Review

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Molecular innovations underlying resistance to nicotine and neonicotinoids in the aphid *Myzus persicae*

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Abstract

The green peach aphid, *Myzus persicae*, is a globally distributed highly damaging crop pest. This species has demonstrated an exceptional ability to evolve resistance to both synthetic insecticides used for control, and natural insecticides produced by certain plants as a chemical defense against insect attack. Here we review work characterizing the evolution of resistance in *M. persicae* to the natural insecticide nicotine and the structurally related class of synthetic neonicotinoid insecticides. We outline how research on this topic has provided insights into long-standing questions of both evolutionary and applied importance. These include questions pertaining to the origins of novel traits, the number and nature of mutational events or 'adaptive steps' underlying the evolution of new phenotypes, and whether host plant adaptations can be co-opted to confer resistance to synthetic insecticides. Finally, research on the molecular mechanisms underlying insecticide resistance in *M. persicae* has generated several outstanding questions on the genetic architecture of resistance to both natural and synthetic xenobiotics, and we conclude by identifying key knowledge gaps for future research.

Keywords: Myzus persicae; resistance; nicotine; neonicotinoids

1 INTRODUCTION

The green peach aphid, *Myzus persicae* (Sulzer, 1776), is an economically important crop pest that feeds on over 400 plant species, including many agricultural and ornamental plants.[1] The species causes damage to plants through direct feeding, the production of honeydew and the transmission of over 100 plant viruses.[2, 3] The control of *M. persicae* on many crops has relied heavily on the use of chemical insecticides, and their intensive use over many years has led to the development of widespread and multiple forms of resistance.[3, 4] Research on the biochemical and molecular basis of resistance in *M. persicae* has uncovered at least eight genetically independent mechanisms of resistance, making it an important case study for molecular evolution in insects.[3, 5] In addition to developing resistance to synthetic insecticides, *M. persicae* is also adept at evolving resistance to natural xenobiotics, such as the secondary metabolites produced by plants as defense against herbivores. The best example of this is the host race associated with tobacco (*Nicotiana tabacum*), formally named as *M. persicae* subsp. *nicotianae*.[6] This subspecies exhibits resistance to the alkaloid nicotine, the potent natural insecticide produced by tobacco, and intriguingly, cross-resistance to neonicotinoid insecticides - synthetic derivatives of nicotine.[7] Both nicotine and neonicotinoids act as agonists of nicotinic acetylcholine receptors (nAChRs); pentameric ion channels that play a key role in signal transduction between nerve cells.[8] However, while nicotine exhibits high affinity towards both vertebrate and invertebrate nAChRs, neonicotinoids show high affinity only for invertebrate receptors.[8]

Over the last decade, understanding of the genetic architecture that underpins the key adaptations that allow *M. persicae* to effectively tolerate nicotine and neonicotinoid insecticides has advanced considerably. Here we review this work and consider how this case study has provided knowledge for both applied aspects of direct relevance to pest management, and more widely for fundamental questions in evolutionary

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biology. Finally, we identify outstanding knowledge gaps for future research.

2 THE GENETIC ARCHITECTURE OF RESISTANCE TO NICOTINE

2.1 Cytochrome P450s belonging to the CYP6CY subfamily confer resistance to nicotine

Because the host shift of *M. persicae* to tobacco occurred recently, direct comparisons of adapted and nonadapted conspecific races offer an excellent opportunity to identify the mechanisms leading to nicotine resistance. Gene expression microarrays were first used to compare the transcriptomes of *M. p. nicotianae* and *M. persicae s.s.*, and identified the cytochrome P450 gene *CYP6CY3* as highly overexpressed in the former.[9] Further study confirmed the causal role of this P450 in resistance by demonstrating that recombinant CYP6CY3 is highly efficient at metabolizing nicotine to less toxic metabolites *in vitro*, compared with the primary human nicotine-metabolizing P450 CYP2A6.[7] This finding was further supported by the demonstration *in vivo* that ectopic expression of CYP6CY3 in transgenic *Drosophila* results in significant desensitization to nicotine.[7]

Initial investigation of the mechanisms leading to the overexpression of *CYP6CY3* revealed that enhanced mRNA levels of this P450 gene in *M. p. nicotianae* result, in part, from amplification of the structural gene.[9] Subsequent publication of the first reference genomes for *M. persicae*[10] allowed a more detailed genetic investigation of the *CYP6CY3* amplification event.[11] These analyses revealed that, in *M. p. nicotianae*, *CYP6CY3* is amplified 3–5-fold (at its native loci) as a tandem array of direct repeats of approximately 325 Kb, adding up to 1.5 Mb to chromosome 3.[11] The large genomic region amplified encompasses several genes in addition to *CYP6CY3*, including two other genes belonging to the *CYP6CY* subfamily (*CYP6CY4* and *CYP6CY23*), the tyrosine-protein kinase gene *Src42A*, a gene encoding a voltage-dependent T-type calcium channel, the last 23 exons of A disintegrin and metalloproteinase with thrombospondin motifs 9 (*ADAMTS9*), and the first two exons of the 40*S* ribosomal protein S11 (*RPS11*) gene.[11] Furthermore, as the segmental duplication occurs as a direct tandem repeat, the chromosomal rearrangement also created a chimeric gene at the junctions between amplicon copies, fusing the regions of the *RPS11* and *ADAMTS9* genes that occur at the amplicon breakpoints.[11] The discovery that multiple genes are co-amplified with *CYP6CY3* raised the possibility that resistance to nicotine may also, in part, result from the increased gene dosage of other genes in the amplicon. Investigation of this hypothesis uncovered evidence that, for some of these genes, enhanced expression may be neutral or deleterious, with nonfunctionalizing mutations observed in certain copies of *Src42A* and the T-type calcium channel gene, that lead to their inactivation.[11] Furthermore, transgenic Drosophila strains expressing either wild-type *ADAMTS9* or the chimeric *RPS11/ADAMTS9* gene showed no increase in tolerance to nicotine compared to flies of the same genetic background lacking a transgene, suggesting they provide no protection to *M. p. nicotianae* from nicotine.[11]

The three *CYP6CY* P450 genes within the amplicon share 72–81% amino acid sequence identity, explaining the very high estimates of *CYP6CY3* copy number in tobacco-adapted clones in early studies using primers that cross-hybridize between these genes.[7, 9, 11] Functional expression of CYP6CY4 and CYP6CY23 *in vitro* revealed that CYP6CY4, but not CYP6CY23, can efficiently metabolize nicotine to it's nontoxic metabolite cotinine.[11] Thus, together with the earlier functional characterization of CYP6CY3, these studies provide convincing evidence that increased expression of *CYP6CY3* and *CYP6CY4* allowed *M. p. nicotianae* to overcome the primary defensive allelochemical produced by tobacco plants.

2.2 Regulation of resistance

2.2.1 Microsatellite repeats and further increases in CYP6CY3 *CNV*

CYP6CY3 and *CYP6CY4* are upregulated as a result of their amplification as part of the large segmental duplication. However, despite their parity in copy number at this loci, RNA-seq analysis revealed that the expression of *CYP6CY3* is more than double that of *CYP6CY4* in *M. p. nicotianae*.[11] This observation suggested that additional mutations that occurred before or after the large segmental duplication may have further elevated *CYP6CY3* expression. In this regard, early work reporting the amplification of *CYP6CY3* also identified a polymorphic AC(n) dinucleotide microsatellite in the putative *CYP6CY3* promoter, comprising 15 repeat units in *M. persicae s.s.*, but increasing to 48 repeat units in *M. p. nicotianae*.[7] Functional investigation of this polymorphism demonstrated that this change results in a doubling of the expression of a reporter gene *in vitro*,[7] and thus likely, in part, explains the enhanced expression of *CYP6CY3* relative to *CYP6CY4*. Furthermore, sequence analysis revealed that all copies of *CYP6CY3* have the microsatellite expansion, suggesting that it predates the amplification event. Subsequent investigation revealed that the expression of *CYP6CY3* is likely further enhanced as a result of additional duplication of this gene outside the large segmental duplication. Specifically, a copy of *CYP6CY3* was identified in *M. p. nicotianae* at a novel locus located on a small (14 Kb) amplicon at least 1.5 Mb away from the native locus.[11] Intriguingly, two DNA transposons, belonging to the *hAT* (*hobo-Ac-Tam3*) and *Tc1/mariner* superfamilies, were also found within the 14 Kb amplicon, neither of which are observed in *M. persicae s.s.* at this position.[11] These transposon sequences occur downstream of *CYP6CY3* and adjacent to the 5′ break point, suggesting they were involved in the mobilization of *CYP6CY3* to the new loci, either indirectly by acting as substrates for nonallelic homologous recombination or directly *via* alternative transposition.[11]

2.2.2 Trans- and post-transcriptional regulation of CYP6CY3

In addition to characterization of the *cis*-regulation of *CYP6CY3*, recent work has also begun to investigate its trans- and post-transcriptional regulation. *CYP6CY3* expression in *M. p. nicotianae* has been shown to be induced (2-fold upregulated) following exposure to 250 μM nicotine.[12] This suggests regulatory regions within or flanking the *CYP6CY3* gene contain xenobiotic responsive elements that are activated by *trans*-acting factors involved in xenobiotic signal transduction. Related to this, investigation of potential trans-regulators of *CYP6CY3* has provided evidence of regulation by the bHLH/PAS transcription factor Aryl hydrocarbon Receptor (AhR), which binds to Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) to form an active heterodimer.[13] Promoter pull down assays, together with reporter gene assays of the *CYP6CY3* promoter with or without the two bHLH/PAS proteins, suggested they regulate its transcription.[13] In the same study *AhR* and *ARNT* were identified as overexpressed in *M. p. nicotianae* compared to *M. persicae s.s.*[13] However, only a single *M. p. nicotianae* clone and two *M. persicae s.s.* clones were used in this study, and transcriptome profiling of a larger number of clones of each

subspecies failed to identify *AhR/ARNT* as consistently upregulated in the tobacco-adapted subspecies.[11] Thus, the importance of the upregulation of these transcription factors in enhancing *CYP6CY3* expression in *M. p. nicotianae* remains unclear.

The post-transcriptional regulation of *CYP6CY3* has been investigated by sequencing microRNAs (miRNAs), a class of non-coding RNAs that play important roles in post-transcriptional gene regulation.[14] This identified the miRNAs *let-7* and *mir-100* as downregulated in a single clone of *M. p. nicotianae* compared to two *M. persicae s.s.* clones.[14] Chemical inhibition of these miRNAs was shown to increase *CYP6CY3* expression and decrease susceptibility to nicotine suggesting they play a role in its regulation.[14] Thus, further investigation of the role of these miRNAs in the regulation of *CYP6CY3* in a greater number of *M. p. nicotianae* and *M. persicae s.s.* clones is required.

2.2.3 *Qualitative changes in* CYP6CY3 *expression*

Work on the regulation of *CYP6CY3* has also served as an excellent illustration that qualitative changes in gene expression can be equally, or even more important, than quantitative changes in enhancing organismal fitness during adaptation to novel conditions. Immunohistochemistry, RNAseq and qPCR analyses revealed that the primary site of *CYP6CY3* expression in *M. persicae s.l.* are the bacteriocytes, representing a previously undescribed tissue-specific expression pattern for an insect P450.[11] Bacteriocytes are specialized aphid cells that house the obligate bacterial endosymbiont *Buchnera aphidicola*, which provides essential amino acids and other nutrients to its host that it does not obtain from its phloem food source.[15] Nicotine has strong antimicrobial properties that extend to Gram-negative bacteria such as *Escherichia coli*, the closest free-living relative of *B. aphidicola*.[16, 17] Assessment of *B. aphidicola* titre following exposure of *M. persicae s.s.* and *M. p. nicotianae* to a diet containing nicotine suggested that enhanced levels of *CYP6CY3* expression in *M. p. nicotianae* may protect *B. aphidicola* from nicotine's inhibitory effects.[11] In addition to the enhanced expression of CYP6CY3 in aphid bacteriocytes, expression analyses further identified the gut as a second site of high expression exclusively in *M. p. nicotianae*, with *CYP6CY3* mRNA levels in this tissue >2500-fold higher in *M. p. nicotianae* than in *M. persicae s.s.*[11] This remodulation of *CYP6CY3* expression in the gut of *M. p. nicotianae* would provide a first line of defense against nicotine ingested during feeding, preventing or delaying it reaching nAChRs in the aphid nervous system. Thus, the pattern of spatial expression of *CYP6CY3* reveals an innovative evolutionary solution, that ensured both the aphid host and its essential symbiont were protected against the primary anti-herbivore defense of tobacco (Fig. 1). In contrast to the tissue-specific expression of *CYP6CY3*, no clear pattern of tissue-specific expression of *CYP6CY4* was observed.[11] Together with the lower levels of expression of this P450 relative to *CYP6CY3*, this finding suggests that overexpression of this P450 may play a secondary role in metabolizing nicotine in tissues outside the gut and bacteriocyte that are exposed to this toxin.

Genomic investigation of the mutations leading to the dramatic increase in *CYP6CY3* expression in the gut by long single-molecule sequencing of the *CYP6CY3* gene locus identified a remarkable series of TE insertions, occurring individually or in combination, in close proximity to amplified copies of the *CYP6CY3* gene.[11] In addition to the *hAT* and *Tc1/mariner* elements described above, these included a transposon with high sequence similarity to the TTAA3_AP element of *Acyrthosiphon pisum*, and a fourth transposon belonging to the Mutator-like element (*MULE*) superfamily of DNA transposons.[11] Transposable element insertions in regulatory regions of P450 genes have been previously shown to lead to both quantitative and qualitative changes in expression.[18, 19] In regards to the latter, examination of the spatial expression of the four elements flanking *CYP6CY3* revealed that, in both *M. persicae* and *M. p. nicotianae*, all are highly and specifically expressed in the gut.[11] Thus, while further experimental validation is required, these TE insertions may have played a role in qualitative changes in the expression of *CYP6CY3* by bringing tissue-specific enhancer sequences into close proximity with this P450 gene.

In summary, the evolution of resistance to nicotine in *M. p. nicotianae* appears to have involved a complex series of mutational events that resulted in profound qualitative and quantitative changes in the expression of P450 genes that can detoxify this toxin. These are outlined in Fig. 2 together with their predicted order of occurrence, based on sequence characterization of the genomic loci involved.

3 CO-OPTION OF A RESISTANCE TRAIT

Resistance monitoring of *M. persicae s. l.* conducted since the commercial launch of the first neonicotinoid, imidacloprid, in Europe and USA consistently showed that tobacco-derived clones exhibit increased tolerance to this insecticide.[20–24] Subsequent studies demonstrated that this resistance extends to other neonicotinoid insecticides.[25] This finding, together with the structural similarity and shared mode of action of nicotine and neonicotinoids, led to the hypothesis that the evolution of resistance to nicotine in *M. p. nicotianae* may have preadapted this subspecies to resist neonicotinoids.[23] The discovery of *CYP6CY3* overexpression in *M. p. nicotianae* allowed this hypothesis to be tested, with functional expression demonstrating that this P450 does indeed metabolize the neonicotinoids imidacloprid and clothianidin to less toxic metabolites *in vitro*, and confers resistance to the latter *in vivo*.[7] Subsequent stable expression of CYP6CY3 in *Drosophila* S2 cells confirmed that CYP6CY3 is active against imidacloprid and clothianidin, as well as acetamiprid and thiacloprid, but had no activity against dinotefuran.[26] Furthermore, and importantly, CYP6CY3 overexpression was identified in two *M. p. nicotianae* clones collected prior to the introduction of neonicotinoids, demonstrating that the mutations leading to the amplification of *CYP6CY3* predate the use of these insecticides.[7] Taken together, these findings unequivocally indicate that *CYP6CY3* overexpression did not arise as a result of selection from the use of neonicotinoid insecticides, but rather that this mechanism evolved during adaptation to tobacco, preadapting *M. p. nicotianae* to resist neonicotinoid insecticides.

CYP6CY3 metabolizes neonicotinoids significantly less efficiently than nicotine,[7] and the levels of resistance conferred by its overexpression are modest,[7, 21] thus the implications of this mechanism for control of *M. p. nicotianae* using this insecticide class are unclear. Tests of the efficacy of the neonicotinoid imidacloprid against *M. p. nicotianae* and *M. persicae s.s.* clones under simulated field conditions showed that both subspecies were well controlled when imidacloprid was applied at the recommended field rate.[21] However, *M. p. nicotianae* was more likely to survive and reproduce when this compound was applied at lower concentrations.[21] Such conditions can arise in neonicotinoid-treated crops from the natural break down of the insecticide over time, or its application at rates below those recommended for aphid control, either as a mechanism to cut costs or to target non-aphid

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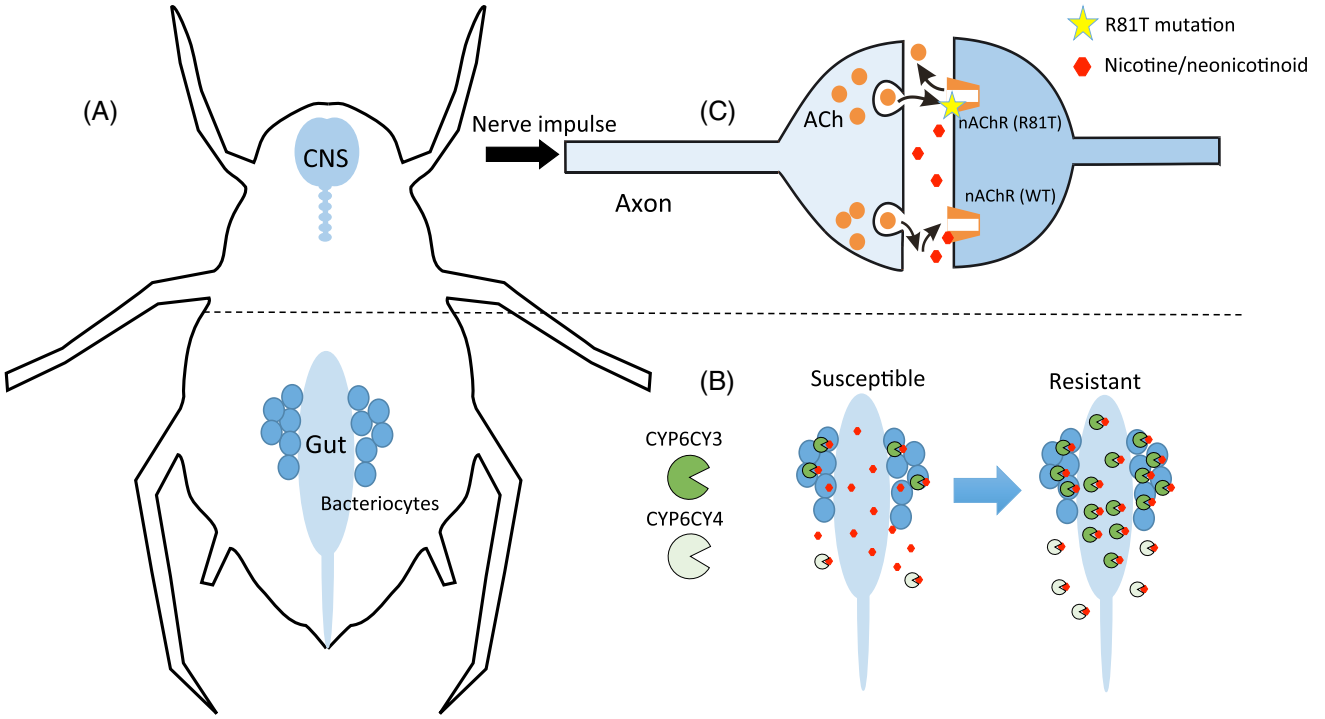

Figure 1. Schematic of the mutations leading to resistance to nicotine and neonicotinoid insecticides in *M. persicae*. (A) Schematic of an aphid showing the primary site of entry (the gut) and action (the nicotinic acetylcholine receptor (nAChR) in the central nervous system (CNS)) of nicotine and neonicotinoid insecticides. Nicotine also has antimicrobial properties that extend to Gram-negative bacteria such as *Escherichia coli*, which is the closest free-living relative of the obligate endosymbiont *Buchnera aphidicola* residing in specialized aphid cells called bacteriocytes. (B) Chromosomal rearrangements result in the increased expression of CYP6CY3 and CYP6CY4 which detoxify nicotine (and in the case of CYP6CY3 certain neonicotinoids). In the case of CYP6CY3 expression is significantly enhanced in bacteriocytes and reprogrammed to include the aphid gut providing two lines of defence that protect *M. p. nicotianae* and its symbiont from nicotine. The high expression of CYP6CY3 in the gut also confers cross-resistance to neonicotinoids. (C) Subsequent mutation (R81T) of the nAChR leads to a reduction in neonicotinoid binding at the receptor, and, in concert with CYP6CY3 overexpression, potent resistance to several members of this insecticide class.

pests present in the crop.[21] Thus, *CYP6CY3* overexpression may provide fitness benefits in neonicotinoid-treated crops that select for this mechanism. If this is the case then an increase in frequency of this mechanism in *M. persicae s.l.* following the introduction of neonicotinoids might be expected, potentially as a result of its transfer from *M. p. nicotianae* to *M. persicae s.s.* Recent population genomic analyses of 127 *M. persicae s.l.* aphid clones collected from multiple host plants around the world has provided strong evidence that selection from neonicotinoid use may indeed have been sufficiently strong for this to have occurred.[5] *CYP6CY3* amplification was found to be ubiquitous in globally sampled clones collected from tobacco, providing further confirmation of the importance of this mechanism in enabling *M. p. nicotianae* to exploit this host plant.[5] However, *CYP6CY3* amplification was also observed at high frequency in clones derived from several other host plants.[5] This suggests that the fitness benefits conferred by this mutation extend beyond nicotine resistance, with its pleiotropic effects on neonicotinoid insecticide sensitivity the most likely explanation. Investigation of the number of evolutionary origins of this mutation revealed that the mechanism of *CYP6CY3* amplification is identical in all clones, regardless of geographical origin.[5] This suggests that *CYP6CY3* amplification had a single origin in *M. p. nicotianae*, with this mechanism subsequently spreading to *M. persicae s.s.* around the world following the introduction of neonicotinoids. Thus, the ability of *CYP6CY3* amplification to confer reduced sensitivity to neonicotinoids, previously a co-incidental pleiotropic effect, appears to have become the major selective force driving the geographic spread of this trait.

4 STANDING GENETIC VARIATION COMBINES WITH *DE NOVO* MUTATION TO CONFER POTENT NEONICOTINOID RESISTANCE

The fact that *M. persicae s.l.* clones overexpressing CYP6CY3 can be controlled when neonicotinoids are applied at recommended rates,[21] provided strong selection for any novel mutations that, in isolation or in combination with CYP6CY3 overexpression, led to more potent neonicotinoid resistance. This scenario was realized 20 years after the launch of neonicotinoids with the discovery of a clone of *M. persicae* that exhibited potent resistance to this insecticide class, easily sufficient to compromise their field effectiveness.[27] This clone was found to carry the mutations leading to CYP6CY3 overexpression, in combination with a novel mutation in the β1 subunit of the nAChR that resulted in an arginine to threonine substitution (R81T).[27] Importantly the amino acid at this position occurs in a region that forms the binding site for the natural ligand acetylcholine and agonists such as neonicotinoids, and is a key determinant of neonicotinoid selectivity for insect nAChRs.[28, 29] Specifically the positively charged arginine usually present at this position in insects forms electrostatic interactions with the distinctive electronegative pharmacophore (nitro

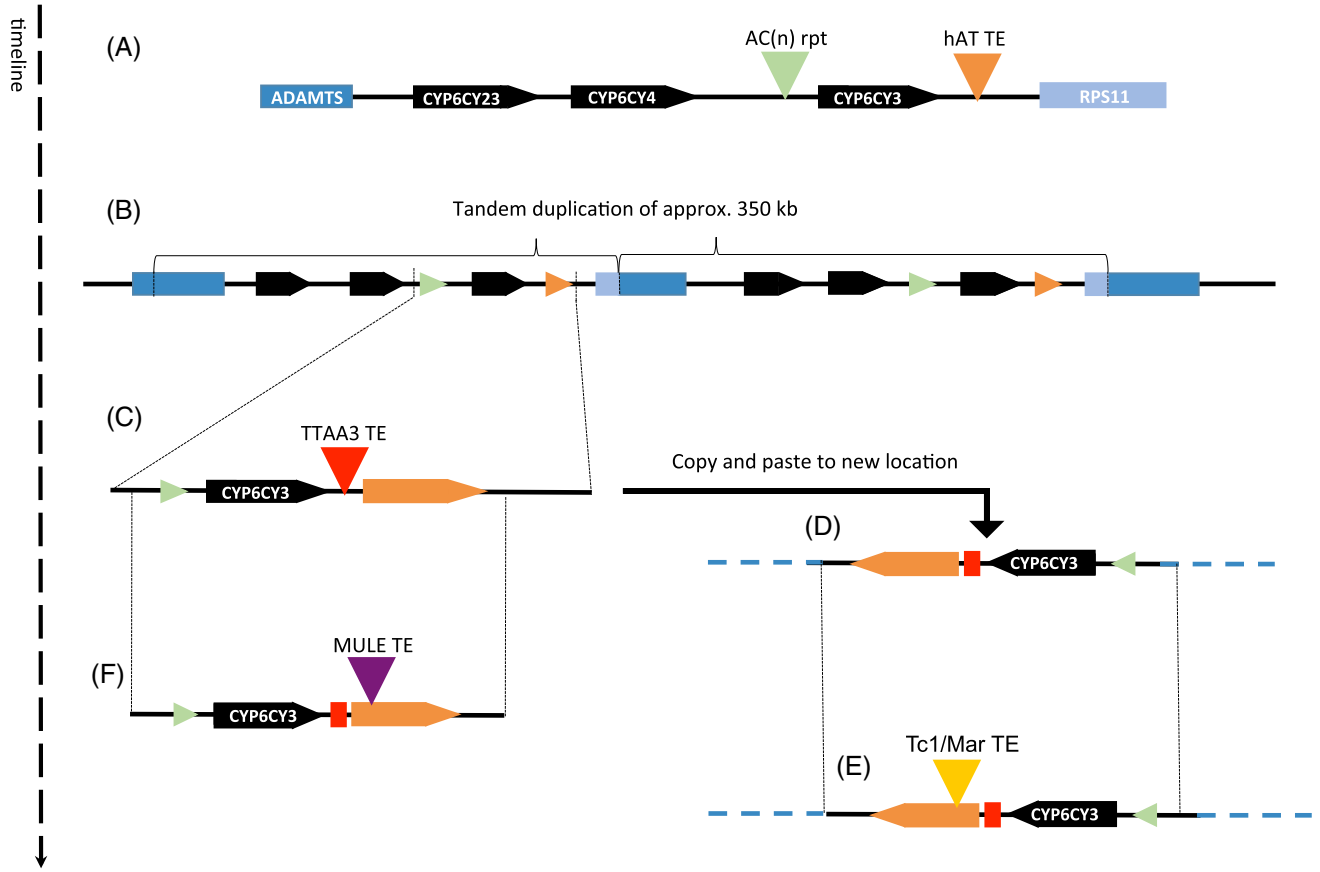

Figure 2. Schematic of mutational events at the *CYP6CY3* locus. (A) Initial genetic modification of the locus included the expansion of an AC(n) microsatellite repeat in the promoter of *CYP6CY3* and the insertion of a hAT transposable element downstream of this gene. (B) A region of approximately 350 Kb in size was then amplified as a series of three to five direct repeats, increasing the copy number of all the genes in the amplicon and creating a chimeric gene (*RPS11/ADAMTS9*). Further remodeling of the 3′ region of *CYP6CY3* continued with the insertion of a TTAA3 element into one of the copies of *CYP6CY3* (C). *A* ~ 14 Kb region encompassing *CYP6CY3* in combination with the hAT and TTAA3 elements was then copied to a new location on the same chromosome (D), after which it was further modified by the insertion of Tc1/mariner element into the existing hAT sequence (E). Remarkably, the latter process appears to have been repeated in the case of the original *CYP6CY3* copy at the native locus but in this case a MULE transposon inserted into the hAT sequence (F). Additional genes included in the 350 Kb insertion and then silenced are not shown for clarity.

or cyano group) of neonicotinoid insecticides.[27–29] In contrast, in the case of vertebrate β subunits these interactions do not form as the amino acid at this position rarely has a positive charge, with a threonine the most common residue observed.[27–29] Thus, the R81T mutation appears to reduce the sensitivity of the nAChR to neonicotinoids by conferring a 'vertebrate-like' quality to the β1 subunit of resistant aphids.[27] Recently, the causal role of R81T in conferring resistance to neonicotinoids was further confirmed by CRISPR-CAS genome editing of *D. melanogaster*.[30] This work also demonstrated that R81T is inherited as an incompletely recessive trait in Drosophila,[30] consistent with the dominance status of this mutation reported for *M. persicae*.[31]

Intriguingly, since its discovery, the R81T mutation has only ever been observed in clones displaying *CYP6CY3* amplification.[3, 5] This suggests that this mutation emerged on a genetic background of *CYP6CY3* overexpression, and the two mechanisms together provide strong fitness benefits against neonicotinoids. Support for this hypothesis has come from two studies. Firstly, co-application of neonicotinoids and the P450 inhibitor piperonyl butoxide to aphids carrying CYP6CY3 overexpression + R81T resulted in a reduction of resistance of up to approximately 9-fold, suggesting a strong P450-mediated component to resistance.[27] Secondly, introduction of R81T into *D. melanogaster* by genome editing resulted in transgenic flies with resistance phenotypes of only 6.5- and 32.6-fold for acetamiprid and imidacloprid, respectively.[30] Thus, the modest levels of resistance conferred by this mutation in isolation is unlikely to fully explain the >1000-fold resistance factors observed in *M. persicae* clones that carry R81T in combination with *CYP6CY3* amplification.[27] While further experimental work is required using the native species, taken together these findings suggest that the evolution of resistance to neonicotinoids in *M. persicae* represents an adaptive walk. In this case, the sequential emergence of novel beneficial mutations comprised a metabolic mechanism arising during adaptation to tobacco followed by target site alteration. Individually, available evidence suggests these confer modest levels of resistance, however, in concert they appear to act synergistically to confer potent resistance to neonicotinoids. In this regard, recent studies of transgenic *Drosophila* lines expressing insecticide metabolizing P450 enzymes of insect crop pests and disease vectors in a genetic background containing target-site resistance mutations has provided clear evidence that these mechanisms may often act synergistically to confer strong resistance.[32]

Finally, given that R81T is spreading through *M. persicae s.l.* populations,[4, 5, 33, 34] it will be interesting to explore if the

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movement of this mechanism into *M. p. nicotianae* provides additional fitness advantages to this subspecies on tobacco by conferring higher levels of resistance to nicotine. In this case, a mechanism that appears to have evolved to resist synthetic insecticides would be recruited to provide additional protection against a natural xenobiotic, demonstrating that the co-option of resistance mechanisms can occur in both directions.

5 EVOLUTIONARY AND APPLIED INSIGHTS

The detailed molecular characterization of resistance in *M. persicae* to natural and synthetic insecticides outlined in this review provides insights of relevance to both fundamental understanding of the evolutionary processes mediating adaptation to novel conditions and applied aspects of pest and resistance management. In this section we briefly outline some of the key fundamental and applied questions this work has informed.

5.1 How many and what type of mutational events or 'adaptive steps' underpin adaptive phenotypes?

The molecular investigation of resistance to nicotine in *M. p. nicotianae* has uncovered a remarkable catalogue of mutations in the genomes of resistant clones (Fig. 2). As described above these include: (i) expansion of microsatellite repeats in gene regulatory regions, (ii) large and small gene duplications that increase the gene dosage of single or multiple genes, (iii) nonfunctionalizing mutations that inactivate amplified gene copies that provide no fitness benefit, and (iv) transposable element insertions that may have been involved in gene duplication events and fundamental changes in gene regulation. Collectively, these findings demonstrate that the evolution of even a relatively simple phenotype (resistance to nicotine) can be underpinned by a remarkable diversity of mutational events. They also provide new insights into the mechanisms by which genetic variation is created, and show that profound genetic alterations, such as chromosomal rearrangements and transposable element insertions, can provide a rich and dynamic source of raw material for adaptation. Indeed, the extensive genetic variation created by these processes may provide greater opportunities for saltatory evolution than stepwise adaptation involving point mutations. The progression of mutations uncovered in this case study also serves as a reminder of the complex processes sometimes involved in adaption. Specifically, the finding that large-scale gene amplification was followed by remodeling to eliminate gene copies that provide no fitness benefit, illustrates how the journey through fitness landscapes can involve the creation of genetic by-products in the form of pseudogenes.

5.2 What is the relative contribution of quantitative *versus* qualitative divergence during the evolution of novel traits?

Study of the role of *CYP6CY3* in resistance has shown that while mutations leading to quantitative changes in gene expression are unquestionably important, in some cases, qualitative changes in gene expression may be just as, or perhaps more, important in conferring the novel phenotype (resistance). Specifically, the profound remodeling of *CYP6CY3* expression to include the aphid gut created a first line of defense against nicotine ingested during feeding.[11] Furthermore, the enhanced expression of this P450 in the specialized cells housing the obligate bacterial symbiont *B. aphidicola* appears to have provided additional protection against the antimicrobial properties of nicotine.[11] Thus, both qualitative and quantitative changes in *CYP6CY3* expression were likely key to ensure the fitness of an essential mutualistic symbiosis was preserved during an insect host shift. Although further experimental confirmation is required, these studies also further implicate the potential role of transposable elements in mediating transcriptional regulation by bringing tissue-specific enhancer sequences in close proximity to key resistance genes.[11, 35]

5.3 What are the origins of resistance traits? Can mechanisms that have evolved to overcome natural xenobiotics be recruited to resist synthetic insecticides?

An important question of applied relevance to pest and resistance management is the extent to which mechanisms arising during insect host plant adaptation can be co-opted to confer resistance to synthetic insecticides. Research on the tobacco-adapted subspecies *M. p. nicotianae* has provided a compelling example that this can occur, with the evolution of mechanisms to overcome the plant alkaloid nicotine preadapting this subspecies to resist neonicotinoids, a globally important class of insecticide. Neonicotinoids and nicotine exhibit similarity in chemical structure, and the active site of CYP6CY3 is thus able to accommodate both plant and man-made insecticides.[3, 7] The observed cross-resistance of *M. p. nicotianae* to these compounds demonstrates that existing detoxification systems can be co-opted to protect insects from insecticides if sufficient similarity exists between their natural substrate(s) and the synthetic compound in question. This finding also highlights the inherent risk of resistance development to insecticidal compounds that share chemical similarity with natural compounds encountered by herbivorous insects.

5.4 Does resistance to synthetic insecticides arise by *de novo* mutation or from standing variation?

As noted above, work on *M. persicae* has shown that the pleiotropic effects of existing adaptations to natural compounds can result in their selection by synthetic insecticides. In this context the polymorphisms leading to *CYP6CY3* overexpression represented standing genetic variation, that is, the polymorphisms were already present in the population prior to the change in selective pressure (the introduction of neonicotinoid insecticides). The levels of neonicotinoid resistance conferred by this mechanism are modest, and thus subsequent *de novo* mutation was required to provide *M. persicae* with greater protection to this insecticide class. Intriguingly, these mechanisms have only ever been observed in combination and appear to act synergistically to confer potent resistance.[3, 5, 27, 30] This suggests that standing genetic variation provided a platform (*i.e.* suitable genetic background) for further evolution by *de novo* mutation. Indeed, the low levels of resistance conferred by the R81T mutation alone,[30] and its synergistic interaction with CYP6CY3 overexpression,[27] suggest that the probability of R81T surviving loss *via* stochastic processes while still rare was enhanced by its emergence in a genetic background of CYP6CY3 overexpression. This research also demonstrates that the evolution of insecticide resistance from standing genetic variation and by *de novo* mutation are not necessarily mutually exclusive, and both sources of variability can act together to provide resistance. This is significant as the relative contribution of *de novo* mutations and standing variation in resistant phenotypes has a number of implications of relevance to resistance management and risk assessment including

repeatability, probability and speed of emergence, and spread of resistance mutations.[36] Finally, it is predicted that *de novo* adaptations to sudden environmental change emerge rapidly, whereas standing variation accumulates over a longer time scale.[36] The latter may allow rarer genetic changes to occur and more complex traits to evolve.[36] The mechanisms of *M. persicae* resistance to neonicotinoids is consistent with these predictions, with the standing variation in this case comprising a complex suite of mutations, in contrast with the single nucleotide alteration arising by *de novo* mutation.

5.5 To what extent do the findings described for *M. persicae* in this review extend to other insect and mite pest species?

Many of the processes and insights into resistance evolution described for *M. persicae* have parallels with recent research conducted on other arthropods. In the case of the genetic architecture of insecticide resistance, while resistance in pest populations directly derived the field, appears to be primarily driven by single genes/mutations of large effect, other, more complex cases have been described. For example in *Drosophila melanogaster* DDT resistance is conferred by overexpression of the P450 gene *Cyp6g1*.[37] Elegant work investigating the upregulation of this gene uncovered successive mutations at the *Cyp6g1* locus, including copy number increases and multiple transposable element insertions in the 5′ regulatory region of the gene.[38] Importantly, this sequence of mutations was shown to lead to step-wise increases in DDT resistance.[38] Further potential parallels of the work on *Cyp6g1* with that of *M. persicae CYP6CY3* relate to qualitative changes in gene expression. Specifically, *cis*-regulatory elements present in the transposable elements in the promoter region of *Cyp6g1* increased its expression in tissues important for detoxification such as the midgut, Malpighian tubules, and the fat body.[35] Changes in gene copy number have also been shown to lead to insecticide resistance in a range of other arthropod pests, in some cases involving large segmental duplications.[39] For example, the *ace-1* gene (encoding the target of organophosphate and carbamate insecticides) was shown to be amplified with 11 other genes as an amplicon of 203 kb in the mosquito *Anopheles gambiae*.[40] Furthermore, as seen at the *CYP6CY3* locus of *M. persicae*, intra-amplicon deletions were identified at the *ace-1* locus that occurred after the duplication that reduced the cost of dosage imbalance for the genes co-amplified with *ace-1*.[40] Taken together, these studies clearly illustrate the important role of transposable elements and gene duplication/amplification in the evolution of insecticide resistance, and demonstrate that these mechanisms are not exclusive to aphids.

The link between adaptation to plant secondary metabolites and resistance to synthetic insecticides described for *M. persicae* is complemented by work on this topic in other generalist pest herbivores, and more recently, non-pest species. For example, recent studies on the glasshouse whitefly and spider mites have shown that transfer of these species to a range of different host plants results in profound changes in gene expression (up to 20% of genes) including in genes encoding detoxification enzymes.[41, 42] Remarkably, these changes in gene expression are associated with significant shifts in the tolerance of host-adapted lines to synthetic insecticides.[41, 42] Together these findings extend the work on *CYP6CY3* of *M. persicae* by demonstrating that induced as well as constitutive changes in the expression of detoxification genes during host plant adaptation can lead to changes in tolerance to synthetic insecticides. In the case of non-pest species, work on bee pollinators has provided additional evidence that the metabolic systems used by insects to detoxify the natural toxins encountered in their environment can preadapt them to tolerate certain synthetic insecticides. Specifically, in honey bees (*Apis mellifera*), bumblebees (*Bombus terrestris*) and red mason bees (*Osmia bicornis*) P450 enzymes belonging to the *CYP9Q* and *CYP9BU* subfamilies were shown to provide protection to certain insecticides from three different classes including *N*-cyanoamidine neonicotinoids.[43–45] Thus these insecticides must be sufficiently similar to the native substrates of these enzymes to allow them to be metabolized. In this regard, although the diversity of natural substrates that these P450 subfamilies can metabolize has not been characterized, all members of the *CYP9Q* subfamily in honeybees can metabolize the plant secondary metabolite quercetin with high efficiency, a flavonoid that is present in pollen and nectar.[46]

Finally, in relation to the evolutionary origins of insecticide resistance, the findings on *M. persicae* are consistent with other insects, where cases of evolution by either *de novo* mutation or selection from standing variation have been described.[36] Examples of resistance evolution *via* a combination of both standing variation and *de novo* mutation, such as in the case of neonicotinoid resistance in *M. persicae*, appear to be much rarer. However, work on the genetics of resistance to organophosphate (OP) insecticides in the sheep blowfly, *Lucilia cuprina*, has provided another example that the two origins are not mutually exclusive.[47, 48] In this case resistance to the OP insecticides diazinon and malathion is conferred by mutations at two sites (G137N and W251L/S/T) in the *Lcα7* gene encoding the esterase E3 enzyme.[47, 48] While, one of these (G137N) was only identified in samples collected after the introduction of diazinon, suggesting it evolved by *de novo* mutation, mutations at the second site (W251) were identified in preserved specimens collected prior to the first use of OPs, providing unambiguous evidence of their presence as standing genetic variation in populations of *L. cuprina*.[47, 48]

6 KNOWLEDGE GAPS AND FUTURE RESEARCH

While the work detailed in this review has advanced understanding of the molecular mechanisms of xenobiotic resistance, a number of unanswered questions on the genetic architecture of resistance in *M. persicae* remain and provide numerous avenues for future research. These are briefly summarized below.

While the causal role of *CYP6CY3* and *CYP6CY4* in nicotine resistance in *M. p. nicotianae* has been established, it is possible that other genes contribute either directly or indirectly. Specifically, further investigation of the role of the *RPS11/ADAMTS9* chimeric gene in *M. p. nicotianae* is warranted, to clarify if it provides any fitness benefits on tobacco or if it represents a neutral polymorphism retained because of its close linkage to other genes in the amplicon that are under positive selection. The finding that transgenic lines of *D. melanogaster* expressing the chimeric gene remain sensitive to nicotine is inconsistent with a direct role in resistance.[11] However, the high expression of *RPS11/ADAMTS9* in *M. p. nicotianae* and absence of nonfunctionalizing mutations in its coding sequence is intriguing, and suggests it might provide alternative fitness benefits on tobacco within its native genetic background.[11] Similarly, transcriptomic analyses identified a glutathione *S*-transferase (GST) belonging to the sigma class as significantly downregulated in *M. p. nicotianae* compared to *M. persicae*

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s.s.[11] GSTs are dimeric enzymes that are most commonly known for their role in cellular detoxification - catalyzing the conjugation of glutathione (GSH) to a wide range of endogenous and xenobiotic agents.[49] However, the downregulation of the GST in *M. p. nicotianae* is not consistent with a fitness benefit *via* a direct role in metabolism, and future work could explore potential non-enzymatic roles in adaptation to nicotine/tobacco. Recent advances in the development of CRISPR-CAS genome editing for the pea aphid, *Acyrthosiphon pisum*, offer a promising avenue for further investigation of the functional characterization of these candidate genes.[50] However, the difficulties associated with the generation and crossing of sexual stages, and the number and development time of eggs produced in *M. persicae* remain obstacles for future adoption of this technology.

The discoveries made on the amplification and regulation of *CYP6CY3* and *CYP6CY4* have also generated further questions and topics for future research. Firstly, the precise mechanisms leading to the large segmental duplication at the native *CYP6CY3/4* locus remain unresolved. Changes in gene copy number can result from non-allelic homologous recombination, non-homologous end joining and/or *via* the activity of transposable elements.[51] The recent publication of two chromosome-scale genome assemblies for *M. persicae* provides a new opportunity to investigate the role of these processes in the amplification of *CYP6CY3/4*.[5, 52] The remarkable expression profile of CYP6CY3 in *M. persicae s.s.* and *M. p. nicotianae* also merits further detailed investigation. The expression of this P450 in the aphid bacteriocyte in both subspecies[11] is unprecedented, and its native role(s) in this specialized tissue is unclear. Further functional analyses are also required to identify the mutations leading to the profound modifications in the tissue-specific expression of *CYP6CY3* in the tobacco-adapted subspecies.[11] In particular, the role of the numerous transposable element insertions occurring in close proximity to *CYP6CY3* in activating expression in the gut requires confirmatory work. More widely, the diverse impacts of transposable elements on host genomic complexity and evolution are now well-established,[19, 53] and transposons are increasingly implicated in resistance evolution.[54, 55] Consequently, transposon load may directly affect the evolvability of pest lineages, particularly when under strong selection, with accompanying implications for insecticide resistance and control. Thus, more research is required to examine the extent to which mobile genetic elements are important players in resistance evolution. Work on these topics will likely also provide further insight into the *trans*-acting factors that regulate *CYP6CY3*.

In terms of neonicotinoid resistance, the precise level of resistance conferred by target-site and metabolic mechanisms individually and in combination in *M. persicae* remains uncertain, and this could be investigated using a conventional genetics approach or by CRISPR-CAS genome editing. Similarly, the impact of R81T and CYP6CY3 overexpression in isolation on the efficacy of control in the field using neonicotinoids remains to be defined for many crops where this insecticide class is widely used. It would also be interesting to examine the fitness of clones carrying the R81T mutation on tobacco to establish if this mechanism provides additional advantages to those conferred by CYP6CY3/4 overexpression. This could also reveal if a resistance mechanism resulting from anthropogenic activity may be co-opted to enhance fitness against a plant defensive compound.

Finally, work is required to establish whether the resistance mechanisms described in this review carry fitness costs in the absence of insecticide selection. In particular, the >2500-fold increase in expression of CYP6CY3 in the aphid gut would be envisaged to incur a significant metabolic cost. Investigation of this topic may also benefit from novel genome editing approaches as these can facilitate fitness comparisons of clones that share the same genetic background but differ in the presence of a specific resistance mechanism.[56] Knowledge generated by such studies is of significant applied value as fitness penalties may result in the restoration of susceptibility to an insect population in the absence of selection, which may be exploited in control and resistant management strategies.

7 CONCLUSION

Over the last decade the development of next generation sequencing technologies and associated post-genomic functional approaches have allowed investigation of the molecular mechanisms involved in resistance to natural and synthetic insecticides at unprecedented resolution. In the case of *M. persicae*, this has revealed the remarkable array of both large-scale and subtle genetic alterations that can be selected by insecticide exposure. Research has illustrated the value of investigation of resistance in providing fundamental insights into the molecular processes by which genetic variation is created and used in the emergence of novel traits. In turn, a mechanistic understanding of resistance evolution has provided knowledge of applied relevance that can be used to inform the design of resistance risk assessments and strategies that aim to prevent, contain or overcome resistance. Finally, this work has generated a range of questions and topics for further investigation providing multiple avenues for exciting research in the years ahead.

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