Insecticide Resistance: Molecular Insight

2

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Contents

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Abstract

Insecticide resistance is one of the major worldwide challenges in insect pest management. Conventional to molecular approaches have been used in identifying insecticide resistance aspects, i.e. behavioural, ecological, physiological and molecular. The molecular mechanisms of insecticide resistance detection are mainly determined by three factors, i.e. gene amplification, upregulation and structural changes in genes. Genome sequencing, DNA barcoding, genome editing, transcriptional control and epigenetic studies have helped in making tremendous progress in insecticide resistance research. The new era of molecular studies has opened more reliable, precise and appropriate options for insecticide resistance recognition and timely management of insect pests.

Keywords

Insecticide resistance · Molecular assay · Gene amplification · Gene upregulation · DNA barcoding · Transposable elements · Epigenetics · Detoxification enzymes

Learning Objectives

1. The molecular studies of insecticide resistance have opened a new era in the assay of insecticide resistance. The availability of insecticide resistance data on time and with high accuracy helps in making timely management strategies and reducing economical losses.
2. The molecular mechanisms of insecticide resistance are mainly determined by three factors, i.e. gene amplification, upregulation and structural changes in genes, and the sequencing of desired genes confirms the aforesaid studies.
3. This chapter has covered molecular aspects of insecticide resistance in addition to the novel epigenetic studies that throw a light on mysteries of recently discovered molecular studies.

2.1 Introduction

Agriculture is an important outcome of human civilization, and insect pests have always been its parallel associate. Synthetic chemical pesticides have protected the crop plants against harmful insects since long, but the recent problem of insecticide resistance has halted this progress. Insecticide resistance is one of the most nuisance and expanding problems, which has become a challenge for scientists working for the development of insect pest management strategies. Resistance is defined as 'the development of an ability in a strain of an organism to tolerate doses of a toxicant, which would prove lethal to the majority of individuals in a normal (susceptible) population of the species' (WHO 1957). However, the term insecticide resistance specifically deals with population of insects, which stops responding to application of recommended doses of insecticides (Javed et al. 2017). Pesticide-resistant insects are modified either by genetic or epigenetic changes, which ultimately leads to

biochemical, physiological and phenotypic differences among them (R4P Network 2016). In one of the recent newsletters of Insecticide Resistance Action Committee (IRAC), data of top 20 countries and top 20 arthropods showing resistance was released, which is very alarming. The crop losses caused by insect pests globally emphasize the threat of insecticide resistance in management practices, and this chapter will help understand the molecular studies associated with insecticide resistance to safeguard chemical management strategies, which is an integral component of integrated pest management (IPM) practices.

2.2 Assays for Detection of Insecticide Resistance (R4P Network 2016)

There are majorly three types of assays, i.e. bioassay, biochemical assay and molecular assay, which focus on the phenotypic, biochemical and genetic modifications.

2.2.1 Bioassay

The aim of bioassays is to determine the doses that affect insects as well as to test the level of resistance (Siqueira et al. 2000) by exposing live insects to determine doses and comparing them with sensitive population, popularly analysed by the dose-response curve. IRAC has formulated different methods of bioassays, but the standard method used is leaf immersion (Bacci et al. 2009). Although there are some limitations (time and space) associated with bioassays, the use of technologies, such as automated imaging platform (Stewart and McDonald 2014), could make a breakthrough by increasing the reliability of these tests.

2.2.2 Biochemical Assay

These assays are used to detect resistance regulated by target enzymes or metabolic enzymes. The measurement of specific activity of enzymes by absorbance or fluorescence reveals the variation in activity of pesticide detoxification enzymes (Reyes et al. 2012). The biochemical assay methodology of some important insecticide-degrading enzymes has been discussed by Kranthi (2005).

2.2.3 Molecular Assay

One of the major constraints of the above two assays was the requirement of live organisms, which in molecular assay is not a limitation. On the basis of technology used, the molecular assays are classified into two major types, i.e. (1) rugged or low-throughput assay and (2) hi-tech or high-throughput assay. Genotyping of

known mutations causing resistance and sequencing of full genotypes to know any level of variations are some examples of molecular assays. Very low detection threshold is the primary advantage of molecular diagnosis of insecticide resistance over all other types of assays (Black and Vontas 2007).

2.3 Molecular Mechanism of Insecticide Resistance

The genomic studies evolved from Mendelian genetics via phases, such as molecular genetics, genomics and most recently epigenetic studies. These studies have played an important role in insect pest management practices beginning from conventional breeding or selection strategies, such as sterile insect techniques (Haymer 2015), to novel techniques, like RNAi. A lot have already been studied about the conventional approaches of insecticide resistance paving a way towards advanced molecular studies. Since the studies up to the level of amino acid was very significant in insecticide resistance hence used the term landmark developments (Perry et al. 2011). There are mainly four aspects of insecticide resistance studies, viz. behavioural, ecological, physiological and molecular, which are further determined by several factors. Gene amplification, upregulation and structural changes in genes encoding detoxification enzymes (P450s, GSTs, esterases) are three factors responsible for molecular mechanism of insecticide resistance (Li et al. 2007) and thus emphasize the role of molecular biology, genomics, epigenetics and bioinformatics tools. Heckel (2003) in his review described the role of genomics in pure and applied biology and comprehensively covered all the fields of genomics, i.e. structural, functional and comparative genomics, and the importance of genomics in entomology.

2.3.1 Gene Amplification

Alteration in the copy number of genes determining the system responsible for detoxification of insecticide encountered by insects is gene amplification (Li et al. 2007), and the transcription and translation of the amplified gene lead to the production of functional proteins responsible for the expression of resistance traits (Feyereisen 1995). Out of the three major detoxification enzymes, the resistance mechanism of gene amplification has been observed in esterases and GSTs; however, more recently, it has been reported for P450s also (Bass and Field 2011). The evidences of gene amplification for insecticide resistance in *Myzus persicae* have been reported by Field et al. (1998); they found that it was due to amplification of gene esterase-4 (E4) or fast-E4 (FE4) (Field et al. 1998).

2.3.2 Upregulation/Altered Expression

Upregulation may be described as increased production of detoxification enzymes or proteins without showing any change in its genomic copy number like in gene amplification (Li et al. 2007), and the mutation in trans- and/or cis-acting regulatory loci has been documented as usual cause of upregulation (Bass and Field 2011). The first example of gene amplification of insecticide target site has been documented for AChE locus in two-spotted spider mite, *Tetranychus urticae* (Kwon et al. 2010). The Northern and Western blot analysis of PxGSTE1 gene in diamondback moth, *Plutella xylostella*, showed that resistance against OP insecticides is due to higher expression of the gene. The molecular reason behind was documented to be upregulation of the gene concerned since there was no evidence of gene amplification from Southern blot results (Sonoda and Tsumuki 2005).

2.3.3 Structural Change

Point mutations, like addition, deletion and substitution, may modify the sequence of DNA responsible for insecticide resistance (Feyereisen 1995). Substitution of one nucleotide with another nucleotide in the coding region may change three-dimensional structural change and may affect resistance against insecticide positively or negatively (Scott 1995).

2.4 Genome Sequencing, Genome Editing and Transcriptional Control

2.4.1 Genome Sequencing

Sequencing is a method for determining the position of nucleotide bases, and genome sequencing identifies every nucleotide in the genome. Early DNA sequencing technologies, also known as 'first-generation sequencing', include sequencing by synthesis (Sanger et al. 1977) and sequencing by cleavage (Gilbert and Maxam 1973), while second-generation sequencing or next-generation sequencing is the novel and highly efficient sequencing technology. Gene amplification and structural changes in the genome have been assayed using these sequencing technologies for both DNA and RNA (Leeuwen et al. 2020). Clarkson et al. (2018) described the role of whole genome sequencing in studying the molecular basis of insecticide resistance, and genomic studies of *Spodoptera litura* by genome sequencing, transcriptome analysis and physical mapping revealed adaptive changes, expansion of selected genes and ecological adaptations (Cheng et al. 2017).

2.4.2 DNA Barcoding

Like the barcodes used in package of any product, DNA barcoding is a system of biological identification by amplifying and sequencing a short reference region of the genome (Hanner et al. 2009). Gene region extensively used in the study of insects is mt-encoded cytochrome c oxidase subunit 1 (cox1, CO1) 648 bp region amplified by primer, and the most probable cause of its wide use is maternal inheritance and wide occurrence, making it suitable for examining population history and easy to isolate, respectively (Cameron 2014). DNA barcoding is applicable in taxonomic identification and early invasion of insects paving the way to apply management strategies on time (Hanner et al. 2009). It is also used in insecticide resistance studies and for the development of selective insecticides to protect natural enemies. In one of such studies on pond wolf spider *Pardosa pseudoannulata*, which is an important natural predatory enemy of rice planthoppers the molecular basis of selectivity of neonicotinoids was observed (Meng et al. 2015a) (Fig. 2.1). There are four major clades in the cytochrome P450 family, viz. CYP2, CYP3, CYP4 and CYPM, and in insects, CYP3 clade contains the majority of detoxifying P450 genes. In *P. pseudoannulata*, CYP2 clade was found to be superior, which is quite different from insects, and thus depicts the difference in resistance mechanism, which could be used for the formation of selective pesticides (Meng et al. 2015b) (Fig. 2.1).

2.4.3 Genome Editing or Genome Engineering

It allows the creation of double-stranded breaks (DSBs), followed by insertion or deletion of foreign DNA sequences. The methods for precise editing of a genome include (1) zinc finger nucleases (ZFNs) technology (Urnov et al. 2010), (2) transcription activator-like effector nucleases (TALENs) (Mussolino et al. 2014) and (3) clustered regularly interspaced short palindromic repeat (CRISPR) or CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) system (Chylinski et al. 2014). CRISPR/Cas9 is the latest technology in genome editing and has been successfully employed in modification of the targeted insect. With the use of this technology, CYP6AE gene cluster was knocked down in *Helicoverpa armigera*, which was responsible for insecticide resistance, and the role of the concerned gene was proven (Wang et al. 2018).

2.4.4 Transcriptional Control

DNA is the carrier of biological information, and the information is transferred to RNA via transcription and is finally expressed by amino acids through the process of translation (Crick 1958). Regulation of genes of various functions occurs at the level of transcription in eukaryotes (Harshman and James 1998). Insecticide exposure induces transcriptional responses in insects that regulate the detoxification

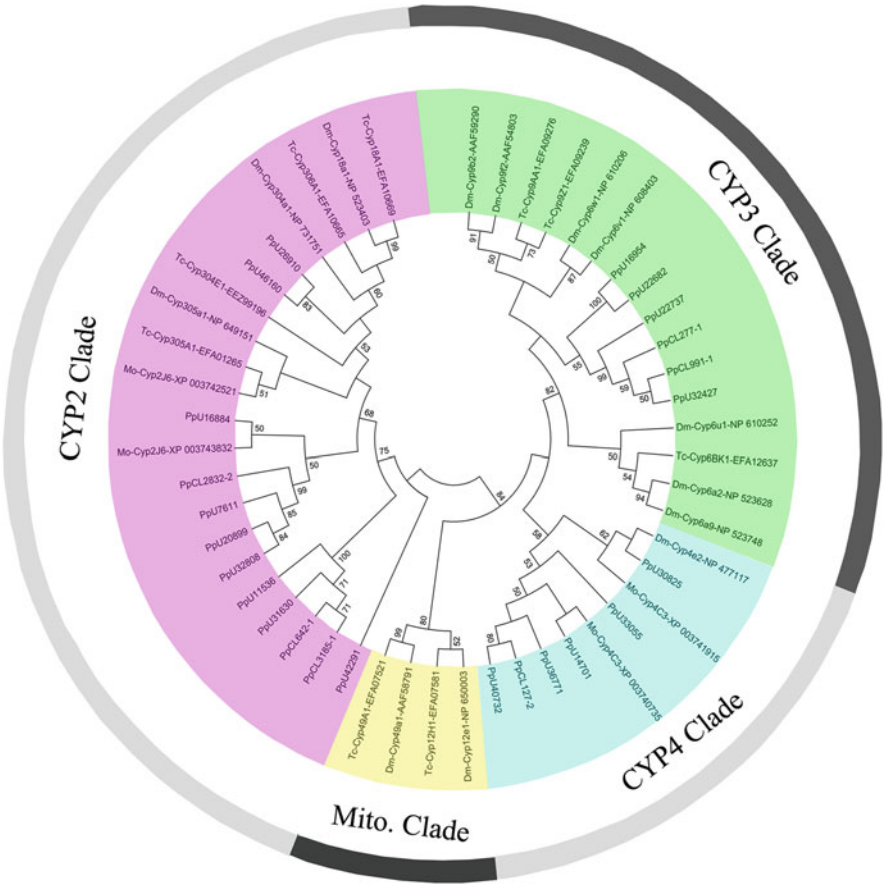

Fig. 2.1 Cytochrome P450 genes in *P. pseudoannulata*. (Source: Meng et al. 2015a)

mechanism (Misra et al. 2011). In peach potato aphid, *Myzus persicae*, insecticide detoxification by amplification of esterase is mainly determined by E4 and FE4 genes. In the absence of selection pressure among laboratory-selected populations, the aphids were reverted to susceptibility even after retaining amplified E4 genes; this was explained because of the decreased transcription in revertant aphids, leading to loss of detoxification enzyme production (Devonshire et al. 1998).

2.5 Transposable Elements (TEs)

Transposons, also known as 'mobile elements' or 'junk DNA' or 'selfish DNA' or 'jumping genes', are DNA sequences that are capable to transpose within the genome (Wilson 1993). It has been reported by Merrell and Underhill (1956) that insecticide resistance is an issue with the population showing more genetic

variability as compared to one with lower variability because more alleles will be available for selection in population with high genetic variability and transposable elements add to the genetic variability of the insect. In regulatory regions of the gene, TE insertion results in upregulation, which is caused because of built-in enhancer sequence in transposable elements (Zhang and Saier 2009). The studies of resistant genes have provided direct and indirect evidence supporting the role of TEs in the molecular resistance mechanism of insecticide (Rostant et al. 2012). For example, it was found in a study that xenobiotic-metabolizing P450 genes of both *Helicoverpa zea* and *Drosophila melanogaster* have TE insertion-rich regions (Chen and Li 2007).

2.6 Molecular Mechanism of Detoxification Enzymes

The FAO in its document on 'Guidelines on Prevention and Management of Pesticide Resistance' has described five categories of insecticide resistance mechanism, viz. (1) metabolic detoxification (enzymatic), (2) reduced sensitivity at target site, (3) reduced penetration, (4) sequestration and (5) behavioural resistance. Metabolic detoxification mechanism of enzymes, such as esterases, cytochrome P450 monooxygenases and glutathione *S*-transferases, is found to occur mainly in insects.

2.6.1 Cytochrome P450 Monooxygenases

This enzyme is a key component of the microsomal oxidase system and mitochondria in insects (Feyereisen 1999). These enzymes have also been mentioned as 'diversozymes' due to a diverse stoichiometry ranging from hydroxylation to epoxidation, *O*-, *N*- and *S*-dealkylations and *N*- and *S*-oxidations (Coon et al. 1996). P450 enzyme categorized into five insects specific six families, viz. CYP6, CYP9, CYP12, CYP18 and CYP28 and one family CYP4 from vertebrate (Feyereisen 1999). Studies on *Drosophila* revealed that when the flies were continuously selected with DDT, there was overexpression of *Cyp6g1* gene and the flies showing overexpression were also found to show cross-resistance with neonicotinoids, OP insecticides and growth regulators, such as lufenuron (Richard et al. 2004; Daborn et al. 2001, 2002).

2.6.2 Esterases

Two major enzymes belonging to the esterase family responsible for detoxification of insecticides are carboxylesterase and acetylcholinesterase (Kranthi 2005). Esterases regulate insecticide resistance by exhibiting insensitivity of the target enzyme (acetylcholinesterase) or by metabolic resistance mediated by carboxylesterase (Cui et al. 2015). Detoxification by esterase occurs via overexpression, which could be due to amplification or upregulation or both in combination (Panini

et al. 2016). Detoxification by amplification has been observed in *Myzus persicae*, *Culex* and *Nilaparvata lugens* (Bass et al. 2014; Hemingway et al. 2004; Small and Hemingway 2000) and by upregulation in *Aphis gossypii* and *Bemisia tabaci* (Cao et al. 2008; Alon et al. 2008).

2.6.3 Glutathione *S*-Transferase (GST)

GST-based insecticide resistance is mediated either directly by Phase I reactions or indirectly by Phase II reactions and ensures detoxification by neutralizing toxic chemicals to water-soluble compounds, finally leading to its excretion from the cells (Mannervik 1985; Habig et al. 1974). According to the location, insect GSTs are of two types, i.e. microsomal and cytosolic; however, it is the cytosolic GST that is vital for insecticide resistance (Panini et al. 2016). The genes related to insect GST can be divided into six families based on sequence similarity and substrate specificity, viz. delta, epsilon, omega, sigma, theta and zeta (Fang 2012). The modern approaches, like transcriptome analysis, forward and reverse genetics techniques and next-generation sequencing studies, have guided in-depth understanding of insecticide resistance mechanism facilitated by GSTs. In a recent study of gene knockdown by RNAi, Bt *GSTd7* gene was discovered to be responsible for imidacloprid resistance in *Bemisia tabaci* (He et al. 2018).

2.7 Epigenetics in Insecticide Resistance

Epigenetics may be described as changes in gene expression (but not gene sequence), ultimately leading to modified phenotype in response to intrinsic or environmental stimuli, which persist after cell division (Yan et al. 2015). There are three major epigenetic inheritance systems (Table 2.1).

Field et al. (1989) reported the first evidence of the role of epigenetics in insecticide resistance for peach potato aphid, *Myzus persicae*. Significance of epigenetics by modification of histone with acetyl group has been observed in honeybee, *Apis mellifera*, regulation of sodium butyrate, which acts as histone deacetylase inhibitor increase honeybee tolerance towards imidacloprid, which was otherwise found to be in low concentration in *A. mellifera* (Hu et al. 2017; Oppold and Muller 2017).

2.8 Genomic Studies of Insecticide Resistance in Some Important Insect Pests

2.8.1 Whitefly, *Bemisia tabaci* (Gennadius 1889)

Whitefly is an important invasive polyphagous pest infesting more than 500 crop plants (Cock 1993) and is a vector of one of the devastating yellow leaf curl and mosaic viral diseases in agronomically vital plants (Scholthof et al. 2011). On the

Table 2.1 Epigenetic inheritance systems (Glastad et al. 2019)

DNA methylation	Histone modification	Noncoding RNAs (ncRNAs)
Mediated by two classes of enzymes: 1. De novo DNA methyltransferase (DNMT3 protein) 2. Maintenance DNA methyltransferase (DNMT1 proteins) 3. DNA methylation occurs by addition of methyl group to cytosine residing in CpG Example: phenotypic plasticity in locusts and honeybees	The association between target histone and underlying DNA can be impacted by addition of acetyl, methyl or phosphorus groups Example: phase change in locusts (migratory and solitary)	These are a heterogeneous class of RNAs that are not translated into proteins: piRNA, microRNA, siRNA and long noncoding RNA Example: silk yield in *Bombyx mori* modulated by differentially expressed lncRNAs

basis of sequences of mitochondrial cytochrome oxidase I (MtCOI) gene, *B. tabaci* has been broadly classified into two globally important pest taxa: Middle East-Asia Minor 1 (MEAM1, formerly biotype B) and Mediterranean (MED, formerly biotype Q) (Liu et al. 2012). Whitefly genomic studies have explained the variability in the pests including the causes of invasiveness and insecticide resistance (Czosnek and Brown 2009). Chen et al. (2016) in their draft of whitefly genome have uncovered genomic mysteries of insecticide resistance in the pest and found that a total of 202 PEBPs are present in *B. tabaci* as compared to a maximum of 16 PEBPs reported in other 15 arthropods. The phosphatidylethanolamine-binding protein (PEBP) gene family has been found to occur in a wide range of organisms and is supposed to have a strong role in rapid evolution against insecticide resistance.

2.8.2 Tobacco Caterpillar, *Spodoptera litura* (Fabricius 1775)

S. litura is a highly polyphagous pest, which feeds on around 120 plant species (CABI Datasheet 2019). The pest has developed high resistance against insecticides and has been ranked at seventh position among the most resistant arthropods by IRAC (Sparks and Nauen 2015). The genomic information of *S. litura* provided an insight into the molecular mechanism of insecticide resistance of detoxification-related gene families. In a comparative study between highly polyphagous *S. litura* and almost monophagous *Bombyx mori*, expansion of chemosensory and detoxification-related gene families was observed in *S. litura* (Fig. 2.2) (Cheng et al. 2017).

Genomic annotation of the P450 genome in *S. litura* showed large expansions of P450 clan 3 and clan 4, and CYP9a especially was expanded greatly compared to other clans on exposure to insecticides (Cheng et al. 2017). It has also been confirmed in recent study the overexpression of *SlituCYP321b1* in the midgut of *S. litura* confirming its role in insecticide resistance (Wang et al. 2017).

	Family	Clan	*S.litura*	*B.mori*
Insecticide-tolerance gene families	**P450**		**138**	**83**
		Clan 3	61	32
		Clan 4	58	33
		Mitochondrial	11	11
		Clan 2	8	7
	GST		**47**	**26**
		ε	21	9
		δ	5	6
		ω	3	4
		σ	7	2
		θ	1	1
		ζ	5	2
		Microsomal	2	1
		Uncharacterized	3	1
	COE		**110**	**76**
		α-esterase	25	15
		Lepidopteran esterase	57	39
		JHE	8	7
		β-esterase	2	2
		Integument esterase	4	2
		Acetylcholinesterase	2	1
		Neuroligin	7	6
		Neurotactin	1	1
		Gliotactin	1	1
		Uncharacterized	3	2
		Clan 3	61	32
		Clan 4	58	33
		Mitochondrial	11	11
		Clan 2	8	7
	APN		**18**	**14**
	ABC		**54**	**51**
Chemosensory gene families	**CSP**		**23**	**21**
	OBP		**36**	**43**
	OR		**73**	**73**
	GR		**237**	**76**

Fig. 2.2 Comparison of detoxification and chemosensory gene families between the extremely polyphagous pest *S. litura* and the almost monophagous *B. mori*. (Source: Cheng et al. 2017)

2.9 Conclusions

Insecticide resistance management (IRM) has become an integral part of insect pest management and is a promising solution to challenging and widespread problem of insecticide resistance. Insecticide resistance gene database has become a boon to the researchers for conducting molecular studies on insect pests. Molecular mechanism

helps in the early detection of resistance in insects as compared to conventional methods and in increasing vigilance to avoid expression of resistance gene and in formulating timely management strategies and better IPM modules. The small sample size required in the molecular studies enhances the effectiveness of detecting resistant individuals, which is not possible with conventional methods and thus paving the way for the specific and accurate approach towards insecticide resistance management practices to overcome losses caused by insect pests and formation of selective solution for problem of insect pests.

Points to Remember

- Insecticide resistance is a new challenge in the management of insect pests and has become a global issue raising concern among the masses directly or indirectly related to its ill effects.
- Molecular assays by genotyping and sequencing have become a precise and timely assay methodology overcoming the limitations of conventional methods of insecticide resistance and described the resistance mechanisms via upregulation, amplification and structural changes.
- The application of first-generation and next-generation sequencing in addition to DNA barcoding opens vast possibilities of insecticide resistance observed in insects. Novel application of genome editing, like CRISPR/Cas9, has been successfully employed in the identification of modified target genes.
- Transposable elements and epigenetic studies comprehensively covered genomic studies of some major insect pests, like *S. litura* and *B. tabaci*, and thoroughly investigated and researched mechanism for insecticide resistance.

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