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Cytochromes P450 of insects: the tip of the iceberg†

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Abstract: The cytochrome P450-dependent monooxygenases are an extremely important metabolic system involved in the metabolism of endogenous compounds and xenobiotics. Collectively, P450 monooxygenases can metabolize numerous substrates and carry out multiple oxidative reactions. The large number of substrates metabolized is due to the plethora of P450 isoforms and to the broad substrate specificity of some isoforms. Monooxygenases of insects have several functional roles, including growth, development, feeding and protection against xenobiotics, including resistance to pesticides and tolerance to plant toxins. This review begins with background information about P450s and their evolution, followed by a discussion of the extraordinary diversity of insect P450s. Given the enormous interest in studying individual P450s, we then provide a synopsis of the different methods that have been used in their isolation and the substrates that are known to be metabolized. We conclude by summarizing the lessons we have learned from the study of individual insect P450s, including their roles in insecticide resistance, plant–insect interactions and insect physiology. However, these studies are just the 'tip of the iceberg'. Our knowledge continues to expand at a rapid pace, suggesting that the next decade will outpace the last in terms of improving our understanding of the cytochromes P450 of insects.

Keywords: insecticide resistance; plant–insect interactions; insect physiology; P450 monooxygenases; xenobiotic metabolism

1 INTRODUCTION

Cytochrome P450-dependent monooxygenases are a very important enzymatic system involved in the metabolism of a phenomenal number of endogenous and exogenous compounds.[1] P450 monooxygenases have been found in all living systems examined. 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. Collectively, P450 monooxygenases are capable of metabolizing numerous substrates and can carry out multiple oxidative reactions.[2] The large number of substrates metabolized by monooxygenases is due to the plethora of P450 isoforms and to the broad substrate specificity of some isoforms. Metabolism by monooxygenases generally results in detoxification of the substrate, although activation is also possible. For example, most of the commonly used organophosphate insecticides require monooxygenase-mediated activation.[3]

The majority of P450s in eucaryotes are located in the endoplasmic reticulum and require the flavoprotein NADPH cytochrome P450 reductase for reducing equivalents. In addition to P450 and reductase, cytochrome b_5 is sometimes needed, depending upon the substrate and/or the P450 isoform involved. Cytochrome b_5 can be important in donating the second electron from NADH to P450 or by allosterically regulating substrate binding to P450.[4–8] Mitochondrial P450s require ferridoxin and an NADPH ferridoxin reductase, and are therefore somewhat similar to the soluble P450 system found in bacteria. P450 is a hemoprotein so named because of its characteristic absorbance peak at 450 nm when the reduced form is combined with carbon monoxide.[9] P450s can be identified by a characteristic sequence (commonly FXXGXXXCXG) in the heme binding region.

2 NOMENCLATURE

P450s are named primarily on the basis of the overall amino acid sequence.[10] A P450 is named CYP followed by a number, a letter and a number. P450s with >40% of the amino acids identical are usually grouped into the same family and members with >55% of the amino acids identical are generally

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grouped into the same sub-family, although there are exceptions to these rules.[10] For example, CYP6A1 and CYP6B2 are both grouped into family 6, but the amino acid identities of these two proteins are less than 40%. In this case the 40% rule was negated because the sequences flanking the conserved cysteine were similar.[11] Given that this nomenclature system is based on the overall amino acid identity, and that a single amino acid change may dramatically alter the substrate specificity of a P450,[12,13] no information regarding the function of a P450 should be assumed from its classification within this system. Alleles are designated *v1, v2*, etc and pseudogenes are designated *p1, p2*, etc.

3 EVOLUTION OF P450S

The presence of P450s in diverse organisms, from bacteria to plants and animals, implies that the P450 superfamily is an extremely ancient enzymatic system and that all the current P450s may have descended from a common ancestral gene.[14,15] The current P450 superfamily is thought to have been formed by gene duplication and adaptive diversification.[16] The rapid increase in the number of new P450s during the past 400 million years may be a reflection of an escalation in animal–plant warfare.[17–19]

The concept of molecular drive has also been used to explain the formation of new P450 genes.[19] Molecular drive is the process by which the genotypic composition of a population can be changed, as a result of the internal dynamics of DNA turnover independent of natural selection.[20] This continuous dynamic DNA turnover includes gene duplication, unequal crossover, gene conversion, transposition, slippage replication and RNA-mediated transfers.[19–21]

During the processes of P450 evolution, a pseudogene may be generated.[17] A pseudogene is a sequence which is similar to a functional gene, but does not produce a functional product (protein in the case of a structural gene). A pseudogene can be identified by either its aberrant coding region or transcriptional silence or both.[22] Pseudogenes are a common phenomenon and are found in many organisms.[22] P450 pseudogenes have been described in various organisms, including four pseudogenes identified in *Drosophila melanogaster* Meig[23,24] and five pseudogenes from *Blattella germanica* (L).[25]

4 DIVERSITY OF INSECT P450S

P450 monooxygenases are a very diverse enzymatic system. This diversity is exemplified by the existence of multiple P450 isoforms within each organism (Section 4.1), the diverse expression patterns among different P450s (Section 4.2) and the wide substrate spectra of some isoforms (Section 6).

4.1 Isoform multiplicity

While the existence of multiple isoforms of P450s in insects has long been known from purification and induction studies,[1] only recently was the first quantitative assessment of the number of P450s in an insect made. Sequencing of the *Drosophila* genome revealed that there were 86 putative P450 genes and four pseudogenes, encompassing 25 different P450 families.[24] Members from families 4 (22) and 6 (23) account for more than half of the P450s in this species. An alignment of the deduced amino acid sequences of the 86 *Drosophila* sequences (DNASTAR, MegAlign program, Clustal method) reveals the highest percentage identity (84.2) for CYP6A17 and CYP6A23. There was >70% identity for only 10 comparisons and the majority of the sequences showed very limited percentage identities. Thus, while all of these genes are similar enough to be classified as P450s, they clearly have very limited homology to each other. Comparison of P450s between species further exemplifies the tremendous diversity of insect P450s. It is common for a new P450 to have no more than 30–50% identity with previously known P450s, even among insects from the same Order. One exception to this is CYP6B8 from *Helicoverpa zea* (Boddie), which shares 99% identity with CYP6B7 from *Helicoverpa armigera* (Huebn).[26] Thus, while it is possible to find related genes between insect species, this appears to be the exception rather than the rule. The term 'diversozymes' coined by Coon *et al*,[27] clearly applies to insects!

4.2 Diversity in expression

P450s are under complex and distinct control by endogenous signals or following exposure to various xenobiotic compounds. As a result, individual P450s can show diverse expression patterns related to life stages, sexes, tissues, strains or diet. For example, some P450s are expressed in all the life stages (eg CYP4D1),[28] some are life-stage specific (eg CYP6D1)[29,30] and some are even expressed only for a portion of a life stage.[31] Most of the insect P450s that have been studied are expressed in both sexes, although at least one is sex-specific (CYP6L1).[32] In insects, monooxygenase activity has been found in many tissues such as fat body, midgut, Malpighian tubules, nervous system[33] and antennae,[34] with the highest activity usually associated with the midgut.[1] Here again, expression of individual P450s varies from ubiquitous (eg CYP6D1)[33,35] to specific (eg CYP6L1 in male reproductive tissues,[32] CYP4G15 in the nervous system[36]). A discussion of the P450s differentially expressed in insecticide-resistant strains was the focus of a recent review[37] and that information will not be repeated here. Information on inducers of P450s can be found in two recent reviews.[38,39]

5 METHODS USED FOR ISOLATION OF INDIVIDUAL INSECT P450S

Several different approaches have been successfully employed to isolate individual insect P450s. In this

section a brief review of the techniques used is presented. There are many examples for most of these approaches, but only one or two representative references are provided.

5.1 Sequencing the entire genome

Sequencing the entire genome of an organism, as was done with *D melanogaster*, is the most systematic approach to isolate P450 genes. Combined with microarray analysis this is a powerful way to gain information about the expression patterns of all P450s in an organism.[31] In addition, the genome sequence provides valuable information, such as flanking sequences, introns and chromosomal location of each P450. However, this approach is not currently feasible for most insects.

5.2 Protein purification

Early attempts to study individual P450 isoforms emphasized the purification of P450 proteins.[1,3] The purified P450 protein can be used to raise antisera which can be used to screen a cDNA expression library. For example, CYP6A1 cDNA from house fly[40] and CYP6A2 cDNA from *D melanogaster*[41] were isolated by screening cDNA expression libraries with a P450 antibody. Alternatively, the purified protein can be microsequenced and serves as the basis for designing gene-specific primers. Short cDNA fragments generated by this method can then be used to screen a cDNA library. CYP6B1 was isolated by this approach.[42] Gene-specific primers can also be used to clone cDNA sequences by the rapid amplification of cDNA ends (RACE) technique.[43] CYP6D1 was cloned using this method.[44] The sequence obtained by these methods is usually the gene for the purified P450. However, it should be remembered that 'related P450s' could be isolated by these methods.

5.3 Using known P450s as probes

Considering the similarity between P450s, a previously identified P450 can be used as a probe to isolate new P450 genes. For example, CYP6B3 was isolated by screening a cDNA library using CYP6B1 cDNA as probe.[45] This approach can even be used across species.[26,46]

5.4 Using degenerate primers

Although very diverse, P450s share some relatively conserved regions at the amino acid level, such as the heme binding region and the I-helix.[47] Degenerate PCR primers based on these regions can be used to obtain partial P450 sequences. PCR products can then be used to screen libraries[48,49] or be used for PCR/RACE.[32,50–52] The degenerate primer itself has even been used as a probe.[45]

5.5 Differential or subtractive screening

Because the expression of some P450s is different between life stages, strains or physiological condition (Section 4.2) it is possible to target a subset of P450s through the use of differential or subtractive screening. For example, CYP4C1 from *B discoidalis* was isolated by screening a cDNA library using differential hybridization designed to select gene sequences regulated by hypertrehalosemic hormone.[53] Although infrequently used, this approach merits serious consideration for researchers interested in targeting P450s with a specific function.

6 SUBSTRATES OF INSECT P450S

Insect monooxygenases metabolize endogenous substrates such as juvenile hormones (JHs), ecdysteroids, pheromones and their analogues, as well as exogenous substrates such as plant allelochemicals, insecticides and promutagens.[1,37,38,54,55] Identification of the specific P450s involved in these reactions has been a subject of intense research. Several approaches, such as isoform-specific antisera, reconstitution experiments and heterologous expression, have shown great promise. The following is a summary of available information on substrates (and non-substrates) for individual insect P450s.

CYP4C7 (*Diploptera punctata* Eschscholtz)

CYP4C7 substrates were investigated using purified CYP4C7 that had been heterologously expressed in *Escherichia coli* Cast & Chalm. CYP4C7 was reconstituted with recombinant house fly P450 reductase and cytochrome b_5. Using this system, CYP4C7 metabolized *(2E, 6E)*-farnesol, *(2E, 6E)*-farnesal, *(2E, 6E)*-farnesoic acid, *(2E, 6E)*-methyl farnesoate, JH I, JH II, JH III, farnesyl methyl ether and geranyl methyl ether. CYP4C7 had no activity towards fatty acids (laurate or palmitate), cyclodiene insecticides (aldrin or heptachlor), geraniol, geranyl geraniol, 2,6,10-trimethyldodecanol, 10,11-epoxy-*(2E, 6E)*-farnesoic acid, monoterpenes or diterpenes.[56]

CYP6A1 (*Musca domestica* L)

In vitro reconstitution experiments using recombinant CYP6A1 and house fly P450 reductase expressed in *E coli* were employed to investigate the substrates for CYP6A1. Substrates for CYP6A1 included aldrin, heptachlor, JH I, JH III, farnesal and a variety of terpenoids. CYP6A1 had little or no detectable activity towards benzphetamine, ecdysone, 7-ethoxycoumarin, farnesol, farnesoic acid, limonene, 7-methoxy-4-methylcoumarin, 7-methoxycoumarin, *p*-chloro-*N*-methylaniline, hydroprene, methoprene, methoxyresorufin or α-pinene.[57,58]

CYP6A2 (*D melanogaster*)

CYP6A2 expressed in lepidopteran (Sf21) cells using baculovirus as expression vectors metabolized aldrin, dieldrin and diazinon.[59] CYP6A2 coexpressed with human P450 reductase in *Saccharomyces cerevisiae* metabolized aflatoxin B_1, 7,12-dimethylbenz[*a*]anthracene and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole.[54]

CYP6B1 (*Papilio polyxenes* Stoll)

CYP6B1 expressed in lepidopteran (Tn5) cells (using baculovirus) metabolized, in order of efficiency, bergapten, xanthotoxin, isopimpinellin, psoralen and 4,5′,8-trimethyl-psoralen. CYP6B1 had little or no activity towards sphondin, angelicin, xanthotoxol and imperatorin.[60,61]

CYP6B4 (*P glaucus*)

The substrates for CYP6B4 expressed in lepidopteran (Tn5) cells (using baculovirus) included, in the order of turnover efficiency, isopimpinellin, imperatorin, bergapten, xanthotoxin and psoralen. CYP6B4 had little or no activity towards sphondin, angelicin, 4,5′8-trimethyl-psoralen or xanthotoxol.[60]

CYP6D1 (*M domestica*)

The substrates of CYP6D1 have been investigated using house fly microsomes and CYP6D1 antibodies and by heterologous expression in yeast. CYP6D1 substrates included methoxyresorufin,[62,63] benzo[*a*]-pyrene,[63] chlorpyrifos, chlorpyrifos-oxon,[64] phenanthrene,[65] deltamethrin[66] and cypermethrin.[5,33] CYP6D1 had little activity towards ethoxyresorufin, pentoxyresorufin or ethoxycoumarin.[63]

CYP12A1 (*M domestica*)

Reconstitution of CYP12A1 expressed in *E coli* together with the bovine mitochondrial adrenodoxin reductase and adrenodoxin demonstrated that CYP12A1 metabolized aldrin, amitraz, azinphos-methyl, diazinon, heptachlor, progesterone, testosterone and 7-pentoxycoumarin. CYP12A1 had little or no activity towards aminopyrine, benzphetamine, cholesterol, cypermethrin, DDT, ecdysteroids, JH analogues, lauric acid or propoxur.[67]

7 LESSONS LEARNED FROM THE STUDY OF INDIVIDUAL INSECT P450S

The monooxygenases of insects have several functional roles, including growth, development, feeding and protection against xenobiotics, including resistance to pesticides and tolerance to plant toxins. Recent reviews have covered some of these topics in detail.[38,39,55] Furthermore, monooxygenases are intimately involved in the synthesis and degradation of insect hormones and pheromones, including 20-hydroxyecdysone and JH.[3,68–70] For example, inducers (or inhibitors) of P450s can produce changes in development, morphology and/or survival of holometabolus insects, undoubtedly because of the disruption of hormone titers via induction (or inhibition) of one or more P450s.[71,72] The amazing ability of P450s to metabolize a wide variety of compounds makes them the subject of active research in many fields. The isolation of individual P450s has (or has potential to) vastly improved our understanding of important areas of insect science. Three such areas are discussed below. We have not tried to make the list or the examples all-inclusive, due to space limitations.

7.1 Insecticide resistance

Insecticide resistance is the development by some insects in a population of an ability to survive doses of a toxicant which would prove lethal to the majority of individuals in a normal population of the same species.[73] Insecticide resistance has serious consequences, such as outright control failure, increased application rates, decreased yields, environmental contamination and wildlife and natural enemy destruction.[74] In monetary terms, the costs related to pesticide resistance are enormous—estimated to be $1.4 billion per year in the USA alone.[75]

P450-mediated detoxification is one of the most important mechanisms by which insects become resistant to insecticides.[1,74,76] This mechanism has been found in many important insect pests and may confer high levels of resistance.[77,78] Due to the broad substrate spectra of P450s, this mechanism may potentially affect several classes of insecticide and thereby confer cross-resistance to unrelated compounds.[1,74,76]

Although P450s have long been associated with insecticide resistance, identification of specific P450 isoforms responsible for insecticide resistance has proved difficult.[37] A recent comprehensive review covered P450s and their role in resistance, and this information will not be repeated here. Our current understanding of monooxygenase-mediated resistance is summarized below using CYP6D1 as an example.

The Learn pyrethroid resistant (LPR) strain of house fly was collected in 1982 from a dairy in New York following the introduction of permethrin for fly control. After permethrin selection the LPR strain became homozygous for the major mechanisms of resistance and attained extremely high levels of resistance to commonly used pyrethroid insecticides.[79] The highest levels of resistance occur towards pyrethroids with a phenoxybenzyl moiety (eg >5000-fold resistance to cypermethrin).[78,79]

In vitro,[5,33,66,78] *in vivo* [66] and synergism[78,80] studies all indicate monooxygenase-mediated detoxification is an important mechanism for pyrethroid resistance in the LPR strain. The two other mechanisms of resistance to pyrethroid insecticides in the LPR strain are target-site insensitivity (*kdr*) and decreased cuticular penetration (*pen*).[78,80,81] Monooxygenase-mediated detoxification appears to be the major mechanism of pyrethroid resistance in this strain.[78] Monooxygenase-mediated resistance maps to both autosomes 1 and 2, while *kdr* and *pen* map to autosome 3.[80–82]

In 1989 a single P450, termed $P450_{1pr}$, was purified from LPR house flies to apparent homogeneity using two HPLC steps.[83] Electrophoresis of house fly microsomes indicated that a protein band corresponding to $P450_{1pr}$ was expressed at elevated levels in LPR

compared to susceptible house flies.[84] An isoform-specific antiserum was raised in rabbits using purified $P450_{1pr}$ protein as the antigen[85] and this antiserum was used to characterize the expression of $P450_{1pr}$ (see below).

The gene for $P450_{1pr}$ was sequenced by PCR amplification using degenerate primers based on known $P450_{1pr}$ polypeptide sequences, and the remainder of the sequence was amplified by PCR.[44] This gene was designated *CYP6D1* within the P450 gene superfamily.[44] A large amount of evidence has accumulated indicating that *CYP6D1* is the $P450_{1pr}$ gene.[30,44,62,86–89]

To ascertain the role of CYP6D1 in pyrethroid resistance in the LPR strain, the P450-dependent metabolism of deltamethrin was investigated. Microsomes from LPR or susceptible house flies were treated with normal rabbit serum or anti-CYP6D1 antiserum. Monooxygenase-dependent deltamethrin metabolism occurred at increased levels in LPR microsomes compared to a susceptible strain.[66] Under these conditions virtually all deltamethrin was recovered unchanged from the susceptible strain in both the normal serum and antiserum treatments. However, 24% of the deltamethrin was metabolized by microsomes from LPR flies. This metabolism of deltamethrin could be reduced to 3% by the addition of anti-CYP6D1 antiserum, indicating that CYP6D1 is the major P450 responsible for deltamethrin metabolism in LPR flies.[66] Similar results have recently been observed for cypermethrin, and the primary CYP6D1-specific metabolite formed *in vitro* was identified by GC–MS as 4′-OH cypermethrin.[90] Thus, CYP6D1 carries out metabolism at a single site on the pyrethroid phenoxybenzyl moiety. This finding helps to explain the reduced levels of resistance to pyrethroids that do not have an unsubstituted phenoxybenzyl moiety.[78]

Using a b_5 antiserum it was demonstrated that b_5 was required for CYP6D1-mediated metabolism of cypermethrin in microsomes from LPR house flies.[5] Therefore, b_5 appears to be directly involved in CYP6D1-mediated pyrethroid resistance. The higher level of b_5 found in the LPR strain[81] and the linkage of this trait to the same autosomes as monooxygenase-mediated resistance[86] suggest that the elevated levels of b_5 may be a requisite for the enhanced metabolism of pyrethroids by CYP6D1 in LPR house flies.

Northern blots of RNA from adults of the LPR and an insecticide-susceptible strain revealed that a CYP6D1 mRNA was expressed at about a ten-fold higher level in LPR flies than in susceptible flies. This agreed with previous results of an eight-fold higher level of CYP6D1 protein in microsomes from LPR relative to those from susceptible flies.[85,86]

Southern blots of genomic DNA hybridized with a *CYP6D1* cDNA probe revealed similar hybridization intensities between LPR and a susceptible strain, indicating that the elevated level of CYP6D1 mRNA in LPR flies is not due to gene amplification.[44] Using allele-specific PCR *CYP6D1* was mapped to autosome 1.[91]

To investigate the role of mRNA stability in the high-level expression of CYP6D1 in the LPR strain, mRNA was isolated and quantified from susceptible and LPR house flies at different times following injection of a transcription inhibitor (actinomycin D). The level of CYP6D1 mRNA decreased over time, indicating that actinomycin D was inhibiting transcription in both strains. The same pattern of decrease in CYP6D1 mRNA abundance (approximate half-life of ~10h) was detected in both LPR and susceptible strains, indicating that the increased expression of *CYP6D1* in LPR is not due to increased stability of the mRNA.[92]

The relative transcription rates of *CYP6D1* in LPR and a susceptible strain were measured using an *in vitro* run-on transcription assay.[92] When nuclei from the susceptible strain were used in the run-on assay only a trace of CYP6D1 mRNA was detected. In contrast, when nuclei from the LPR strain were used, abundant CYP6D1 mRNA was detected. Quantitation of the relative intensities of the CYP6D1 signals between a susceptible strain and LPR revealed approximately a ten-fold difference.[92] This is comparable with the differences in *CYP6D1* expression observed by Northern hybridization.[30,86] This was the first direct evidence for increased transcription as an underlying cause of insecticide resistance.[92]

The increased rate of transcription of *CYP6D1* was due to factors on autosomes 1 and 2 in the LPR strain.[92] This is consistent with what is known about the linkage of monooxygenase-mediated resistance[80,81] and overexpression of CYP6D1 mRNA and protein levels.[86] It is interesting that resistance (via increased transcription of *CYP6D1*) is mediated both by *cis* and *trans* regulatory factors.[91]

Recently the CYP6D1 5′ flanking sequences from LPR and five pyrethroid-susceptible strains were reported[93] and some differences were observed that could play a role in the differential expression of CYP6D1 in these strains. However, much more study will be needed before the mechanisms underlying the increased transcription of *CYP6D1* in LPR are fully understood.

In addition to the LPR strain, *CYP6D1* was sequenced from four pyrethroid susceptible strains of house flies. Comparison of the five *CYP6D1* alleles reveals that the deduced protein sequence from the LPR allele differs from that of the CS, aabys, ISK and Rutgers (strain not homozygous) alleles by 8, 11, 7 and 6–7 amino acids, respectively.[30] Among them, five amino acids are the same in CS, aabys, ISK and Rutgers, but are different from LPR: Asp to Ala (150), Ile to Leu (153), Thr to Ser (165), Glu to Gln (218) and Met to Ile (227).[30] The observed amino acid substitutions occur at two highly variable regions among cytochromes P450 in family 6, and the changes at residues 218, 220, 225 and 227 are close to a proposed substrate binding region.[94] Whether or not

the amino acid differences among the CYP6D1 proteins result in different catalytic activity towards pyrethroid insecticides remains to be elucidated.

CYP6D1 mRNA expression is developmentally regulated with no CYP6D1 mRNA detectable in eggs, larvae or pupae.[88] High levels of mRNA were found in adults from 1 to 6 days old. The low levels of expression observed in day 3 pupae may be attributed to pharate adults in this sample. This pattern matches that observed for the CYP6D1 protein.[29] Results from studies on individual tissues indicate that CYP6D1 protein is found in many tissues throughout the house fly abdomen.[35,95] The relative abundance of CYP6D1 protein in microsomes from 3- to 5-day-old female house flies decreased in the following order: fat bodies >proximal intestine > reproductive system > rectum in both LPR and susceptible strains.[35,95] There was more CYP6D1 protein detected in each of the tissues from LPR than in those from the susceptible strain.[35,95] Recently it was demonstrated that CYP6D1 is expressed in all tagmata and in thoracic ganglia of the house fly. The level of expression of CYP6D1 at each of these sites was higher in LPR than in susceptible strains.[33] These results suggest that there is no single tissue or site within the LPR house fly that is responsible for pyrethroid resistance.

CYP6D1 was found to be strongly inhibited by xanthotoxin, chlorpyrifos, β-naphthoflavone, piperonyl butoxide, 5-methoxypsoralen,[96] alkynylpyrenes and substituted phenanthrenes.[97] The highest selectivity for inhibition of CYP6D1 (compared to other P450s) was seen for 5-methoxypsoralen, xanthotoxin, β-naphthoflavone, chlorpyrifos-oxon, isosafrole and psoralen.[96] These studies demonstrated that identification of isoform selective inhibitors of P450s within an insect, and between species, was possible. In addition, isosafrole and verbutin were shown to be potent synergists of pyrethroid insecticides in adult house flies.[96,97]

CYP6D1-mediated pyrethroid resistance was recently discovered in house flies from Georgia, where this mechanism had apparently evolved over the last 15 years.[98] This suggests that this resistance mechanism may have evolved once and then spread, as has been proposed for esterase-mediated resistance in mosquitoes.[99] However, it is unknown how geographically widespread (beyond the Eastern USA) CYP6D1-mediated pyrethroid resistance is.

7.2 Plant–insect interactions

Plants and herbivores are constantly at war in an evolutionary arms race. Plants evolve to become less hospitable to herbivores, while herbivores evolve to cope with changes in their host plants or to find ways to exploit new hosts. Due to their abundance, diversity and highly variable ranges of host specificity, insects are an ideal group of organisms for researchers studying this process of co-evolution. P450s are important in this process of 'plant–insect warfare' because they are used both by plants (to produce toxins) and insects (as a means of detoxification).[39] While the role of P450s as a mechanism of defense against plant allelochemicals has been well documented,[100,101] identification of the individual P450s involved in the detoxification of plant allelochemicals is quite recent. Perhaps the best-studied P450s involved in metabolism of host plant allelochemicals are those in the *CYP6B* subfamily. These studies are briefly summarized below.

Furanocoumarins are plant allelochemicals found in at least eight plant families. They are highly toxic to a wide variety of organisms, including bacteria, plants, insects, fish, birds and mammals because they are capable of reacting directly and irreversibly with pyrimidine bases in DNA after photoactivation.[102,103] Two types of furanocoumarin occur in plants: linear (eg xanthotoxin and bergapten) and angular (eg angelicin and sphondin). Despite this chemical defense mechanism, insects in the genus *Papilio* are still able to feed on furanocoumarin-containing plants. Insects adapted to feeding on plants containing linear furanocoumarins are not necessarily adapted to feeding on plants containing angular furanocoumarins.[102]

P polyxenes feeds almost exclusively on plants containing furanocoumarins and can tolerate a diet containing up to 0.1% xanthotoxin.[104] In this species, xanthotoxin can be detoxified by P450 monooxygenases,[105] and it also induces P450 activities approximately five-fold.[106] In 1992, *CYP6B1* was sequenced from *P polyxenes* and was shown to be xanthotoxin-inducible.[42] Two *CYP6B1* alleles (deduced amino acid sequences differ by nine amino acids) have been described.

Expression of *CYP6B1v1* and *CYP6B1v2* in lepidopteran cell lines using a baculovirus expression system indicates that both alleles code for P450s that metabolize linear furanocoumarins, such as xanthotoxin and bergapten, but not angular furanocoumarins such as angelicin and sphondin.[61] This provides clear evidence for the role of CYP6B1 in the detoxification of xanthotoxin and in tolerance to plant allelochemicals. RNase protection assays and Northern blot analysis indicated that transcription of *CYP6B1* in larvae could be significantly induced by xanthotoxin, slightly induced by bergapten, but could not be induced by angular furanocoumarins.[107,108] Given that angular furanocoumarins can be metabolized by *P polyxenes*, these results suggest the presence of another P450(s) capable of metabolizing angular furanocoumarins.

CYP6B3 was the second P450 sequenced from *P polyxenes*. This P450 is 88% identical to *CYP6B1* (based on deduced amino acid sequences). Reverse transcription-PCR Southern analysis indicated that CYP6B3 transcripts were induced by both linear and angular furanocoumarins.[109] The substrates of CYP6B3 have not yet been reported. Thus, *P polyxenes* appears to have adapted to feeding on toxin-containing host plants through a diversification of the P450s

involved in detoxification and through its furanocoumarin-responsive regulatory cascades.

To investigate the evolutionary relationships between furanocoumarin-metabolizing P450s, comparisons were made between the *CYP6B* P450s of a species with a relatively narrow host range (*P polyxenes*) and a species with a much broader host range (*P glaucus*). CYP6B4 and CYP6B5, two nearly identical P450s (99.8% identical, based on deduced amino acid sequence), are present in *P glaucus*[110] and are 61–63% similar to CYP6B1 and CYP6B3 from *P polyxenes*. Comparisons of the structure and promoter sequence of these genes reveal that they all have the same intron/exon arrangement and similar promoter sequence.[110] The similarities in the structural and promoter regions suggest that these insect *CYP6B* genes are derived from a common ancestral gene.

7.3 Insect physiology

Insect hormones (eg JHs, ecdysteroids, etc) are very important in insect growth, development and reproduction[111] and often P450s play critical roles in the metabolism of these compounds. One of the best examples is the conversion of ecdysone to 20-OH ecdysone. Although it is unclear if this P450 activity is found in microsomes,[112] mitochondria,[113–115] or both,[116] identification of the P450(s) involved will help to enhance our understanding of the regulation of this important hormone. Unfortunately, efforts so far have not succeeded in identifying the P450(s) responsible for the conversion of ecdysone to 20-hydroxyecdysone (P450s with this putative function have been identified). To date, only one individual P450, CYP4C7 from *D punctata*, has been clearly shown to be involved in the metabolism of an insect hormone (JHs and their analogues).[56] Clearly, given the importance of P450s in the metabolism of insect hormones, this is an area that deserves substantial attention. The recent isolation of the first sex-specific and tissue-specific insect P450s,[32,36] as well as the use of microarrays to investigate P450 expression during development[31] suggests that many breakthroughs in this area are on the horizon.

8 CONCLUSIONS

In the last decade we have seen an explosion in our knowledge of insect P450s. Determination of the number of P450s in an insect species, a feat that once seemed impossible, has now been accomplished. But these studies are just the 'tip of the iceberg'. Our knowledge continues to expand at a rapid pace, suggesting that the next decade will outpace the last in terms of improving our understanding of the cytochrome P450s of insects.

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