The Role of Cytochrome P450s in Insect Toxicology and Resistance

Ralf Nauen,[1*] Chris Bass,[2] René Feyereisen,[3,4] and John Vontas[5,6]

[1] Bayer AG, Crop Science Division, R&D, Alfred Nobel Str. 50, D-40789 Monheim, Germany; ralf.nauen@bayer.com

[2] College of Life and Environmental Sciences, Biosciences, University of Exeter, Penryn Campus, Penryn, Cornwall, UK; C.Bass@exeter.ac.uk

[3] Department of Plants and Crops, Faculty of Bioscience Engineering, Coupure Links 653, Ghent University, B-9000 Ghent, Belgium; rene.feyereisen@gmail.com

[4] Department of Plant and Environmental Sciences, University of Copenhagen, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Copenhagen, Denmark

[5] Department of Crop Science, Agricultural University of Athens, 75 Iera Odos Street, GR-11855 Athens, Greece; vontas@imbb.forth.gr

[6] Institute of Molecular Biology & Biotechnology, Foundation for Research & Technology Hellas, 100 N. Plastira Street, GR-700 13 Heraklion, Crete, Greece

*Corresponding author:

Dr Ralf Nauen, email: ralf.nauen@bayer.com

ORCID: 0000-0002-7525-8589

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Abstract

Insect cytochrome P450 monooxygenases (P450s) perform a variety of important physiological functions but it is their role in the detoxification of xenobiotics, such as natural and synthetic insecticides, that is the topic of this review. Recent advances in insect genomics and post-genomic functional approaches have provided an unprecedented opportunity to understand the evolution of insect P450s and their role in insect toxicology. These approaches also have been harnessed to provide new insights on the genomic alterations that lead to insecticide resistance, the mechanisms by which P450s are regulated and the functional determinants of P450-mediated insecticide resistance. In parallel, an emerging body of work on the role of P450s in defining the sensitivity of beneficial insects to insecticides has been developed. The knowledge gained from these studies has applications for the management of P450-mediated resistance in insect pests and can be leveraged to safeguard the health of important beneficial insects.

Keywords: cytochrome P450, resistance, tolerance, insect, insecticide, toxicology

1. INTRODUCTION

Cytochrome P450s (encoded by the *CYP* genes) are a remarkable superfamily of enzymes found in all kingdoms of life that can catalyze a diverse array of oxidative transformations of both endogenous and exogenous substrates (78). This includes the detoxification of pesticides in crop pests and disease vectors leading to resistance (23, 112), and in bees (7), where P450s have been recently shown to act as key determinants of insecticide selectivity (6, 65). The recent explosion in research on the complete complement of P450 genes, the "CYPome", in arthropods is, in part, a reflection of the dramatic increase in the number of invertebrate genomes sequenced (86). Furthermore, the development of advanced protein and genetic approaches for the functional characterization of P450s has provided unprecedented opportunities for researchers to study their regulation, molecular physiology and functional relevance for xenobiotic metabolism.

2. GENOMIC INSIGHTS INTO THE DIVERSITY OF P450s ASSOCIATED WITH PESTICIDE RESISTANCE

The "CYPome" of most arthropods generally comprises between 60-100 genes, from lows of 23 in the eriophyoid mite *Aculops lycopersici* and 36 in the body louse *Pediculus humanus* to well over 200 in some ticks, collembolans and culicine mosquitoes (23). CYPomes are commonly composed of few CYP families with many genes and many CYP families with few, often single copy genes. In insects, most of the single copy genes belong to the CYP2 clan and mitochondrial CYP clan, and most of the multiple copy paralogs belong to the CYP3 and CYP4 clans (23, 29), and these are often arrayed in clusters on chromosomes (100). Many of the closely paralogous genes are part of lineage-specific family expansions, called P450 blooms (28). The P450s that have been implicated in xenobiotic metabolism and pesticide resistance are often found in such blooms.

These notably include: members of the CYP6 and CYP9 families in *Anopheles* and *Aedes* mosquito disease vectors (112); members of the lepidopteran-specific CYP6AE subfamily in *Helicoverpa armigera* (101, 116); members of the CYP392 family in *Tetranychus urticae* (108); *Tribolium castaneum* CYP6BQ9 (125); *Bemisia tabaci* CYP6CM1 (50); *Myzus persicae nicotianae* CYP6CY3 (5) and *Apis mellifera* CYP9Q3 and CYP9Q2 (65). A variety of different P450s have been associated with resistance across mosquito species and geographical regions (112). In contrast, pesticide resistance mediated by P450s in several agricultural pests appears to more commonly result from the same P450s being selected across different settings and continents (47, 87, 113). There is no apparent relationship between the phylogenetic relatedness and the catalytic competence of pesticide/xenobiotic metabolizing P450s (23). While pesticide/xenobiotic metabolizing P450s are currently found mostly in the CYP6 and CYP9 families of the CYP3 clan, this is biased by early work on fly and mosquito insecticide resistance. In fact, such P450s are present in all four major clans, like the CYP2 clan in mites, often in very close phylogenetic proximity with P450s acting on endogenous substrates and biosynthetic pathways, i.e. there is no distinct clade for P450s involved in pesticide resistance (23).

Because assigning functions to P450 enzymes is technically demanding (see section 4), it is difficult to predict which P450 is most likely to metabolize a pesticide in any of the many insect pest species. The majority of the P450s in the insect model *Drosophila* are of unknown function (i.e. "orphans"), but about a third of the CYPome has been implicated in xenobiotic metabolism (96). Some P450 blooms (and genomic clusters) may be relatively ancient, so that they can be recognized as orthologous, and indeed, resistance linked P450s are found in both the *Ceratitis capitata* and *Musca domestica* CYP6A blooms. This genomic clustering can facilitate functional screening by knockout (116) as will be

described below. More importantly perhaps, only a subset of P450 genes are transcriptionally inducible by xenobiotics, or constitutively overexpressed in pesticide-resistant strains, thus often restricting the search for genes involved in xenobiotic tolerance or resistance.

3. CHANGES RESPONSIBLE FOR P450-MEDIATED RESISTANCE OR TOLERANCE

Changes leading to P450-based pesticide resistance (a selected, heritable change) or tolerance (a reversible, physiological change) may result from constitutive or induced changes in P450 expression (respectively) that increase the amount of P450 available to metabolize an insecticide or via qualitative changes that enhance the capacity of a P450 to utilize an insecticide as a substrate (Figure 1). Less frequent, and only recently detected at the molecular level, downregulation of a P450 involved in pro-pesticide activation can also mediate resistance.

3.1 Quantitative changes

3.1.1 Changes responsible for constitutively higher P450 expression levels

Constitutive quantitative changes in the expression of one or more P450 genes is one of the most common mechanisms underpinning insect resistance to xenobiotics and may result from *cis*-acting and/or *trans*-acting regulatory factors or gene duplication/amplification (Figure 1). *Cis*-acting regulators are sequence elements located in proximity to the gene itself, while *trans* regulators are diffusible elements, often transcription factors, that may be encoded anywhere in the genome, and may affect more than one target gene.

3.1.1.1 *Cis*-regulatory transcriptional factors

A landmark study of P450 resistance mediated by *cis*-regulatory change was the characterization of mechanisms leading to the overexpression of CYP6G1 in DDT resistant *Drosophila* (20, 97; reviewed in 56). Overexpression was initially reported to be mediated by the insertion of an Accord transposable element upstream of the CYP6G1 gene, with subsequent work revealing alleles of this P450 with additional transposable element insertions (20, 97). Since this study, *in silico* analyses have revealed that transposable elements (TEs) are commonly enriched within, or in close proximity to, xenobiotic metabolizing P450 genes (10, 13). A range of other mutations in P450 regulatory regions have also been shown to lead to P450 overexpression and resistance (84, 114, 127). These include point mutations, such as the single nucleotide substitution located near the transcription start site of CYP9M10 (44), and larger indels such as the expansion of a dinucleotide microsatellite in the promoter of CYP6CY3 in nicotine and neonicotinoid resistant peach potato aphid, *M. p. nicotianae* (5).

3.1.1.2 *Trans*-acting regulating factors

More than two decades ago studies first linked the overexpression of P450s that confer insecticide resistance to *trans*-acting factors. For example, in *M. domestica*, constitutive overexpression of CYP6A1, which confers diazinon resistance and maps to chromosome 5, was shown to be controlled by one or more loci located on chromosome 2 (11) at, or close to, the ali-esterase (MdαE7) gene (93). However, the precise *trans*-acting genetic change(s) involved has not been definitively identified. Surprisingly, despite advances in insect genomics, the nature of the specific *trans*-acting mutations

that lead to constitutive P450 upregulation continues to remain an important knowledge gap. Despite this, recent work has significantly enhanced our understanding of the role of transcription factors and their binding sites in regulating P450s and other detoxification genes implicated in resistance (reviewed in 2). These are found in three main superfamilies: nuclear receptors (NRs), basic-helix-loop-helix/per-ARNT-SIM (bHLH-PAS) and basic-leucine zipper (bZIP) (73). RNAi knockdown of members of all three superfamilies in the red flour beetle, *T. castaneum*, revealed that it is the bZIP transcription factor Cap 'n' Collar isoform-C (CncC) and its heterodimer partner Muscle Aponeurosis Fibromatosis (Maf), which regulate P450s of the CYP6BQ subfamily that confer resistance to pyrethroids (48, 49). Since then CncC/Maf have been implicated in the upregulation of P450s in a range of insect and mite species (recently reviewed in 79, 119), further emphasizing their important role as key regulators of xenobiotic metabolizing P450s. Whether the CncC/Maf pathway chiefly regulates some P450s in response to oxidative stress caused by the toxicant as recently reported in *Spodoptera litura* (64), or whether it also operates in induction of P450s by non-toxic chemicals is currently unknown. Interestingly, CncC, and indeed other transcription factors, are often themselves constitutively upregulated in resistant arthropods that overexpress resistance-conferring P450s (see 119 and references therein). Other transcription factors (73) have been shown or implicated in the regulation of insect P450s, including the nuclear receptor HR96 (54). Most recently, cAMP-response element binding protein (CREB) (122), has been shown to regulate CYP6CM1, a P450 that confers resistance to several insecticides in the whitefly, *B. tabaci* (50). Like CncC/Maf, CREB is constitutively upregulated in a resistant strain (122) but, as for previous studies, the genetic alteration leading to the observed overexpression remains unknown.

3.1.1.3 Upstream regulators and signal transduction

The transcription factors that regulate P450s may be themselves regulated by upstream signaling pathways. Studies of pyrethroid resistance in the mosquito, *Culex quinquefasciatus*, first implicated Rhodopsin-like G protein-coupled receptors (GPCRs), membrane proteins that detect a range of molecules outside the cell and activate signal transduction pathways, in regulating P450s involved in resistance (57-59). Subsequent studies suggested that GPCRs can upregulate P450s via a signaling pathway comprising GPCR/ Gs *alpha* subunit protein (Gαs)/ adenylyl cyclase (ACs)/ 3,5-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKAs). In the case of the regulation of CYP6CM1 in *B. tabaci* by the transcription factor CREB, a marked increase was observed in the activated (phosphorylated) form of this transcription factor in resistant *B. tabaci* (122). Further work with inhibitors, RNAi and biochemical assays provided strong evidence that the mitogen-activated protein kinases (MAPK) ERK and p38 positively regulate CYP6CM1 expression by phosphorylating CREB (122), implicating the MAPK signaling pathway in the regulation of this P450.

3.1.1.4 Gene duplication and amplification

Several studies have demonstrated that structural duplication or amplification of P450 genes can play an important role in the evolution of insecticide resistance (5, 43, 97, 117, 120, 127). In most of these cases gene duplication/amplification has an adaptive role by increasing gene dosage.

The mechanisms by which P450s may be duplicated or amplified in resistant strains are not fully understood, however, recent work has shown they can be copied as part of large or small amplicons. For example, *CYP9M10* is duplicated as part of an amplicon of ~100 kb in length in resistant *C. quinquefasciatus* (43), and *CYP6CY3* and *CYP6CY4* are amplified 3-5-fold as a part of a region approximately 325 kb in length in *M. p. nicotianae* (102). In the latter example additional copies of

CYP6CY3 were found at a novel locus as part of a much smaller (~14 kb) amplicon associated with two transposable element insertions. These nested insertions occur immediately adjacent to the 5' breakpoint, strongly suggesting they played a role in the mobilization of *CYP6CY3* to the new loci, either indirectly by acting as substrates for non-allelic homologous recombination or directly via alternative transposition (102). The work on *CYP6CY3* and other insect P450s has also demonstrated that duplication may act in concert with other cis-acting mutational events to progressively increase the expression of key resistance genes. An elegant example of this comes from work on *Drosophila* CYP6G1, where an allelic series comprising at least four sequential mutations including gene duplication and at least three transposable element insertions were shown to progressively increase resistance to DDT (97). Another, more recent, study in *Spodoptera frugiperda* (fall armyworm) suggested that the adaptive evolution of CYP9A genes by copy number variation mediates deltamethrin resistance (32).

3.1.2 P450 induction

P450 genes may also be induced upon exposure to a xenobiotic (19, 33, 60, 62, 109). However, while induced tolerance to plant allelochemicals is common (109), a similar protection afforded by exposure to a pesticide is not. This is likely because insecticides applied at (high) recommended label rates may not allow sufficient time for protective P450s to be upregulated in the window between exposure and irreversible toxicity. In contrast, in the case of exposure to host plant toxins, insects may control (i.e. limit) dose by modifying feeding behavior (104). Induction of insect P450s by plant chemicals has long been known (8), and there are now several well characterized examples of P450 induction to overcome plant chemical defenses, such as the CYP6B genes in *Papilio* (swallowtail butterflies) by furanocoumarins which have xanthotoxin-responsive elements in their promoters (17, 31, 40, 61, 68), and CYP6AE89 of parsnip webworm, *Depressaria pastinacella*, which is induced by bergapten (9). Examples of induced expression of P450s leading to marked changes in toxicity of synthetic insecticides are rarer. While a range of insecticides have been shown to induce P450 genes (usually when applied at sub-lethal doses) (39; see 62 and references therein), the practical significance of the level of resistance conferred has been less well established. Furthermore, if differential induction is not a heritable trait in resistant strains, then induction is not resistance *sensu stricto*, but transient tolerance.

Recent work has also revealed a link between insect and mite host plant adaptation, P450 induction and sensitivity to synthetic insecticides. In both the two-spotted spider mite, *T. urticae*, and the whitefly *B. tabaci*, transcriptome profiling revealed that transfer to more or less challenging host plants resulted in significant changes in tolerance to synthetic insecticides with up to 50-fold shifts in sensitivity observed to certain compounds (22, 85). Finally, a growing body of work has also demonstrated that P450s can be induced by non-insecticidal xenobiotics in the environment. For example, exposure of larvae of the mosquito *Aedes aegypti* to xenobiotics including the herbicide atrazine, the polycyclic aromatic hydrocarbon fluoranthene and copper induced a range of P450 genes and increased the tolerance of larvae to subsequent pyrethroid exposure (albeit modestly) (83).

3.1.3 Decreased P450 expression mediated resistance

A coumaphos-resistant *Varroa destructor* population was recently shown to escape organophosphate toxicity via reduced pro-insecticide activation, by downregulating one of the only 26 P450s in its genome, CYP4EP4, leading to resistance to the organophosphate coumaphos (110). This case study is discussed further in section 4.2.2.

3.2 Qualitative changes in P450 coding sequences

3.2.1 P450 allelic variants

Qualitative changes that cause resistance by modifying the catalytic activity/metabolic profile of P450s are less commonly reported than quantitative changes in P450 expression. An early report of point mutations enabling DDT resistance in a *Drosophila* P450 (3) has been invalidated by a transgenic approach (29, 81). However, work on the mosquito *Anopheles funestus* has identified allelic variants of CYP6P9a and CYP6P9b in resistant populations with multiple amino acid alterations that enhance their activity against pyrethroids (41). Work on CYP6ER1, which is overexpressed in brown planthopper, *Nilaparvata lugens*, populations that are resistant to neonicotinoid insecticides, has provided another example of how qualitative changes in P450s can evolve from ancestral enzymes that lack the capacity to break down insecticides (127). In this case the variants overexpressed in resistant populations across Asia are characterized by profound amino-acid alterations in substrate recognition sites that confer the capacity to detoxify the neonicotinoid imidacloprid. In addition, CYP6ER1 is duplicated in resistant strains with resistant individuals carrying one copy with the gain-of-function mutations and one without. This observation strongly suggests that gene duplication was required to free CYP6ER1 from functional constraint and permit the acquisition of mutations that led to the novel function (resistance). Interestingly, in this example of P450 resistance by neofunctionalization the 'resistant' CYP6ER1 copy is highly overexpressed relative to the wild-type copy in resistant individuals. This finding, together with previous studies (41, 71), demonstrates that qualitative and quantitative changes are not mutually exclusive and may act in tandem to enhance P450-mediated resistance.

3.2.2 Gene conversion

Improvement or acquisition of pesticide-metabolizing activity via qualitative changes may be constrained by the number of mutations required for gain-of-function, or the requirement to preserve the native function of the enzyme. Recent work has highlighted novel mechanisms by which P450s have escaped these constraints. More complex than a single non-silent nucleotide change, a recent example of gene conversion involving two adjacent, recently duplicated P450 genes was reported in the cotton bollworm *H. armigera*. Resistance to pyrethroids was found to result from a chimeric P450 gene, CYP337B3 (47), which has arisen multiple times (87, 113) by unequal crossing-over between two parental P450 genes. The unique amino acid sequence of CYP337B3 is directly responsible for its ability to metabolize pyrethroids, as neither parental enzyme has this ability *in vitro* (47).

4. FROM GENE TO FUNCTION: TOOLS AND MECHANISMS OF RESISTANCE

Despite the substantial progress achieved in the molecular analysis of P450-mediated insecticide resistance in arthropods and the identification of several P450s that are associated with the trait, their exact role and their actual contribution to the phenotype remains largely unknown. *In silico* approaches, recombinant expression and functional *in vitro* characterization of P450s, RNA interference (RNAi)-based reverse genetics, *in vivo* overexpression or genetic knock out have provided different levels of validation for the involvement of P450s in insecticide resistance.

4.1 Tools for functional characterization of P450s

4.1.1. *In vitro* validation by recombinant protein expression

Several expression systems and strategies including modifications of the protein sequence or electron delivery method have been employed for successful P450 functional expression in arthropods. These have been recently reviewed in (77), including a critical summary of the importance of selection of host organisms, strains and vectors, N-terminal modifications and optimization of NADPH-cytochrome P450 reductase (CPR) coupling.

Some recent examples of successful expression and biochemical characterization of recombinant P450s in different systems include *A. gambiae* CYP6P3, CYP6M2 and CYP9K1, which were expressed in *E. coli* with a N-terminus OmpA signal peptide to direct the P450 to the bacterial membranes (72, 111, 123); the entire *A. mellifera* CYP3 clan (encompassing 27 CYP genes) including CYP9Q1, CYP9Q2, CYP9Q3 (65, 66), *N. lugens* CYP6ER1 (127), *Laodelphax striatellus* CYP6AY3v2 (115), *P. humanus* CYP6CJ1 (53) and the successful expression of *H. armigera* CYP6AEs in insect cells using the baculovirus expression system (101); *H. armigera* CYP337B3 (47) and *B. tabaci* CYP6CM1 expressed in stable cell lines (35), and *A. aegypti* CYP6Z8 which was expressed in *Saccharomyces cerevisiae*, in combination with *A. aegypti* CPR in the yeast genome (14).

These studies illustrate how *in vitro* characterization approaches can be used to validate differentially regulated P450s or different allelic variants associated with resistance. However, the precise prediction of the resistance phenotype conferred by a P450 variant *in vivo* from its activities *in vitro* and levels of differential expression is not a trivial task (112).

4.1.2 Approaches for *in vivo* functional validation

Functional validation of the role of P450s in resistance *in vivo* has been achieved by RNAi-based suppression, transgenic overexpression or P450 gene knockouts. For example, RNAi silencing of the pyrethroid metabolizer CYP6BQ9 in *T. castaneum* resulted in a dramatic drop (>100 fold) in resistance (125). Silencing of *CYP6ER1* by RNAi in *N. lugens* was also used to suggest that the expression of CYP6ER1 is sufficient to confer neonicotinoid resistance (80, 106). While RNAi is a quick and easy way to provide reliable functional links between certain P450s and resistance *in vivo*, it does not work equally efficiently in all insect orders (18, 52).

The integration of the molecular genetic toolbox developed in *Drosophila* (24, 99) into P450 resistance research has facilitated approaches such as conditional expression *in vivo* for the validation of P450 genes (reviewed in 38, 82). One important tool is the bipartite GAL4/UAS expression system (reviewed in 25) which allows the temporal and tissue-specific ectopic expression of *CYP* genes in *Drosophila*. Examples include the ectopic expression of the *A. gambiae* P450s CYP6M2 and CYP6P3, *A. funestus* CYP6P9a and CYP6P9b (27, 41, 91), and P450s from agricultural pests, such as CYP6CM1 (*B. tabaci*), CYP6AY3v2 (*L. striatellus*) and CYP6ER1 (*N. lugens*) (82, 115, 127). Although this approach has been shown to be a useful tool for validating the functional role of candidate pest and pollinator P450s (e.g. 65, 107), the altered levels of toxicity achieved in transgenic *Drosophila* are frequently much lower than those observed in the native species (69). Recently Samantsidis et al. (95) generated transgenic *Drosophila* lines expressing pyrethroid metabolizing P450s, along with engineered mutations in the voltage-gated sodium channel (*para*) and showed that these mechanisms acted synergistically. This confirmed previous hypotheses that combinations of P450s with other co-selected resistance factors may be necessary to provide high levels of resistance (103).

The utility of GAL4/UAS-based tools to characterize P450s directly in non-model species has been demonstrated and allows the resistance-conferring capacity of a candidate P450 to be examined in its native environment. For example, these tools validated insecticide resistance phenotypes conferred

by increased expression of two P450 genes (*CYP6M2* and *CYP6P3*) in *A. gambiae* (1). In agricultural pests, a genome editing CRISPR/Cas9-based reverse genetics approach was used in the cotton bollworm *H. armigera* to knock out a cluster of nine genes of the CYP6AE subfamily, and showed that this significantly increases sensitivity against two classes of insecticides and phytochemicals (116).

4.2 Functional/biochemical mechanisms of P450-mediated resistance

4.2.1 Increased metabolism of insecticides to less toxic metabolites

In general, P450 metabolism converts a substrate into a more polar product or introduces a functional group facilitating conjugation, thus rendering the insecticide molecule more excretable and less toxic (29). Overexpression of a P450 that metabolizes an insecticide can therefore tip the toxicokinetic balance towards resistance. For instance, populations of the pollen beetle, *Meligethes aeneus*, can vary widely in their resistance levels to pyrethroids, and increasing expression levels of a single P450 gene, *CYP6BQ23* are sufficient to explain both increasing deltamethrin detoxification and resistance levels (126).

The cross-catalytic spectrum of P450s involved in insecticide resistance is variable and unpredictable. For example, *A. gambiae* CYP6P3 metabolizes both α-cyano and non–α-cyano pyrethroids (72), as well as the carbamate bendiocarb (123), while CYP6M2 metabolizes pyrethroids and DDT (70). The activity of ten *H. armigera* CYP6AEs towards ten different substrates is highly variable, apparently following no pattern of sequence similarity (23, 101, 116). Similarly, *B. tabaci* CYP6CM1 has a wide spectrum of activity against many, but not all, neonicotinoids (92), as well as pyriproxyfen and pymetrozine (75, 76). However, in the mite *T. urticae*, certain P450s seem to have a specialized catalytic role against specific acaricides, for example, CYP392A16, CYP392A11 and CYP392E7 metabolize abamectin, METIs and spirodiclofen, respectively (21, 88-90), but not other compounds from different insecticide classes. Interestingly, four members of the large CYP9J subfamily in *A. aegypti*, can metabolize pyrethroids with similar catalytic efficiencies (105), demonstrating that these P450s have a considerable degree of functional redundancy in terms of xenobiotic metabolism.

4.2.2. Reduced propesticide activation

Propesticides need to be bioactivated either *in planta* or pests to become intrinsically active (94). Decreased bioactivation of propesticides as a mechanism of resistance is rare, but a growing tendency in propesticide development may change this. Early reports provided biochemical support for such a mechanism (55), however, it has only recently been unequivocally demonstrated. Investigation of resistance to the acaricide coumaphos in *Varroa* mites (110) by transcriptome analysis revealed the underexpression of *CYP4EP4* in resistant mites. Subsequent functional validation by RNAi-mediated silencing of CYP4EP4 in the susceptible population, to mimic underexpression, prevented coumaphos activation, and substantially decreased coumaphos toxicity, confirming that the suppression of the P450-mediated activation step caused resistance (110). Similarly, bioactivation of the neonicotinoid nitenpyram by CYP12A5, a mitochondrial P450 in *Drosophila* (36), suggested that underexpression of such P450s may be found to underlie nitenpyram resistance.

4.2.3. Identification of key amino acid determinants of insecticide metabolism

Despite progress in expression and characterization of arthropod P450s, there are very few studies identifying the key amino acid determinants of insecticide metabolism or explaining the functional

contribution of point mutations to insecticide resistance (98). Docking of fenvalerate isomers into the active center of the *H. armigera* CYP337B3 by comparison with SRSs (substrate recognition sites) of CYP6Z1 of *A. gambiae* suggested 9-10 amino acids that could explain differences in substrate specificity with its parental enzymes, of which Thr102 may be essential for fenvalerate recognition and binding (47). Site-directed mutagenesis and functional analyses demonstrated that three amino acid changes (Val109Ile, Asp335Glu and Asn384Ser) from the resistant allele of the *A. funestus* CYP6P9b were key mutations for inducing high metabolic efficiency (41). Unfortunately, our current ability to predict and understand the role of key determinants in the substrate specificity of a P450 for insecticides is hindered by the lack of a crystal structure for any insect P450.

4.2.4. Indirect mechanisms of resistance

In the examples described above, P450s play a role in pesticide resistance by direct metabolism of the toxin. However, recent work has provided evidence that they can also play indirect roles in insecticide resistance. Transcriptome profiling of the mosquitos *A. gambiae* and *A. arabiensis* has shown that two CYP4G subfamily genes, *CYP4G16* and *CYP4G17* are frequently overexpressed in resistant populations (42, 46). These P450s were subsequently shown to be oxidative decarbonylases that catalyze the last step in cuticular hydrocarbon (CHC) synthesis (4, 51). Biochemical analysis revealed that the cuticle of resistant mosquitoes is thicker and has a significantly increased CHC content compared to susceptible mosquitoes, and this was associated with a significantly reduced rate of pyrethroid penetration through the cuticle (4). Taken together, these and other findings (reviewed in 30) strongly suggest that the overexpression of P450s of the CYP4G subfamily, mostly in insect oenocytes, may play an indirect role in resistance by enhancing CHC production which was shown to reduce insecticide penetration. Furthermore, control of CYP4G expression by other P450s, such as *CYP303A1* in *Locusta migratoria* (121) suggests a regulatory cascade in CHC production and thus indirectly in insecticide penetration rates.

5. LOCALISATION AND PHYSIOLOGY OF P450s MEDIATING RESISTANCE

5.1 Spatial expression of P450s associated with resistance

Just as important as how much of an insecticide metabolizing P450 is expressed, is where and when that P450 is expressed. Indeed, recent work has illustrated the extraordinary spatial- and temporal-specific expression exhibited by some insecticide metabolizing P450s and how this can change under insecticide selection (e.g. 39). Studies on several insect species have revealed that P450s associated with insecticide metabolism are not only expressed in “first line of defense" tissues that are involved in xenobiotic detoxification, but also at sites of insecticide action. Thus, *CYP6G1* is highly expressed in the midgut, Malpighian tubules (MT), and fat body in resistant *D. melanogaster* – all tissues that play an important role in the biotransformation of xenobiotics in insects (16). In contrast *CYP6BQ9* is predominantly expressed in the brain in pyrethroid resistant *T. castaneum*, a tissue enriched in the target protein of this insecticide class – the voltage gated sodium channel (125). Variable levels of expression in different tissues and life-stages were also demonstrated for *CYP6* genes suggested to be involved in insecticide sensitivity in *L. migratoria* (124). Tissue-specific expression was also observed for honey bee *CYP9Q3*, known to metabolize *N*-cyano neonicotinoids; here the expression levels were significantly higher in brain (and MTs) compared to midgut tissue, suggesting higher detoxification capacity at the site of neonicotinoid action in honey bee brain (65).

Recent work on *CYP6CY3* in *M. persicae* revealed that the native sites of expression of this P450 are aphid bacteriocytes - specialized aphid cells which house the obligate endosymbiont *Buchnera aphidicola* that provides essential amino acids and other nutrients to its host (102). Enhanced expression of *CYP6CY3* in this tissue was observed in the tobacco-adapted subspecies *M. p. nicotianae*. Furthermore, very high levels of *CYP6CY3* were also observed in the gut of this subspecies (>2500-fold higher than in guts of *M. persicae s. s.*). Together these changes in expression appear to protect both the aphid host and its essential symbiont from the toxic/inhibitory effects of nicotine (102). In combination with previous studies of *CYP6G1* in *D. melanogaster*, these findings also illustrate how the spatial expression of insect P450s can be fundamentally altered during the evolution of resistance. Interestingly in both cases this appears to have been mediated by transposable elements bringing tissue-specific enhancer sequences in close proximity to the P450 genes (16, 102).

Some evidence of tissue specificity of P450s associated with insecticide resistance is also available for *A. gambiae* (42). A key pyrethroid metabolizer, CYP6P3, was highly expressed in the midgut of the resistant strain, whereas CYP6M2 had a broader up-regulation in midgut, MT, and the abdomen and CYP6Z2 was overexpressed in the MT and gut (42). Intriguingly, transgenic overexpression of CYP6M2 or CYP6P3 in the midgut does not result in resistant phenotypes, despite achieving high levels of protein expression in this tissue. Furthermore, it was concluded that the insecticides were not metabolized in the MTs (1). These studies thus suggest that P450 expression in unidentified tissues may also be critical for insecticide detoxification in mosquitoes.

5.1 Temporal expression of P450s associated with resistance

P450s involved in resistance may exhibit marked temporal changes in their expression with implications for the resistance exhibited by different insect life stages. In the best example of this, *CYP6CM1* expression in *B. tabaci* was shown to be higher in adult whiteflies than nymphs and this was correlated with age-specific resistance to imidacloprid (45, 74). Similar mechanisms might also operate in other species, where the age-specific expression of resistance is of major operational importance, such as for example in *Anopheles* vectors of malaria, where resistance seems to be compromised by mosquito age (15).

6. P450s AS DETERMINANTS OF INSECTICIDE SELECTIVITY

In common with pest insects, beneficial insects have evolved P450s that can detoxify many of the natural xenobiotics they encounter in their environment (7), an asset to be exploited in the design and development of pest-selective insecticidal compounds (12). A recent, but growing body of work, has demonstrated that some of these P450s are preadapted to protect bees from certain insecticides from multiple classes. In the honey bee, CYP9Q2 and CYP9Q3 are highly expressed in the brain and MTs (65), but also the hind legs that collect pollen in foraging bees (67). They readily detoxify thiacloprid and acetamiprid (but not imidacloprid) by hydroxylation and *N*-demethylation, respectively (65). Similar work on bumblebees, *Bombus terrestris*, and red mason bees, *Osmia bicornis*, has identified CYP9Q4, CYP9Q6 and the related CYP9BU1 as functional orthologs of honeybee CYP9Q2/3 and key metabolic determinants of neonicotinoid sensitivity in these species (6, 65, 107). Thus, these P450s explain, in part, why these bee species are orders of magnitude more sensitive to imidacloprid than to *N*-cyano neonicotinoids. However, in the alfalfa leafcutter bee, *Megachile rotundata*, the CYP9Q/9BU

subfamilies are absent, and all neonicotinoids are equally toxic (37). These examples show that while P450s that are close in sequence can maintain some degree of functional conservation, the dynamics of births and deaths of P450 genes in species-rich lineages such as Hymenoptera makes it hazardous to extrapolate findings across species. Moreover, honey bee CYP9Q1–3 metabolize the pyrethroid *tau*-fluvalinate and the organophosphate coumaphos, as well as some phytochemicals (66, 98), so while they can be seen as generalist P450s, it is also hazardous to predict their specificity, as shown by their differential activity towards neonicotinoids. Screening of functionally expressed pollinator P450s may become a standard procedure in future insecticide development, just as screening of the major human liver P450s is now standard in the development of new drugs (63). For instance, screening of azole fungicides revealed that those synergizing neonicotinoids *in vivo* are also potent inhibitors of bee CYP9Q2 and CYP9Q3 (34).

7. CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

7.1 Conclusions

Our understanding of the role of P450s in insect toxicology over the last decade has advanced greatly. There has been an explosion in the number of genomes that have been sequenced and CYPomes annotated, and as outlined in this review, a large body of evidence supports the importance of P450-mediated adaptation to pesticides. This research has revealed a lack of correlation between CYPome size and xenobiotic tolerance. Furthermore, while xenobiotic metabolizing P450s are present in all four major CYP clans, there is no clear distinction from P450s that metabolize endogenous substrates. Despite the difficulty of P450 biochemistry and the great diversity of P450s in arthropod pests, emerging evidence suggests that only a subset of P450 genes are associated with pesticide resistance. Cases of differential up and/or down regulation of P450s responsible for resistance have been resolved, while recent work has also demonstrated that qualitative (i.e. amino acid differences in the coding sequence) and quantitative changes may act in tandem to enhance P450-mediated resistance. *Cis*-, *trans*- and signal transduction mechanisms have been studied extensively in recent years, resulting in the identification of promotor changes, transcription factors (such as CncC/Maf) and the elucidation of gene amplification events. A number of *in vitro* and *in vivo* tools have been developed to validate and/or measure the role and contribution of certain P450s, alone or in combination with other mechanisms in the resistance phenotype, with partial success and limitations (in vitro systems, RNAi, and *Drosophila* model) or more robust outcomes (genome-editing of non-model organisms). Finally, one of the most exciting findings in the last decade, the documentation of P450-based pesticide selectivity in bee pollinators at the molecular level, has opened novel molecular options for mechanistic pesticide risk assessment in honey bees using recombinant P450 libraries (34). Similar tool sets have been developed for pest species, which can be used to test the metabolic lability of novel insecticidal leads (12) and screens for synergists to block resistance, or even explore negative cross resistance concepts (69, 112).

7.2 Key knowledge gaps - priorities for future research

The diversity of arthropod CYPomes has only been broadly outlined, and even in some major crop pests and disease vectors the key P450s that determine pesticide sensitivity have not all been identified. Global and unbiased biochemical and genomic approaches will help identify which P450s

are likely to mediate resistance, facilitating the implementation of simple diagnostics to study the spread of resistance alleles.

The precise mutational events that are responsible for the constitutive trans-regulation of P450 genes up- (or down-) in pesticide-resistant strains remain obscure, despite the plethora of such documented cases.

Standardization of insect P450 expression is also desirable, to exploit the technological potential of insect P450s for industrial/research applications, such as the construction of libraries of recombinant P450s (e.g. libraries of all 57 human P450s (26)).

The further development and use of efficient genetic transformation systems (e.g. CRISPR/Cas9) in non-model organisms will facilitate our understanding of the role of P450s in pesticide resistance either alone or in combination with other mechanisms.

Insect P450 structures co-crystallized in complex with ligands will be needed to predict and understand the determinants of substrate specificity of the enzymes, because researchers currently rely on homology models based on distant vertebrate P450 structures.

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SUMMARY POINTS

1. Arthropods have widely diverse CYPomes, with xenobiotic metabolizing P450s present in all four major P450 clans, with no clear distinction from P450s catalyzing endogenous physiological reactions.
2. Assigning functions to P450 enzymes remains difficult by heterologous expression of recombinant enzymes *in vitro*, but complementary *in vivo* tools have been used to validate the role of P450s in resistance, with partial success and limitations (RNAi, and transgenic expression in *Drosophila*) or more robust outcomes (genome-edited non-model organisms).
3. Qualitative and quantitative changes in P450s may act individually or in tandem to enhance P450-mediated resistance.
4. The predictive value of P450-based diagnostics needs careful consideration, as epistasis (i.e. different phenotype depending on the genetic background) is present and many resistance markers may be required for diagnosis in each case.
5. *Cis*-, *trans*- and signal transduction or gene amplification mechanisms have been studied extensively in recent years, resulting in the identification of promotor changes, transcription factors (such as CncC /Maf) and the elucidation of gene amplification events.
6. The documentation of P450-based pesticide selectivity in bee pollinators at the molecular level has opened novel molecular options for mechanistic pesticide risk assessment in bees by exploiting recombinant P450 libraries.

FUTURE ISSUES

1. Global and unbiased biochemical and genomic approaches will facilitate the further investigation of which P450s are likely to mediate resistance.
2. The reconstruction or destruction of complex resistance phenotypes by functional genetic approaches in non-model insects will enhance our ability to elucidate the contribution of each individual molecular mechanism in the resistance phenotype.
3. Extensive lineage specific genome sequencing and cell-based functional work is needed to decipher the mutational events that are responsible for the regulation of P450 genes involved in pesticide resistance.
4. Standardization of insect P450 expression will increase the technological potential of insect P450s for industrial applications, e.g. exploitation for the discovery of selective insecticides and risk assessment.
5. The development and use of efficient genetic transformation systems in non-model organisms will facilitate our understanding of the function and physiology of P450-mediated resistance.
6. Crystal structures from insect P450s in complex with ligands are needed to predict and understand the determinants of substrate specificity.

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RELATED RESOURCES

1. Feyereisen R, Dermauw W, Van Leeuwen T. 2015. Genotype to phenotype, the molecular and physiological dimensions of resistance in arthropods. *Pestic. Biochem. Physiol.* 121: 61-77

2. Lu K, Song Y, Zeng R. 2021. The role of cytochrome P450-mediated detoxification in insect adaptation to xenobiotics. *Curr. Opin. Insect Sci*. 43: 103-7

Annotations for selected references

1. Overexpression of individual P450s in genetically modified *Anopheles gambiae*, determines their contribution in resistance, when expressed in different tissues

5. P450 enzymes that detoxify plant defense chemistry can be preadapted to confer resistance to synthetic insecticides

34. First description of a mechanistic approach for bee pollinator risk assessment and azole-mediated neonicotinoid synergism by CYP9Q enzyme inhibition

37. P450s that are preadapted to detoxify certain insecticides are not ubiquitous across all managed bee species

47. Novel P450s that confer resistance to insecticides can arise from unequal crossing-over between two parental P450 genes, resulting in a chimeric enzyme

65. Recombinant expression of the entire honey bee CYP3 clan revealed CYP9Q2/3 as the key determinants of bee sensitivity to neonicotinoids

66. First description of CYP9Q enzymes involved in honey bee sensitivity to acaricides

71. This study showed that cis-regulatory P450 variants confer operationally relevant pyrethroid resistance in *Anopheles funestus*

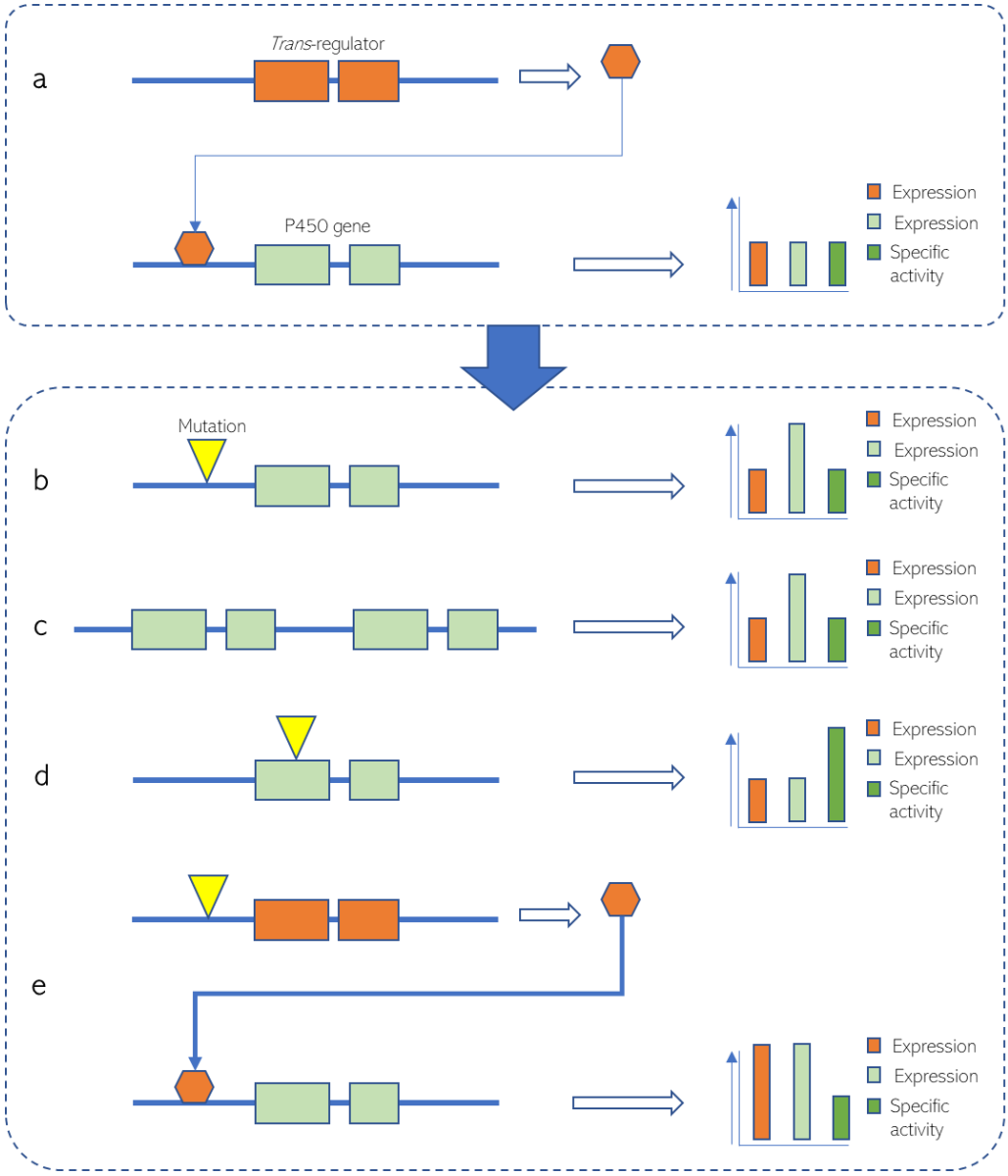
110. Reduced pro-insecticide activation as a mechanism of resistance in *Varroa destructor* by underexpression of a P450

116. First report of a complete P450 gene cluster knock-out in a global lepidopteran pest of agricultural importance

Figure 1. Molecular mechanisms of P450-mediated resistance to xenobiotics

a) Regulation of a P450 gene in a wild type (insecticide susceptible) insect strain by a *trans*-acting transcription factor. Please note: Activity in the bar chart refers to P450 specific catalytic activity (not total activity).

b-**e**) A variety of mutations can result in quantitative or qualitative changes in insect P450s leading to xenobiotic resistance. These include: **b**) *cis*-acting mutations in regulatory regions that increase P450 expression **c**) gene duplications or gene amplification which increase P450 gene dosage **d**) mutations that alter P450 coding sequence and enhance activity against xenobiotics; these can be one or more point mutations or affect larger portions of the coding sequence by gene conversion and **e**) mutations that affect the expression of *trans*-acting factors that regulate P450 genes. In the example shown in **e**) the mutation leads to the increased expression of a positive regulator leading to increased P450 expression, however a mutation that decreased the expression of a negative regulator would have the same effect. Similarly, a mutation in the coding sequence of a *trans*-regulator may affect its binding to *cis*-regulatory elements. In b), c) and e), the specific activity of the P450 is unchanged, whereas in d), P450 expression is unchanged, but the specific activity is increased.


a
Trans-regulator
P450 gene
Expression
Expression
Specific activity
Mutation
b
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