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The evolution of insecticide resistance in the peach potato aphid, *Myzus persicae*

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ABSTRACT

The peach potato aphid, *Myzus persicae* is a globally distributed crop pest with a host range of over 400 species including many economically important crop plants. The intensive use of insecticides to control this species over many years has led to populations that are now resistant to several classes of insecticide. Work spanning over 40 years has shown that *M. persicae* has a remarkable ability to evolve mechanisms that avoid or overcome the toxic effect of insecticides with at least seven independent mechanisms of resistance described in this species to date. The array of novel resistance mechanisms, including several 'first examples', that have evolved in this species represents an important case study for the evolution of insecticide resistance and also rapid adaptive change in insects more generally. In this review we summarise the biochemical and molecular mechanisms underlying resistance in *M. persicae* and the insights study of this topic has provided on how resistance evolves, the selectivity of insecticides, and the link between resistance and host plant adaptation.

1. Introduction

The peach potato or green peach aphid, *Myzus persicae* Sulzer (1776) (Hemiptera: Aphididae) is the most economically important aphid crop pest worldwide (van Emden and Harrington, 2007). There are a number of factors that have enhanced the status of this species as a pest, including its distribution, host range, mechanisms of plant damage, life cycle, capacity to disperse and ability to evolve resistance to insecticides. *M. persicae* is both an extremely cosmopolitan species with a worldwide distribution (see www.cabi.org for its current distribution) and highly polyphagous, with a host range of more than 400 species in 40 different plant families, including many economically important crop plants (Blackman and Eastop, 2000). The exceptional ability of *M. persicae* to adapt to new host plants has, in some cases, led to the formation of host races, the best documented of these being associated with tobacco, and formally named as *M. persicae* subsp. *nicotianae* (Blackman, 1987). *M. persicae* causes damage to its host by direct feeding, the transmission of plant viruses and the production of honeydew. The economic importance of these mechanisms varies depending on crop plant but in many cases the primary route of damage stems from the efficiency of this species as a virus vector; it is capable of transmitting over 100 different plant viruses (Blackman and Eastop, 2000).

The life cycle of *M. persicae* depends on the climate, the availability of its primary winter host *Prunus* spp., especially *P. persica* (peach), and the genotypic lineage (Blackman, 1974). In temperate regions of the world, where *P. persica* is available and autumn temperatures are low enough, *M. persicae* is usually heteroecious and holocyclic, with the winter sexual phase on *P. persica* and the parthenogenetic (asexual) summer generations on a wide number of secondary herbaceous summer hosts. However, in many countries where peach is absent and/or a warmer climate permits, the life cycle is often anholocyclic (continual parthenogenesis throughout the year). This latter life-cycle, combined with a short generation time, allows populations to increase rapidly under favourable conditions and quickly reach damaging numbers. In addition, this mode of reproduction has significant implications for population genetics and therefore relevance to the topic of this review.

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The control of *M. persicae* on many crops has relied almost exclusively 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. The first report of resistance in this species dates back to 1955 (Anthon, 1955) with resistance now reported to most classes of insecticide, including the organophosphates, carbamates, pyrethroids, cyclodienes, and neonicotinoids (Fig. 1), making *M. persicae* one of the most widely and strongly resistant species worldwide (www.pesticideresistance.com). The focus of this review is the biochemical and molecular mechanisms underlying resistance in *M. persicae* that have been characterised to date and the insights this body of work has provided into resistance and adaptive evolution in insects more generally. The last review of this topic was published in 1998 (Devonshire et al., 1998) and included details of the three resistance mechanisms that had been described up to that point, since then at least four additional mechanisms of resistance have been described (Fig. 2).

2. Biochemical and molecular mechanisms of resistance in *M. persicae*

2.1. Overproduction of carboxylesterases and resistance to organophosphate and carbamate insecticides

The first mechanism of resistance to insecticides described in *M. persicae* was the enhanced production of carboxylesterases that confer broad spectrum resistance to members of the organophosphate (OP), (mono-methyl) carbamate and, to a much lesser extent, pyrethroid classes (Fig. 2). This mechanism was first implicated over 40 years ago when it was demonstrated biochemically that the esterases of OP resistant aphids had an enhanced ability to hydrolyse a model substrate (1-naphthyl acetate) for this enzyme class (Needham and Sawicki, 1971). Subsequent work showed that this was due to the overproduction of one of two possible carboxylesterases, E4 or FE4, that both hydrolyse and sequester the insecticide before it can reach the target site in the insect nervous system (Devonshire and Moores, 1982; Devonshire et al., 1983). Further studies implicated and then confirmed that amplification of the structural E4 and FE4 genes (with just one of the two paralogues usually amplified in individual aphids) was the genetic basis of overproduction (Devonshire and Sawicki, 1979; Field et al., 1988). The level of amplification was shown to be highly correlated with the resistance phenotype, with a serial four-fold increase in gene copy number (up to a maximum of approximately 80 copies), leading to successively more resistant aphids (R_1, R_2 and R_3 phenotypes) (Field et al., 1999). Fluorescence *in situ* hybridisation (FISH) was used to demonstrate that amplification of the E4 gene is closely linked to a chromosomal translocation (the autosomal 1,3 translocation event), and that amplified genes are situated at a single heterozygous site on autosome 3, as a tandem array of head-to-tail amplicons (Blackman et al., 1995; Field and Devonshire, 1997). In contrast, amplified FE4 genes are not associated with any visible chromosomal rearrangement and are present at multiple loci in the genome (Blackman et al., 1995, 1999). An interesting aspect of the resistance resulting from amplified E4 genes is that it can be unstable, with 'revertant' clones displaying a sudden loss of both esterase gene expression and insecticide resistance within a single generation (ffrench-Constant et al., 1988). In revertants, the large reduction in expression is associated with the loss of 5-methylcytosine (5 mC) present in CpG doublets within the E4 genes, resulting in gene silencing via demethylation (Field, 2000; Field et al., 1989). This allows the costly production of esterase enzyme (up to 3% of total protein in R_3 aphids) to be 'switched off' in the absence of insecticide selection. Although the amplification of E4 is clearly associated with the chromosomal translocation, exactly how this results in amplification of the E4 gene is not fully understood. Even less is known about the mechanism underlying the amplification of FE4 with speculation that it may have occurred by inversions, reciprocal exchanges or in association with transposable elements remaining unconfirmed (Blackman et al., 1999).

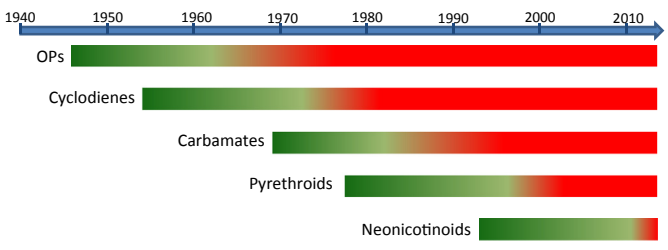

Fig. 1. Timeline of resistance development in *M. persicae*. Green bars indicate years where insecticides provide good control. Red bars indicate the development of control compromising resistance. OPs: organophosphates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

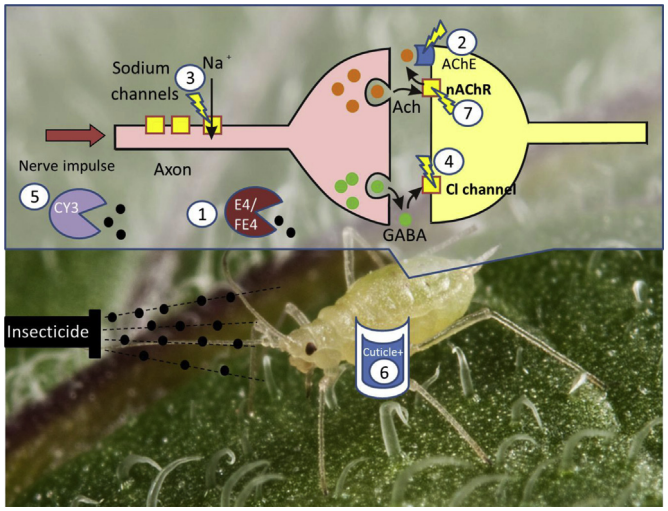

Fig. 2. Cartoon of seven resistance mechanisms characterised in *M. persicae*. Resistance mechanisms are numbered 1–7, many of these involve mutation of insecticide targets in the insect nervous system and a cartoon on an insect synapse is depicted at the top of the figure. Enhanced expression of E4/FE4 esterase confers resistance to organophosphates (OPs), (mono-methyl) carbamates and, to much a lesser extent, pyrethroids by sequestering and metabolising these insecticides before they reach the nervous system. 2. Mutation (S431F) of the acetylcholinesterase enzyme (AChE) results in resistance to dimethyl carbamates. 3. Mutation (L1014F, M918T, M918L) of the voltage-gated sodium channel confers resistance to pyrethroids. 4. Mutation (A302G) of the GABA gated chloride channel confers resistance to cyclodienes. 5. Enhanced expression of the P450 CYP6CY3 confers resistance to nicotine and cross-resistance to neonicotinoids. 6. Reduced penetration through the cuticle confers resistance to neonicotinoids. 7. Mutation (R81T) of the nicotinic acetylcholine receptor (nAChR) confers resistance to neonicotinoids.

2.2. Mutation of the acetylcholinesterase enzyme and insensitivity to dimethyl carbamate insecticides

Certain members of the carbamate class of insecticides, such as the dimethylcarbamates pirimicarb and triazamate, retain good efficacy against *M. persicae* populations with high levels of esterase resistance and have excellent selectivity profiles as aphicides (Foster et al., 2002). However, in the early 1990s significant resistance to these insecticides was detected in populations of *M. persicae* from Greece (Moores et al., 1994). Biochemical inhibition assays demonstrated that resistance results from insensitivity of

the target site, the acetylcholinesterase enzyme (AChE), a serine hydrolase that terminates nerve impulse transmission by rapidly hydrolysing the neurotransmitter acetylcholine at cholinergic synapses (Fig. 2). Modified AChE (MACE) leads to a specific >100-fold insensitivity to the dimethyl carbamate, pirimicarb (Moores et al., 1994). However, initial attempts to identify the molecular basis of MACE by cloning the gene encoding AChE (*ace*) by homology with the dipteran *Drosophila melanogaster/Musca domestica ace* locus failed to reveal any amino acid differences between resistant and susceptible aphid clones (Javed et al., 2003). This paradox was resolved when several studies revealed that many insects, including *M. persicae*, have two *ace* genes and the gene encoding the insecticide target (termed *ace-1*) in these species is not the orthologue of the *D. melanogaster/M. domestica* gene (termed *ace-2*) (Andrews et al., 2004). Sequencing of the *ace-1* gene of resistant and susceptible *M. persicae* clones revealed a point mutation which causes a single amino acid substitution S431F in the predicted protein sequence of the insensitive enzyme that correlated with resistance to pirimicarb (Andrews et al., 2002; Nabeshima et al., 2003). Subsequent recombinant expression of modified aphid *ace-1* provided functional evidence that this mutation strongly affects pirimicarb binding to AChE (Benting and Nauen, 2004). The S431F substitution is located in the acyl pocket of AChE which determines the orientation of ligands in the active site. The substitution of a small serine with a large, hydrophobic phenylalanine would be predicted to disturb both the space and hydrophobicity, thus preventing pirimicarb from interacting with at least one member of the catalytic triad at the centre of the active site (Andrews et al., 2004; Nabeshima et al., 2003). An interesting aspect of the S431F substitution seen in the AChE of pirimicarb resistant *M. persicae* is that it is the reverse of the situation reported in non-aphid insect species and vertebrates, where a highly conserved phenylalanine is the norm at the same position (Fig. 3). Sequencing of ten additional aphid species confirmed that serine is the default residue in wild-type aphid AChEs, with the phenylalanine mutation associated with pirimicarb-insensitivity bringing the amino acid back in line with other organisms (Andrews et al., 2004). This finding indicates that the choice of amino acid residue at this position has a strong influence on the selectivity of pirimicarb and that it is the presence of the native serine in aphids that causes sensitivity to the dimethylcarbamates such as pirimicarb and underlies the excellent aphicidal specificity of this insecticide.

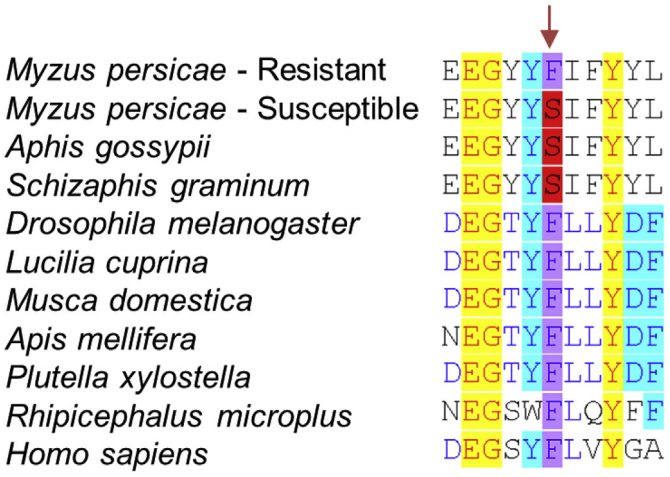

Fig. 3. Amino acid alignment of the AChE sequence of aphids, other insects/mites and humans. The arrow highlights the amino acid at position 431 where a substitution S431F is observed in *M. persicae* clones that are resistance to pirimicarb. Genbank numbers of sequences: *Myzus persicae* (AY147797); *Aphis gossypii* (AJ748114); *Schizaphis graminum* (AF321574); *Drosophila melanogaster* (X05893); *Lucilia cuprina* (U88631); *Musca domestica* (AJ310134); *Apis mellifera* (AF213012); *Plutella xylostella* (AY061975); *Rhicephalus microplus* (AJ223965); *Homo sapiens* (M55040).

2.3. Mutation of the voltage-gated sodium channel and resistance to pyrethroid insecticides

Although overproduction of esterases confers modest cross-resistance to most pyrethroid insecticides, it was subsequently demonstrated that this is secondary to resistance caused by a target-site resistance mechanism termed `knockdown resistance' or kdr (Martinez-Torres et al., 1999) (Figs. 2 and 4). Kdr, alongside an enhanced allelic form 'super-kdr' was initially identified in houseflies (Sawicki, 1978) and was subsequently shown to be conferred by mutations in the voltage-gated sodium channel (Williamson et al., 1996), a transmembrane ion channel that plays an essential role in the initiation and propagation of action potentials in neurons, and the target of pyrethroids. The kdr mechanism was first reported in *M. persicae* in 1997 when a leucine-to-phenylalanine replacement (L1014F) in transmembrane segment IIS6 of the sodium channel was identified in several pyrethroid resistant clones (Martinez-Torres et al., 1997, 1999). Several of these clones had been continuously reared in the laboratory for over twenty years indicating that this mechanism had been present in *M. persicae* populations for some time prior to its discovery. In *M. persicae* kdr alone confers 35-fold resistance to the pyrethroid deltamethrin and cross resistance to DDT (which shares the same target-site) but resistance is enhanced up to a further 15-fold in aphids with additional high levels of esterase (Martinez-Torres et al., 1999). *In vitro* expression in *Xenopus laevis* oocytes has confirmed that insect sodium channels with the kdr mutation are up to 17-fold less sensitive to the toxic effects of pyrethroids (Vais et al., 2000). Molecular modelling of insect sodium channels (O'Reilly et al., 2006), based on the crystal structure of the rat brain Kv1.2 potassium channel (Long et al., 2005) suggested that the site of the L1014F mutation is not part of the pyrethroid binding site and may confer resistance via a conformational effect that makes the sodium channel less likely to open (Davies and Williamson, 2009). An alternative hypothesis that has recently been suggested based on computer modelling and mutational analysis of a mosquito (*Aedes aegypti*) sodium channel is that insect sodium channels possess two pyrethroid binding sites with L1014F mapping to the second site (Du et al., 2013). In the absence of a crystal structure of a eukaryotic sodium channel with bound pyrethroids, further evidence is required to demonstrate if the *M. persicae* sodium channel has a dual pyrethroid binding site or a single site that is allosterically modified by L1014F.

Subsequently, a second mutation, M918T, was identified in *M. persicae* alongside the L1014F mutation within the nearby IIS4–S5 intracellular linker (Eleftherianos et al., 2008). This mutation corresponds to the original super-kdr mutation first identified in the housefly and was shown to significantly enhance the phenotypic expression of kdr resistance (Eleftherianos et al., 2008). Functional expression of the *Drosophila para* gene with M918T + L1014F was shown to reduce the pyrethroid sensitivity of sodium channels by approximately 100-fold (Vais et al., 2000). Molecular modelling has indicated that M918T likely confers resistance through the loss of polar interactions between pyrethroids and M918 on the IIS4–S5 linker that are critical for their toxicity (particularly in the case of type II pyrethroids) (O'Reilly et al., 2006). Both mutations have now been identified in populations of *M. persicae* worldwide, although M918T has never been observed in the absence of L1014F, and haplotype analysis has suggested that kdr and super-kdr have arisen as a result of multiple

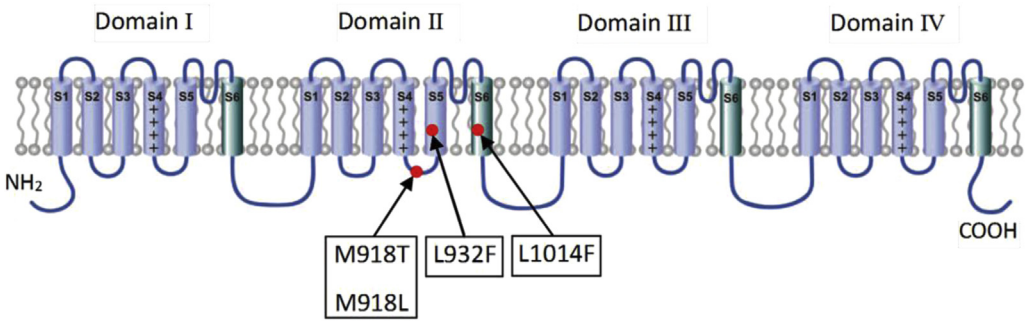

Fig. 4. Schematic of the voltage-gated sodium channel, target of the pyrethroid insecticides, highlighting the position of kdr/super-kdr mutations that have been associated with pyrethroid resistance in *M. persicae*.

independent *de novo* mutations (Anstead et al., 2005). More recently an alternative super-kdr variant (M918L) has been identified in *M. persicae* on oilseed rape in France (Fontaine et al., 2011) and was linked to resistance to *lambda*-cyhalothrin. In this study a further polymorphism in the sodium channel at position 932 causing an L932F amino acid substitution was identified in individuals with the L1014F mutation (Fontaine et al., 2011). However, the level of resistance conferred by L1014F + L932F in *M. persicae* has yet to be determined using insecticide bioassays. In most insect species kdr/super-kdr mutations have been shown to be inherited as largely recessive traits, i.e. little or no phenotype is observed in heterozygotes (Davies et al., 2007). Intriguingly, this does not appear to hold true in *M. persicae* where L1014F, M918T and M918L heterozygotes all display a resistant phenotype to a range of pyrethroids (Eleftherianos et al., 2008; Fontaine et al., 2011).

2.4. Duplication and mutation of the GABA receptor subunit gene and resistance to cyclodiene insecticides

Although the use of the cyclodiene insecticide endosulfan has now been largely phased out, for many years it was used as an aphicide on a range of crops and provided a rotation option for resistance management programs as it was not cross-resisted by other mechanisms (e.g. esterases/kdr). After many years of endosulfan use, resistance was reported in *M. persicae* populations collected from peach and nectarine orchards in the US (Unruh et al., 1996). In most insect species resistance to cyclodienes results from mutations in the GABA receptor, a ligand-gated chloride channel that responds to the neurotransmitter γ-aminobutyric acid (GABA) inhibiting the firing of new action potentials (ffrench-Constant et al., 2000) (Fig. 2). Substitutions at a single residue (A302) in the M2 region of the receptor encoded by the *Rdl* (resistance to dieldrin – another cyclodiene insecticide) gene have been identified in a wide range of insect species, with two resistant alleles, A302S and A302G, most commonly described (ffrench-Constant et al., 2000). In *M. persicae* the situation appears to be more complex with up to four different *Rdl* alleles found in individual clones, the wild-type allele A (encoding A302), allele G (glycine302), allele S (serine^{TCG}302) and allele S′ (serine^{AGT}302) (Anthony et al., 1998). Southern blot analyses indicated a recent duplication of the *Rdl* locus in *M. persicae* with two independent *Rdl* loci identified, one carrying allele A or G and the other allele S or S′. Interestingly, insecticide bioassays showed that only allele G (locus one) appears to confer resistance to endosulfan, with heterozygous A/G clones showing an intermediate level of resistance (Anthony et al., 1998). The replacement of alanine 302 with a glycine is thought to confer resistance by both directly affecting the binding site and allosterically destabilising the insecticide-preferred conformation of the receptor (ffrench-Constant et al., 1998). To date, the functional significance of the two *Rdl* loci in *M. persicae* remains unknown.

2.5. Overexpression of the cytochrome P450 CYP6CY3 and resistance to nicotine and neonicotinoid insecticides

As a result of the widespread resistance to OPs, carbamates, cyclodienes and pyrethroids, the neonicotinoids, which are unaffected by resistance mechanisms that had evolved to the older compounds, rapidly became a mainstay for control of *M. persicae* on many crops after their introduction in the early 1990s (Nauen and Denholm, 2005). Interestingly, low level resistance to members of this chemical class described as 'natural tolerance' was observed very soon after the introduction of the first neonicotinoid imidacloprid in certain *M. persicae* populations, especially the tobacco adapted *M. persicae nicotianae* (Devine et al., 1996; Nauen et al., 1996). In 2007 a clone of *M. persicae nicotianae* (5191A) was collected from tobacco in Greece that exhibited 30–60-fold resistance (in topical bioassays) to different neonicotinoids when compared to a reference susceptible strain (Philippou et al., 2009; Puinean et al., 2010). Insecticide bioassays using enzyme inhibitors suggested P450-mediated detoxification plays a primary role in resistance although additional mechanisms may contribute (Philippou et al., 2009; Puinean et al., 2010). Microarray analysis, using an array populated with probes corresponding to all known detoxification genes in *M. persicae*, revealed constitutive overexpression (22-fold) of a single P450 gene (*CYP6CY3*) (Fig. 2) in the 5191A clone and quantitative PCR using genomic DNA as template showed that the over-expression is due, at least in part, to gene amplification (Puinean et al., 2010).

The overexpression of CYP6CY3 was initially identified in a tobacco-adapted clone and the possibility that this P450 also plays a role in providing protection from the plant secondary metabolite nicotine was explored in a subsequent study (Bass et al., 2013). Previous work had shown that *M. persicae nicotianae* shows a reduced sensitivity to the plant alkaloid nicotine and cross-resistance to neonicotinoids (Devine et al., 1996; Nauen et al., 1996) and this was confirmed using an artificial feeding assay for five clones of *M. persicae nicotianae* from three continents ((Bass et al., 2013) and our unpublished data). Quantitative PCR showed that *CYP6CY3* was highly overexpressed (10–75-fold) in all five clones compared with *M. persicae sensu strictu* and the level of expression was significantly correlated with observed mortality to nicotine in bioassays. Significantly, two of the *M. persicae nicotianae* clones tested had been collected prior to the introduction of neonicotinoids, categorically indicating that the use of these insecticides had not selected for *CYP6CY3* overexpression. Functional expression of CYP6CY3 in an insect cell line revealed that it is remarkably efficient at metabolizing nicotine to less toxic

metabolites *in vitro* and can also detoxify the neonicotinoids imidacloprid and clothianidin although with much lower efficiency (Bass et al., 2013). When compared with CYP2A6 the main high-affinity metabolizer of nicotine in humans, CYP6CY3 was found to metabolise both nicotine and neonicotinoids with greater efficiency. This may result from the larger active site of CYP6CY3 being more easily able to accommodate these substrates (see Fig. 5). Confirmation that CYP6CY3 confers resistance *in vivo* came from transgenic expression in *D. melanogaster*, with flies expressing the transgene displaying significant resistance to both nicotine and clothianidin. Together these findings suggest that overexpression of CYP6CY3 was a prerequisite for the host shift of *M. persicae* to tobacco and has preadapted *M. persicae nicotianae* to resist neonicotinoid insecticides.

The fact that the host shift of *M. persicae* to tobacco occurred recently (Margaritopoulos et al., 2009) allowed the genetic basis of CYP6CY3 overexpression to be explored (Bass et al., 2013). Examination of *CYP6CY3* gene copy number revealed that overexpression resulted, at least in part, from gene amplification with the five *M. persicae nicotianae* clones carrying 14–100 copies of the gene (compared to 2 copies in clones of *M. persicae s.s.*). Sequencing of the *CYP6CY3* coding sequence and 5′ flanking region supported a single origin and then global spread of the amplification event and also suggested it occurred recently. Both these findings are consistent with previous studies of the global genetic structure of *M. persicae s.l.* populations which have suggested that adaptation to tobacco arose as a recent, single evolutionary event, originating in East Asia where tobacco-adapted races were first described (Margaritopoulos et al., 2009). Interestingly for all but one of the five *M. persicae nicotianae* clones the increase in gene copy number was approximately half the increase seen in the level of mRNA expression, suggesting that other factors in addition to gene amplification may be involved in the enhanced expression of this gene in at least four of the clones. Genome walking and sequencing of the 5′ putative promoter region of *CYP6CY3* revealed that an AC(n) dinucleotide microsatellite present in *M. persicae s.s.* has expanded in four of the *M. persicae nicotianae* clones from 15 to 48 repeat units. The functional significance of this polymorphism was investigated using reporter gene assays which revealed that the promoter with the longer AC(n) repeat drives around twofold greater expression than the shorter *M. persicae s.s.* insert. Sequence analysis of *CYP6CY3* amplicons suggest that the microsatellite expansion predates the amplification of this gene and has 'hitch-hiked' on the amplification event, effectively doubling the level of expression of the gene as a result.

2.6. Reduced penetration of insecticides through the cuticle and resistance to neonicotinoid insecticides

The initial study of the neonicotinoid resistant clone 5191A suggested that the overexpression of CYP6CY3 was not the only mechanism involved in the resistant phenotype. Firstly, susceptibility could not be completely restored using enzyme inhibitors (Philippou et al., 2009; Puinean et al., 2010), secondly the resistance phenotype (LC_{50} value) assessed in feeding assays was 17 times lower than that calculated in topical application bioassays using the neonicotinoid clothianidin (Puinean et al., 2010). Finally, the level of resistance in topical assays of the 5191A clone and a second clone 926B, that have similar expression levels of CYP6CY3, is significantly different (10-fold vs. 50 fold) to imidacloprid but very similar in oral feeding assays with nicotine and clothianidin ((Bass et al., 2013) and our unpublished data). Together these findings suggested that reduced penetration of insecticide was an additional mechanism of resistance in the 5191A clone (Fig. 2). A further piece of evidence supporting this idea came from the results of the microarray comparison of 5191A with the susceptible clone 4106A which showed that a large number of ESTs (expressed sequence tags) encoding cuticular proteins (CPs) are up-regulated in 5191A (Puinean et al., 2010). Although it was difficult to ascertain the exact number of unique genes represented by these ESTs, as many were short reads generated by 454 sequencing, all three members of the CPR group of CPs (the largest structural CP family in arthropods) were represented along with several CPs of the CPG family. In the aphid *Acyrthosiphon pisum*, CP genes were found to be clustered in the genome and may also be co-regulated which may explain why several ESTs encoding CPs were identified as being over-expressed in the *M. persicae* 5191A clone (Gallot et al., 2010). Finally *in vivo* penetration assays using [^{3}H] imidacloprid confirmed that there are significant differences in the penetration of insecticide through the cuticle of the 5191A *M. persicae* clone compared with the susceptible clone with only 22% of the initial imidacloprid dose recovered from the cuticle after 50 h for the susceptible and more than half the initial dose recovered for the resistant clone (Puinean et al., 2010). The mechanism by which insecticide penetration through

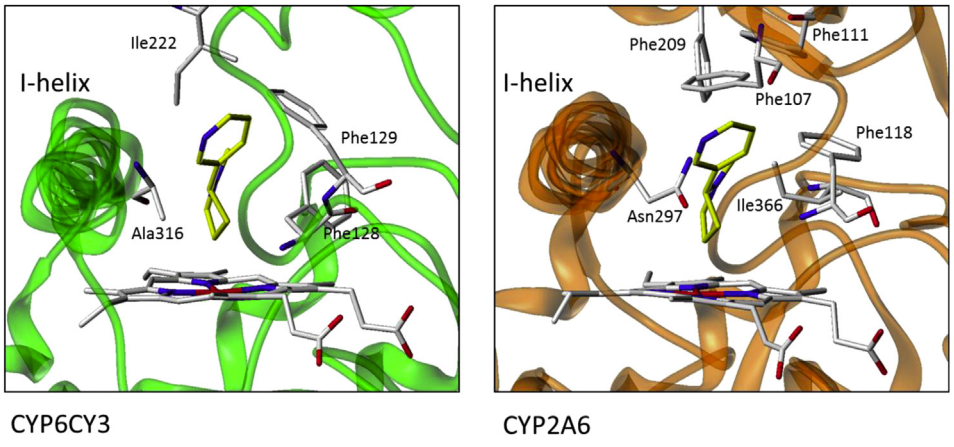

Fig. 5. Crystal structure of the human P450 CYP2A6 (orange) and homology model of the aphid P450 CYP6CY3 (green) each with nicotine docked in the active site. The active site is shown in enlarged view with those amino acids closest in distance to the docked nicotine ligand highlighted. The active site of CYP6CY3 (volume estimate: 1556 Å^3) is considerably larger than the corresponding CYP2A6 cavity (volume estimate: 458 Å^3). Nicotine is shown as a capped-stick model (carbon = yellow, nitrogen = blue). The homology model of CYP6CY3 was constructed using the crystal structure of human CYP3A4 (PDB-Id 1TQN) as a template (due to the closer sequence similarity with this P450 than CYP2A6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the cuticle is altered in the 5191A clone has not been determined and may result from changes to the cuticle structure, composition or both.

2.7. Mutation of the nicotinic acetylcholine receptor (nAChR) and resistance to neonicotinoid insecticides

Although enhanced CYP6CY3 expression and/or reduced penetration confer moderate levels of resistance to neonicotinoids in topical bioassays, their practical significance is limited as they are insufficient to impair the field effectiveness of these insecticides when they are applied at recommended rates. However, this situation changed significantly when in 2009 a clone of *M. persicae* (FRC) was collected from peach in Southern France that was shown to exhibit extremely potent resistance to neonicotinoids, easily sufficient to compromise the field effectiveness of members of this insecticide class (Bass et al., 2011; Slater et al., 2011). Synergist bioassays implied the presence of two resistance mechanisms in FRC, one based on enhanced detoxification by cytochrome P450s, and another unaffected by piperonyl butoxide (PBO) (Bass et al., 2011). Furthermore, microarray and quantitative real-time PCR analyses showed that *CYP6CY3* was overexpressed but at a level not significantly different from that exhibited by other clones with modest resistance (Bass et al., 2011). These findings suggested additional mechanism(s) of resistance underlie the extreme resistance phenotype of the FRC clone, with alteration of the neonicotinoid target-site, the nicotinic acetylcholine receptor (nAChR) a neurotransmitter-gated ion channel that plays an important role in nerve signalling at the post-synaptic membrane, an obvious possibility. Binding of [^{3}H]-imidacloprid to membrane preparations of FRC aphids revealed that the high affinity [^{3}H]-imidacloprid binding site present in susceptible *M. persicae* is completely lost in this clone and the remaining lower affinity site is overexpressed and slightly altered compared to susceptible clones resulting in a substantial overall reduction in binding affinity (Bass et al., 2011). Interestingly, the observed binding site overexpression was lost after maintaining the FRC strain under laboratory selection conditions for two years, however, this was shown to have no effect on neonicotinoid resistance levels (Beckingham et al., 2013).

Subsequent sequencing of genes encoding the nAChR, a pentameric ligand-gated ion channel that may be assembled from α and non-α subunits, identified a point mutation in the loop D region of the β1 subunit that causes an arginine to threonine substitution (R81T) (Fig. 2). Loop D is one of three regions (D, E and F) of the β1 subunit that in combination with loops A, B and C of α subunits form the binding site for the natural ligand acetylcholine and certain agonists including neonicotinoids (Grutter and Changeux, 2001). The amino acid at the position corresponding to R81 in *M. persicae* is highly conserved in insect β1 receptors with a positively charged arginine normally present; in contrast, vertebrate *β* subunits rarely have a positively charged amino acid at this position with a threonine the most common residue observed (Bass et al., 2011). Indeed, the selectivity of neonicotinoids for insect nAChRs is thought to be directly influenced by interactions between positively charged residues in loop D (with R81 particularly implicated) of β subunits and the distinctive electronegative pharmacophore (nitro or cyano group) of these insecticides (Shimomura et al., 2006; Tomizawa and Casida, 2003, 2005) (Fig. 6). The importance of R81 in neonicotinoid binding had been highlighted by site-directed mutagenesis and homology modelling studies of vertebrate and insect recombinant receptors in earlier studies (Shimomura et al., 2006). Substituting the threonine residue in the chicken β2 subunit at this position with arginine or another basic residue was shown to greatly enhance the affinity of recombinant nAChRs (such as *D. melanogaster* Dα2/chicken β2 hybrids) for imidacloprid. Modelling suggested this resulted from direct interaction of the nitro group of imidacloprid with the introduced basic residue at this position (Shimomura et al., 2006). The R81T mutation therefore appears to confer a 'vertebrate-like' quality to the β1 subunit of resistant aphids resulting in reduced sensitivity of the nAChR to neonicotinoids through the loss of direct electrostatic interactions of the electronegative pharmacophore with the basic arginine residue at this key position within loop D. More recent work has demonstrated that other insecticide classes

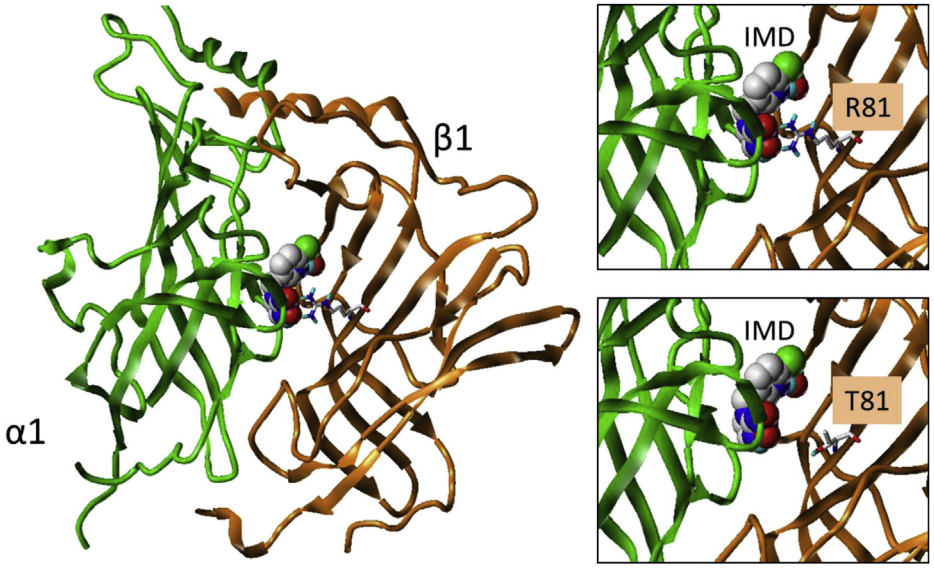

Fig. 6. Homology model of the *M. persicae* α1β1 nAChR dimer. Model was constructed using the crystal structures of the acetylcholine binding protein (AChBP) from *Lymnea stagnalis* (PDB-Id 1UW6) and *Aplysia californica* (PDB-Id 2BYQ) with imidacloprid docked into the ligand binding site formed by the two subunits. Models were produced using SYBYLx2.0 and are inspired by figures in (Shimomura et al., 2006). Only imidacloprid, T81, and R81 are shown in a space-filling representation (atom colours: carbon = grey; nitrogen = blue; oxygen = red; chlorine = green), while the protein backbone of the α1 and β1 subunit is illustrated by green and brown ribbons, respectively. Enlarged views show the electrostatic interaction of the imidacloprid nitro moiety with the basic arginine residue (R81) 81 within loop D of the β1 subunit that is lost when replaced with a threonine (T81). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that act on the nAChR are also affected by the R81T mutation including the sulfoxamines (Sulfoxaflor) and butenolides (Flupyradifurone) ((Cutler et al., 2013) and our unpublished data).

2.8. Other potential mechanisms of resistance

2.8.1. Induced resistance

All of the resistance mechanisms described above are constitutive (with the revertant esterase phenomenon a possible exception) and no inducible mechanism of resistance has been convincingly demonstrated for *M. persicae*. A single study has examined the potential role of induced resistance to insecticide in this species (Silva et al., 2012). In this study the transcriptional response of three aphid clones (a lab susceptible clone, a clone with the kdr L1014F mutation and a clone with the kdr mutation and the MACE mutation) was compared after the application of a sub-lethal dose of pirimicarb. The greatest transcriptional response was observed in the lab susceptible clone, however, changes in gene expression were low and likely represent a general stress response to pirimicarb exposure rather than anything that would result in a measurable resistant phenotype. Regretfully, the pirimicarb resistance status of aphids after 'priming' with pirimicarb was not examined which may have confirmed or refuted this interpretation. Interestingly, the two clones with resistance mutations showed very few significant changes in gene expression, demonstrating that inter-clone variation in response to insecticide exposure exists. In the case of the clone with MACE it is likely that the protection this mutation confers from pirimicarb toxicity limited the stress response observed. However, the reason for the low level response of the clone with the kdr mutation (which confers no cross resistance to pirimicarb) is unclear, although with only one clone representing each of these resistance types there is a danger of over-interpreting the significance of differences in their responses.

2.8.2. Behavioural resistance

Several studies have described the behaviour modifying effects of insecticides on aphids (Nauen, 1995; Nauen and Elbert, 1997) but more recently behavioural 'avoidance' has been suggested as a mechanism of resistance of *M. persicae* to neonicotinoids (Fray et al., 2014). In this study a comparison of the dispersal behaviour of the neonicotinoid resistant FRC and 5191A clones with that of an insecticide susceptible clone (US1L) revealed that FRC aphids spent a greater proportion of time on untreated leaf tissue than insecticide treated tissue, compared to the other two clones in a choice test. Such behaviour could confer resistance by allowing such aphids to more rapidly disperse from neonicotinoid treated leaves to untreated plant material (Fray et al., 2014). Although this has interesting implications for aphid management further work is required to confirm this hypothesis as only a single susceptible clone was used in the study. In earlier work a susceptible strain of aphids also showed a significant migratory response away from imidacloprid treated leaves to untreated leaves, suggesting differences in avoidance behaviour may not necessarily be linked to an aphid's resistance status (Nauen, 1995).

3. Fitness costs associated with resistance mechanisms

Most of the resistance mechanisms detailed above involve the increased production of metabolic enzymes or alteration of important receptors in the insect nervous system and might be expected to reduce aphid fitness in the absence of insecticide selection because they are energetically costly or alter the efficiency of a receptor to carry out its native function. This may be especially likely for *M. persicae* where the accumulation of multiple resistance mechanisms in individual clones might be hypothesised to lead to a similar accumulation of fitness handicaps. Some of the most well characterised examples of fitness penalties associated with insecticide resistance come from a series of studies examining the relative fitness of *M. persicae* with and without different resistance mechanisms, particularly under times of stress. Evidence of a fitness cost for carboxylesterase overproduction was initially suggested by monitoring data of UK *M. persicae* populations which showed a reduction in the frequency of aphids with the esterase amplification over several winters compared to the previous autumns (Furk et al., 1990). In a series of field studies it was demonstrated that aphid clones with higher levels of esterase production overwintered more poorly than susceptible aphids and differences in survival were most pronounced during colder, wetter winters (Foster et al., 1996). The behaviour of esterase overproducing aphids was shown to be modified, with these aphids migrating away from deteriorating leaves significantly more slowly than susceptible aphids (Foster et al., 1997). A second behavioural change associated with both enhanced esterase production and the kdr mutation was an altered response to the aphid alarm pheromone (*E*)-β-farnesene (Foster et al., 1999, 2003). This pheromone is released by the cornicles of *M. persicae* when disturbed by natural enemies and provides a signal to neighbouring aphids to stop feeding and disperse. However, aphids with kdr or esterase resistance showed a significantly reduced response to (*E*)-β-farnesene compared with susceptible aphids and, as a result, were significantly more likely to be parasitized by the aphid parasitoid, *Diaeretiella rapae* in predation bioassays (Foster et al., 2011, 2007, 1999). Beyond behavioural changes, aphids producing the highest levels of esterase (R_3 clones) and aphids with MACE have been shown to have a reduced reproductive fitness (intrinsic rate of increase) (Foster et al., 2000), although a more recent study of *M. persicae* from Chile found no evidence for energy or reproductive fitness costs associated with total esterase activity or MACE (Castaneda et al., 2011). In the latter study the total esterase activity measured in the resistant Chilean genotypes were only at R_1 levels which likely explains the lack of a fitness cost associated with esterase resistance in these clones. The differences between this study and earlier studies is more difficult to explain for MACE but may be related to the different host plants on which aphids fed (Chinese cabbage vs. pepper).

In the case of carboxylesterase resistance, the molecular basis of the fitness penalties described may relate to the significant investment of resources in extreme over-production of the carboxylesterase enzyme, which in R_3 forms represents about 3% of total body protein (Devonshire and Moores, 1982). For the kdr mechanism, there is growing evidence that resistance mutations have a direct effect upon nerve function through the associated alteration to sodium channel proteins in insect nerves. Neurophysiological studies incorporating kdr mutations into the insect *para* gene, which codes for this protein, and expressing them *in vitro* using *Xenopus* oocytes, have shown that the resulting single amino acid substitution has two effects. Not only does it confer resistance through reduced sensitivity to pyrethroids and DDT but it also alters sodium channel gating properties resulting in an abnormal elevation in action potential thresholds (Vais et al., 2001, 2000). *In vivo* this would cause a general reduction in the excitability of an insect's nervous system and thereby potentially disrupt the perception and behavioural response to various other stimuli that are important for survival such as semiochemicals and plant compounds.

4. Insights into adaptive evolution and future directions

The significant body of work carried out on the development of resistance in a single aphid species has provided a number of major

advances in our understanding of how rapid adaptive traits evolve in insects. Together these studies have broadly confirmed that resistance to synthetic and natural insecticides most commonly arises through enhanced metabolic detoxification or target-site insensitivity.

For metabolic resistance, gene amplification appears to be a key evolutionary driver in *M. persicae* in rapidly increasing the production of specific enzymes that detoxify or sequester man-made or plant toxins and this may be a more common mechanism of adaptive evolution in arthropods than previously anticipated. Study of gene amplification in this species has also demonstrated that the increased enzyme production resulting from this process is not necessarily 'irreversible' but can be regulated by epigenetic mechanisms avoiding the energetic cost incurred in producing large amounts of enzyme in the absence of any selective advantage (Field and Blackman, 2003). To date, the mechanism(s) by which detoxification genes are amplified in *M. persicae* has not been identified and further work is required to identify precisely how genes are copied and then moved around the genome. Sequencing of the regions flanking the break points of amplified gene copies and interrogation of the sequence obtained against the recently sequenced genome of this species will likely shed light on this subject.

In addition to gene amplification, study of the molecular basis of CYP6CY3 overexpression in *M. persicae* has confirmed that *cis*-acting factors in promoter regions play a role in enhancing the expression of detoxification genes and provided evidence that such factors may include dinucleotide microsatellites. Microsatellites such as $AC_{(n)}$ repeats have been historically considered non-functional neutral markers or 'junk DNA'. However more recent studies on humans and yeast have provided evidence that they may be important *cis*-regulatory DNA sequences that have direct influence on both gene expression and phenotypic variation (Rockman and Wray, 2002; Vinces et al., 2009) and the work on *M. persicae* has now demonstrated that dinucleotide microsatellite variation is also a driver of adaptive change in gene expression during insect evolution. It would be extremely interesting to examine the frequency and location of $AC_{(n)}$ repeats in the *M. persicae* genome and other insect genomes more generally to examine if they are observed at greater frequency in gene promoter regions where they might influence transcription. In the *M. persicae* example $AC_{(n)}$ repeats appear to work in concert with gene amplification to drive high levels of gene expression and the frequency of this association could also be explored in other insects.

Work on metabolic resistance in *M. persicae* has also provided clear support for the hypothesis that the capacity of insects and mites to evolve metabolic resistance to synthetic insecticides/acaricides may result from the recruitment of detoxification mechanisms that have evolved to process plant allelochemicals (Dermauw et al., 2013). Study of the host shift of *M. persicae* to tobacco has shown that the mechanisms that had evolved to detoxify nicotine also pre-adapted such aphids to resist neonicotinoid insecticides, with the active site of CYP6CY3 able to accommodate both plant and man-made insecticides (Bass et al., 2013). This finding highlights the potential risk of resistance development to insecticides that have structural similarity to natural compounds encountered by phytophagous insects. *M. persicae* is highly polyphagous and it has been suggested that generalist herbivores may have a particular propensity to evolve insecticide resistance as a result of the broad spectrum of natural defence compounds they encounter in their diet (Dermauw et al., 2013). In support of this idea it is noteworthy that *M. persicae* has at least 40% more genes encoding cytochrome P450s than the specialist pea aphid *A. pisum* (Ramsey et al., 2010).

The study of resistance in *M. persicae* has also demonstrated the importance of target-site mechanisms in conferring high levels of resistance by reducing insecticide binding to receptors in the insect nervous system. However, beyond this, such work has also provided significant insights into the selectivity of different classes of insecticides, and has revealed that key amino acid residues that underlie the selectivity of insecticides are often evolutionary targets for change, leading to target-site resistance due to a loss or reduction of species or insect selectivity (Andrews et al., 2004; Bass et al., 2011; Nabeshima et al., 2003). In a remarkable example of this, the amino acid at position 81 of the *M. persicae* nAChR β1 subunit was predicted as a key determinant of neonicotinoid selectivity for insect nAChRs and hence a resistance 'hotspot' (Shimomura et al., 2006) five years prior to the discovery of the R81T mutation. Furthermore, this position in loop D has also been suggested to confer imidacloprid selectivity between *M. persicae* and its natural enemy *Pardosa pseudoannulata* which has a polar glutamine residue at position R81 (Song et al., 2009).

Although the *M. persicae* case study has revealed that resistance may come with a fitness cost in the absence of selection, it has also shown that several resistance mechanisms can coexist in the same aphid individual resulting in populations of *M. persicae* that are resistant to multiple insecticides (Panini et al., 2013). In two examples (pyrethroid and neonicotinoid resistance) it appears that low level resistance has evolved first as a result of metabolic mechanisms, followed by target site mutation to confer much higher levels of resistance. In the case of neonicotinoid resistance, a target site change appears to have evolved in a genetic background of enhanced P450 production (Bass et al., 2011) and indeed, to date, these two mechanisms have never been found in isolation (our unpublished work). As a result the relative role of the two mechanisms in determining the resistance phenotype is currently unknown and future work is required to investigate if these mechanisms act additively or synergistically. Furthermore, although the fitness of aphid lineages with one or two mechanisms has been examined further work is urgently required to assess the fitness of individuals carrying three or more mechanisms. Such information is valuable, as fitness penalties may result in the restoration of susceptibility to an insect population in the absence of selection and this may be exploited in control strategies. Unfortunately, manipulation of *M. persicae* genetics (e.g. to establish different combinations of resistance alleles in a common genetic background) is greatly hampered by the frequency of anholocycly and the time and effort required to produce progeny from crosses between aphids that can be induced to form sexual stages under laboratory conditions.

5. Implications and prospects for *M. persicae* resistance management

Another legacy of work on *M. persicae* has been a reinforcement of the dangers of widespread and long-term reliance on a very limited supply of insecticidal classes for aphid control. For almost 50 years, control of *M. persicae* depended on three chemical classes that encompass just two modes of action, i.e. OPs and carbamates acting on AChE, and pyrethroids acting on the voltage-gated sodium channel. The introduction of neonicotinoid insecticides (Table 1) in principle expanded the portfolio of chemistry/modes of action in many agricultural systems and diversified the selection pressure being imposed by insecticides. Unfortunately, by this point widespread resistance had been reported to OPs and carbamates, and although for some time pyrethroids retained efficacy against *M. persicae*, resistance to this insecticide class followed. In addition, OPs have widely fallen out of favour due to their unfavourable environmental profile and were replaced rather than complemented by neonicotinoids. The ensuing reliance on neonicotinoids, particularly for certain crops, must over many years have

Table 1
Major insecticide modes of action and chemical classes marketed globally for *Myzus persicae* control (registrations and availability of individual modes of action or chemical classes may differ regionally).

IRAC group[a]	Target site	Chemical class	Introduction	Example
1	AChE	Organophosphates	1940s	Dimethoate
		Carbamates	1950s	Pirimicarb
3	VGSC	Pyrethroids	1970s	Deltamethrin
4	nAChR	Neonicotinoids	1991	Imidacloprid
		Sulfoximines	2012	Sulfoxaflor
		Butenolides	2014	Flupyradifurone
9	Chordotonal organs	Pyridine azomethines	1990s	Pymetrozine
		Carboxamides	2010	Flonicamid
23	ACCase	Tetramic acids	2007	Spirotetramat
28	RyR	Diamides	2013	Cyantraniliprole

[a] IRAC – Insecticide Resistance Action Committee (www.irac-online.org) (Nauen et al., 2012).

exerted strong selection for resistance, but despite this it took 20 years for resistance to emerge (R81T mutation in the nAChR β1 subunit) that was sufficiently potent to result in control failure. Target-site resistance to neonicotinoids is currently restricted to peach growing regions in southern France, Spain and Italy but in the latter has very recently been observed in a small number of samples collected from herbaceous hosts (Panini et al., 2013; Slater et al., 2011) . Control strategies in these regions are now reliant on insecticides with novel modes of action that are not cross-resisted by existing resistance mechanisms in *M. persicae*. These include pymetrozine and flonicamid acting on insect chordotonal organs, and spirotetramat acting on acetyl-CoA carboxylase (Ausborn et al., 2005; Nauen et al., 2008). A further compound recently developed for *M. persicae* control is the diamide insecticide cyantraniliprole which acts on insect ryanodine receptors (Foster et al., 2012; Selby et al., 2013). There are currently 10 different chemical classes of insecticides addressing six different modes of action that are potentially available for use against *M. persicae* (Table 1). However, half of these modes of action can be compromised by known metabolic and/or target-site resistance mechanisms. In order to ensure effective and sustainable control of this pest it is important to identify and exploit simultaneously all the insecticide classes that are approved and known to be effective on a particular crop and in a particular region. Strategies based on the alternation of new mode of action groups and carefully tailored to local conditions (including the prevailing pest complex) have the potential to keep aphids below economic damage thresholds and to reduce the intensity of selection for new resistance mechanisms. We recommend that such regional resistance management guidelines are developed and disseminated through appropriate channels of communication, and monitoring programmes are implemented to detect shifts in susceptibility that may herald the appearance of novel resistance genes and mechanisms. The history of resistance development in *M. persicae* as detailed in this paper provides ample warning against complacency when contending with such an adaptable species.

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