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The Role of Cytochrome P450s in Insect Toxicology and Resistance

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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 postgenomic functional approaches have provided an unprecedented opportunity to understand the evolution of insect P450s and their role in insect toxicology. These approaches have also been harnessed to provide new insights into 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.

1. INTRODUCTION

Cytochrome P450 monooxygenases (P450s; encoded by the *CYP* genes) make up 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 (79). This includes the detoxification of pesticides in crop pests and disease vectors, leading to resistance (22, 113), and in bees (7), where P450s have recently been shown to act as key determinants of insecticide selectivity (6, 66). 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 (87). 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 and 100 genes but ranges from lows of 23 in the eriophyoid mite *Aculops lycopersici* and 36 in the body louse *Pediculus humanus* to highs of well over 200 in some ticks, collembolans, and culicine mosquitoes (22). 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 the mitochondrial CYP clan, and most of the multiple-copy paralogs belong to the CYP3 and CYP4 clans (22, 29) and are often arrayed in clusters on chromosomes (101). 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 (113), members of the lepidopteran-specific CYP6AE subfamily in *Helicoverpa armigera* (102, 116), members of the CYP392 family in *Tetranychus urticae* (109), *Tribolium castaneum* CYP6BQ9 (126), *Bemisia tabaci* CYP6CM1 (51), *Myzus persicae nicotianae* CYP6CY3 (5), and *Apis mellifera* CYP9Q3 and CYP9Q2 (66). A variety of different P450s have been associated with resistance across mosquito species and geographical regions (113). 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, 88, 114). There is no apparent relationship between the phylogenetic relatedness and the catalytic competence of pesticide- and xenobiotic-metabolizing P450s (22). While pesticide- and xenobiotic-metabolizing P450s have to date been found mostly in the CYP6 and CYP9 families of the CYP3 clan, these findings are 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 (22).

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 approximately a third of the CYPome has been implicated in xenobiotic metabolism (97). Some P450 blooms (and genomic clusters) may be relatively ancient, such 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 is described below. Perhaps more importantly, 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 frequently, in a process only recently detected at the molecular level, downregulation of a P450 involved in propesticide activation can also mediate resistance.

3.1. Quantitative Changes

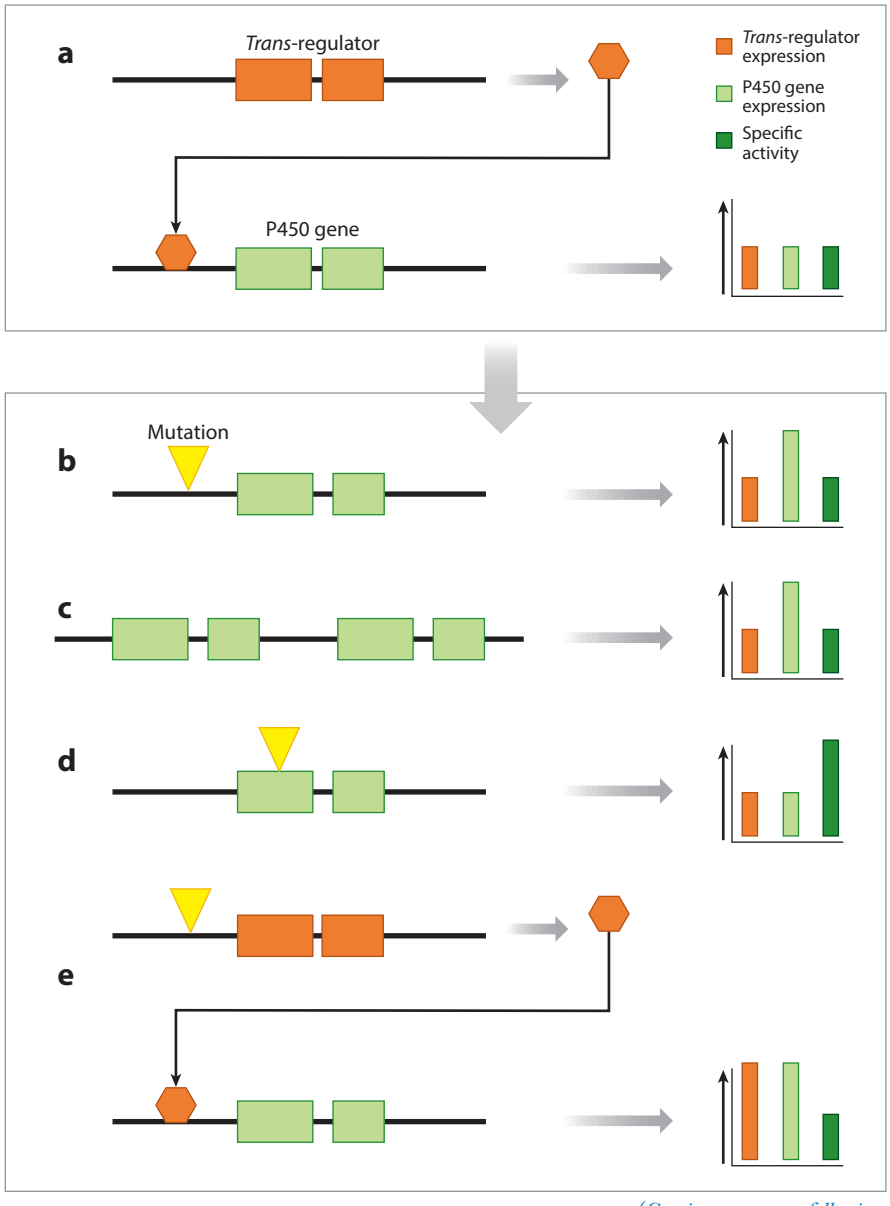
Metabolic resistance has long been associated with elevated enzyme activities; however, more recently, the diversity of mechanisms underpinning quantitative changes in P450 expression has become clearer.

3.1.1. Changes responsible for constitutively higher P450 expression levels. Constitutive quantitative change 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 from gene duplication or amplification (**Figure 1**). *Cis*-acting regulators are sequence elements located in proximity to the gene itself, while *trans*-acting 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 characterized the mechanisms leading to the overexpression of CYP6G1 in DDT-resistant *Drosophila* (20, 98; for a review, see 57). Overexpression was initially reported to be mediated by the insertion of an Accord transposable element (TE) upstream of the CYP6G1 gene, with subsequent work revealing alleles of this P450 with additional TE insertions (20, 98). Since this study, in silico analyses have revealed that 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 (85, 115, 120, 128). These include point mutations, such as the single-nucleotide substitution located near the transcription start site of *CYP9M10* (44, 120), 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. persicae 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 (94). However, the precise *trans*-acting genetic change(s) involved have 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 (for a review, see 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) (74). RNA interference (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) that regulate P450s of the CYP6BQ subfamily that confer resistance to pyrethroids (49, 50). Since then, CncC and Maf have been implicated in the upregulation



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

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. 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 amplifications that increase P450 gene dosage; (*d*) mutations that alter P450 coding sequence and enhance activity against xenobiotics, which can be one or more point mutations or affect larger portions of the coding sequence via gene conversion; and (*e*) mutations that affect the expression of *trans*-acting factors that regulate P450 genes. In the example shown in panel *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 panels *b*, *c*, and *e*, the specific activity of the P450 is unchanged, whereas in panel *d*, P450 expression is unchanged, but the specific activity is increased.

of P450s in a range of insect and mite species (for reviews, see 80, 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* (65), or whether it also operates in induction of P450s by nontoxic 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 (74) have been implicated in the regulation of insect P450s, including the nuclear receptor HR96 (55). Most recently, 3,5-cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB) (123) has been shown to regulate CYP6CM1, a P450 that confers resistance to several insecticides in the whitefly *B. tabaci* (51). Like CncC and Maf, CREB is constitutively upregulated in a resistant strain (123), but, as in 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 (58–60). Subsequent studies suggested that GPCRs can upregulate P450s via a signaling pathway comprising GPCR/G_s alpha subunit protein/adenylyl cyclase/cAMP-protein kinase A. 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* (123). Further work with inhibitors, RNAi, and biochemical assays provided strong evidence that the mitogen-activated protein kinases (MAPKs) ERK and p38 positively regulate CYP6CM1 expression by phosphorylating CREB (123), 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, 98, 118, 121, 128). In most of these cases, gene duplication or amplification results in adaptation to insecticide selection 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 that they can be copied as part of large or small

amplicons. For example, *CYP9M10* is duplicated as part of an amplicon approximately 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. persicae nicotianae* (103). In the latter example, additional copies of *CYP6CY3* were found at a novel locus as part of a much smaller (approximately 14 kb) amplicon associated with two TE insertions. These nested insertions occur immediately adjacent to the 5′ breakpoint, strongly suggesting that they played a role in the mobilization of *CYP6CY3* to the new loci, either indirectly by acting as substrates for nonallelic homologous recombination or directly via alternative transposition (103). 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 TE insertions, was shown to progressively increase resistance to DDT (98). 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, 61, 63, 110). However, while induced tolerance to plant allelochemicals is common (110), 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) the dose by modifying feeding behavior (105). 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, 62, 69), and CYP6AE89 of the 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 sublethal doses) (39; see 63 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 instead is transient tolerance.

Recent work has also revealed a link among 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 to certain compounds observed (23, 86). 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) (84).

3.1.3. Decreased P450 expression–mediated resistance. A coumaphos-resistant *Varroa destructor* population was recently shown to escape organophosphate toxicity via reduced proinsecticide activation by downregulating one of the only 26 P450s in its genome, CYP4EP4, leading to resistance to the organophosphate coumaphos (111). This case study is discussed further in Section 4.2.2.

3.2. Qualitative Changes in P450 Coding Sequences

Measuring the levels of P450 transcripts or activity is much easier than measuring the biochemical characterization of recombinant P450s expressed in vitro. It is therefore not surprising that evidence for a causal relationship between changes in P450 coding sequence and resistance has only emerged recently.

3.2.1. P450 allelic variants. Qualitative changes that cause resistance by modifying the catalytic activity or 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, 83). 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 the activity of these variants 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 (128). 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, 72), 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 by the requirement to preserve the native function of the enzyme. Recent work has highlighted novel mechanisms by which P450s have escaped these constraints. In a process more complex than a single nonsilent 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 (48), which has arisen multiple times (88, 114) 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

4.1. Tools for Functional Characterization of P450s

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, the exact role of P450s and their actual contribution to the phenotype remain largely unknown. In silico approaches; recombinant expression; and functional in vitro characterization of P450s, RNAi-based reverse genetics, in vivo overexpression, and genetic knockout have provided different levels of validation for the involvement of P450s in insecticide resistance.

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 Reference 78, 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 *Anopheles gambiae* CYP6P3, CYP6M2, and CYP9K1, which were expressed in *Escherichia coli* with an N-terminal OmpA signal peptide to direct the P450 to the bacterial membranes (73, 112, 124); the entire *A. mellifera* CYP3 clan (encompassing 27 CYP genes), including CYP9Q1, CYP9Q2, and CYP9Q3 (66, 67); *N. lugens* CYP6ER1 (128), *Laodelphax striatellus* CYP6AY3v2 (117), *P. humanus* CYP6CJ1 (53), and the successful expression of *H. armigera* CYP6AEs in insect cells using the baculovirus expression system (102); *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 (113).

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, and P450 gene knockouts. For example, RNAi silencing of the pyrethroid metabolizer CYP6BQ9 in *T. castaneum* resulted in a dramatic drop (>100-fold) in resistance (126). Silencing of *CYP6ER1* by RNAi in *N. lugens* was also used to suggest that the expression of CYP6ER1 is sufficient to confer neonicotinoid resistance (81, 107). 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, 54).

The integration of the molecular genetic toolbox developed in *Drosophila* (24, 100) into P450 resistance research has facilitated approaches such as conditional expression in vivo for the validation of P450 genes (for reviews, see 38, 82). One important tool is the bipartite GAL4/UAS expression system (for a review, see 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, 92); and P450s from agricultural pests, such as CYP6CM1 (*B. tabaci*), CYP6AY3v2 (*L. striatellus*), and CYP6ER1 (*N. lugens*) (82, 117, 128). Although this approach has been shown to be a useful tool for validating the functional role of candidate pest and pollinator P450s (e.g., 66, 108), the altered levels of toxicity achieved in transgenic *Drosophila* are frequently much lower than those observed in the native species (70). Recently, Samantsidis et al. (96) 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 (104).

The utility of GAL4/UAS-based tools to characterize P450s directly in non-model species has been demonstrated, and these tools allow 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 knockout significantly increases sensitivity against two classes of insecticides and phytochemicals (116).

4.2. Functional and Biochemical Mechanisms of P450-Mediated Resistance

Many P450s upregulated in resistance phenotypes have been functionally described as directly degrading insecticides and thus conferring insecticide resistance. In contrast, P450-mediated insecticide resistance linked to reduced levels of P450 expression and indirect P450-driven mechanisms affecting pesticide penetration were less common.

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 toward 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 (127).

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 (73), as well as the carbamate bendiocarb (124), while CYP6M2 metabolizes pyrethroids and DDT (71). The activity of 10 *H. armigera* CYP6AEs toward 10 different substrates is highly variable, apparently following no pattern of sequence similarity (22, 102, 116). Similarly, *B. tabaci* CYP6CM1 has a wide spectrum of activity against many, but not all, neonicotinoids (93), as well as pyriproxyfen and pymetrozine (76, 77). 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, 89–91), 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 (106), 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 in pests to become intrinsically active (95). 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 (56); however, it has only recently been unequivocally demonstrated. Investigation of resistance to the acaricide coumaphos in *Varroa* mites (111) 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 (111). 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, very few studies have identified the key amino acid determinants of insecticide metabolism or explained the functional contribution of point mutations to insecticide resistance (99). Docking of fenvalerate isomers into the active center of the *H. armigera* CYP337B3, in comparison with 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 via 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 *Anopheles 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, 52). 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 (for a review, see 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* (122), suggests a regulatory cascade in CHC production and thus indirectly in insecticide penetration rates.

5. LOCALIZATION 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 expression can change under insecticide selection (e.g., 39). Studies on several insect species have revealed that P450s associated with insecticide metabolism are expressed not only 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 (MTs), and fat body in resistant *Drosophila 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 of pyrethroid-resistant *T. castaneum* in a tissue enriched in the target protein of this insecticide class—the voltage gated sodium channel (126). 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* (125). Tissue-specific expression was also observed for honey bee *CYP9Q3*, known to metabolize *N*-cyano neonicotinoids; in this case, the expression levels were significantly higher in brain (and

MT) compared to midgut tissue, suggesting higher detoxification capacity at the site of neonicotinoid action in the honey bee brain (66).

Recent work on *CYP6CY3* in *M. persicae* revealed that the native sites of expression of this P450 are aphid bacteriocytes—specialized aphid cells that house the obligate endosymbiont *Buchnera aphidicola*, which provides essential amino acids and other nutrients to its host (103). Enhanced expression of *CYP6CY3* in this tissue was observed in the tobacco-adapted subspecies *M. persicae nicotianae*. Furthermore, very high levels of *CYP6CY3* were also observed in the gut of this subspecies (>2,500-fold higher than in guts of *M. persicae* sensu stricto). Together, these changes in expression appear to protect both the aphid host and its essential symbiont from the toxic or inhibitory effects of nicotine (103). 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 alteration appears to have been mediated by TE bringing tissue-specific enhancer sequences into close proximity with the P450 genes (16, 103).

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; in contrast, CYP6M2 had a broader upregulation in the midgut, MTs, and abdomen, and CYP6Z2 was overexpressed in the MTs and gut (42). Intriguingly, transgenic overexpression of CYP6M2 or CYP6P3 in the midgut does not result in resistant phenotypes, despite leading to 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.

5.2. 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 in nymphs, and this was correlated with age-specific resistance to imidacloprid (45, 75). Similar mechanisms might also operate in other species where the age-specific expression of resistance is of major operational importance, such as 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 that they encounter in their environment (7), an asset that could 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 (66) but also in the hind legs, which collect pollen in foraging bees (68). CYP9Q2 and CYP9Q3 readily detoxify thiacloprid and acetamiprid (but not imidacloprid) by hydroxylation and *N*-demethylation, respectively (66). 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, 66, 108). 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 (67, 99); thus, while they can be seen as generalist P450s, it is also hazardous to predict their specificity, as shown by their differential activity toward 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 (64). 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 past decade has advanced greatly. There has been an explosion in the numbers of genomes that have been sequenced and CYPomes that have been 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 downregulation 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 and transcription factors (such as CncC and Maf) and the elucidation of gene amplification events. Several 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 past decade, the documentation of P450-based pesticide selectivity in bee pollinators at the molecular level, has opened up 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 that can be used to test the metabolic lability of novel insecticidal leads (12) and screen for synergists to block resistance, or even to explore negative cross-resistance concepts (70, 113).

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 and 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, as researchers currently rely on homology models based on distant vertebrate P450 structures.

SUMMARY POINTS

1. Arthropods have widely diverse CYPomes, with xenobiotic-metabolizing P450s intermingled with P450s catalyzing endogenous physiological reactions in all four major CYP clans.
2. Assigning functions to P450 enzymes remains difficult using 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 phenotypes 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 and transcription factors (such as CncC and 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 up 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 are 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., their 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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