We have tried different time pre- and post transfection, different concentrations of siRNA, different P450s, different time-course of xenobiotic exposition but could never reproduce twice the results that we obtained in the first place. However encouraging, these results need to be validated and raise the question about RNAi efficiency in *S. frugiperda*.

RNAi is a powerful technique for unveiling gene functions in many organisms and has been used on numerous insect species. Its use in non-drosophilids is reviewed in (Belles, 2010). *In vivo* injection of dsRNA in *Tribolium castaneum* for example has allowed the functional identification of 19 nuclear receptors (Tan and Palli, 2008) and injection of dsRNA targeted against aminopeptidase N in *Spodoptera litura* gave evidence of the function of this protein as a receptor for *Bacillus thuringiensis* insecticidal proteins (Rajagopal et al., 2002). Moreover, RNAi techniques have also been used in insect cell line such as in *S. frugiperda* Sf21 cell line through siRNA transfection to study the effect of baculovirus-expressed aminopeptidase (Agrawal et al., 2004), in Sf9 cells by stable expression of dsRNA through baculovirus-mediated expression vector (Lin et al., 2006) and in *Bombyx mori* cells using long-hairpin RNA-expressing plasmid DNA (Fujita et al., 2009).

In *Spodoptera frugiperda*, there are only two recorded studies in the literature showing evidence of successful RNAi silencing by injection and feeding of dsRNA to *S. frugiperda* larvae to study the role of allatostatin in JH signaling mechanisms (Griebler et al., 2008; Meyering-Vos et al., 2006). This suggests that this species is likely to be non sensitive to RNAi *in vivo* techniques as negative results are often not reported. Species sensitivity is a major drawback in RNAi functional analysis and it has been hypothesized that less derived species such as *Tribolium castaneum* are more sensitive to systemic RNAi than more derived species such as Lepidoptera (Belles, 2010). In addition, some tissues might be more resistant to respond to RNAi resulting from poor penetration and transmission of the interfering signal through cells and tissues, and RNAi can also have gene-specific sensitivity (Palli, R., personal communication). All these observations might help to understand our lack of reproducible results in *S. frugiperda* larvae and cells.

2. Bombyx mori transgenic construction

In an attempt to circumvent pitfalls encountered in *S. frugiperda* using RNAi techniques, we have worked in collaboration with the National Institute of Agrobiological Sciences in Tsukuba, Japan, to construct transgenic *Bomby mori* that would express non-functional HR96 receptors in order to study the effect of HR96 impairment in this species. Dr. Tamura's team at the NIAS has developed a robust protocol to achieve stable genetic transformation of *Bombyx mori* larvae, this insect being used as a model organism to represent lepidopteran insects. The entire genomic sequence of *B. mori* was recently published (International Silkworm Genome Consortium, 2008). In *B. mori*, the techniques of *piggyBac* transposon-mediated transgenesis were established by the injection of DNA into preblastodermal embryos (Tamura et al., 2000). The binary GAL4/UAS system was constructed in *B. mori* (Imamura et al., 2003). In the system, the yeast transcriptional activator, GAL4, activates the gene downstream of UAS target sequence (see figure 27). Using the GAL4/UAS system, enhancer trap system in the silkworm has been developed recently (Uchino et al., 2008). The enhancer trap lines allow the study of gene expression or repression by RNAi in specific tissues/organ and at specific stages. Indeed, the lines that expressed GAL4 gene in the midgut were successfully used to determine the function of the yellow brood gene *Y* (Sakudoh et al., 2007) and densovirus-resistance gene *nsd-2* (Ito et al., 2008).

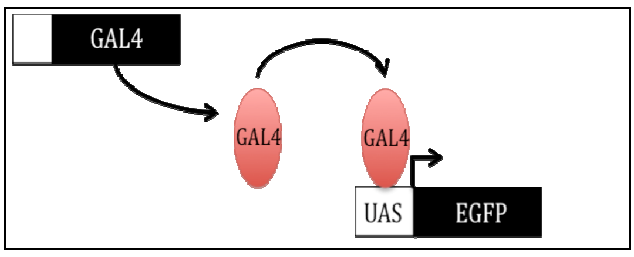


Figure 27 : the GAL4/UAS system principle. GAL4 transcription produces the GAL4 transcription factor that will bind to the specific UAS promoting sequence upstream of reporter gene, e.g. EGFP, inducing its transcription.

We have therefore taken benefit from NIAS technical knowledge to knockdown HR96 in *B. mori* by two approaches.

First, we have conducted transgenic RNAi with GAL4/UAS system using a transgene of UAS-driven inverted repeat (UAS-IR) constructed by cloning the gene fragment of *BmHR96* as an inverted repeat, using *piggyBac* as a vector and *EGFP* gene as an eye marker (see figure 28 for the plamid map). The DNA of the UAS-IR transgene is then injected into the eggs of the silkworm with helper plasmid and mRNA of the transposase for germline transformation. When the UAS-IR transgenic line is obtained, it will be crossed with a midgut-GAL4 line. By this approach, we will knock-down the messenger of BmHR96 in transgenic silkworms by expressing BmHR96 IR under the control of UAS-GAL4 promotor.

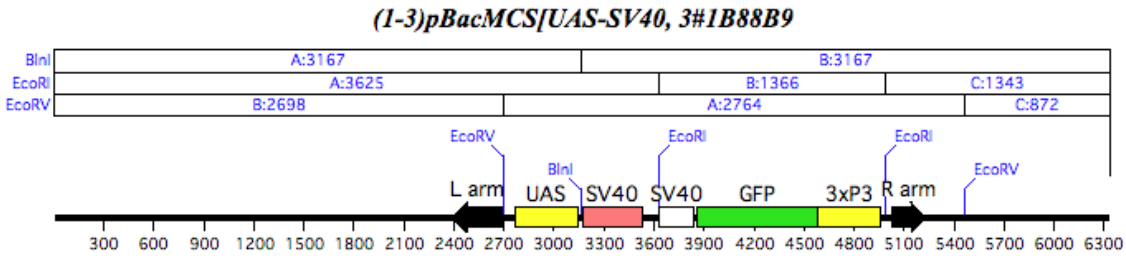


Figure 28: restriction map of the plasmid DNA used as a vector for generating transgenic *B. mori*. ORF of BmHR96 in inserted into BlnI site.

Second, the same methodology was used to clone dominant negative (ΔAF-2) fragments of *BmHR96* into the *piggyBac* vector. Indeed, Kocarek and colleagues (2002) have shown that the deletion of the N-terminal AF-2 region of nuclear receptors is sufficient to prevent the formation of active transcription factor complex (Kocarek et al., 2002). They constructed expression plasmid carrying PXR, CAR and LXR nuclear receptor truncated from 8 to 10 amino acids in their AF-2 domain. When co-transfected with reporter plasmids, they showed a decrease in the expression of receptors-regulated genes, such as P450s. We have therefore conducted the same approach in *B. mori* with BmHR96 receptor. First, we have successfully cloned the full sequence of BmHR96, which shows 79% identity with its *S. frugiperda* ortholog (figure 29). Then, we designed specific primers that allowed the deletion of 18 amino acids from the C-terminal part of the receptor by PCR technique, as it is shown in figure 29. The full-length and dominant negative ΔAF-2 fragments were then cloned into the piggyBac vector the same way than for IR of BmHR96. By this approach, we will knock-down the function of the protein bu expressing non-functional dominant negative receptors under the control of UAS/GAL4 promotor into transgenic silkworms.

The resulting G0 moths are intercrossed to produce the G1 silkworm. The transgenic insects are obtained in the G1 silkworm by the marker screening. The UAS-IR strain is then crossed with the GAL4 strains that express GAL4 gene in the midgut.

```
BmHR96          MDNIGENKTENVSQKKELPLNLQKICLVCGDKALGYNFNAISCESCKAFFRRNALASKEF
SfHR96          MDNNSDKKDDASAQKKEVLSSLQKICLVCGDKALGYNFNAISCESCKAFFRRNALASKEF
                *** .::* :  :****:  .**************************************

BmHR96          KCPFTNNCVITVVTRRFCQKCRLEKCFAVGMVKEFIMSEEDKAEKRRKIEENRARKRKSD
SfHR96          KCPFTNNCVITVVTRRFCQKCRLEKCLSIGMVKEFIMSEEDKAEKRRKIEENRARKRQRD
                ***************************:::*****************************: *

BmHR96          CDEAVSSSKSMKKDDENLTSNYPPES--IQYDVLNSTTCSPSSTVQSPLNNDMESSIQSP
SfHR96          PDDAVTSSKNLKRDDEGDTGLIPLQDTVIQYDTL-STTCSPSSTVQSPLASDLEASLHSP
                 *:**:***.:*:***. *.  * :.  ****.* ************** .*:*:*::**

BmHR96          PMYNYAPIPPVQP---EYNMKVFPIDEKQIMDQALYEQRMMDSYKMYDNVESN--TYMEP
SfHR96          -LYNYTPAPPVPTNEQPYPMKVYPIDEKSIINRQIYDQRMMDSYNMYENVDSNNMYPVEP
                 :***:* ***  .    * ***:*****.*::: :*:*******:**:**:**    :**

BmHR96          PKQNSIRQILTNGDTATAFKETKQQHVCEEIPSTSTDPDVNKARDILQDVERIEPNSMES
SfHR96          PKQNSIRSILTNSDKP-AAREARPQHVCEEIPSTSNNPDVNKARDILQDVERIEPNSMES
                *******.****.*.. * :*:: ***********.:**********************

BmHR96          ILCEAIKLEFESYASVNSCSGSSRELNEVERAKLNELIVANKALNAPIDDDISQLVDSAS
SfHR96          ILCEAIKLEFEAYTSVSPCSGSSRELNEVERAKLNELIVANKALHAPIDDDVTQLVGETA
                ***********:*:**..*************************:******::***..::

BmHR96          AEPG-----GKHDPRLIRLVNLTAVAIRRLIKIAKKISAFKNMCEEDQVALLKGGCIEMM
SfHR96          SSAGFKGGDGKHDPRLITIVNLTAVAIRRFIKMAKKINAFKNMCEEDQVALLKGGCIEMM
                :..*     ******** :**********:**:****.**********************

BmHR96          VLRSTMTYDGQRNQWKIPHCQEQFGRIGTDVLKLAKGDIYRSHEAFISTFCARWRTDESV
SfHR96          VLRSTMTYDGQRKQWKIPHCQEQFGSIRTDVLKLAKGNIYRSHDSFIRSFEARWRTDEHV
                ************:************ * *********:*****::** :* ******* *

BmHR96-WT       ILILSAILLFAPHRPRLVHRDVVKLEQNSYYYLLRRYLESVYPGCEAKSTFLKLIQKVLE
SfHR96          ILIMSAILLFTPDRPKVVHPDVIKLEQNSYYYLLRRYLESVYPGCEAKSTFLKLIQKILE
                ***:******:*.**::** **:**********************************:**

BmHR96          LRKLAEEITDVYLDVNPLEPLLMEIFDLKHHGGRD
SfHR96          LRKLAEEVTGVYLDVNPIEPLLMEIFDLKHHAA--
                *******:*.*******:*************..
```

Figure 29 : alignment of the deduced amino acid sequence of BmHR96 with SfHR96. Conserved DBD (pink) and LBD (green) domains are indicated. The red boxed sequence of 18 amino acids at the C-terminal part represent the truncated part in dominant negative ΔAF-2 BmHR96.

The cloning and construction of the full length and dominant negative mutants was realized during my 3 months stay in Dr Shinoda's laboratory at NIAS. Transgenic silkworms will be available in the upcoming weeks and experiments on P450 induction in dominant negative and RNAi transgenic *B. mori* will be conducted by the NIAS team members, in a continuing collaboration with our INRA laboratory.

As results, we would expect to see a decrease in induction level of P450 in dominant negative and RNAi transgenic *B. mori*. Such results will validate the function of HR96 as a key regulator in detoxification gene induction in insects.

GENERAL CONCLUSION

The overall objective of this work was to study xenobiotic detoxification mechanisms in *Spodoptera frugiperda* and in particular to identify and characterize potential xenosensors in this polyphagous lepidopteran pest.

Phytophagous insect species encounter in their diet many plant allelochemicals and insecticides used in pest management. They have therefore developed sophisticated mechanisms to detoxify these potentially toxic compounds and survive in this chemically adverse environment. Such mechanisms are mainly based on the activity of detoxification enzymes, one of the best known being the cytochrome P450s. It is now well established that insect P450s are induced by and metabolize toxic plant allelochemicals and have been involved in insecticide resistance. However, a link is still missing between the ingestion of the toxin and the induction of P450s. Nuclear receptors have been shown in many vertebrate studies to be key transcription factors that would recognize xenobiotics and induce the expression of P450s. However, unlike the situation in vertebrate, to date there has been no clear evidence of such xenosensing receptor in insects. Our work aimed to fill this gap in our knowledge.

The detoxification gene response

As a first step in studying the xenobiotic response in insects, we have identified patterns of gene expression in the model species *Drosophila melanogaster* by studying the expression of a specific P450, CYP6A2 in larval and adult tissues of *Drosophila* and in response to various xenobiotics. Our results compared to the available literature P450 induction that could be gleaned from microarray studies indicate that only a third of the "CYP-ome" is inducible by xenobitocis and that there are distinct subsets of inducers/induced genes, suggesting multiple xenobiotic transduction mechanisms.

We have then extended our study to the xenobiotic response of a polyphagous pest, *S. frugiperda* by identifying patterns of gene expression in response to various plant allelochemicals and xenobiotics using microarray analysis and measuring specific P450 gene induction by qRT-PCR analysis. Our results confirm the first study in *Drosophila* as only a small subset of P450s were induced by the different xenobiotics, showing different patterns of induction in *S. frugiperda* larvae and Sf9 cells. Overall, even though Sf9 cells could not recreate what is happening *in vivo*, both systems showed detoxification gene induction

responses as microarray analysis revealed with the expression of several detoxification genes being affected by the different treatments.

Our results allowed us to identify which type of xenobiotic induced which type of P450. With this information being catalogued, it would now be particularly interesting to study the ability of these P450s to metabolize effectively the inducing compounds. Heterologous expression of P450s has been largely used to study the ability of these enzymes to metabolize inecticide and plant compounds. For example, CYP6B1 from *Helicoverpa zea* expressed in Sf9 cells through baculovirus-mediated expressing vector is able to metabolize different plant allelochemicals, such as quercetin and xanthotoxin as well as insecticide such as cypermethrin (Li et al., 2004). We have already undertaken the construction of yeast heterologous expression of the most induced P450s in *S. frugiperda* larvae. The analysis of their ability to efficiently metabolize each of their inducing compounds may show that the xenobiotic response of *S. frugiperda* is based on the induction of P450 metabolizing enzymes that allow this species to feed on toxic plant hosts.

The xenosensing nuclear receptors

In the second step of this work, we have studied the role of nuclear receptors in the xenobiotic response of *S. frugiperda* by first looking at the effects of two hormone agonists on Sf9 cells, methoprene and methoxyfenozide. Methoprene is an analog of the Juvenile Hormone (JH) and methoxyfenozide is an agonist of the moulting hormone 20-hydroxyecdysone (20E). Both compounds have been widely used in pest management controls as they mimic the action of the two major hormones controlling metamorphosis and development in homometabolous insects, thus interfering with hormone signaling and leading to defect in development of insects. Our results show that both insecticides induce cell growth arrest in the G1 phase for the methoxyfenozide and in the G2/M phase for the methoprene. Microarray analysis showed that the regulation of specific genes involved in cell cycle progression was affected by both of the insecticides.

If the mode of action of 20E is well-known, it remains more enigmatic for JH due to the lack of knowledge of its receptor. 20E exerts it action through the binding to a nuclear receptor heterodimer consisting of an ecdysone receptor (EcR) and ultraspiracle (USP). We found that EcR was induced by the 20E agonist methoxyfenozide and by methoprene, the JH

analog induced the expression of USP. Our results suggets therefore two different signalling pathways involved in Sf9 cells.

We showed that Sf9 cells respond to insecticide compounds that mimic the action of two main hormones by stopping cell cycle progression and that nuclear receptors EcR and USP might be involved in this signaling mechanism.

In the last step, we have therefore tried to identify nuclear receptors that would act as xenosensors in *S. frugiperda* and regulate the expression of P450 genes in response to xenobiotic rather than hormonal exposure. We have chosen a candidate gene approach by focusing on the ortholog of DHR96, the vertebrate ortholog of CAR and PXR, for which xenosensing function is well established and involvement in P450 expression well identified. We have cloned the full-length sequence of SfHR96. We have shown that it was expressed both in *S. frugiperda* midgut, fat body and Malpighian tubules, as well as in Sf9 cells. In the latter case, this is not surprising as Sf9 cells have been shown to express both EcR and USP as well as other nuclear receptors (Chen et al., 2002). However, SfHR96 expression was found very low, with no changes throughout the developmental stages, except for a light peak of expression just before the last instar moult, concomitant with a high expression of SfUSP-2. In addition, SfHR96 expression was not induced by any of the xenobiotics tested, suggesting that this receptor might be constitutively expressed and therefore able to be constantly activated by xenobiotic compounds.

Our attempts to silence the expression of SfHR96 by RNAi experiments failed both *in vitro* and *in vivo*, still raising the question of whether this receptor is involved in the P450 gene induction. Further RNAi experiments should be undertaken in Sf9 cells with stable DNA vector based expression of dsRNA as it has already been proven to be successful (Lin et al., 2006). Moreover, dsRNA transfection presents advantages over siRNA as in the latter case the silencing effects are often transient and the transfection efficiency of siRNA would influence the silencing effects in target cells (Agrawal et al., 2004). In addition, RNAi effects should be tested on many different xenobiotic compounds and different induced P450 genes as RNAi could be gene specific and we could have missed effects on P450 gene expression by focusing on only a few responding genes.

The work on transgenic *Bombyx mori* in collaboration with NIAS in Japan will bring new insights into the functional identification of HR96 in the detoxification response of Lepidoptera. Although *B. mori* is a specialist species in contrats to *S. frugiperda*, there is no doubt that we will find potent compounds that will induce the expression of some P450 genes in a similar way that in *Spodoptera* and study the effect of BmHR96 silencing on this induction. In addition, NIAS teams have developed technical knowledge to express DNA vector-based long hairpin RNA in *B. mori* cell line, allowing stable RNAi silencing of different genes (Fujita et al., 2009). We could therefore use this model to study the effect of HR96 impairment in *B. mori* cells if Sf9 cells remain resistant to that technique.

We think that SfHR96 is a good candidate for xenosensors in insects. Further characterization of the genes regulated by this receptor will help to validate our hypothesis.

DHR96 has already been shown to be involved on the regulation of phenobarbital-regulated genes (King-Jones et al., 2006) and despite the fact that this receptor has been involved as a sterol sensor in the regulation of cholesterol metabolism (Horner et al., 2009). The fact that HR96 mutants are fully viable in *Tribolium castaneum* (Tan and Palli, 2008) show that the presence of this receptor is actually not essential for metamorphosis and development and support the fact that this receptor might have two non-exclusive functions, not necessary only related to steroid mechanisms.

The characterization of this orphan receptor would also have to go through the identification of potential ligands as well as potential hetero-dimerization partners. Indeed, most nuclear receptors function as homo- or heterodimer with another member of the NR superfamily. This is for example the case for the EcR/USP complex involved in ecdysteroid mechanisms. In the case of HR96, no binding partners have yet been identified. It is known that USP can interact with another nuclear receptor, DHR38 (Baker et al., 2000), suggesting that USP might as well be involved as a heterodimerization partner for other nuclear receptors. USP is the vertebrate ortholog of the Retinoid X receptor (RXR), which is known to bind to CAR and PXR. As HR96 is the insect ortholog of these receptors, we can assume that USP would bind HR96 in similar manner. We have found that the expression of SfUSP-2 was somehow correlated with the peak of expression of SfHR96 in late 5^{th} instar larvae, as it has already been shown in *B. mori* (Cheng et al., 2008). However further experiments are needed to

validate our hypothesis of USP being a dimerization partner for SfHR96. RNAi technique could for example be used against SfUSP together with the extinction of SfHR96 in order to monitor if the presence of USP is necessary for the induction of P450 genes by SfHR96. This hypothesis is particularly attractive, as the recruitment of USP by HR96 upon xenobiotic exposure would implicate that USP is less available for binding with EcR. Thus xenobiotic effects would interfere with ecdysteroid signaling mechanisms and that could explain the developmental and metamorphic effects of some of these xenobiotics. Protein binding assay is another technique that could be used to measure the ability of USP to bind to SfHR96 as well as identifying potential ligands for the latter receptor. High-throughput techniques have been made available with the development of Surface Plasmon resonance (SPR) analysis known as the BIAcore technology, which allow the study of real-time protein-protein interactions and binding affinity. The use of such technology could help to find potential ligands for SfHR96 by measuring the binding affinities of a virtually infinite number of molecules, in addition to testing the ability of USP and SfHR96 to form a heterodimer.

Overall, the identification of SfHR96 as a xenosensor involved in the regulation of P450s in *S. frugiperda* will help to understand xenobiotic responses in insects by making the link between the ingestion of the toxin and the induction of detoxification mechanisms. In addition to unveiling how phytophagous insects survive in a chemically adverse environment, our findings could have potential implications in pest management. Indeed, xenosensing by intracellular receptors offers an innovative target as interfering with an insect's ability to induce the detoxification of plant secondary chemicals can have a beneficial impact. Lack of induction (antagonists) can cause toxicity or feeding arrest (and thus increased exposure to natural enemies), whereas enhanced induction (agonists) can favor the activation of plant pro-toxins. Furthermore, the sustainability of host plant resistance programs, often based on the genetic improvement of the plant chemical defenses, could be enhanced by xenosensor antagonists that would act as synergists. Elaboration on such strategies (plant-based or spray-based) is pointless without a thorough understanding of the insect xenosensors.

REFERENCES

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., *et al*. (2000). The genome sequence of *Drosophila melanogaster*. Science **287**, 2185-2195.

Agrawal, N., Malhotra, P., and Bhatnagar, R.K. (2004). siRNA-directed silencing of transgene expressed in cultured insect cells. Biochem Biophys Res Commun **320**, 428-434.

Antebi, A., Yeh, W.H., Tait, D., Hedgecock, E.M., and Riddle, D.L. (2000). daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. Genes Dev **14**, 1512-1527.

Aranda, A., and Pascual, A. (2001). Nuclear hormone receptors and gene expression. Physiol Rev **81**, 1269-1304.

Ashok, M., Turner, C., and Wilson, T.G. (1998). Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. Proc Natl Acad Sci USA **95**, 2761-2766.

Baker, K.D., Warren, J.T., Thummel, C.S., Gilbert, L.I., and Mangelsdorf, D.J. (2000). Transcriptional activation of the *Drosophila* ecdysone receptor by insect and plant ecdysteroids. Insect Biochem Mol Biol **30**, 1037-1043.

Belles, X. (2010). Beyond *Drosophila*: RNAi in vivo and functional genomics in insects. Annu Rev Entomol **55**, 111-128.

Berenbaum, M.R. (1990). Evolution of specialization in insect-umbellifer associations. Annu Rev Entomol **35**, 319-343.

Berenbaum, M.R. (2002). Postgenomic chemical ecology: from genetic code to ecological interactions. J Chem Ecol **28**, 873-896.

Berenbaum, M.R., Favret, C., and Schuler, M.A. (1996). On defining "key innovations" in an adaptive radiation: cytochrome P450s and papilionidae. Am Nat **148**, S139-S155.

Bitra, K., and Palli, S.R. (2009). Interaction of proteins involved in ecdysone and juvenile hormone signal transduction. Arch Insect Biochem Physiol **70**, 90-105.

Bonneton, F., Chaumot, A., and Laudet, V. (2008). Annotation of *Tribolium* nuclear receptors reveals an increase in evolutionary rate of a network controlling the ecdysone cascade. Insect Biochem Mol Biol **38**, 416-429.

Bourguet, W., Germain, P., and Gronemeyer, H. (2000). Nuclear receptor ligand-binding domains: three-dimensional structures, molecular interactions and pharmacological implications. Trends Pharmacol Sci **21**, 381-388.

Brattsten, L.B. (1979). Ecological significance of mixed-function oxidations. Drug Metab Rev **10**, 35-58.

Brattsten, L.B., Evans, C.K., Bonetti, S., and Zalkow, L.H. (1984). Induction by carrot allelochemicals of insecticide-metabolising enzymes in the southern armyworm *Spodoptera eridania*. Comp Biochem Physiol C **77**, 29-37.

Brown, R.P., McDonnell, C.M., Berenbaum, M.R., and Schuler, M.A. (2005). Regulation of an insect cytochrome P450 monooxygenase gene (CYP6B1) by aryl hydrocarbon and xanthotoxin response cascades. Gene **358**, 39-52.

Brun, A., Cuany, A., Le Mouel, T., Berge, J., and Amichot, M. (1996). Inducibility of the *Drosophila melanogaster* cytochrome P450 gene, CYP6A2, by phenobarbital in insecticide susceptible or resistant strains. Insect Biochem Mol Biol **26**, 697-703.

Bujold, M., Gopalakrishnan, A., Nally, E., and King-Jones, K. Nuclear receptor DHR96 acts as a sentinel for low cholesterol concentrations in *Drosophila melanogaster*. Mol Cell Biol **30**, 793-805.

Bull, D.L., Ivie, G.W., Beier, R.C., and Pryor, N.W. (1986). *In vitro* metabolism of a linear furanocoumarin (8-methoxypsoralen, xanthotoxin) by mixed function oxidases of larvae of black swallowtail butterfly and fall armyworm. J Chem Ecol **12**, 885-892.

Chen, J.H., Turner, P.C., and Rees, H.H. (2002). Molecular cloning and induction of nuclear receptors from insect cell lines. Insect Biochem Mol Biol **32**, 657-667.

Cheng, D., Xia, Q., Duan, J., Wei, L., Huang, C., Li, Z., Wang, G., and Xiang, Z. (2008). Nuclear receptors in *Bombyx mori*: insights into genomic structure and developmental expression. Insect Biochem Mol Biol **38**, 1130-1137.

Claessens, F., and Gewirth, D.T. (2004). DNA recognition by nuclear receptors. Essays Biochem **40**, 59-72.

Cohen, M.B., Berenbaum, M.R., and Schuler, M.A. (1989). Induction of cytochrome P450-mediated detoxification of xanthotoxin in the black swallowtail. J Chem Ecol **15**, 2347-2355.

Cohen, M.B., Schuler, M.A., and Berenbaum, M.R. (1992). A host-inducible cytochrome P-450 from a host-specific caterpillar: molecular cloning and evolution. Proc Natl Acad Sci U S A **89**, 10920-10924.

Cruz, J., Sieglaff, D.H., Arensburger, P., Atkinson, P.W., and Raikhel, A.S. (2009). Nuclear receptors in the mosquito *Aedes aegypti*: annotation, hormonal regulation and expression profiling. FEBS J **276**, 1233-1254.

Daborn, P., Boundy, S., Yen, J., Pittendrigh, B., and ffrench-Constant, R. (2001). DDT resistance in *Drosophila* correlates with *Cyp6g1* over-expression and confers cross-resistance to the neonicotinoid imidacloprid. Mol Genet Genomics **266**, 556-563.

Daborn, P.J., Lumb, C., Boey, A., Wong, W., Ffrench-Constant, R.H., and Batterham, P. (2007). Evaluating the insecticide resistance potential of eight *Drosophila melanogaster* cytochrome P450 genes by transgenic over-expression. Insect Biochem Mol Biol **37**, 512-519.

Daborn, P.J., Yen, J.L., Bogwitz, M.R., Le Goff, G., Feil, E., Jeffers, S., Tijet, N., Perry, T., Heckel, D., Batterham, P., et al. (2002). A single p450 allele associated with insecticide resistance in *Drosophila*. Science **297**, 2253-2256.

Denison, M.S., Fisher, J.M., and Whitlock, J.P., Jr. (1988). The DNA recognition site for the dioxin-Ah receptor complex. Nucleotide sequence and functional analysis. J Biol Chem **263**, 17221-17224.

Denison, M.S., and Nagy, S.R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol **43**, 309-334.

Despres, L., David, J.P., and Gallet, C. (2007). The evolutionary ecology of insect resistance to plant chemicals. Trends Ecol Evol **22**, 298-307.

Dhadialla, T.S., Retnakaran, A., and Smagghe, G. (2005). Insect growth- and development-disrupting insecticides. In Comprehensive molecular insect science (Elsevier), pp. 55-115.

Dicke, M. (2000). Chemical ecology of host-plant selection by herbivorous arthropods: a multitrophic perspective. Biochem Syst Ecol **28**, 601-617.

Dobson, C.M. (2004). Chemical space and biology. Nature **432**, 824-828.

Duncan, D.M., Burgess, E.A., and Duncan, I. (1998). Control of distal antennal identity and tarsal development in *Drosophila* by spineless-aristapedia, a homolog of the mammalian dioxin receptor. Genes Dev **12**, 1290-1303.

Dunkov, B.C., Guzov, V.M., Mocelin, G., Shotkoski, F., Brun, A., Amichot, M., Ffrench-Constant, R.H., and Feyereisen, R. (1997). The *Drosophila* cytochrome P450 gene Cyp6a2: structure, localization, heterologous expression, and induction by phenobarbital. DNA Cell Biol **16**, 1345-1356.

Emmons, R.B., Duncan, D., Estes, P.A., Kiefel, P., Mosher, J.T., Sonnenfeld, M., Ward, M.P., Duncan, I., and Crews, S.T. (1999). The spineless-aristapedia and tango bHLH-PAS proteins interact to control antennal and tarsal development in *Drosophila*. Development **126**, 3937-3945.

Enmark, E., and Gustafsson, J.A. (2001). Comparing nuclear receptors in worms, flies and humans. Trends Pharmacol Sci **22**, 611-615.

Escriva, H., Delaunay, F., and Laudet, V. (2000). Ligand binding and nuclear receptor evolution. Bioessays **22**, 717-727.

Escriva, H., Safi, R., Hanni, C., Langlois, M.C., Saumitou-Laprade, P., Stehelin, D., Capron, A., Pierce, R., and Laudet, V. (1997). Ligand binding was acquired during evolution of nuclear receptors. Proc Natl Acad Sci U S A **94**, 6803-6808.

Estabrook, R.W., Cooper, D.Y., and Rosenthal, O. (1963). The Light Reversible Carbon Monoxide Inhibition of the Steroid C21-Hydroxylase System of the Adrenal Cortex. Biochem Z **338**, 741-755.

Feyereisen, R. (1999). Insect P450 enzymes. Annu Rev Entomol **44**, 507-533.

Feyereisen, R. (2005). Insect cytochrome P450. In Comprehensive molecular insect science (Elsevier), pp. 1-77.

Feyereisen, R. (2006). Evolution of insect P450. Biochem Soc Trans **34**, 1252-1255.

Feyereisen, R., Koener, J.F., Farnsworth, D.E., and Nebert, D.W. (1989). Isolation and sequence of cDNA encoding a cytochrome P-450 from an insecticide-resistant strain of the house fly, *Musca domestica*. Proc Natl Acad Sci U S A **86**, 1465-1469.

Fisk, G.J., and Thummel, C.S. (1995). Isolation, regulation, and DNA-binding properties of three *Drosophila* nuclear hormone receptor superfamily members. Proc Natl Acad Sci U S A **92**, 10604-10608.

Fujii-Kuriyama, Y., and Mimura, J. (2005). Molecular mechanisms of AhR functions in the regulation of cytochrome P450 genes. Biochem Biophys Res Commun **338**, 311-317.

Fujita, K., Sagisaka, A., Tomimoto, K., Ishibashi, J., Imanishi, S., Yamakawa, M., and Tanaka, H. (2009). DNA vector-based RNA interference in cell lines derived from *Bombyx mori*. Biosci Biotechnol Biochem **73**, 2026-2031.

Garfinkel, D. (1958). Studies on pig liver microsomes. I. Enzymic and pigment composition of different microsomal fractions. Arch Biochem Biophys **77**, 493-509.

Gatehouse, J.A. (2002). Plant resistance towards insect herbivores: a dynamic interaction. New Phytol **156**, 145-169.

Germain, P., Staels, B., Dacquet, C., Spedding, M., and Laudet, V. (2006). Overview of nomenclature of nuclear receptors. Pharmacol Rev **58**, 685-704.

Giguere, V. (1999). Orphan nuclear receptors: from gene to function. Endocr Rev **20**, 689-725.

Gilbert, L.I., and Warren, J.T. (2005). A molecular genetic approach to the biosynthesis of the insect steroid molting hormone. Vitam Horm **73**, 31-57.

Girardot, F., Monnier, V., and Tricoire, H. (2004). Genome wide analysis of common and specific stress responses in adult *Drosophila melanogaster*. BMC Genomics **5**, 74.

Gould, F., and Hodgson, E. (1980). Mixed function oxidase and glutathione transferase activity in last instar *Heliothis virescens* larvae. Pest Biochem Physiol **13**, 34-40.

Graham, S.E., and Peterson, J.A. (1999). How similar are P450s and what can their differences teach us? Arch Biochem Biophys **369**, 24-29.

Griebler, M., Westerlund, S.A., Hoffmann, K.H., and Meyering-Vos, M. (2008). RNA interference with the allatoregulating neuropeptide genes from the fall armyworm *Spodoptera frugiperda* and its effects on the JH titer in the hemolymph. J Insect Physiol **54**, 997-1007.

Gronemeyer, H., Gustafsson, J.A., and Laudet, V. (2004). Principles for modulation of the nuclear receptor superfamily. Nat Rev Drug Discov **3**, 950-964.

Gueguen, Y., Mouzat, K., Ferrari, L., Tissandie, E., Lobaccaro, J.M., Batt, A.M., Paquet, F., Voisin, P., Aigueperse, J., Gourmelon, P., et al. (2006). [Cytochromes P450: xenobiotic metabolism, regulation and clinical importance]. Ann Biol Clin (Paris) **64**, 535-548.

Guengerich, F.P. (2001). Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. Chem Res Toxicol **14**, 611-650.

Gunderson, C.A., Brattsten, L.B., and Fleming, J.T. (1986). Microsomal oxidase and glutahtione transferase as factors influencing the effects of pulegone in southern and fall armyworm larvae. Pest Biochem Physiol **26**, 238-249.

Hahn, M.E. (2002). Aryl hydrocarbon receptors: diversity and evolution. Chem Biol Interact **141**, 131-160.

Handschin, C., and Meyer, U.A. (2003). Induction of drug metabolism: the role of nuclear receptors. Pharmacol Rev **55**, 649-673.

Hartmann, T. (2004). Plant-derived secondary metabolites as defensive chemicals in herbivorous insects: a case study in chemical ecology. Planta **219**, 1-4.

Helvig, C., Koener, J.F., Unnithan, G.C., and Feyereisen, R. (2004). CYP15A1, the cytochrome P450 that catalyzes epoxidation of methyl farnesoate to juvenile hormone III in cockroach corpora allata. Proc Natl Acad Sci U S A **101**, 4024-4029.

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., Nusskern, D.R., Wincker, P., Clark, A.G., Ribeiro, J.M., Wides, R., *et al*. (2002). The genome sequence of the malaria mosquito *Anopheles gambiae*. Science **298**, 129-149.

Honkakoski, P., and Negishi, M. (2000). Regulation of cytochrome P450 (CYP) genes by nuclear receptors. Biochem J **347**, 321-337.

Honkakoski, P., Zelko, I., Sueyoshi, T., and Negishi, M. (1998). The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. Mol Cell Biol **18**, 5652-5658.

Horner, M.A., Pardee, K., Liu, S., King-Jones, K., Lajoie, G., Edwards, A., Krause, H.M., and Thummel, C.S. (2009). The Drosophila DHR96 nuclear receptor binds cholesterol and regulates cholesterol homeostasis. Genes Dev **23**, 2711-2716.

Hung, C.F., Harrison, T.L., Berenbaum, M.R., and Schuler, M.A. (1995a). CYP6B3: a second furanocoumarin-inducible cytochrome P450 expressed in *Papilio polyxenes*. Insect Mol Biol **4**, 149-160.

Hung, C.F., Prapaipong, H., Berenbaum, M.R., and Schuler, M.A. (1995b). Differential induction of cytochrome P450 transcripts in *Papilio polyxenes* by linear and angular furanocoumatins. Insect Mol Biol **25**, 89-99.

Imamura, M., Nakai, J., Inoue, S., Quan, G.X., Kanda, T., and Tamura, T. (2003). Targeted gene expression using the GAL4/UAS system in the silkworm *Bombyx mori*. Genetics **165**, 1329-1340.

International Silkworm Genome Consortium (2008). The genome of a lepidopteran model insect, the silkworm *Bombyx mori*. Insect Biochem Mol Biol **38**(12):1036-45.

Ito, K., Kidokoro, K., Sezutsu, H., Nohata, J., Yamamoto, K., Kobayashi, I., Uchino, K., Kalyebi, A., Eguchi, R., Hara, W., et al. (2008). Deletion of a gene encoding an amino acid transporter

in the midgut membrane causes resistance to a *Bombyx* parvo-like virus. Proc Natl Acad Sci U S A **105**, 7523-7527.

Jakoby, W.B., and Ziegler, D.M. (1990). The enzymes of detoxication. J Biol Chem **265**, 20715-20718.

Jindra, M., Huang, J.Y., Malone, F., Asahina, M., and Riddiford, L.M. (1997). Identification and mRNA developmental profiles of two ultraspiracle isoforms in the epidermis and wings of *Manduca sexta*. Insect Mol Biol **6**, 41-53.

Johnson, E.F., Wester, M.R., and Stout, C.D. (2002). The structure of microsomal cytochrome P450 2C5: a steroid and drug metabolizing enzyme. Endocr Res **28**, 435-441.

Jones, G., Jones, D., Teal, P., Sapa, A., and Wozniak, M. (2006). The retinoid-X receptor ortholog, ultraspiracle, binds with nanomolar affinity to an endogenous morphogenetic ligand. Febs J **273**, 4983-4996.

Jones, G., and Sharp, P.A. (1997). Ultraspiracle: an invertebrate nuclear receptor for juvenile hormones. Proc Natl Acad Sci U S A **94**, 13499-13503.

Kawamoto, T., Sueyoshi, T., Zelko, I., Moore, R., Washburn, K., and Negishi, M. (1999). Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the *CYP2B* gene. Mol Cell Biol **19**, 6318-6322.

King-Jones, K., Horner, M.A., Lam, G., and Thummel, C.S. (2006). The DHR96 nuclear receptor regulates xenobiotic responses in *Drosophila*. Cell Metab **4**, 37-48.

King-Jones, K., and Thummel, C.S. (2005). Nuclear receptors - a perspective from *Drosophila*. Nat Rev Genet **6**, 311-323.

Kliewer, S.A., Moore, J.T., Wade, L., Staudinger, J.L., Watson, M.A., Jones, S.A., McKee, D.D., Oliver, B.B., Willson, T.M., Zetterstrom, R.H., et al. (1998). An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell **92**, 73-82.

Klingenberg, M. (1958). Pigments of rat liver microsomes. Arch Biochem Biophys **75**, 376-386.

Kocarek, T.A., Shenoy, S.D., Mercer-Haines, N.A., and Runge-Morris, M. (2002). Use of dominant negative nuclear receptors to study xenobiotic-inducible gene expression in primary cultured hepatocytes. J Pharmacol Toxicol Methods **47**, 177-187.

Koch, M.A., Schuffenhauer, A., Scheck, M., Wetzel, S., Casaulta, M., Odermatt, A., Ertl, P., and Waldmann, H. (2005). Charting biologically relevant chemical space: a structural classification of natural products (SCONP). Proc Natl Acad Sci U S A **102**, 17272-17277.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., and Hogness, D.S. (1991). The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell **67**, 59-77.

Kretschmer, X.C., and Baldwin, W.S. (2005). CAR and PXR: xenosensors of endocrine disrupters? Chem Biol Interact **155**, 111-128.

Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J.M., and Chambon, P. (1986). The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. EMBO J **5**, 891-897.

Kuhnel, K., Ke, N., Cryle, M.J., Sligar, S.G., Schuler, M.A., and Schlichting, I. (2008). Crystal structures of substrate-free and retinoic acid-bound cyanobacterial cytochrome P450 CYP120A1. Biochemistry **47**, 6552-6559.

Kumar, R., and Litwack, G. (2009). Structural and functional relationships of the steroid hormone receptors' N-terminal transactivation domain. Steroids **74**, 877-883.

Kumar, R., and Thompson, E.B. (1999). The structure of the nuclear hormone receptors. Steroids **64**, 310-319.

Laudet, V., and Bonneton, F. (2005). evolution of nuclear hormone receptors in insects. In Comprehensive molecular insect science (Elsevier), pp. 287-318.

Lavery, D.N., and McEwan, I.J. (2005). Structure and function of steroid receptor AF1 transactivation domains: induction of active conformations. Biochem J **391**, 449-464.

Le Goff, G., Boundy, S., Daborn, P.J., Yen, J.L., Sofer, L., Lind, R., Sabourault, C., Madi-Ravazzi, L., and ffrench-Constant, R.H. (2003). Microarray analysis of cytochrome P450 mediated insecticide resistance in *Drosophila*. Insect Biochem Mol Biol **33**, 701-708.

Le Goff, G., Hilliou, F., Siegfried, B.D., Boundy, S., Wajnberg, E., Sofer, L., Audant, P., ffrench-Constant, R.H., and Feyereisen, R. (2006). Xenobiotic response in *Drosophila melanogaster*: sex dependence of P450 and GST gene induction. Insect Biochem Mol Biol **36**, 674-682.

Lehmann, J.M., McKee, D.D., Watson, M.A., Willson, T.M., Moore, J.T., and Kliewer, S.A. (1998). The human orphan nuclear receptor PXR is activated by compounds that regulate *CYP3A4* gene expression and cause drug interactions. J Clin Invest **102**, 1016-1023.

Li, W., Petersen, R.A., Schuler, M.A., and Berenbaum, M.R. (2002). CYP6B cytochrome p450 monooxygenases from *Papilio canadensis* and *Papilio glaucus*: potential contributions of sequence divergence to host plant associations. Insect Mol Biol **11**, 543-551.

Li, W., Schuler, M.A., and Berenbaum, M.R. (2003). Diversification of furanocoumarin-metabolizing cytochrome P450 monooxygenases in two papilionids: Specificity and substrate encounter rate. Proc Natl Acad Sci U S A **100** Suppl 2, 14593-14598.

Li, X., Baudry, J., Berenbaum, M.R., and Schuler, M.A. (2004). Structural and functional divergence of insect CYP6B proteins: From specialist to generalist cytochrome P450. Proc Natl Acad Sci U S A **101**, 2939-2944.

Li, X., Schuler, M.A., and Berenbaum, M.R. (2007). Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu Rev Entomol **52**, 231-253.

Lin, C.C., Hsu, J.T., Huang, K.L., Tang, H.K., and Lai, Y.K. (2006). Stable RNA interference in *Spodoptera frugiperda* cells by a DNA vector-based method. Biotechnol Lett **28**, 271-277.

Lindblom, T.H., Pierce, G.J., and Sluder, A.E. (2001). A *C. elegans* orphan nuclear receptor contributes to xenobiotic resistance. Curr Biol **11**, 864-868.

Lipinski, C., and Hopkins, A. (2004). Navigating chemical space for biology and medicine. Nature **432**, 855-861.

Ma, R., Cohen, M.B., Berenbaum, M.R., and Schuler, M.A. (1994). Black swallowtail (*Papilio polyxenes*) alleles encode cytochrome P450s that selectively metabolize linear furanocoumarins. Arch Biochem Biophys **310**, 332-340.

Maglich, J.M., Stoltz, C.M., Goodwin, B., Hawkins-Brown, D., Moore, J.T., and Kliewer, S.A. (2002). Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. Mol Pharmacol **62**, 638-646.

Mangelsdorf, D.J., and Evans, R.M. (1995). The RXR heterodimers and orphan receptors. Cell **83**, 841-850.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., et al. (1995). The nuclear receptor superfamily: the second decade. Cell **83**, 835-839.

Mansuy, D. (1998). The great diversity of reactions catalyzed by cytochromes P450. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol **121**, 5-14.

Mao, W., Rupasinghe, S.G., Johnson, R.M., Zangerl, A.R., Schuler, M.A., and Berenbaum, M.R. (2009). Quercetin-metabolizing CYP6AS enzymes of the pollinator *Apis mellifera* (Hymenoptera: Apidae). Comp Biochem Physiol B Biochem Mol Biol **154**, 427-434.

McDonnell, C.M., Brown, R.P., Berenbaum, M.R., and Schuler, M.A. (2004). Conserved regulatory elements in the promoters of two allelochemical-inducible cytochrome P450 genes differentially regulate transcription. Insect Biochem Mol Biol **34**, 1129-1139.

McEwan, I.J. (2004). Molecular mechanisms of androgen receptor-mediated gene regulation: structure-function analysis of the AF-1 domain. Endocr Relat Cancer **11**, 281-293.

Meyering-Vos, M., Merz, S., Sertkol, M., and Hoffmann, K.H. (2006). Functional analysis of the allatostatin-A type gene in the cricket *Gryllus bimaculatus* and the armyworm *Spodoptera frugiperda*. Insect Biochem Mol Biol **36**, 492-504.

Miura, K., Oda, M., Makita, S., and Chinzei, Y. (2005). Characterization of the *Drosophila* Methoprene -tolerant gene product. Juvenile hormone binding and ligand-dependent gene regulation. FEBS J **272**, 1169-1178.

Moore, D.D., Kato, S., Xie, W., Mangelsdorf, D.J., Schmidt, D.R., Xiao, R., and Kliewer, S.A. (2006). International Union of Pharmacology. LXII. The NR1H and NR1I receptors: constitutive androstane receptor, pregnene X receptor, farnesoid X receptor alpha, farnesoid X receptor beta, liver X receptor alpha, liver X receptor beta, and vitamin D receptor. Pharmacol Rev **58**, 742-759.

Moreau, A., Maurel, P., Vilarem, M.J., and Pascussi, J.M. (2007). Constitutive androstane receptor-vitamin D receptor crosstalk: consequence on CYP24 gene expression. Biochem Biophys Res Commun **360**, 76-82.

Murataliev, M.B., Guzov, V.M., Walker, F.A., and Feyereisen, R. (2008). P450 reductase and cytochrome b5 interactions with cytochrome P450: effects on house fly *CYP6A1* catalysis. Insect Biochem Mol Biol **38**, 1008-1015.

Nakagawa, Y., and Henrich, V.C. (2009). Arthropod nuclear receptors and their role in molting. FEBS J **276**, 6128-6157.

Neal, J.J. (1987). Metabolic costs of mixed-function oxidase induction in *Heliothis zea*. Entomol exp appl **43**, 175-179.

Nebert, D.W., Adesnik, M., Coon, M.J., Estabrook, R.W., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Kemper, B., Levin, W., et al. (1987). The P450 gene superfamily: recommended nomenclature. DNA **6**, 1-11.

Nebert, D.W., Dalton, T.P., Okey, A.B., and Gonzalez, F.J. (2004). Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. J Biol Chem **279**, 23847-23850.

Nebert, D.W., and Nelson, D.R. (1991). P450 gene nomenclature based on evolution. Methods Enzymol **206**, 3-11.

Nebert, D.W., Nelson, D.R., Adesnik, M., Coon, M.J., Estabrook, R.W., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Kemper, B., et al. (1989). The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. DNA **8**, 1-13.

Nebert, D.W., and Russell, D.W. (2002). Clinical importance of the cytochromes P450. Lancet **360**, 1155-1162.

Nelson, D.R. (1999). Cytochrome P450 and the individuality of species. Arch Biochem Biophys **369**, 1-10.

Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C., Gotoh, O., et al. (1993). The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. DNA Cell Biol **12**, 1-51.

Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., et al. (1996). P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics **6,** 1-42.

Nuclear Recetors Nomenclature Committee (1999). A unified nomenclature system for the nuclear receptor superfamily. Cell **97**, 161-163.

Olefsky, J.M. (2001). Nuclear receptor minireview series. J Biol Chem **276**, 36863-36864.

Omura, T., and Sato, R. (1964). The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. J Biol Chem **239**, 2370-2378.

Oppenoorth, F.J. (1984). Biochemistry of insecticide resistance. Pest Biochem Physiol **22**, 187-193.

Oro, A.E., McKeown, M., and Evans, R.M. (1990). Relationship between the product of the *drosophila* ultraspiracle locus and the vertebrate retinoid X receptor. Nature **347**, 298-301.

Palanker, L., Necakov, A.S., Sampson, H.M., Ni, R., Hu, C., Thummel, C.S., and Krause, H.M. (2006). Dynamic regulation of Drosophila nuclear receptor activity *in vivo*. Development **133**, 3549-3562.

Pascussi, J.M., Gerbal-Chaloin, S., Duret, C., Daujat-Chavanieu, M., Vilarem, M.J., and Maurel, P. (2008). The tangle of nuclear receptors that controls xenobiotic metabolism and transport: crosstalk and consequences. Annu Rev Pharmacol Toxicol **48**, 1-32.

Petersen, R.A., Niamsup, H., Berenbaum, M.R., and Schuler, M.A. (2003). Transcriptional response elements in the promoter of CYP6B1, an insect P450 gene regulated by plant chemicals. Biochim Biophys Acta **1619**, 269-282.

Petersen, R.A., Zangerl, A.R., Berenbaum, M.R., and Schuler, M.A. (2001). Expression of CYP6B1 and CYP6B3 cytochrome P450 monooxygenases and furanocoumarin metabolism in different tissues of *Papilio polyxenes* (Lepidoptera: Papilionidae). Insect Biochem Mol Biol **31**, 679-690.

Petryk, A., Warren, J.T., Marques, G., Jarcho, M.P., Gilbert, L.I., Kahler, J., Parvy, J.P., Li, Y., Dauphin-Villemant, C., and O'Connor, M.B. (2003). Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. Proc Natl Acad Sci U S A **100**, 13773-13778.

Poulos, T.L., Finzel, B.C., and Howard, A.J. (1986). Crystal structure of substrate-free *Pseudomonas putida* cytochrome P-450. Biochemistry **25**, 5314-5322.

Prapaipong, H., Berenbaum, M.R., and Schuler, M.A. (1994). Transcriptional regulation of the *Papilio polyxenes* *CYP6B1* gene. Nucleic Acids Res **22**, 3210-3217.

Rajagopal, R., Sivakumar, S., Agrawal, N., Malhotra, P., and Bhatnagar, R.K. (2002). Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. J Biol Chem **277**, 46849-46851.

Rates, S.M. (2001). Plants as source of drugs. Toxicon **39**, 603-613.

Rausher, M.D. (2001). Co-evolution and plant resistance to natural enemies. Nature **411**, 857-864.

Ravichandran, K.G., Boddupalli, S.S., Hasermann, C.A., Peterson, J.A., and Deisenhofer, J. (1993). Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. Science **261**, 731-736.

Ray, J.W. (1967). The epoxidation of aldrin by housefly microsomes and its inhibition by carbon monoxide. Biochem Pharmacol **16**, 99-107.

Remmer, H., and Merker, H.J. (1963). Drug-Induced Changes in the Liver Endoplasmic Reticulum: Association with Drug-Metabolizing Enzymes. Science **142**, 1657-1658.

Rendic, S. (2002). Summary of information on human CYP enzymes: human P450 metabolism data. Drug Metab Rev **34**, 83-448.

Rewitz, K.F., Rybczynski, R., Warren, J.T., and Gilbert, L.I. (2006). The Halloween genes code for cytochrome P450 enzymes mediating synthesis of the insect moulting hormone. Biochem Soc Trans **34,** 1256-1260.

Richards, S., Gibbs, R.A., Weinstock, G.M., Brown, S.J., Denell, R., Beeman, R.W., Gibbs, R., Bucher, G., Friedrich, M., Grimmelikhuijzen, C.J., et al. (2008). The genome of the model beetle and pest *Tribolium castaneum*. Nature **452**, 949-955.

Riddiford, L.M., Cherbas, P., and Truman, J.W. (2001). Ecdysone Receptors and Their Biological Actions. Vitam Horm **60**, 1-73.

Robinson-Rechavi, M., Carpentier, A.S., Duffraisse, M., and Laudet, V. (2001). How many nuclear hormone receptors are there in the human genome? Trends Genet **17**, 554-556.

Robinson-Rechavi, M., and Laudet, V. (2003). Bioinformatics of nuclear receptors. In Methods in enzymology, Elsevier, ed., pp. 95-118.

Sabourault, C., Guzov, V.M., Koener, J.F., Claudianos, C., Plapp, F.W., Jr., and Feyereisen, R. (2001). Overproduction of a P450 that metabolizes diazinon is linked to a loss-of-function in the chromosome 2 ali-esterase (MdalphaE7) gene in resistant house flies. Insect Mol Biol **10**, 609-618.

Sakudoh, T., Sezutsu, H., Nakashima, T., Kobayashi, I., Fujimoto, H., Uchino, K., Banno, Y., Iwano, H., Maekawa, H., Tamura, T., et al. (2007). Carotenoid silk coloration is controlled by a carotenoid-binding protein, a product of the Yellow blood gene. Proc Natl Acad Sci U S A **104**, 8941-8946.

Sasorith, S., Billas, I.M., Iwema, T., Moras, D., and Wurtz, J.M. (2002). Structure-based analysis of the ultraspiracle protein and docking studies of putative ligands. J Insect Sci **2**, 25.

Schuler, M.A. (1996). The role of cytochrome P450 monooxygenases in plant-insect interactions. Plant Physiol **112**, 1411-1419.

Scott, E.E., and Halpert, J.R. (2005). Structures of cytochrome P450 3A4. Trends Biochem Sci **30**, 5-7.

Shimada, T., and Fujii-Kuriyama, Y. (2004). Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. Cancer Sci **95**, 1-6.

Sluder, A.E., and Maina, C.V. (2001). Nuclear receptors in nematodes: themes and variations. Trends Genet **17**, 206-213.

Snyder, M.J., Hsu, E.L., and Feyereisen, R. (1993). Induction of cytochrome P-450 activities by nicotine in the tobacco hornworm, *Manduca sexta*. J Chem Ecol **19**, 2903-2916.

Snyder, M.J., Stevens, J.L., Andersen, J.F., and Feyereisen, R. (1995). Expression of cytochrome P450 genes of the CYP4 family in midgut and fat body of the tobacco hornworm, *Manduca sexta*. Arch Biochem Biophys **321**, 13-20.

Stegeman, J.J., and Livingstone, D.R. (1998). Forms and functions of cytochrome P450. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol **121**, 1-3.

Stevens, J.L., Snyder, M.J., Koener, J.F., and Feyereisen, R. (2000). Inducible P450s of the CYP9 family from larval *Manduca sexta* midgut. Insect Biochem Mol Biol **30**, 559-568.

Strong, D.R. (1988). Special features: insect host range. Editorial. Ecology **69**, 885.

Sueyoshi, T., Kawamoto, T., Zelko, I., Honkakoski, P., and Negishi, M. (1999). The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. J Biol Chem **274**, 6043-6046.

Sueyoshi, T., and Negishi, M. (2001). Phenobarbital response elements of cytochrome P450 genes and nuclear receptors. Annu Rev Pharmacol Toxicol **41**, 123-143.

Sullivan, A.A., and Thummel, C.S. (2003). Temporal profiles of nuclear receptor gene expression reveal coordinate transcriptional responses during *Drosophila* development. Mol Endocrinol **17**, 2125-2137.

Sutherland, J.D., Kozlova, T., Tzertzinis, G., and Kafatos, F.C. (1995). Drosophila hormone receptor 38: a second partner for Drosophila USP suggests an unexpected role for nuclear receptors of the nerve growth factor-induced protein B type. Proc Natl Acad Sci U S A **92**, 7966-7970.

Sutherland, T.D., Unnithan, G.C., Andersen, J.F., Evans, P.H., Murataliev, M.B., Szabo, L.Z., Mash, E.A., Bowers, W.S., and Feyereisen, R. (1998). A cytochrome P450 terpenoid hydroxylase linked to the suppression of insect juvenile hormone synthesis. Proc Natl Acad Sci U S A **95**, 12884-12889.

Talbot, W.S., Swyryd, E.A., and Hogness, D.S. (1993). *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell **73**, 1323-1337.

Tamura, T., Thibert, C., Royer, C., Kanda, T., Abraham, E., Kamba, M., Komoto, N., Thomas, J.L., Mauchamp, B., Chavancy, G., et al. (2000). Germline transformation of the silkworm *Bombyx mori* L. using a piggyBac transposon-derived vector. Nat Biotechnol **18**, 81-84.

Tan, A., and Palli, S.R. (2008). Identification and characterization of nuclear receptors from the red flour beetle, *Tribolium castaneum*. Insect Biochem Mol Biol **38**, 430-439.

Thummel, C.S. (2002). Ecdysone-regulated puff genes 2000. Insect Biochem Mol Biol **32**, 113-120.

Tijet, N., Helvig, C., and Feyereisen, R. (2001). The cytochrome P450 gene superfamily in *Drosophila melanogaster*: annotation, intron-exon organization and phylogeny. Gene **262**, 189-198.

Timsit, Y.E., and Negishi, M. (2007). CAR and PXR: the xenobiotic-sensing receptors. Steroids **72**, 231-246.

Tompkins, L.M., and Wallace, A.D. (2007). Mechanisms of cytochrome P450 induction. J Biochem Mol Toxicol **21**, 176-181.

Uchino, K., Sezutsu, H., Imamura, M., Kobayashi, I., Tatematsu, K., Iizuka, T., Yonemura, N., Mita, K., and Tamura, T. (2008). Construction of a piggyBac-based enhancer trap system for

the analysis of gene function in silkworm *Bombyx mori.* Insect Biochem Mol Biol **38**, 1165-1173.

Velarde, R.A., Robinson, G.E., and Fahrbach, S.E. (2006). Nuclear receptors of the honey bee: annotation and expression in the adult brain. Insect Mol Biol **15**, 583-595.

Waxman, D.J., and Azaroff, L. (1992). Phenobarbital induction of cytochrome P-450 gene expression. Biochem J **281** (Pt 3), 577-592.

Wen, Z., Pan, L., Berenbaum, M.R., and Schuler, M.A. (2003). Metabolism of linear and angular furanocoumarins by *Papilio polyxenes* CYP6B1 co-expressed with NADPH cytochrome P450 reductase. Insect Biochem Mol Biol **33**, 937-947.

Werck-Reichhart, D., and Feyereisen, R. (2000). Cytochromes P450: a success story. Genome Biol **1**(6), 3003.1-3003.9.

Wheat, C.W., Vogel, H., Wittstock, U., Braby, M.F., Underwood, D., and Mitchell-Olds, T. (2007). The genetic basis of a plant-insect coevolutionary key innovation. Proc Natl Acad Sci U S A **104**, 20427-20431.

Wheeler, G.S., Slansky, F.J., and Yu, S.J. (1993). Fall armyworm sensitivity to flavone: limited role of constitutive and induced enzyme activity. J Chem Ecol **19**, 645-667.

Whitlock, J.P., Jr. (1999). Induction of cytochrome P4501A1. Annu Rev Pharmacol Toxicol **39**, 103-125.

Willoughby, L., Chung, H., Lumb, C., Robin, C., Batterham, P., and Daborn, P.J. (2006). A comparison of *Drosophila melanogaster* detoxification gene induction responses for six insecticides, caffeine and phenobarbital. Insect Biochem Mol Biol **36**, 934-942.

Willson, T.M., and Kliewer, S.A. (2002). PXR, CAR and drug metabolism. Nat Rev Drug Discov **1**, 259-266.

Wittstock, U., and Gershenzon, J. (2002). Constitutive plant toxins and their role in defense against herbivores and pathogens. Curr Opin Plant Biol **5**, 300-307.

Woodcroft, K.J., and Novak, R.F. (1998). Xenobiotic-enhanced expression of cytochromes P450 2E1 and 2B in primary cultured rat hepatocytes. Drug Metab Dispos **26**, 372-378.

Yao, T.P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., McKeown, M., Cherbas, P., and Evans, R.M. (1993). Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. Nature **366**, 476-479.

Yao, T.P., Segraves, W.A., Oro, A.E., McKeown, M., and Evans, R.M. (1992). *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell **71**, 63-72.

Yu, S.J. (1982). induction of microsomal oxidases by host plants in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). Pest Biochem Physiol **17**, 59-67.

Yu, S.J. (1983). Induction of detoxifying enzymes by allelochemicals and host plants in the fall armyworm. Pest Biochem Physiol **19**, 330-336.

Yu, S.J. (1985). microsomal sulfoxidation of phorate in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). Pest Biochem Physiol **23**, 273-281.

Yu, S.J. (1986). Microsomal oxidation of allelochemicals in generalist (*Spodoptera frugiperda*) and semispecialist (*Ancarsia gemmatalis*) insect. J Chem Ecol **13**, 423-435.

Yu, S.J., Berry, R.E., and Terriere, L.C. (1979). Host plant stimulation of detoxifying enzymes in a phytophagous insect. Pest Biochem Physiol **12**, 280-284.

Zhang, Z., Burch, P.E., Cooney, A.J., Lanz, R.B., Pereira, F.A., Wu, J., Gibbs, R.A., Weinstock, G., and Wheeler, D.A. (2004). Genomic analysis of the nuclear receptor family: new insights into structure, regulation, and evolution from the rat genome. Genome Res **14**, 580-590.

Zhou, X., Ma, C., Li, M., Sheng, C., Liu, H., and Qiu, X. (2009). CYP9A12 and CYP9A17 in the cotton bollworm, *Helicoverpa armigera*: sequence similarity, expression profile and xenobiotic response. Pest Manag Sci. **66** (1), 65-73

Zhu-Salzman, K., Bi, J.-L., and Liu, T.-X. (2005). Molecular strategies of plant defense and insect counter-defense. insect Science **12**, 3-15.

Je voudrais dédier cette these à mes meilleurs ennemis, les insectes et aux 23 457 larves de Spodoptera mortes pour la science…