Molecular mechanisms of insecticide resistance in the glasshouse whitefly, *Trialeurodes vaporariorum*

Submitted by

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..……  ……..

Acknowledgements

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Abstract

The whitefly *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) is a serious pest of protected vegetable and ornamental crops in most temperate regions of the world. Neonicotinoids, pymetrozine (a feeding blocker), spiromesifen (a tetronic acid derivative), bifenthrin (a pyrethroid), and pyriproxyfen (a juvenile hormone mimic) are among the most important insecticides used to control this species.

Bioassays were used to quantify responses of recently-collected strains of *T. vaporariorum* to three neonicotinoids (imidacloprid, thiamethoxam, and acetamiprid), pymetrozine, spiromesifen, bifenthrin, and pyriproxyfen. 454 pyrosequencing was exploited to generate the first transcriptome for this species. PCR-sequencing was used to identify mutations in the target proteins of spiromesifen and bifenthrin potentially associated with resistance to these compounds. Microarray sequencing technology was employed to investigate differences in gene expression associated with pyriproxyfen resistance.

Resistance to neonicotinoids was age-specific in expression and consistently associated with resistance to pymetrozine, supporting a hypothesis of metabolic resistance analogous to that in the tobacco whitefly, *Bemisia tabaci*. Bioassays also showed moderate to high level resistance to spiromesifen, bifenthrin and pyriproxyfen in some strains. Analysis of the transcriptome identified genes encoding enzymes involved in the detoxification of xenobiotics (cytochrome P450s, carboxyl/cholinesterases, and glutathione-s transferases) and ones encoding insecticide targets: acetyl-coA carboxylase (ACCase), the target of spiromesifen and the voltage-gated sodium channel protein targeted by pyrethroids. PCR-sequencing revealed a single nucleotide polymorphism in the ACCase gene, which was consistently associated with spiromesifen resistance. Three amino-acid substitutions in the sodium channel of pyrethroid-resistant *T. vaporariorum* were found in positions previously implicated in pyrethroid resistance in *B. tabaci*. Microarray sequencing disclosed that a cytochrome P450 gene (*CYP4G61*) was overexpressed in a strain selected for increased pyriproxyfen resistance. The implications of these results and opportunities for further work are discussed.

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Chapter 1

Introduction

1.1 INSECTICIDE USE - PAST AND PRESENT

Insecticides are toxic compounds used against all developmental stages of insect pests of agriculture, humans and livestock. Even before 1000 BC, the burning of sulphur to fumigate houses against pests and diseases was mentioned by Homer (van Emden & Service, 2004). The organochlorine dichlorodiphenyltrichloroethane (DDT) was first synthesized in 1874 and started being used as an insecticide during World War II to control human disease vectors. DDT and related compounds were then adopted for agricultural use, leading to a revolution in the development of new insecticides. Nicotine is an important naturally-occurring insecticidal compound with a long history, which later prompted the development of a major group of synthetic molecules, the neonicotinoids (Yamamoto, 1999). Nicotine is still in use today as a fumigant and a foliar spray of a range of horticultural pests.

Insecticides are one of the main reasons for the increase in agricultural productivity in the 20th century (Maredia *et al*., 2003), along with the development of high yielding crops and new fertilizers. However, DDT and other insecticides have been banned in many countries, because of their persistence, damage to the environment and/or their toxicity to mammals and non-target organisms (van Emden & Service, 2004). Today, the approval and use of insecticides is subject to increasingly stringent regulation. It is necessary to balance agricultural needs for development of efficient and profitable insecticides, with environmental and health issues. Many modern agricultural systems

often still rely on multiple applications of insecticides for the control of insect pests. However, there are many drawbacks to the overuse of insecticides. In particular, after prolonged exposure to a limited number of insecticides, pests can adapt to survive insecticides' toxic effects.

1.2 INSECTICIDE RESISTANCE AND MECHANISMS OF RESISTANCE

Insecticide resistance is an evolutionary phenomenon. It is a heritable trait selected by insecticides that allows a pest to survive chemical control tactics. More specifically, insecticide resistance allows an insect to survive doses of insecticides that would normally kill susceptible populations of the same species (Onstad, 2008). Melander (1914) was the first author who described the occurrence of insecticide resistance. Georghiou and Lagunes-Tejeda (1991) reported that by 1989, more than 500 species of insects, mites and other arthropods were known to have developed resistance to one or more pesticides. This problem is continuously increasing, affecting almost all the chemical classes.

According to the Insecticide Resistance Action Committee (IRAC) insecticide resistance is "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" (IRAC, 2007). Insecticide resistance is caused by mutations that arise randomly and protect individuals that possess them from insecticide applications. The rate of development of resistance depends mainly on the selection pressure imposed upon the pest by the insecticide; increased exposure of a population to a toxin increases the frequency of resistant individuals in that population. Resistance can increase faster in the confined

environment of greenhouses, were the pests reproduce more rapidly with very little or no immigration of susceptible individuals.

Insecticide resistance can result from two main types of mechanism: 1) reduced binding of the insecticide to its target through target site mutations (e.g. acetylcholinesterase for organophosphates/carbamates, the voltage-gated sodium channel for pyrethroids and the nicotinic acetylcholine receptor for neonicotinoid insecticides) (Pittendrigh *et al*., 2008) and 2) increased detoxification or sequestration of insecticides (Ranson *et al*., 2002; Pittendrigh *et al*., 2008) by enzymes such as carboxyl-cholinesterases (CCEs) (Oakeshott *et al*., 2005), glutathione-S-transferases (GSTs) (Ranson & Hemingway, 2005) and cytochrome P450 monooxygenases (Feyereisen, 2005). Also, there are cases of resistance caused by reduced toxin penetration, increased toxin excretion or even behavioural resistance (avoidance of insecticide). A pest can develop more than one mechanism of resistance (multiple-resistance) to one or more different compounds. Cross resistance, occurs when the genetic mutation that made the pest resistant to one pesticide also makes it resistant to other pesticides, more commonly affecting compounds with similar modes of action (Pittendrigh *et al*., 2008).

1.3 WHITEFLIES AS AGRICULTURAL PESTS

Whiteflies are major pests of many agricultural crops worldwide. They belong to the family Aleyrodidae of the order Hemiptera. More than 1450 species have been described as belonging to two subfamilies and the most economically important pest species are members of the Aleyrodinae (Bink-Moenen & Mound, 1990; Martin *et al*., 2000). The most damaging species are the cotton whitefly, *Bemisia tabaci* (Gennadius) and the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood).

Whiteflies are small phloem-feeding insects, most of them measuring between 1-3mm. They usually have short life cycles, which are temperature-dependent and have multiple generations per year (Martin *et al*., 2000).

Whiteflies are partially parthenogenetic, arrhenotokous insects. Haploid males are produced asexually and contain half the chromosomes of the sexually produced females, which are diploids and contain the full genetic material. This means that unfertilised females lay haploid eggs, resulting only in male progeny (Byrne & Devonshire, 1996; Calvitti *et al*., 1998; Carrière, 2003). Thus, males can never be heterozygous, as they possess only half of the alleles of their mother (Horowitz *et al*., 2003). Haplodiploid breeding systems encourage the selection of pesticide resistance genes, leading to rapid and widespread pesticide resistance development. This happens because resistance genes arising by mutations are exposed to selection from the outset in hemizygous males, irrespective of intrinsic dominance or recessiveness (Denholm *et al*., 1998; Horowitz *et al*., 2003). However, the assumption that pesticide resistance evolves rapidly in haplodiploid arthropods is based on the belief that resistant males are as tolerant as resistant females (Carrière, 2003). Results from studies with the sweet potato whitefly and the greenhouse whitefly, indicate that males are less tolerant to pesticides than females (Horowitz *et al*., 1988; Sanderson & Roush, 1992; Carrière, 2003). Further research toward that aspect is necessary as these findings could have important implications in pesticide resistance development and management.

1.3.1 The greenhouse whitefly, *Trialeurodes vaporariorum*

Trialeurodes vaporariorum Westwood (Hemiptera: Aleyrodidae) (Figure 1.1), also known as the greenhouse or glasshouse whitefly, is a cosmopolitan species of

temperate regions. It is widely distributed throughout Europe, although in northern countries and the UK it is primarily found in crops grown under glass or plastic (Martin *et al*., 2000). It is a highly polyphagous pest that causes economic damage to Brassicaceae (watercress), Fabaceae (bean, soybean and pea), Cucurbitaceae (cucumber, melons, pumpkin and edible gourds), Rosaceae (rose, strawberry), Asteraceae (lettuce), Euphorbiaceae (poinsettia) and Solanaceae (tomato, potato, aubergine) plant families. Many weed species serve as alternate hosts for *T. vaporariorum* (Mound & Halsey, 1978; Byrne *et al*., 1990; Brødsgaard & Albajes, 1999).

The importance of *T. vaporariorum* as a pest is fuelled by its wide host range, small size (adults are 1-2 mm long) and rapid reproductive rate, due to a short developmental time and high fecundity. It congregates on the underside of leaves, which makes pesticide delivery difficult (Brødsgaard & Albajes, 1999). The life cycle of *T. vaporariorum* is shown in Figure 1.2. Females deposit their eggs on the undersides of leaves in characteristic circles, as the insect rotates about her rostrum while continuing to feed (Martin *et al*., 2000). Females are capable of mating less than 24 hours after emergence and can lay up to 30 eggs per day. The newly hatched larvae, often known as crawlers, are mobile, while the two subsequent scale-like immature stages are immobile and difficult to distinguish with the naked eye. During the fourth and final immature stage, known as the puparium, the larva thickens and the eyes and other body tissues become visible (Curry & Pimentel, 1971; Gill, 1990).

Figure 1.1 Adults, nymphs and eggs of *Trialeurodes vaporariorum* (Photograph by Kevin Gorman)

All the life stages, apart from the egg can cause damage to crops either by direct feeding or by the excretion of honeydew, which leads to sooty mould contamination. Large populations can result in stunted plants and leaf necrosis (Byrne *et al*., 1990). *T. vaporariorum* is also a vector of numerous plant viruses, with all of them belonging in the Closteroviridae. It transmits Beet pseudo-yellow virus (BPYV) that affects cucumber, melon, lettuce and sugar beet. It also transmits Melon yellow virus (MYV), Potato yellow vein virus (PYVV), Tomato infectious chlorosis virus (TICV) and Tomato chlorosis virus (ToCV) (Byrne *et al*., 1990; Wisler *et al*., 1998; Jones, 2003; Antignus, 2004; Khurana & Garg, 2004).

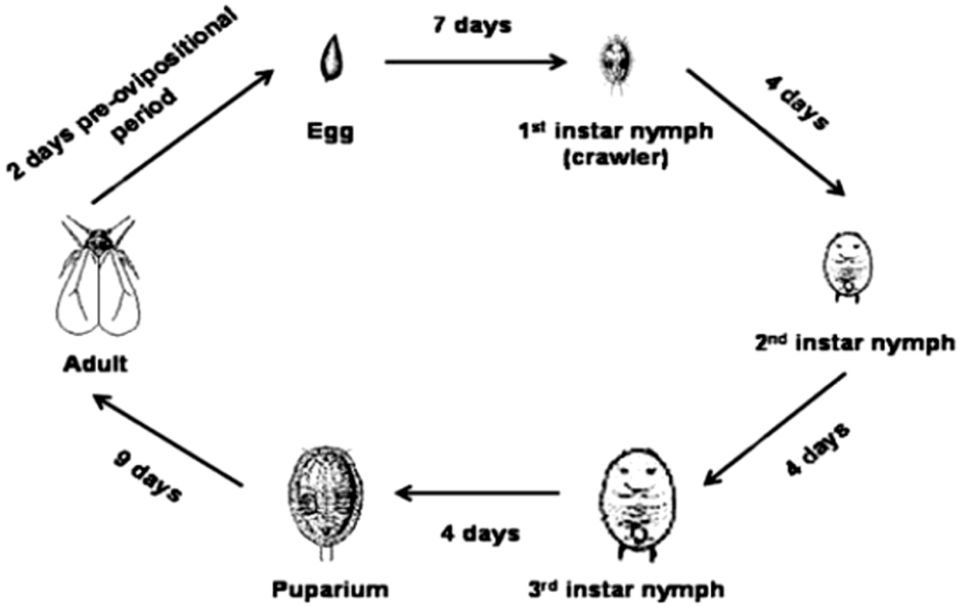


Figure 1.2 Life cycle of *Trialeurodes vaporariorum* at 22^{o}C

1.3.2 The tobacco whitefly, *Bemisia tabaci*

Bemisia tabaci Gennadius (Hemiptera: Aleyrodidae), also known as cotton, tobacco or sweet-potato whitefly, is a cosmopolitan species found outdoors in tropical and sub-tropical regions. In temperate countries it can be found only in crops grown under protection (Martin *et al*., 2000). It is a highly polyphagous pest, affecting hundreds of different plant species (Byrne *et al*., 1990; Martin *et al*., 2000) and transmitting more than 110 plant viruses, belonging in the Begomovirus, Crinivirus and Carlavirus genera (Jones, 2003). Most of those viruses can cause severe damage to crops and so even very low populations of this pest can result in major crop failures. Adults are smaller than those of *T. vaporariorum* (1 mm long). Several biotypes of *B. tabaci* have been identified; the two currently of most importance to agricultural environments are termed biotypes B and Q (Perring, 2001).

1.4 CONTROL OF *TRIALEURODES VAPORARIORUM*

1.4.1 Biological control and Integrated Pest Management (IPM) of *Trialeurodes vaporariorum*

In glasshouses, whiteflies can potentially be controlled by the use of biological control agents, such as species of parasitic Hymenoptera, predatory Hemiptera and Coleoptera (Gerling, 1990), as well as by the use of entomopathogenic fungi (Fransen, 1990). In greenhouses throughout Europe, effective biological control of the greenhouse whitefly is achieved by the use of parasitoids such as *Encarsia* spp. and *Eretmocerus* spp. (Hymenoptera: Aphelinidae), the use of predators *Macrolophus* spp. (Heteroptera: Miridae) and entomopathogens such as *Verticillium*, *Paecilomyces* and *Aschersonia* spp. (van Lenteren & Martin, 1999; van Lenteren, 2003).

IPM strategies for the control of whiteflies in greenhouses have long since been advocated (Dowell, 1990) and represent the most flexible and effective approach. These growing systems integrate physical and cultural control tactics with the use of biological control and selective pesticides. Underpinning measures include the physical exclusion of whiteflies from greenhouses, the elimination of any source of whitefly infestation (such as weeds and plant debris) and preventative releases of natural enemies. Compatible insecticides should ideally be used only if they are essential and always in the context of Insecticide Resistance Management (IRM). For accurate decision making, frequent monitoring of pest and natural enemy populations is required.

1.4.2 Chemical control of *Trialeurodes vaporariorum*

Today, there are a limited number of either systemic or contact insecticides that are available for whitefly control, with most of them belonging in one of the following broad groups: insect growth regulators, neonicotinoids, pyrethroids and organophosphates. Compounds of low specificity, such as carbamates, organochlorines and pyrethroids are becoming less favourable due to high persistence, resistance development and incompatibility with beneficial organisms (van Emden & Service, 2004). Other chemical classes used for whitefly control include the spinosyns, with spinosad being the forerunner of the class (Ishaaya *et al*., 2007; Millar & Denholm, 2007) and the more recently introduced tetronic acid derivatives.

1.4.2.1 Organophosphate and carbamate insecticides

Organophosphates (OPs) are a group of broad-spectrum insecticides that bind to acetylcholinesterase (AChE) and inhibit irreversibly the hydrolysis of the neurotransmitter acetylcholine. Acetylcholine therefore accumulates at the synapses, resulting in paralysis and death (Fukuto, 1990). There are a number of OPs available for whitefly control, such as malathion and chlorpyrifos (Figure 1.3). Carbamates are another class of insecticides that target the AChE of insects by acting as agonists for binding to the enzyme's substrate surface (Fukuto, 1990). Although organophosphates and carbamates were once the most widely used chemical groups worldwide, they have high mammalian toxicity and long persistence. This led to a severe reduction in their commercial availability, with most carbamates being banned and only a very limited number of organophosphates being in the approved list of the EU pesticide database.

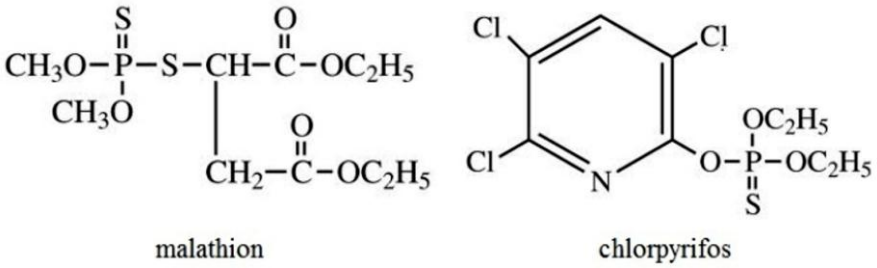
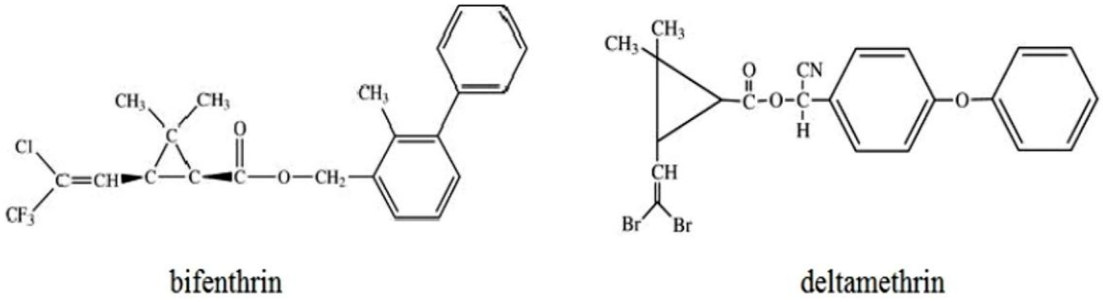


Figure 1.3 Chemical structures of the organophosphate insecticides malathion and chlorpyrifos

1.4.2.2 Pyrethroid insecticides

Pyrethroid insecticides were invented at Rothamsted Research during the early 1970s. They are synthetic analogues of natural pyrethrins produced by the pyrethrum daisy *Tanacetum cinerariifolium* L. and are one of the most successful chemical classes of insecticides, due to their efficacy, low mammalian toxicity and high photo-stability (Casida & Quistad, 1995; Elliott, 1996). Pyrethroids are axonic poisons that work by binding to the voltage-gated sodium channels in the neuronal membranes of insects. The sodium channel is a small pore through which sodium ions are permitted to enter the axon and cause excitation. Pyrethroids initially stimulate nerve cells to produce repetitive discharges and as the nerves cannot de-excite, the insect is paralyzed (Ware & Whitacre, 2004). The first widely used synthetic pyrethroid was permethrin followed by cypermethrin and deltamethrin (Davies *et al*., 2007). Bifenthrin (Figure 1.4) was one of the pyrethroids most widely used against *T. vaporariorum* (Bi & Toscano, 2007) but it was recently withdrawn due to an EU pesticides review (http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=activesubstance.selection).



bifenthrin deltamethrin

Figure 1.4 Chemical structures of the pyrethroid insecticides bifenthrin and deltamethrin

1.4.2.3 Insect growth regulators

Insect growth regulators (IGRs) are chemicals that disrupt insect growth and development, and due to their usual high specificity, are well placed for incorporation in IPM programs. There are mainly two types commercially available: the chitin synthesis inhibitors (DeCock & Degheele, 1993; DeCock *et al*., 1995) and the juvenile hormone analogues (JHA) (Eto, 1990; Ishaaya & Horowitz, 1992). The first of these inhibit the production or deposition of chitin, resulting in an interruption of the moulting process and the death of the insect within the old cuticle (DeCock & Degheele, 1993; DeCock *et al*., 1995). In Europe, the chitin synthesis inhibitors buprofezin and teflubenzuron (Figure 1.5) are the most widely used IGRs for the control of *T. vaporariorum* (Hegazy *et al*., 1990; DeCock & Degheele, 1993; DeCock *et al*., 1995; Ishaaya *et al*., 2007). The novel JHAs compete for juvenile hormone (JH) binding site receptors, regulating embryogenesis and metamorphosis (Eto, 1990; Ishaaya & Horowitz, 1992; Ishaaya *et al*., 1994; Ishaaya & Horowitz, 1995; Horowitz & Ishaaya, 2004; Ishaaya *et al*., 2007). Pyriproxyfen (Figure 1.5) is the most widely used JHA against whiteflies (Ishaaya *et al*., 2007). The insecticidal action of JHAs (such as methoprene and pyriproxyfen) is still not understood due to the lack of a known signalling pathway and/or a receptor molecule. However, the *Methoprene-*

tolerant gene (*Met*) (also known as *Resistance to juvenile hormone*) is a good candidate for the JH receptor as it has been shown to confer resistance to toxic doses of JH when mutated (Shemshedini & Wilson, 1990; Dubrovsky, 2005).

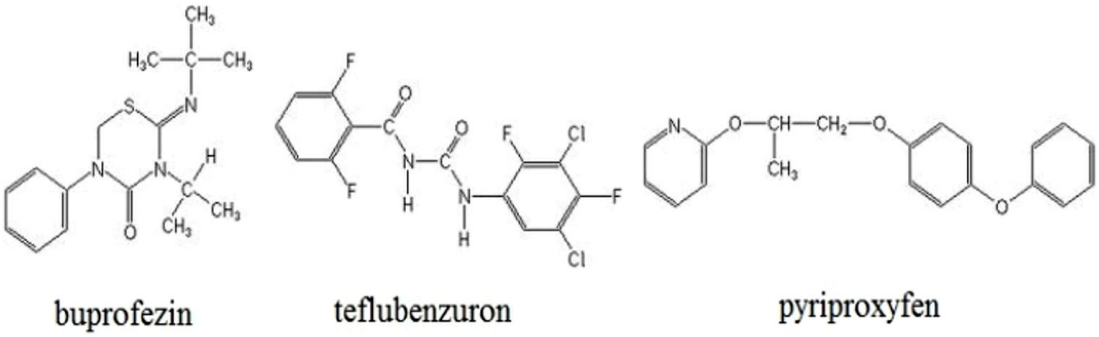


Figure 1.5 Chemical structures of the chitin synthesis inhibitors, buprofezin and teflubenzuron, and of the juvenile hormone analogue, pyriproxyfen

1.4.2.4 Neonicotinoid insecticides

Neonicotinoid insecticides target the nicotinic acetylcholine receptor (nAChR), causing paralysis followed by death (Millar & Denholm, 2007). There is an increasing range of compounds within this chemical class, formulated for foliar, systemic, stem and soil applications. The most widely used neonicotinoid compound is imidacloprid, followed by thiamethoxam and acetamiprid (Figure 1.6). As well as having strong insecticidal activity against sucking pests like aphids, whiteflies and planthoppers, the high systemicity of specific neonicotinoids enables them to be more effectively targeted at the pest, reducing impacts on beneficial organisms and improving their compatibility with IPM programs (Ishaaya *et al*., 2007; Millar & Denholm, 2007). Consequently, there is the potential for these compounds to be used as rotational partners within contemporary protected cropping systems.

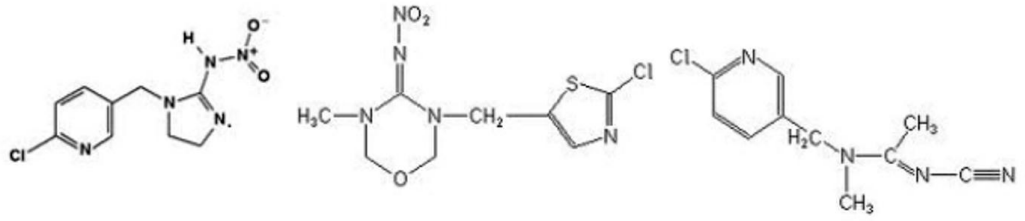


Figure 1.6 Chemical structures of the neonicotinoid insecticides imidacloprid, thiamethoxam and acetamiprid

1.4.2.5 Novel insecticides

Over recent decades there have been many attempts to develop novel compounds with selective properties and different modes of action from conventional insecticides. Pymetrozine (Figure 1.7), a triazine derivative, is a relatively narrow-spectrum insecticide that disrupts the feeding behaviour of sucking insects, leading to starvation and death (Harrewijn & Kayser, 1997; Polston & Sherwood, 2003; Ausborn *et al*., 2005). Although pymetrozine has strong insecticidal activity against whiteflies, it has no appreciable effect on natural enemies and the environment and thus it is considered as highly compatible with IPM programs (Ishaaya *et al*., 2007). In a study with the peach potato aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae) it was revealed that pymetrozine acts via a novel mechanism that is linked to the signalling pathway of serotonin (Kaufmann *et al*., 2004). Studies with the migratory locust *Locusta migratoria* L. (Orthoptera: Acrididae) revealed that pymetrozine affects chordotonal mechanoreceptors. The femoral chordotonal organ, which monitors joint position and movement, and the chordotonal sensillae in general appear to be the sites of pymetrozine action in locusts (Ausborn *et al*., 2005).

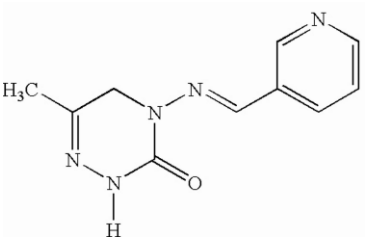


Figure 1.7 Chemical structure of the triazine derivative pymetrozine

The spirocyclic tetronic and tetramic acid derivatives (spirodiclofen, spiromesifen and spirotetramat) are a new class of effective insecticides and acaricides, with spiromesifen and spirotetramat becoming increasingly important for whitefly control (Figure 1.8). They act as inhibitors of acetyl-CoA carboxylase (ACCase) and cause significant reductions in total lipid biosynthesis (Nauen *et al*., 2003; Nauen, 2005; Nauen *et al*., 2005; Kontsedalov *et al*., 2009).

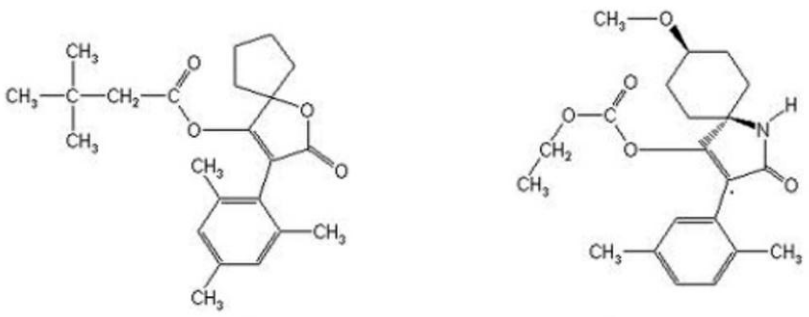


Figure 1.8 Chemical structures of the tetronic acid derivatives spiromesifen and spirotetramat

1.5 INSECTICIDE RESISTANCE AND CROSS-RESISTANCE IN *TRIALEURODES VAPORARIORUM*

Trialeurodes vaporariorum has developed resistance to several insecticide groups. The first documented occurrence of resistance involved pyrethroid and organophosphate insecticides during the 70s and 80s. By the mid-80s, this resistance had become widespread in the UK (Wardlow *et al*., 1976; Wardlow, 1985; Wardlow, 1987). Furthermore, resistance to the insect growth regulators buprofezin and teflubenzuron has been documented in Northern Europe and the Mediterranean region (De Cock *et al*., 1995; Gorman *et al*., 1998; Gorman *et al*., 2002). Organophosphate resistance in whiteflies arises most likely through changes in target site (AChE) sensitivity and/or insecticide detoxification by CCEs or P450s (Cahill *et al*., 1995; Gorman *et al*., 1998; Javed *et al*., 2003; Alon *et al*., 2008). The status and mechanisms of pyrethroid resistance in the greenhouse whitefly is explored in Chapter 4.

Imidacloprid resistance (Cahill *et al*., 1996; Nauen & Denholm, 2005) was first documented in Spanish populations of Q-biotype tobacco whitefly, *B. tabaci*. Resistance to imidacloprid was more recently found in *T. vaporariorum* strains from the UK, The Netherlands and the USA (Gorman *et al*., 2007; Bi & Toscano, 2007) and may be becoming widespread due to increased reliance on neonicotinoid compounds.

In *B. tabaci*, cross-resistance has been documented between neonicotinoids (Nauen *et al*., 2002; Rauch & Nauen, 2003). Furthermore, there is evidence in *B. tabaci* for the presence of cross resistance between neonicotinoids and pymetrozine (Gorman *et al*., 2010). There are no previous reports of the presence of cross-resistance within the

class of neonicotinoids neither for the greenhouse whitefly, nor between neonicotinoids and other compounds with different modes of action such as pymetrozine. The status of resistance to neonicotinoid insecticides and the unrelated pyridine azomethine pymetrozine is explored further in Chapter 2.

1.6 AIMS, OBJECTIVES AND THESIS FORMAT

The main aim of this project was to investigate the status of insecticide resistance in UK and European populations of *T. vaporariorum* and to investigate underlying mechanisms. Furthermore, parallels with resistance in *B. tabaci* were studied. The work presented in the following chapters is published or has been submitted for publication in peer-reviewed journals. Each experimental chapter consists of an introduction, complete materials and methods, and results/discussion and a conclusion section. Supplementary materials (additional files in chapters 3-6) are included in a Compact Disc in a folder on the back of this thesis. In the following paragraphs, the main objectives and author contributions for each chapter are discussed.

The objective of Chapter 2 was to characterise and quantify the phenotypic expression of neonicotinoid and pymetrozine resistance in UK and European strains of the glasshouse whitefly and to investigate any parallels with that already established for *B. tabaci*. This work was published in the journal Pest Management Science (Karatolos *et al*., 2010). In this work, Nikos Karatolos (NK), Ian Denholm (ID), Martin Williamson (MW), Ralf Nauen (RN) and Kevin Gorman (KG) conceived the study. NK did the experimental work, data analysis and constructed the initial manuscript. All authors contributed to the preparation of the final version of the manuscript.

In Chapter 3, 454-based pyrosequencing technology was used to generate a substantial EST dataset of the *T. vaporariorum* transcriptome in order to identify and characterise genes encoding detoxification enzymes and insecticide target proteins. These data will elucidate further the molecular mechanisms underlying insecticide resistance. This chapter was published in the journal BMC Genomics (Karatolos *et al*., 2011a). ID, Richard ffrench-Constant (RFC), MW, KG and NK conceived the study. Yannick Pauchet (YP) generated the cDNA libraries. YP and Paul Wilkinson (PW) conducted preliminary data curation and transcriptome assembly. NK and Ritika Chauhan (RC) annotated, and manually curated genes of interest. David Nelson (DN) named the manually curated P450 genes. NK analysed the data (gene homology searches and annotation) using BLAST2GO software. NK constructed the initial manuscript and all authors contributed to the preparation of the final version.

The work presented in Chapter 4 correlates the phenotypic expression of resistance to the pyrethroid bifenthrin in *T. vaporariorum* with the presence of domain II mutations in the voltage-gated sodium channel, implicating target site insensitivity as a major mechanism of pyrethroid resistance in this species. This work was accepted for publication in the journal Pest Management Science (Karatolos *et al*., 2011b). In this work, NK, ID, MW and KG conceived the study. NK did the experimental work, data analysis and constructed the initial manuscript. All authors contributed to the preparation of the final version of the manuscript.

In Chapter 5, the effect of the tetronic acid derivative spiromesifen was evaluated on field populations of *T. vaporariorum* and moderate resistance was found in some populations. Nucleotide sequencing was used to search for point mutations within the target of spiromesifen acetyl-CoA carboxylase (ACCase), which correlated with

spiromesifen resistance. This work is under review in the journal Insect Molecular Biology (Karatolos *et al*., 2011c). NK, ID, KG, MW and RN conceived the study. NK did the experimental work, data analysis and constructed the initial manuscript. All authors contributed to the preparation of the final version of the manuscript.

The aim of the work presented in Chapter 6 was to exploit the data generated by 454-based pyrosequencing to investigate potential mechanisms of pyriproxyfen resistance in a laboratory selected strain which shows more than 4000-fold resistance to pyriproxyfen. For this reason microarray technology was used to investigate differences in the gene-expression of the selected strain compared to a susceptible strain. This work is under review in the journal PLoS ONE (Karatolos *et al*., 2011d). NK and Chris Bass (CB) conceived the study. NK did the experimental work and data analysis. NK constructed the initial manuscript. All authors contributed to the preparation of the final version of the manuscript.

Chapter 2

Incidence and characterisation of resistance to neonicotinoid insecticides and pymetrozine in the greenhouse whitefly, *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae)

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Abstract

BACKGROUND: *Trialeurodes vaporariorum* (Westwood), also known as the greenhouse whitefly, is a serious pest of protected vegetable and ornamental crops in most temperate regions of the world. Neonicotinoid insecticides are used widely to control this species, although resistance has been reported and may be becoming widespread.

RESULTS: Mortality rates of UK and European strains of *T. vaporariorum* to a range of neonicotinoids and pymetrozine, a compound with a different mode of action, were calculated and significant resistance was found in some of those strains. A strong association was found between neonicotinoids and pymetrozine and reciprocal selection experiments confirmed this finding. Expression of resistance to the neonicotinoid imidacloprid and pymetrozine was age-specific and resistance in nymphs did not compromise recommended application rates.

CONCLUSION: This study indicates strong parallels in the phenotypic characteristics of neonicotinoid resistance in *T. vaporariorum* and the tobacco whitefly *Bemisia tabaci*, suggesting possible parallels in the underlying mechanisms.

2.1 INTRODUCTION

Trialeurodes vaporariorum Westwood (Hemiptera: Aleyrodidae), also known as the greenhouse or glasshouse whitefly, is an important pest of protected vegetable and ornamental crops in temperate regions of the world (Wardlow *et al*., 1976; Byrne *et al*., 1990; Brødsgaard & Albajes, 1999). Its pest status reflects a high degree of polyphagy combined with a rapid reproductive rate and potential for inadvertent transfer between sites on infested plant material. *T. vaporariorum* causes damage by direct feeding or by excretion of honeydew, and large infestations can result in stunted plants and leaf necrosis (Byrne *et al*., 1990), and it also transmits numerous plant viruses belonging to the Closteroviridae family (Jones, 2003; Khurana & Garg, 2004).

Neonicotinoid insecticides, including imidacloprid, thiamethoxam, thiacloprid and acetamiprid, have become important agents for controlling *T. vaporariorum*. These compounds target nicotinic acetylcholine receptors (nAChRs) in the insect central nervous system, causing paralysis followed by death (Millar & Denholm, 2007). The increasing number of commercialised neonicotinoids can be formulated as foliar, systemic, stem and soil applications. The unrelated pyridine azomethine pymetrozine is a whitefly control agent that disrupts feeding behaviour, leading to starvation and death (Harrewijn & Kayser, 1997; Ausborn *et al*., 2005). Both neonicotinoids and pymetrozine have the potential to be used in combination with natural enemies in coordinated integrated pest management programmes (Harrewijn & Kayser, 1997; Ausborn *et al*., 2005; Millar & Denholm, 2007). Consequently, there is a need to optimise their use to exploit IPM compatibility, as well as minimise risks of resistance that have compromised effective control of *T. vaporariorum* in the past (Wardlow *et al*., 1976; Gorman *et al*., 2002; Gorman *et al*., 2007).

Resistance to imidacloprid and other neonicotinoids was first characterised in Spanish populations of the tobacco whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Cahill *et al*., 1996; Nauen *et al*., 2002; Rauch & Nauen, 2003; Nauen & Denholm, 2005). Resistance to imidacloprid was reported more recently in *T. vaporariorum* strains from the United Kingdom, the Netherlands and the United States following increasing reliance on these compounds for whitefly control (Bi & Toscano, 2007; Gorman *et al*., 2007). Other insect species known to have developed neonicotinoid resistance are the rice brown planthopper, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) (Zewen *et al*., 2003; Gorman *et al*., 2008), the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (Zhao *et al*., 2000; Alyokhin *et al*., 2007), and the peach potato aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae) (Foster *et al*., 2003).

In *B. tabaci*, the primary mechanism of neonicotinoid resistance is enhanced detoxification due to overexpression of a gene (*CYP6CM1*) encoding a cytochrome P450-dependent monooxygenase enzyme (Karunker *et al*., 2008; Karunker *et al*., 2009). This mechanism shows the unusual phenomenon of age specificity, with resistance becoming more potent in the adult stage (Nauen *et al*., 2008). Another feature of this mechanism is unexpected cross-resistance to pymetrozine, in spite of structural and functional differences from neonicotinoids (Gorman *et al*., 2010). The objective of this study was to characterise further the phenotypic expression of resistance in *T. vaporariorum* and to investigate any parallels with that already established for *B. tabaci*.

2.2 MATERIALS AND METHODS

2.2.1 Insect strains

Eight strains of *T. vaporariorum* including a laboratory susceptible reference strain (TV1) were used in this study (Table 2.1). These encompassed five countries of origin and both edible and ornamental plant hosts. All strains were reared at Rothamsted without exposure to insecticides on French bean plants, *Phaseolus vulgaris* L., cv. 'Canadian Wonder' (Fabaceae), under a 16:8 h light:dark photoperiod at 24 °C.

Table 2.1 *Trialeurodes vaporariorum* strains, origins, years of collection and original hosts

Strain	Country of origin	Year of collection	Original host
TV1	UK	1971	French bean
TV2	UK	2004	Ornamentals
TV3	UK	2008	Roses
TV4	UK	2008	Ornamentals
TV5	Spain	2008	Tomato
TV6	Turkey	2006	Vegetables
TV7	China	2008	Aubergine
TV8	Germany	2008	Ornamentals

2.2.2 Insecticides

All insecticides were used as commercially-available formulations. These included three neonicotinoids, imidacloprid 200 g L^{-1} SL (Confidor®; Bayer CropScience), thiamethoxam 250 g kg^{-1} WG (Actara®; Syngenta) and acetamiprid 200 g kg^{-1} SP (Gazelle®; Syngenta), as well as pymetrozine 500 g kg^{-1} WG (Plenum®; Syngenta). All compounds were diluted to the required AI concentrations in distilled water containing 0.1 g L^{-1} of the non-ionic wetter Agral® (Syngenta).

2.2.3 Bioassays

Responses of adults and second instar nymphs were quantified using leaf-dip bioassays (Nauen *et al*., 2008). For testing adults, leaf discs were cut from bean plants and dipped for 20 s into the required concentration of insecticide or into 0.1 g L^{-1} Agral® for controls. Treated leaf discs were placed on 1 % agar inside petri dishes and allowed to air-dry, and 20-30 adult females of the required strain were then placed in each petri dish and confined with an absorbent cellulose pad and lid in order to minimise static and humidity. Three replicates were used for each concentration. All bioassays were maintained at 24 °C and mortality was scored after 72 h for neonicotinoids and 96 h for pymetrozine. Nymphs were tested by trimming leaves on intact bean plants into rectangles of approximately 40 mm x 50 mm. The trimmed plants were placed in cages with at least 200 adult whiteflies for 24 h to obtain a synchronised cohort of eggs, following which the adults were removed. Leaves were dipped after 11 days (when the majority of immature whiteflies had reached second instar) for 15 s in the required concentration of insecticide or into 0.1 g L^{-1} Agral® for controls. Three replicates were used for each concentration. All bioassays were maintained at 24 °C and mortality was scored after 22-25 days after counting dead immature stages and pupae from which adults had emerged.

2.2.4 Reciprocal selection experiment

Potential cross-resistance between imidacloprid and pymetrozine was investigated by selecting whiteflies of the TV6 strain with each of the insecticides and testing for a correlated change in response to the other. A total of 2000 adult whiteflies were exposed in a leaf-dip bioassay to 300 mg L^{-1} of either imidacloprid or pymetrozine, and survivors were reared for a single untreated generation to build up numbers.

Adults of the next generation were selected further with 1000 mg L^{-1} of either imidacloprid or pymetrozine, and survivors were reared to obtain the next generation for dose-response bioassays with both insecticides.

2.2.5 Statistical analysis

Concentration-mortality relationships were fitted by probit analysis, using the software GenStat 12th edition (VSN International Ltd, Hertfordshire, UK). Resistance factors were calculated by dividing LC_{50} values for field-collected strains by those for the susceptible standard (TV1). Selection factors (SFs) were calculated by dividing LC_{50} values of selected strains by those of the unselected parental strain. Lack of overlap of 95 % confidence limits on fitted LC_{50} values denoted significant differences between responses of strains. Correlations between responses to different compounds were investigated using Kendall's coefficient of concordance, calculated from ranking each of the estimated LC_{50} values.

2.3 RESULTS

2.3.1 Resistance of adults to neonicotinoids and pymetrozine

Resistance factors (RFs) for imidacloprid ranged from 2.6 (TV4) to 22 (TV8) in adults (Table 2.2). Five strains (TV3, TV5, TV6, TV7 and TV8) were significantly less susceptible at LC_{50} than TV1. There was a clear association between resistance factors for imidacloprid, thiamethoxam and acetamiprid, with the same four strains (TV3, TV5, TV7 and TV8) being consistently the most resistant. All field strains were significantly less susceptible than TV1 to pymetrozine, with TV3, TV5, TV7 and TV8 again being the most resistant, yielding RF values between 7.5 and 20.

Table 2.2 Responses of *Trialeurodes vaporariorum* adults to imidacloprid, thiamethoxam, acetamiprid and pymetrozine. Resistance factors (RFs) relative to TV1 are given for all the field strains.

Chemical	Strain	LC50 (mg L^{-1}) * (95 % CL)	Slope	RF
Imidacloprid	TV1	17.2 (9.30-32.6) a	0.99±0.16	1
	TV2	47.9 (21.4-99.4) ab	0.73±0.12	2.78
	TV3	358 (170-827) bc	0.80±0.14	20.8
	TV4	44.9 (26.1-74.2) ab	1.06±0.14	2.61
	TV5	185 (66.2-278) bc	2.43±0.86	10.8
	TV6	102 (54.0-189) b	1.11±0.17	5.92
	TV7	214 (142-324) bc	1.43±0.20	12.4
	TV8	374 (227-638) c	0.87±0.12	21.8
Thiamethoxam	TV1	18.6 (11.4-29.5) a	1.51±0.27	1
	TV2	25.1 (14.5-43.6) a	1.33±0.24	1.35
	TV3	169 (80.6-361) bc	1.06±0.20	9.09
	TV4	38.2 (23.0-62.5) a	1.35±0.21	2.05
	TV5	283 (175-444) bc	1.48±0.24	15.2
	TV6	40.4 (23.8-68.5) a	2.04±0.53	2.17
	TV7	379 (235-641) c	1.08±0.15	20.4
	TV8	119 (78.2-183) b	1.64±0.28	6.41
Acetamiprid	TV1	44.2 (27.0-71.1) a	1.24±0.17	1
	TV2	114 (60.5-211) ab	0.81±0.11	2.57
	TV3	374 (210-710) bc	0.82±0.11	8.45
	TV4	153 (83.9-263) b	0.83±0.11	3.45
	TV5	822 (437-1798) c	0.94±0.17	18.6
	TV6	41.7 (23.4-69.4) a	1.43±0.24	0.94
	TV7	185 (119-300) b	1.17±0.16	4.18
	TV8	165 (101-260) b	1.44±0.25	3.73
Pymetrozine	TV1	38.8 (23.8-63.7) a	1.43±0.24	1
	TV2	160 (106-237) b	1.47±0.22	4.13
	TV3	290 (177-511) bc	1.57±0.32	7.48
	TV4	269 (169-450) bc	1.19±0.17	6.93
	TV5	646 (454-934) c	2.03±0.33	16.6
	TV6	204 (122-343) bc	1.13±0.17	5.25
	TV7	792 (568-1107) c	1.73±0.24	20.4
	TV8	484 (329-715) c	1.70±0.28	12.5

* Different letters indicate significant difference between strains, based on overlapping 95 % CL of LC_{50} values.

Kendall's coefficient (KC) for the three neonicotinoids (KC = 0.815, χ^2 = 17, 1, df = 7, P = 0.017) showed a strong concordance in the ranking of LC_{50} values. A strong

association was also found between the three neonicotinoids and pymetrozine (KC = 0.833, χ^2 = 23.3, df = 7, $P \leq 0.001$), supporting a hypothesis of cross-resistance between neonicotinoids and pymetrozine.

2.3.2 Response of nymphs to imidacloprid and pymetrozine

No significant reduction in susceptibility of second-instar nymphs to imidacloprid was observed with any of the field strains (Table 2.3). Although TV3, TV5, TV7 and TV8 still yielded the four highest LC_{50} values, resistance factors were consistently less than 4. Furthermore, the highest LC_{50} values for nymphs did not significantly exceed that of susceptible adults. Similarly, there was no significant resistance of second-instar nymphs to pymetrozine in any of the field strains (Table 2.4).

Table 2.3 Responses of *Trialeurodes vaporariorum* second-instar nymphs to imidacloprid. Resistance factors (RFs) relative to TV1 are given for all strains

Strain	LC_{50} (mg L^{-1}) * (95 % CL)	Slope	RF
TV1	7.54 (3.67-30.0) a	1.26±0.39	1
TV2	9.67 (5.59-19.7) a	0.85±0.15	1.28
TV3	13.7 (7.02-28.2) a	1.19±0.30	1.81
TV4	4.65 (3.34-6.21) a	1.25±0.11	0.62
TV5	28.0 (22.4-36.3) a	1.47±0.19	3.71
TV6	10.2 (5.27-24.2) a	0.74±0.13	1.35
TV7	20.3 (15.2-27.4) a	1.53±0.24	2.70
TV8	17.3 (12.3-25.5) a	1.00±0.13	2.30

* Different letters indicate significant difference between strains, based on overlapping 95 % CL of LC_{50} values.

Table 2.4 Responses of *Trialeurodes vaporariorum* second-instar nymphs to pymetrozine. Resistance factors (RFs) relative to TV1 are given for all the field strains.

Strain	LC_{50} (mg L^{-1}) * (95 % CL)	Slope	RF
TV1	126 (54.6-345) a	0.15±0.02	1
TV2	171 (105-304) a	0.36±0.03	1.35
TV3	213 (127-396) a	0.34±0.03	1.69
TV4	208 (134-346) a	0.36±0.03	1.65
TV5	54.0 (29.9-101) a	0.28±0.03	0.43
TV6	78.0 (47.8-139) a	0.35±0.04	0.62
TV7	207 (114-437) a	0.29±0.03	1.64
TV8	120 (70.4-224) a	0.31±0.03	0.95

* Different letters indicate significant difference between strains, based on overlapping 95 % CL of LC_{50} values.

2.3.3 Reciprocal selection with imidacloprid and pymetrozine

Exposure of adults to 300 and subsequently 1000 mg L^{-1} imidacloprid increased resistance of TV6 to both imidacloprid and pymetrozine (43- and 3.8-fold respectively). Selection with the same concentrations of pymetrozine increased resistance to both compounds more than 14-fold (Table 2.5). These results support those from cross-resistance bioassays, demonstrating a correlated response to imidacloprid and pymetrozine.

Table 2.5 Post-selection responses of imidacloprid- and pymetrozine-selected *Trialeurodes vaporariorum* adults to both compounds. Selection factors (SFs) relative to the unselected TV6 are given.

Selection regime	Imidacloprid		Pymetrozine	
	LC_{50} (mg L^{-1})[a] (95 % CL)	SF	LC_{50} (mg L^{-1})[a] (95 % CL)	SF
TV6 unselected	43.2 (27.1-65.7)	1	116 (60-213)	1
TV6 imidacloprid selected	1860 (864-5670) *	43.1	444 (234-702) *	3.83
TV6 pymetrozine selected	1090 (296-14600) *	25.2	1650 (536-9550) *	14.2

[a] * indicates significant difference compared with the unselected TV6 (based on overlapping 95 % CL of the LC_{50} values)

2.4 DISCUSSION

Results from bioassays extend those reported previously demonstrating the occurrence of resistance to neonicotinoids in *T. vaporariorum* (Bi & Toscano, 2007; Gorman *et al.*, 2007). The highest resistance factor recorded for any of the three neonicotinoids (imidacloprid, thiamethoxam and acetamiprid) was 22, for a German strain (TV8) tested with imidacloprid. Although this is substantially lower than the very high resistance (up to 1000-fold) documented for some strains of *B. tabaci* populations (Rauch & Nauen, 2003; Nauen & Denholm, 2005), it was associated with claims of control failures and significant crop losses at the site of collection. All the strains used in this study were taken from sites where neonicotinoid insecticides had been used for whitefly control. Cross-resistance between neonicotinoids has already been reported for *B. tabaci* (Nauen *et al.*, 2002; Rauch & Nauen, 2003; Horowitz *et al.*, 2004; Prabhaker *et al.*, 2005), *N. lugens* (Zewen *et al.*, 2003), *L. decemlineata* (Alyokhin *et al.*, 2007), and peach potato aphid, *M. persicae* (Foster *et al.*, 2008).

Correlations between data for the three neonicotinoids used in this study demonstrate the presence of intragroup cross-resistance in *T. vaporariorum* also.

Although none of the field strains had any known previous exposure to pymetrozine, treatment histories were not always complete, and this cannot be discounted. Bioassay results demonstrated at least a low level of reduced susceptibility to pymetrozine in all strains; the highest observed resistance factor was again approximately 20 (TV7). Results of cross-resistance bioassays and selection experiments were consistent in showing a correlation in responses to neonicotinoids and pymetrozine, implying a common resistance mechanism in spite of differences in chemical structure and mode of action. This finding closely parallels results for *B. tabaci* (Gorman *et al*., 2010), but there is no evidence of such cross-resistance in other species, including *M. persicae* (Foster *et al*., 2002). Given clear similarities in the phenotypic characteristics of resistance in *T. vaporariorum* and *B. tabaci*, including cross-resistance between neonicotinoids and pymetrozine (Gorman *et al*., 2010) and its differential expression across life-stages (Nauen *et al*., 2008), there are likely to be parallels in the underlying mechanism(s). Resistance in *B. tabaci* primarily reflects overexpression of a monooxygenase enzyme (Karunker *et al*., 2008) that has been confirmed to facilitate the breakdown of imidacloprid to its major metabolite (Karunker *et al*., 2009). Work is now underway to isolate genes encoding detoxification enzymes in *T. vaporariorum*, to investigate whether overexpression of a homologue of *CYP6CM1* in *B. tabaci* or a related monooxygenase gene is responsible for neonicotinoid and pymetrozine resistance in *T. vaporariorum* also.

Chapter 3

Pyrosequencing the transcriptome of the greenhouse whitefly, *Trialeurodes vaporariorum* reveals multiple transcripts encoding insecticide targets and detoxifying enzymes

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Abstract

BACKGROUND: The whitefly *Trialeurodes vaporariorum* is an economically important crop pest in temperate regions that has developed resistance to most classes of insecticides. However, the molecular mechanisms underlying resistance have not been characterised and, to date, progress has been hampered by a lack of nucleotide sequence data for this species. Here, we use pyrosequencing on the Roche 454-FLX platform to produce a substantial and annotated EST dataset. This 'unigene set' will form a critical reference point for quantitation of over-expressed messages via digital transcriptomics.

RESULTS: Pyrosequencing produced around a million sequencing reads that assembled into 54,748 contigs, with an average length of 965 bp, representing a dramatic expansion of existing cDNA sequences available for *T. vaporariorum* (only 43 entries in GenBank at the time of this publication). BLAST searching of non-redundant databases returned 20,333 significant matches and those gene families

potentially encoding gene products involved in insecticide resistance were manually curated and annotated. These include, enzymes potentially involved in the detoxification of xenobiotics and those encoding the targets of the major chemical classes of insecticides. A total of 57 P450s, 17 GSTs and 27 CCEs were identified along with 30 contigs encoding the target proteins of six different insecticide classes.

CONCLUSION: Here, we have developed new transcriptomic resources for *T. vaporariorum*. These include a substantial and annotated EST dataset that will serve the community studying this important crop pest and will elucidate further the molecular mechanisms underlying insecticide resistance.

3.1 INTRODUCTION

Whiteflies (Hemiptera: Aleyrodidae) are important pests of agriculture that feed on and transmit viruses to a wide range of crops. The two most damaging and widespread species are the tobacco or cotton whitefly (*Bemisia tabaci* Gennadius) and the greenhouse whitefly (*Trialeurodes vaporariorum* Westwood).

One factor enhancing the pest status of whiteflies is their ability to evolve resistance to insecticides. Both *B. tabaci* and *T. vaporariorum* are known to exhibit resistance to several insecticide groups including the neonicotinoids, the most widely-used compounds for whitefly control (Gorman *et al*., 2010; Karatolos *et al*., 2010). Insecticide resistance commonly arises through two main mechanisms 1) reduced binding of the insecticide to its target through target site mutation (Pittendrigh *et al*., 2008) (e.g. acetylcholinesterase for organophosphates/carbamates, the voltage-gated sodium channel for pyrethroids) and 2) enhanced metabolism or sequestration of insecticide by enzymes such as carboxyl-cholinesterases (CCEs), glutathione-S-transferases (GSTs) and cytochrome P450 monooxygenases (Ranson *et al*., 2002;

Feyereisen, 2005; Oakeshott *et al*., 2005; Ranson & Hemingway, 2005; Pittendrigh *et al*., 2008).

CCEs, GSTs and P450s are encoded by large and diverse gene families that are difficult to fully characterise by traditional biochemical methods. Identification and cloning of genes encoding insecticide target sites composed of multiple subunit proteins (such as the nicotinic acetylcholine receptor) by degenerate PCR is also a lengthy and sometimes difficult process. The recent and rapid growth of the use of next generation sequencing has made it easier to study large complex genes or gene families such as insecticide target sites and those involved in detoxification of xenobiotics via the de novo sequencing of whole insect transcriptomes (Pauchet *et al*., 2009; Pauchet *et al*., 2010). Although there is a significant amount of genomic data for *B. tabaci* in this regard, including an expressed sequence tag (EST) library (Wang *et al*., 2010) and an ongoing genome project (Leshkowitz *et al*., 2006), very little comparable data for *T. vaporariorum* exist, with only 43 nucleotide sequences currently available at NCBI.

Cost-effective high-throughput DNA sequencing technologies such as 454-based pyrosequencing of ESTs are a powerful new approach to characterise the transcriptome of insect species that lack a fully sequenced genome (Papanicolaou *et al*., 2009; Pauchet *et al*., 2009; Pauchet *et al*., 2010). The amount of sequence information generated by these methods also facilitates the global analysis of gene expression by providing a reference transcriptome for cDNA microarray design and/or Serial Analysis of Gene Expression (SAGE) (Morozova *et al*., 2009; Puinean *et al*., 2010; Wang *et al*., 2010). Here, we have used 454-based pyrosequencing to

generate a substantial EST dataset of the *T. vaporariorum* transcriptome and then characterised genes encoding detoxification enzymes and insecticide target proteins.

3.2 MATERIALS AND METHODS

3.2.1 Insects and RNA extraction

Whiteflies for the generation of cDNA libraries were obtained from two different strains of *T. vaporariorum*. One was an insecticide susceptible standard strain (TV1) and the other was a strain from Turkey (TV6) selected with a 1000 ppm dose of the neonicotinoid insecticide, imidacloprid (Confidor; Bayer CropScience). TV6 was collected from a greenhouse with a history of intensive insecticide use, although the complete treatment history is unknown for this strain. Insects were maintained on French bean plants, *Phaseolus vulgaris* L., cv. 'Canadian Wonder' (Fabaceae), under a 16h photoperiod at 24 °C. More than 2000 adults of each strain were collected in two separate 2 ml Eppendorf tubes and flash frozen in liquid nitrogen. Samples were sent to the University of Exeter (Cornwall Campus, Penryn, UK) in dry ice and stored at -80 ℃ prior to RNA extraction.

RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Genomic DNA contamination was removed by DNase treatment (TURBO DNAse, Ambion) for 30 min at 37 ℃, RNA was further purified (RNeasy MinElute Clean up Kit, Qiagen) following the manufacturer's protocol and eluted in 20 µl of RNA storage solution (Ambion).

3.2.2 cDNA library preparation, sequence pre-processing and assembly

Two cDNA libraries were used for two main reasons. Firstly, in order to identify as many genes encoding detoxification enzymes as possible. This may have been

influenced by differences in gene expression levels in the two libraries, despite the fact that both libraries were normalised. Another reason for the use of the two cDNA libraries was to look for potential SNPs in target-site genes associated with insecticide resistance. Full-length, enriched, cDNAs were generated from 2 μg total RNA (SMART PCR cDNA synthesis kit, BD Clontech) following the manufacturer's protocol. Reverse transcription was performed using the PrimeScript reverse transcription enzyme (Takara) for 60 min at 42 °C and 90 min at 50 °C. In order to reduce over-abundant transcripts, double-stranded cDNAs were normalised using the Kamchatka crab duplex-specific nuclease method (Trimmer cDNA normalisation kit, Evrogen) (Zhulidov *et al*., 2004). Two aliquots, one of each of the normalised cDNA libraries, were 454 sequenced at the Advanced Genomics facility at the University of Liverpool. The two cDNA libraries were tagged prior to sequencing using molecular barcodes (Multiplex Identifiers, Roche Applied Sciences). A single full plate run (using both the TV1 and TV6 cDNA tagged libraries) was performed on the 454 GS-FLX Titanium series pyrosequencer (Roche Applied Science) using 3 μg of normalised cDNAs processed by the "shotgun" method. For raw reads pre-processing (removal of Poly-A tails and SMART adapters) and assembly, the custom pipeline est2assembly was used (Papanicolaou *et al*., 2009). A pool of the processed reads from both cDNA libraries (TV1 and TV6) were clustered using the MIRA v2.9.26x3 assembler with the "de novo, normal, EST, 454" parameters, specifying a minimum read length of 40 nucleotides, a minimum sequence overlap of 40 nt, and a minimum percentage overlap identity of 80 %.

3.2.3 Blast homology searches and sequence annotation

Blast homology searches and sequence annotations were carried out following a method that was successfully used for a midgut transcriptome of the tomato hornworm, *Manduca sexta* Linnaeus (Lepidoptera: Sphingidae) (Pauchet *et al*., 2010). BLAST2GO software v.2.3.1 (http://www.blast2go.org) was used to perform several analyses of the EST assembly (contigs) (Conesa & Götz, 2008). Initially, homology searches were performed remotely on the NCBI server through QBLAST in a sequential strategy. Firstly, contig sequences were searched via BLASTx against the NCBI non-redundant (nr) database, using an E-value cut-off of $1E^{-3}$ and selecting predicted polypeptides of a minimum length of 10 amino acids. Secondly, the sequences that did not receive any BLASTx hit were searched via BLASTn against the NCBI nr nucleotide database using an E-value cut-off of $1E^{-10}$. Also, BLASTx searches with an E-value cut-off of $1E^{-5}$ were performed against the *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) uniprot (100) database. For gene ontology mapping (GO; http://www.geneontology.org), the program extracts the GO terms associated with homologies identified with NCBI's QBLAST and returns a list of GO annotations represented as hierarchical categories of increasing specificity. BLAST2GO allows the selection of a significance level for the false discovery rate, here used at a 0.05 % probability level cut-off. GO terms were modulated using the annotation augmentation tool ANNEX (Myhre *et al*., 2006), followed by GOSlim. GOSlim consists of a subset of the GO vocabulary encompassing key ontological terms and a mapping function between the full GO and the GOSlim. Here, we used the 'generic' GOSlim mapping term (goslim_generic.obo) available in BLAST2GO. Enzyme classification (EC) codes, and KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic pathway annotations, were generated from the direct mapping of

GO terms to their enzyme code equivalents. Finally, InterPro (InterProScan, EBI) searches were performed remotely from BLAST2GO via the InterPro EBI web server. Potential ORFs (open reading frames) were identified using the ORF-predictor server (http://proteomics.ysu.edu/tools/OrfPredictor.html) (Min *et al*., 2005). An ORF cut-off of 200 bp was used.

3.2.4 Manual curation of genes of interest, phylogenetic analysis and SNP identification

Contigs that had a protein motif of a cytochrome P450 or a protein domain of a CCE or a GST, as well as contigs that corresponded to the target sites of the most important chemical classes of insecticides were searched by BLASTn against all the assembled processed reads (http://www.rfc.ex.ac.uk/iceblast/iceblast.php) using an E-value cut-off of $1E^{-4}$. Each contig was reassembled from the reads that returned a BLAST hit and manually curated using Geneious software v.4.8.5 (Biomatters Ltd, Auckland, New Zealand). Nucleotide sequences were dynamic translated using the EXPASY Proteomics Server (http://www.expasy.ch/tools/dna.html, Swiss Institute of Bioinformatics). All the identified sequences were searched by BLASTx against all the assembled contigs in the iceblast server using an E-value cut-off of $1E^{-4}$ and the results with more than 99 % similarity with the query sequence were eliminated as allelic variants (note that from those sequences, only the longest contigs with the best coverage were manually curated). MEGA 4.0 software (Tamura *et al*., 2007) was used to perform multiple sequence alignment of P450s, CCEs, GSTs and nAChRs to construct consensus phylogenetic trees using the neighbour-joining method. Bootstrap analysis of 1,000 replication trees was performed in order to evaluate the branch strength of each tree. The manually curated re-assembled contigs that encoded

an insecticide target were investigated for the presence of SNPs arising due to nucleotide divergence between the two strains.

3.2.5 Sequence submission

The raw nucleotide reads obtained by 454 sequencing were submitted to the Sequence Read Archive (SRA) database at NCBI with accession number SRA024353.1. An assembly of the T. vaporariorum data as well as the unassembled reads was uploaded to the InsectaCentral database (http://www.insectacentral.org/) and is searchable by BLAST at the following URL: http://www.rfc.ex.ac.uk/iceblast/iceblast.php. InsectaCentral is a central repository of insect transcriptomes, similar to the ButterflyBase, produced using traditional capillary sequencing or 454 pyrosequencing (NGS) (Papanicolaou *et al*., 2008). Note that the names of the validated enzymes (see additional files 3.5-3.8) are made from the letters Tv followed by the number of the contig from the InsectaCentral database (For example IC88556AaEcon23678 is called Tv23678).

3.3 RESULTS AND DISCUSSION

3.3.1 454 pyrosequencing and assembly

Over-abundant 0.6-6 kb transcripts were reduced by normalisation of the whitefly cDNAs and an even distribution of transcripts ranging from 0.5 to 6 kb in size was produced. 454 pyrosequencing of two libraries (from the insecticide-susceptible TV1 and the imidacloprid selected TV6 *T. vaporariorum* strains) resulted in a total of 1,104,651 reads. After quality scoring of the reads, 990,945 high-quality reads with an average length of 362 bp were entered to assembly (est2assembly). One pooled assembly was done incorporating both libraries (52,832,938 bp of sequencing), which

resulted in 54,748 contigs with an average length of 965 bp (Table 3.1). 55.8 % (30,552 contigs) of these had an ORF (open reading frame) ≥ 200 bp, with an average length of 540 bp. The characteristics of the assembled *T. vaporariorum* 454 contigs and BLASTx alignments against the *D. melanogaster* uniprot database are shown in Additional file 3.1. Figure 3.1 demonstrates that contigs that were assembled from up to 200 reads displayed a linear relationship between sequence read number and contig length ($R = 0.716$, $P < 0.001$).

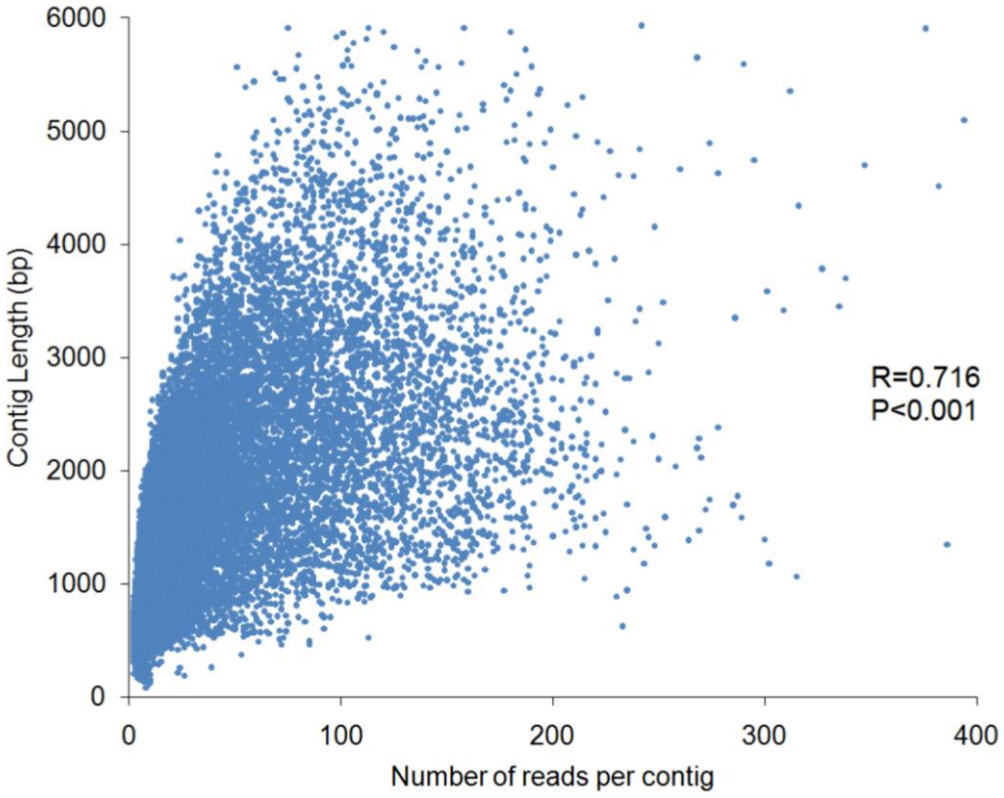

Figure 3.1 Scatter plot of number of reads representing a contig versus the contig length. Summary of correlation statistics is shown.

Table 3.1 Summary statistics for *Trialeurodes vaporariorum* EST assembly and annotation.

Assembly	
Total number of reads	1,104,651
Number of reads after pre-processing	990,945
Average read length after pre-processing	362 bp
Total number of contigs	54,748
Average contig length	965 bp
Sequencing length	52,832,938 bp
Contigs with ORF ≥ 200 bp (average length)	30,552 (540bp)
Average read coverage per contig	4.34x
Average GC % content of contigs	37.77 %
Annotation	
% contigs with at least 1 GO term	30.01 %
% contigs with an EC number	6.10 %
% contigs with at least 1 IPR	33.27 %
Contigs with at least 1 blast hit against nr	
Total number	20,333
Average length	1,394 bp
% of those contigs with at least 1 IPR	42.61 %
% of those contigs with at least 1 GO term	78.43 %
Contigs with ORF ≥ 200 bp (average length)	18,080 (704 bp)
Contigs with no blast hits	
Total number	34,416
Average length	712 bp
% of those contigs with at least 1 GO term	1.41 %
% of those contigs with at least 1 IPR	28.01 %
Contigs with ORF ≥ 200 bp (average length)	12,472 (301 bp)

* nr: non-redundant database, GO: gene ontology term, EC: enzyme commission number, IPR: inter-pro result, ORF: open reading frame.

3.3.2 Homology searches, gene ontology and protein classification

Approximately 37 % (20,333 sequences) of the contigs returned an above cut-off BLAST hit to the NCBI nr database ($1E^{-3}$ for BLASTx resulted in 19,983 and $1E^{-10}$ for BLASTn resulted in 350 additional BLAST results) (Additional file 3.2). The average read length of these contigs was 1,394 bp and E-value and sequence

similarity distributions are detailed in Additional file 3.3. As expected, the pea aphid *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) is the species that returned the most BLAST hits (16 %) with the *T. vaporariorum* contigs (Figure 3.2A), since this species' genome was recently fully sequenced (International Aphid Genomics Consortium, 2010) and currently represents the vast majority of hemipteran sequences available in GenBank.

The remaining 34,416 contigs that did not return a significant BLAST result against the NCBI nr database, had an average read length of 712 bp. More than 36 % of those contigs (12,472 sequences) were found to have a significant ORF ≥ 200 bp with an average length of 301 bp. 28 % of these contigs (9,553 sequences) returned an InterPro result and 1.4 % (488 sequences) returned a GO term (Table 3.1). These results give some indication of the limitation of BLAST comparison as a tool for inferring the relevant biological function of tentative unique genes assembled from sequencing data for species with very limited existing transcriptomic information. However, it is likely that the rapid expansion in sequence data from ongoing small and large scale insect sequencing projects will facilitate the future annotation of these genes.

GO terms were used for the classification of the functions of the predicted whitefly proteins, producing 21,899 terms for biological process categories, 15,571 for molecular function categories, and 14,966 for cellular component categories. Enzyme classification shows that hydrolases account for the largest proportion of *T. vaporariorum* enzymes (38 %), followed by transferases (32 %) and oxidoreductases (15 %) (Figure 3.2B). Most of the molecular function GO terms (Figure 3.3A) were involved in binding (45 %) followed by catalytic activity (35 %). Metabolic and

cellular processes were involved with more than a half of the biological process GO terms (Figure 3.3B). The overall distribution suggests that the sequencing provided a comprehensive representation of the *T. vaporariorum* transcriptome and that 454 pyrosequencing of ESTs can achieve a great number and depth of sequence contigs.

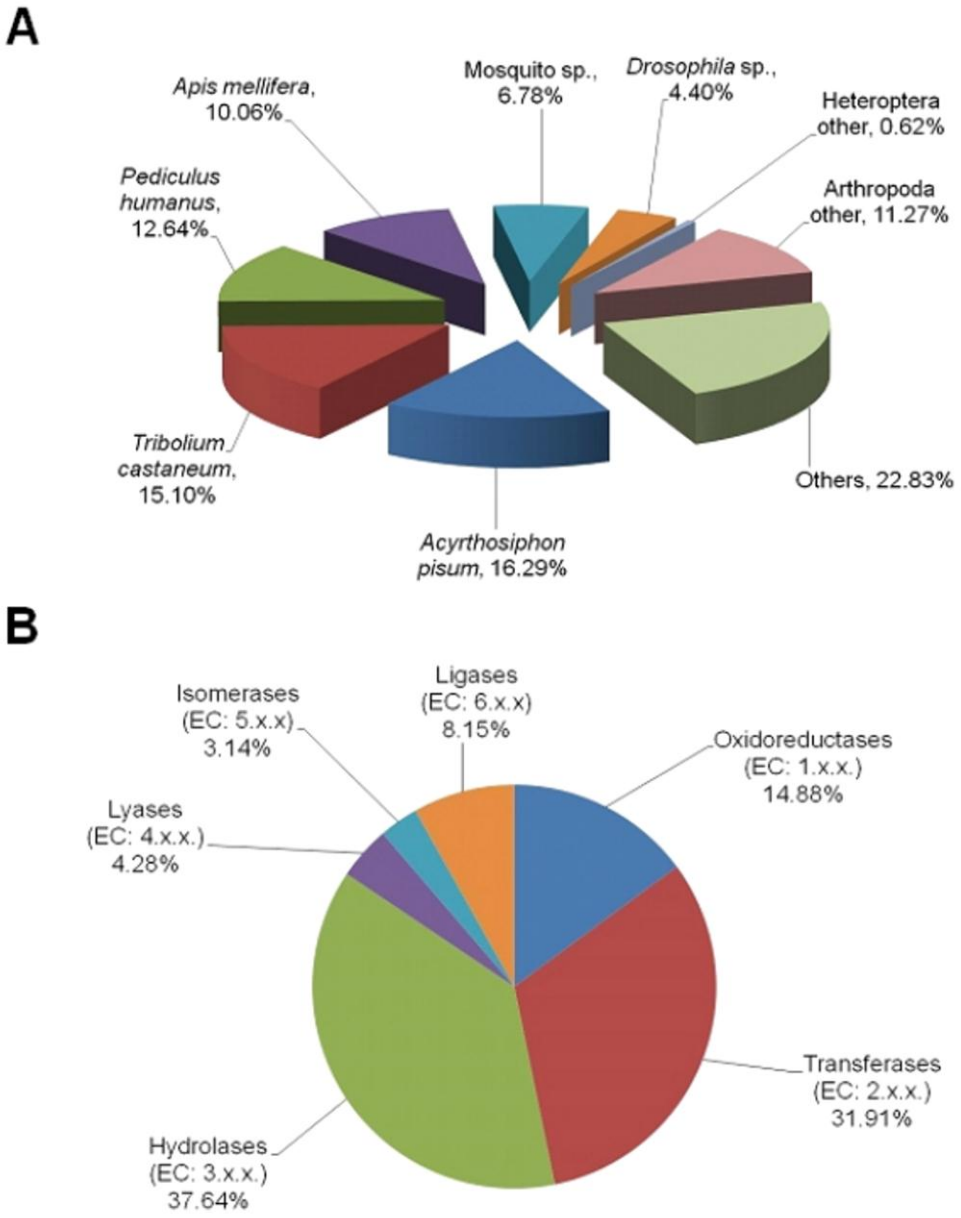

Figure 3.2 Species distribution of the top BLAST hit in the nr database (A) and general Enzyme Classification (EC) terms (B) for the contigs of *Trialeurodes vaporariorum*.

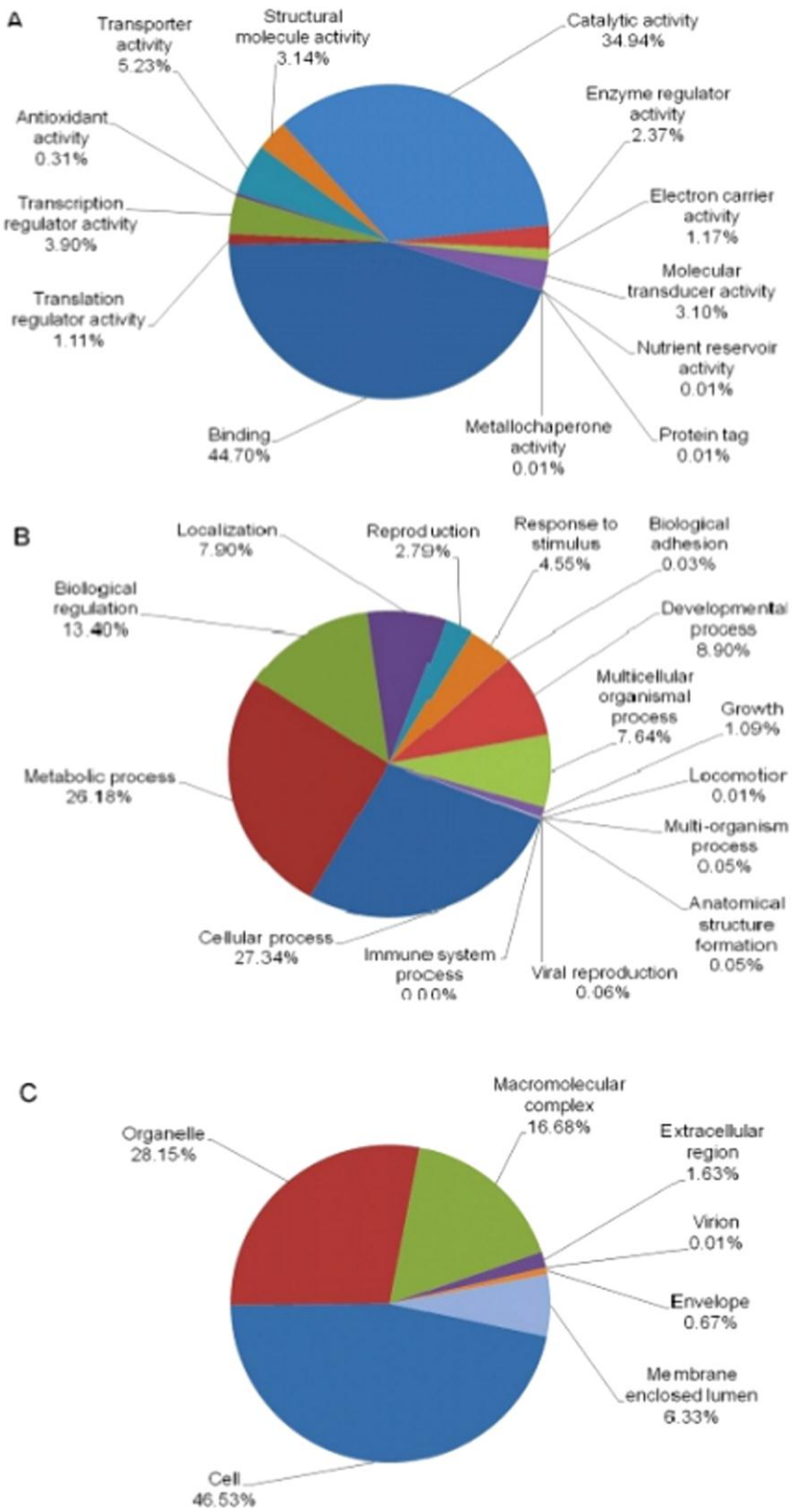

Figure 3.3 Gene ontology (GO) assignments for the *Trialeurodes vaporariorum* transcriptome. A. Molecular function GO terms, B. Biological process GO terms, C. Cellular component GO terms. The data presented represent the level 2 analysis, illustrating general functional categories.

3.3.3 Transcripts encoding genes involved in insecticide detoxification

Trialeurodes vaporariorum, like most insect species, metabolises xenobiotics such as secondary plant chemicals and insecticides using a suite of detoxification enzymes such as P450s, GSTs and CCEs. Representatives of all three enzyme families were identified in the *T. vaporariorum* transcriptome and the average sequence length and coverage obtained for members of these gene families using one full plate of 454 sequencing was comprehensive (Table 3.2).

Table 3.2 Summary information for the identified cytochrome P450s, carboxyl/cholinesterases (CCEs) and glutathione-S transferases (GSTs) in the *Trialeurodes vaporariorum* transcriptome.

Genes	Contigs	Average contig size	Average reads per contig	Average coverage per contig	Average ORF length	
					Trialeurodes vaporariorum [1]	*Acyrthosiphon pisum* [2]
P450s	123	1,532 bp	37	6.3x	1,442 bp	1,508 bp
CCEs	78	1,394 bp	27	4.9x	1,487 bp	1,768 bp
GSTs	44	1,111 bp	44	11.3x	672 bp	756 bp

[1] The average ORF length of all the manually curated genes belonging to a certain family identified in *Trialeurodes vaporariorum*; [2] The average ORF length of the full-length genes belonging to a certain family identified in the fully-annotated genome of *Acyrthosiphon pisum*. The sequences were taken from http://www.aphidbase.com/aphidbase.

Table 3.2 reveals that the average ORF length of each of these gene families obtained for *T. vaporariorum* was more than 84 % of that of the same gene families in the fully-annotated genome of *A. pisum*. Candidate contigs were manually curated to identify allelic variants of the same gene or those with a high number of sequencing errors. Most contigs were assembled from at least 4 sequencing reads and were identified in both *T. vaporariorum* libraries (TV1 and TV6). The only exception was

contig 12863, which was assembled from 19 reads from only the imidacloprid resistant strain (TV6) library and had a protein motif of a CCE.

3.3.3.1 Transcripts encoding putative P450s

A total of 123 P450 related contigs were identified in the transcriptome. Of these, 57 were manually curated (Additional file 3.4 and Additional file 3.5) as the remainder were found to be either allelic variants of the same P450 gene or contained too many sequencing errors. These 57 P450 sequences were named by Dr David Nelson in accordance with the P450 nomenclature committee convention (http://drnelson.uthsc.edu/cytochromeP450.html) (Nelson, 2009) and 40 of them were found to represent full length ORFs. Based on the closest BLAST hits in the NCBI nr database, and when possible, by phylogenetic analyses with other known insect P450 genes, P450s were assigned to appropriate CYP clades and families. Representatives of all 4 major insect CYP clades (CYP2-4 and mitochondrial) were found in this dataset (Figure 3.4). A majority of identified P450s belonged to the CYP3 family (34/57 P450s), 13 to the CYP4 family, and the rest to the CYP2 and mitochondrial families (3 and 7 respectively) (Table 3.3). Phylogenetic analysis of the *T. vaporariorum* P450s with those of *A. pisum* (Figure 3.4) revealed significant divergence in this gene family between these two species with only a few putative *A. pisum* orthologues identified (Additional file 3.4). Two sequences were considered orthologues if they were paired in the phylogeny with bootstrap support greater than 50 %. Duplication events specific to *T. vaporariorum* are also apparent from the phylogeny, with the best example being the three CYP4-type sequences *CYP4G59*, *CYP4G60* and *CYP4G61*. The potential role of these duplication events in insecticide resistance warrants further investigation as amplification of a P450 gene has recently

been implicated in insecticide resistance in *Myzus persicae* Sulzer (Hemiptera: Aphididae) (Puinean *et al*., 2010).

CYP3 and CYP4 P450 families in other insect species are implicated in the metabolism of plant secondary metabolites and synthetic insecticides (Feyereisen, 2005). In the other hemipterans *B. tabaci* and *M. persicae*, over-expression of cytochrome P450s (*CYP6CM1* and *CYP6CY3* respectively) contribute to resistance to neonicotinoid insecticides (Karunker *et al*., 2008; Puinean *et al*., 2010). The closest hits of these two P450s in *T. vaporariorum* are *CYP6CM2*, *CYP6CM 3* (68 % and 67 % similarity to *CYP6CM1* respectively) and *CYP6DP1*, *CYP6DZ1* (60 % and 59 % similarity to *CYP6CY3* respectively). These genes and the other CYP3 and CYP4 P450 genes identified in this study are candidates for a potential role in neonicotinoid resistance in *T. vaporariorum*.

Although the number of P450s in the *T. vaporariorum* transcriptome (57) is within the range of P450s identified in other insect species (46-164) (Oakeshott *et al*., 2010), additional P450 genes may await discovery due to their absence from the current transcriptomic dataset. Analysis of fully sequenced insect genomes have identified 164 P450s in *Aedes aegypti* Linnaeus (Diptera: Culicidae), 106 in *Anopheles gambiae* Giles (Diptera: Culicidae), 85 in *D. melanogaster*, 115 in the green peach aphid *M. persicae*, 83 in the green pea aphid *A. pisum*, and 46 in the western honey bee *Apis mellifera* Linnaeus (Hymenoptera: Apidae) (Adams *et al*., 2000; Holt *et al*., 2002; Claudianos *et al*., 2006; The Honeybee Sequencing Consortium, 2006; Strode *et al*., 2008; Oakeshott *et al*., 2010; Ramsey *et al*., 2010). The current number of 57 P450s in *T. vaporariorum* is at the lower end of this range, almost half of that for *M. persicae*.

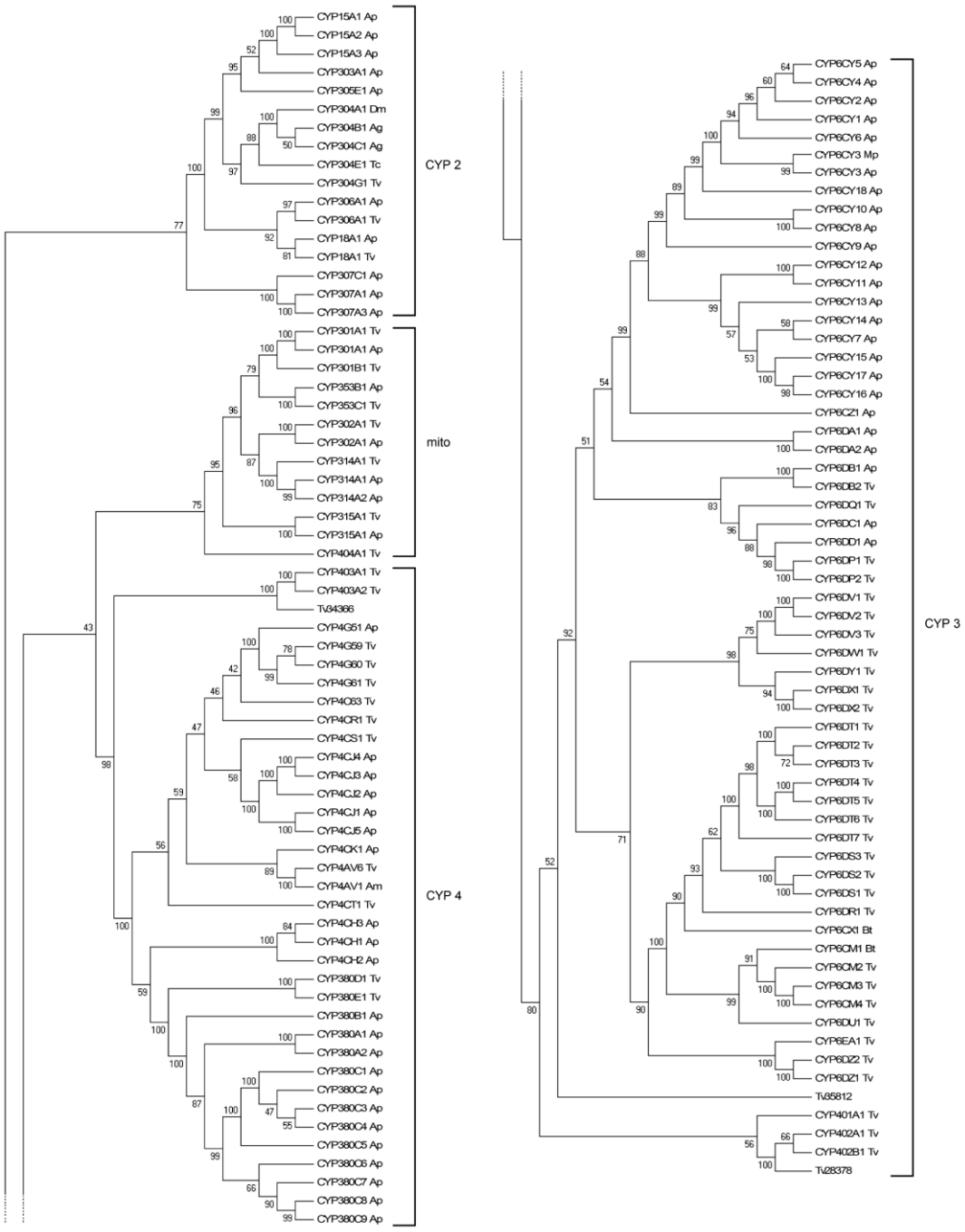

Figure 3.4 Neighbour-joining phylogenetic analysis of cytochrome P450s from *Trialeurodes vaporariorum* (Tv) and other insect species. Bootstrap values next to the nodes represent the percentage of 1,000 replicate trees that preserved the corresponding clade. Positions containing alignment gaps and missing data were eliminated with pairwise deletion. *Acyrthosiphon pisum* (Ap), *Bemisia tabaci* (Bt), *Myzus persicae* (Mp), *Drosophila melanogaster* (Dm), *Anopheles gambiae* (Ag), *Tribolium castaneum* (Tc) and *Apis mellifera* (Am) sequences were taken from http://drnelson.uthsc.edu/aphid.htm (Nelson, 2009).

Table 3.3 Number of validated GSTs, CCEs and cytochrome P450s annotated in *Trialeurodes vaporariorum* (this study), *Acyrthosiphon pisum*, *Myzus persicae* (Ramsey *et al*., 2010) and *Apis mellifera* (Claudianos *et al*., 2006; The Honeybee Sequencing Consortium, 2006) genomes and their distribution across classes and clades.

Enzymes/Class	**Gene numbers**			
	Trialeurodes vaporariorum	*Acyrthosiphon pisum*	*Myzus persicae*	*Apis mellifera*
Cytochrome P450s				
CYP2	3	10	3	8
CYP3	34	33	63	28
CYP4	13	32	48	4
Mitochondrial P450s	7	8	1	6
Total P450s	**57**	**83**	**115**	**46**
Carboxyl/cholinesterases				
Dietary class				
A clade	11	5	5	8
B clade	0	0	0	0
C clade	1	0	0	0
Hormone/semiochemical processing				
D clade	0	0	0	1
E clade	6	18	12	3
F clade	0	0	0	0
G clade	0	0	0	1
Neurodevelopmental				
H clade	1	1	0	0
I clade	1	0	1	2
J clade	2	2	3	2
K clade	1	1	1	1
L clade	3	3	0	5
M clade	1	0	0	1
Total CCEs	**27**	**30**	**22**	**24**
Cytosolic GSTs				
Delta	9	10	8	1
Epsilon	1	0	0	0
Omega	0	0	0	1
Sigma	5	6	8	4
Theta	0	2	2	1
Zeta	1	0	0	1
Microsomal	1	2	2	2
Total cytosolic GSTs	**17**	**20**	**20**	**10**

3.3.3.2 Transcripts encoding putative CCEs

A total of 78 contig sequences with a protein motif of a CCE were identified. Of these, 27 were manually curated (Additional file 3.4 and Additional file 3.6) as some of the original sequences were found to be either allelic variants of the same CCE gene or contained too many sequencing errors, and 14 were found to be full length. Based on the closest BLAST hits in the NCBI nr database and when possible by phylogenetic analyses with other known CCE genes from other insect species, these enzymes were assigned to three known classes of CCEs (Figure 3.5; Table 3.3). Known CCEs can be divided into 13 clades, nine of which are represented in *T. vaporariorum*. Clades without identifiable *T. vaporariorum* homologues are clade B (alpha esterase), integument esterases (D) and lepidopteran juvenile hormone esterase (F and G). Esterases involved in the detoxification of insecticides belong to clades A-C and 12 sequences were assigned to these clades. A phylogenetic analysis indicates a potential expansion of *T. vaporariorum* CCEs in clade A (compared to known, aphid CCEs). There is also evidence of a contraction in clade E, which contains the vast majority of aphid CCEs, although it is difficult to ascertain if genes in this clade have been lost from the genome or simply remain to be discovered (Figure 3.5; Table 3.3). In addition, divergence in the CCEs of *A. pisum* and *T. vaporariorum* is apparent in the same clades (Figure 3.5). The potential *A. pisum* and *B. tabaci* orthologues of *T. vaporariorum* CCEs are detailed in Additional file 3.4. Clade A contains the largest number of identified *T. vaporariorum* CCEs (11 sequences), twice as many as in two other hemipteran species *A. pisum* and *M. persicae* (5 sequences each) (Ramsey *et al*., 2010).

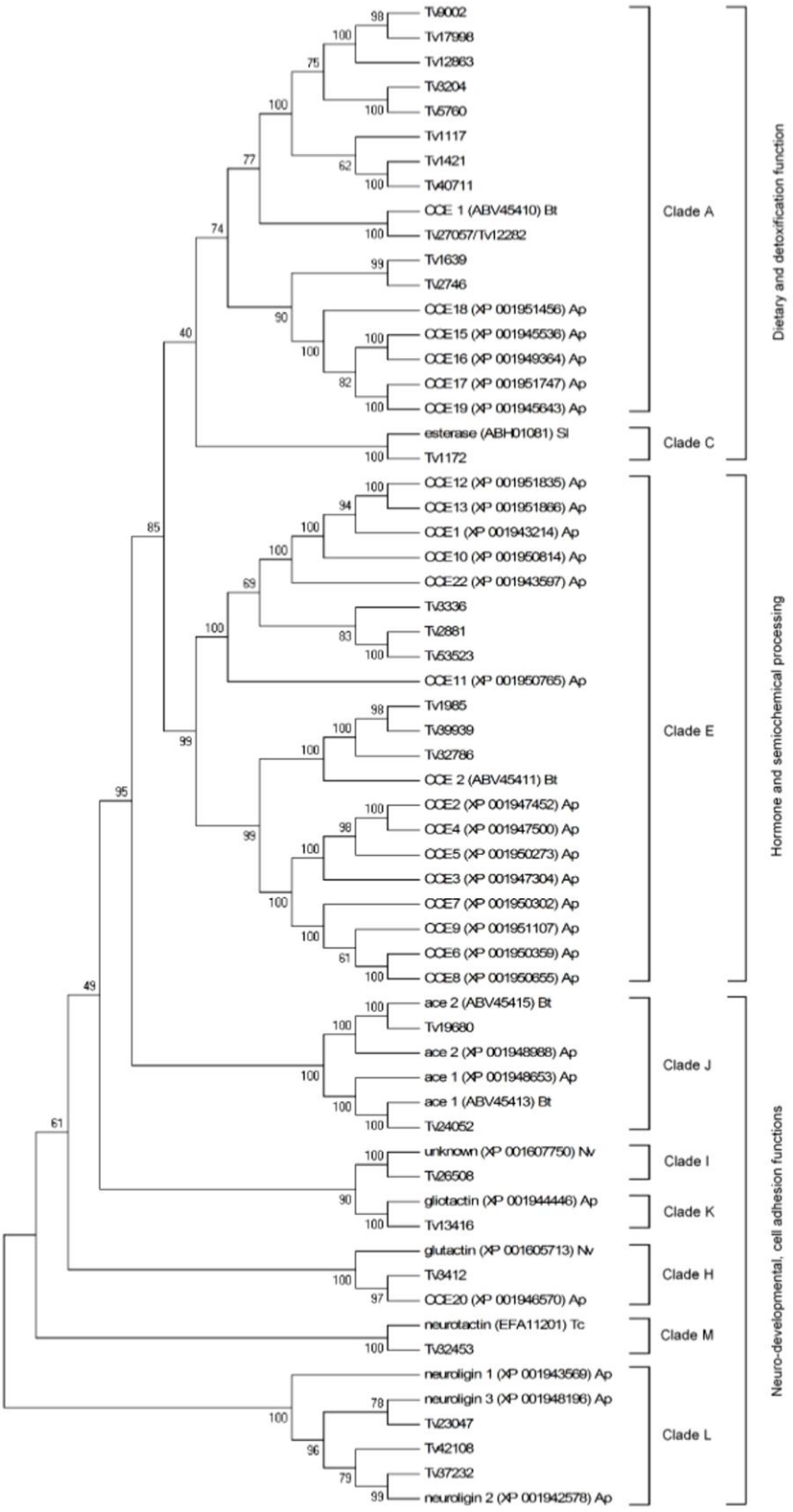

Figure 3.5 Neighbour-joining phylogenetic analysis of carboxyl/cholinesterases from *Trialeurodes vaporariorum* (Tv) and other insect species (accession numbers are given). Bootstrap values next to the nodes represent the percentage of 1,000 replicate trees that preserved the corresponding clade. Positions containing alignment gaps and

missing data were eliminated only with pairwise deletion. *Acyrthosiphon pisum* (Ap), *Bemisia tabaci* (Bt), *Nasonia vitripennis* (Nv), *Spodoptera littoralis* (Sl), *Tribolium castaneum* (Tc). *Acyrthosiphon pisum* sequences were taken from http://www.aphidbase.com/aphidbase.

Of these, one CCE sequence (contig 12282) had a high homology to a carboxylesterase gene in *B. tabaci* (COE1; accession ABV45410), which is over-expressed in organophosphate-resistant strains (Alon *et al*., 2008) and another (contig 12863) was identified only in the imidacloprid resistant TV6 library and is therefore a candidate gene for a potential role in the neonicotinoid resistance of this strain. One identified sequence (contig 1172) had high homology to Lepidoptera-specific alpha esterase (C). Six sequences had homology to beta esterase (E) and two contigs were identified as acetylcholinesterases (AChE, clade J), which are the targets for organophosphate and carbamate insecticides. One of these, contig 19680, corresponds to a known AChE sequence of *T. vaporariorum* (*ace-2*; accession number CAE11223). Finally, other clades with identified *T. vaporariorum* homologues are glutactin (H), gliotactin (K), neuroligin (L), neurotactin (M) and an uncharacterised group (I).

3.3.3.3. Transcripts encoding putative GSTs

A total of 44 GST-related contig sequences were identified, 17 of which were unique and manually curated (Additional file 3.4 and Additional file 3.7), and thirteen of these were full length. Based on the closest BLAST hits in the NCBI nr database and when possible by phylogenetic analysis these contigs were assigned to the Delta, Epsilon, Omega, Sigma, Theta, Zeta, and microsomal classes (Figure 3.6; Table 3.3). Phylogenetic comparison of *A. pisum* and *T. vaporariorum* GSTs revealed significant divergence in this gene family between the two species (Figure 3.6). Most of the

identified GSTs were assigned to the Delta class (9 sequences), members of which are known to play a role in insecticide detoxification in other insect species (Claudianos *et al*., 2006). The number of Delta class GSTs in *T. vaporariorum* (9 sequences) is close to that in *A. pisum* were 10 sequences were identified (Ramsey *et al*., 2010). Although the Epsilon and Zeta classes are absent in *A. pisum* and *M. persicae* (Ramsey *et al*., 2010), two contigs (one for each class) were identified in *T. vaporariorum*. One contig, namely Tv7290, was found to encode a microsomal GST.

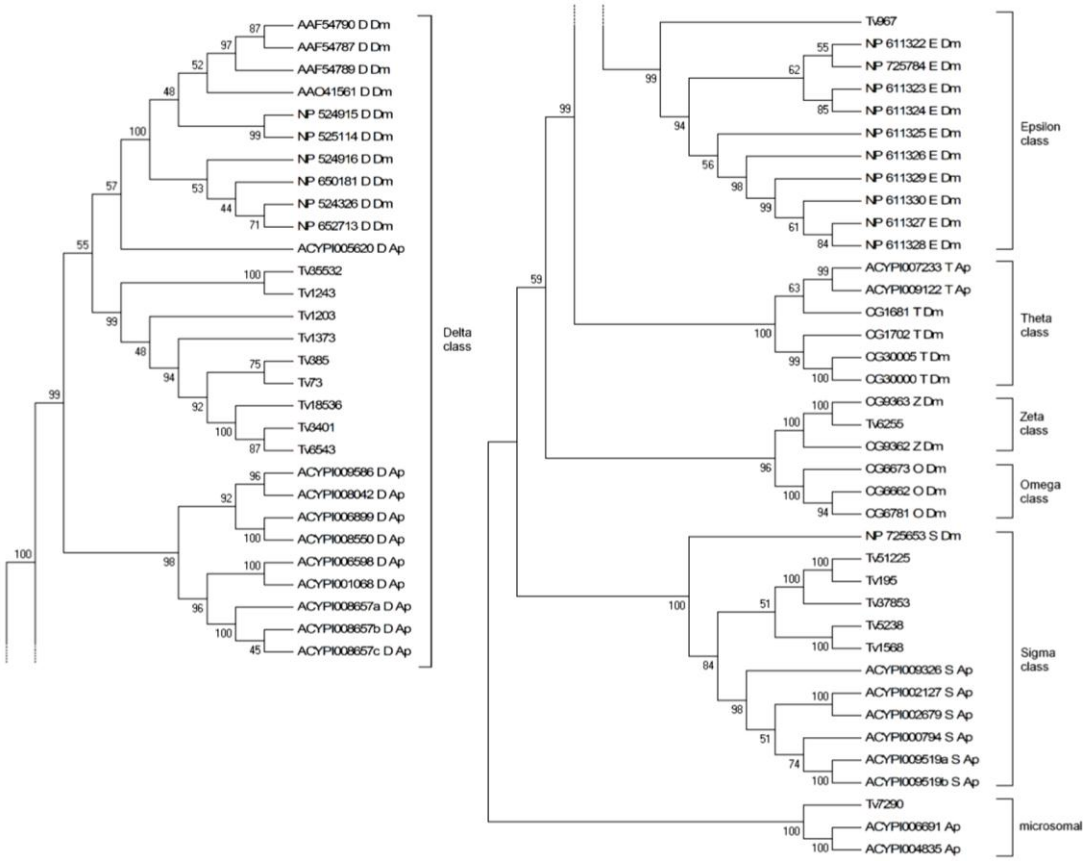

Figure 3.6 Neighbour-joining phylogenetic analysis of glutathione-S-transferases from *Trialeurodes vaporariorum* (Tv) and other insect species (accession numbers are given). Bootstrap values next to the nodes represent the percentage of 1,000 replicate trees that preserved the corresponding clade. Positions containing alignment gaps and missing data were eliminated only with pairwise deletion. *Acyrthosiphon pisum* (Ap), *Drosophila melanogaster* (Dm). *Acyrthosiphon pisum* sequences were taken from http://www.aphidbase.com/aphidbase.

3.3.4 Detection of gene sequences encoding insecticide targets

A number of contigs encoding insecticide target proteins were identified in the *T. vaporariorum* transcriptome. These include the acetylcholinesterase enzyme (AChE), nicotinic acetylcholine receptor subunits (nAChRs), the acetyl-CoA carboxylase (ACCase), the voltage-gated sodium channel (VGSC), the γ-aminobutyric acid (GABA) receptor, the glutamate-gated chloride channel (GluCl) and the ryanodine receptor (RyR) (Table 3.4, Figure 3.7 and Additional file 3.8). All contigs that were assembled from more than 3 reads and the vast majority of the contigs with lower coverage were identified in both *T. vaporariorum* libraries (TV1 and TV6). Although many of these contigs are not full length, they will nevertheless facilitate further characterisation of these targets by PCR and/or RACE. As the two cDNA libraries were tagged prior to sequencing, we investigated the occurrence of SNPs in contigs encoding insecticide target-sites between the resistant and susceptible *T. vaporariorum* strains. A limited number of non-synonymous SNPs were observed between the two strains and these are listed in Additional file 3.9. A number of, often highly conserved, mutations have been described in many of these target proteins that lead to varying degrees of insensitivity such as mutations within the active-site gorge of the AChE enzyme (Russell *et al*., 2004), in domains II or III of the VGSC (Davies *et al*., 2007), in the pore lining M2 region of the GABA receptor (ffrench-Constant *et al*., 2000) and within two alpha subunits of the nAChR (Liu *et al*., 2005).

Table 3.4 Validated genes related to insecticide target sites in *Trialeurodes vaporariorum*.

Insecticide class	Target site	Gene name	Contig Number	Coverage
Organophosphates, Carbamates	Acetylcholinesterase (AChE)	AChE 1	24052	1.59
		AChE 2	19680	1.63
neonicotinoids	Nicotinic acetylcholine receptor (nAChR)	nAChR alpha 2 subunit	19430	2.87
			21473	1.66
		nAChR alpha 3 subunit	20111	3.16
		nAChR alpha 4 subunit	16361	2.47
		nAChR alpha 5 subunit	22076	2.01
			35554	1.33
			21985	1.70
		nAChR alpha 6 subunit	20921	1.89
			29179	3.37
			22598	1.46
		nAChR alpha 7 subunit	12555	3.50
		nAChR alpha 10 subunit	1918	15.0
		nAChR beta 1 subunit	31493	1.55
			36485	1.37
Tetronic & Tetramic acid derivatives	Acetyl-CoA carboxylase (ACCase)	ACCase	28490	1.84
			41433	1.18
			17359	1.84
			1349	10.7
Pyrethroids, Pyrethrins	Voltage-gated sodium channel (VGSC)	VGSC	22691	3.98
			37637	1.96
			21272	2.65
Organochlorines, Phenylpyrazoles (Fiproles)	GABA receptor	GABA receptor	16203	4.37
			37638	1.47
	Glutamate-gated chloride channel (GluCl)	GluCl	35107	1.36
			15229	2.62
			35534	1.69
Diamides (chlorantraniliprole, cyanthraniliprole, flubendiamide)	Ryanodine receptor (RyR)	RyR	35833	1.09
			7799	6.30

Additional file 3.7 includes their nucleotide sequences.

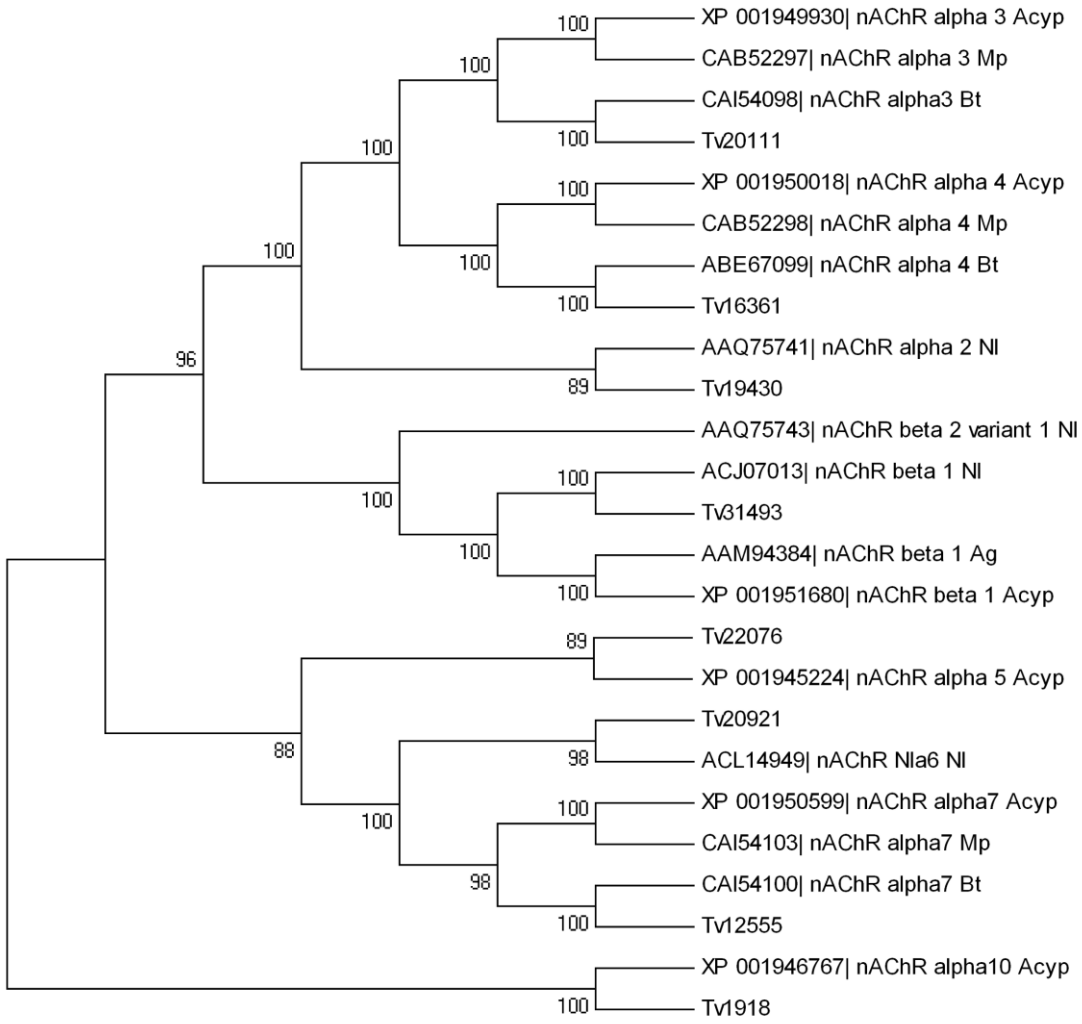

Figure 3.7 Neighbour-joining phylogenetic analysis of nicotinic acetylcholine receptors (nAChR) from *Trialeurodes vaporariorum* (Tv) and other insect species (accession numbers are given). Bootstrap values next to the nodes represent the percentage of 1,000 replicate trees that preserved the corresponding clade. Positions containing alignment gaps and missing data were eliminated only with pairwise deletion. *Acyrthosiphon pisum* (Acyp), *Myzus persicae* (Mp), *Bemisia tabaci* (Bt), *Nilaparvata lugens* (Nl) and *Aphis gossypii* (Ag).

Where possible we examined the two *T. vaporariorum* libraries for previously described mutations at known 'hot-spots' in other arthropod species but none were observed. However, several non-synonymous mutations at alternative positions in many of the target site genes were found (Additional file 3.9) and these clearly warrant further investigation. Pyrosequencing or TaqMan® assays can now be rapidly

developed and used to screen additional whitefly populations with different resistance phenotypes to determine the consistency of the correlation of these SNPs with resistance.

3.4 CONCLUSION

T. vaporariorum is an important agricultural pest that has developed resistance to several insecticides used for whitefly control. To date, the lack of genomics data available for this species has hampered characterisation of the molecular mechanisms underlying resistance. The ~55,000 non-redundant EST contigs described in this study represent a dramatic expansion of existing cDNA sequence available for *T. vaporariorum*. We have identified the genes and gene families that are potential candidates for conferring insecticide resistance in *T. vaporariorum* including those encoding enzymes putatively involved in metabolic detoxification of xenobiotics and those encoding the target proteins of the major chemical classes of insecticides. The EST contig library developed in this study can be used as a reference transcriptome for analysis of gene expression using cDNA microarray and/or SAGE. We plan to use these genomic resources to investigate the role of detoxifying enzymes and target-site modification in *T. vaporariorum* populations that are resistant to insecticides. However, more broadly the annotated EST library will facilitate the investigation of the fundamental biology of *T. vaporariorum* and its interactions with host plants. *T. vaporariorum* has a similar biology to *B. tabaci*, offering the prospect of sharing information on resistance mechanisms and other biological traits between these major crop pests.

3.5 ADDITIONAL FILES

Additional file 3.1 Characteristics of assembled *Trialeurodes vaporariorum* 454 contigs and BLASTx alignments against *Drosophila melanogaster*. (A,B) length and coverage of contigs, (C,D) percent identity and deduced amino acid alignment length for all blast hits to *D. melanogaster* predicted proteins (Additional file 3.1.pdf)

http://www.biomedcentral.com/content/supplementary/1471-2164-12-56-s1.pdf

Additional file 3.2 Top BLAST hits in the NCBI nr database for each unique contig. Note that only the contigs that returned a BLAST result are shown in this file (Additional file 3.2.xls)

http://www.biomedcentral.com/content/supplementary/1471-2164-12-56-s2.xls

Additional file 3.3 E-value (A) and percentage similarity (B) distributions of the top BLAST hit for each contig of *Trialeurodes vaporariorum* (Additional file 3.3.tiff)

http://www.biomedcentral.com/content/supplementary/1471-2164-12-56-s3.tiff

Additional file 3.4 Names, corresponding contig numbers, amino-acid sequences and *Acyrthosiphon pisum* or *Bemisia tabaci* orthologues of contigs that encode detoxifying enzymes (Additional file 3.4.xls)

http://www.biomedcentral.com/content/supplementary/1471-2164-12-56-s4.xls

Additional file 3.5 P450s nucleotide sequences (Additional file 3.5.txt)

http://www.biomedcentral.com/content/supplementary/1471-2164-12-56-s5.txt

Additional file 3.6 CCEs nucleotide sequences (Additional file 3.6.txt)

http://www.biomedcentral.com/content/supplementary/1471-2164-12-56-s6.txt

Additional file 3.7 GSTs nucleotide sequences (Additional file 3.7.txt)

http://www.biomedcentral.com/content/supplementary/1471-2164-12-56-s7.txt

Additional file 3.8 Nucleotide sequences of target sites of the most important insecticide classes (Additional file 3.8.txt)

http://www.biomedcentral.com/content/supplementary/1471-2164-12-56-s8.txt

Additional file 3.9 Single nucleotide polymorphisms (SNPs) arising due to nucleotide divergence between the two strains (TV1 and TV6) (Additional file 3.9.xls)

http://www.biomedcentral.com/content/supplementary/1471-2164-12-56-s9.xls

Chapter 4

Mutations in the sodium channel associated with pyrethroid resistance in the greenhouse whitefly, *Trialeurodes vaporariorum*

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Abstract

BACKGROUND: *Trialeurodes vaporariorum* Westwood is an important pest of protected crops in temperate regions of the world. Resistance to pyrethroid insecticides is long established in this species but the molecular basis of the mechanism(s) responsible has not previously been disclosed.

RESULTS: Mortality rates of three European strains of *T. vaporariorum* to the pyrethroid bifenthrin were calculated and each possessed significant resistance (up to 662-fold) when compared to a susceptible reference strain. Direct sequencing revealed three amino-acid substitutions in the *para*-type voltage gated sodium channel (the pyrethroid and DDT target site) of bifenthrin-resistant *T. vaporariorum*, at positions previously implicated with pyrethroid or DDT resistance (M918L, L925I and T929I) in other related species.

CONCLUSION: This study indicates that resistance to bifenthrin in *T. vaporariorum* is associated with target-site insensitivity, and that the specific mutations in the sodium channel causing resistance may differ between localities.

4.1 INTRODUCTION

The greenhouse whitefly *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae), is an important pest of protected vegetable and ornamental crops in temperate regions of the world (Byrne *et al*., 1990). All feeding life stages cause damage through the extraction of nutrients and the excretion of honeydew, and large infestations can result in stunted plants and leaf necrosis (Byrne *et al*., 1990). *T. vaporariorum* also transmits numerous plant viruses of the Closteroviridae family (Jones, 2003).

T. vaporariorum is the target of many insecticides and is known to have developed resistance to numerous chemical classes including pyrethroids. Pyrethroid resistance and cross-resistance was first documented in UK populations of *T. vaporariorum* in the 1970s (Wardlow *et al*., 1976; Wardlow, 1985) and continues to compromise efficacy. Pyrethroid insecticides share a similar mode of action to the organochlorine compound DDT and act by altering the normal functioning of neuronal voltage-gated sodium channels causing paralysis and ultimately death of the insect (Narahashi, 1992).

Resistance to pyrethroids can result from two distinct types of mechanism; either increased detoxification commonly conferred by cytochrome P450 monooxygenases (Bergé *et al*., 1998), carboxylesterases (Wheelock *et al*., 2005) and occasionally glutathione-S transferases (Vontas *et al*., 2001); or alternatively as a consequence of mutations in the *para*-type sodium channel gene (VGSC) that confer target-site insensitivity through traits commonly known as knockdown resistance (*kdr* and super-*kdr*) (Taylor *et al*., 1993; Williamson *et al*., 1993; Dong & Scott, 1994). The sodium channel is a large membrane protein consisting of 4 internally repeating

homologous domains (I–IV), each with 6 membrane-spanning segments (S1–S6) (Catterall, 2000). Previous studies of a range of insect, mite and tick species have shown that the mutations that cause these resistant phenotypes are predominantly clustered within two regions of the channel protein, The first being domain II S4-S5, S5 and S6 segments, encompassing the 'original' *kdr* mutation (L1014F) (Miyazaki *et al*., 1996; Williamson *et al*., 1996) that gives moderate (10-30 fold) levels of resistance, and a series of commonly more potent 'super-*kdr*' mutations at residues M918, L925, T929 and L932 (Davies *et al*., 2007; Soderlund, 2008). The second region is IIIS6 and includes residues F1538, F1534 and G1535 (Davies *et al*., 2007; Soderlund, 2008). The clustering of mutations in these two regions is consistent with recent homology modelling studies (based on the structures of closely-related potassium channels) that predict that the IIS4-S5, IIS5 and IIIS6 regions of the channel contribute to a hydrophobic binding pocket for pyrethroids and DDT, with many of the residues identified in resistance forming key side-chain interactions that stabilise the high affinity binding of these compounds (O'Reilly *et al*., 2006).

The present study aimed to investigate potential correlations between the phenotypic expression of bifenthrin resistance in *T. vaporariorum* and the presence of domain II mutations in the voltage-gated sodium channel, thereby assessing the likelihood of target site insensitivity as a major mechanism of pyrethroid resistance in this species.

4.2 MATERIALS AND METHODS

4.2.1 Insect strains

Four strains of *T. vaporariorum* including an insecticide susceptible reference strain (TV1) were used in this study (Table 4.1). These encompassed three countries of origin and both edible and ornamental plant hosts. All strains were reared at

Rothamsted Research without exposure to insecticides on French bean plants, *Phaseolus vulgaris* L., cv. "Canadian Wonder" (Fabaceae), under a 16 h photoperiod at 24 °C.

Table 4.1 *Trialeurodes vaporariorum* strains, origins, years of collection and original hosts

Strain	Country of origin	Year of collection	Original host
TV1	UK	1971	French bean
TV3	UK	2008	Ornamentals
TV6	Turkey	2006	Vegetables
TV8	Germany	2008	Ornamentals

4.2.2 Insecticides and bioassays

The pyrethroid bifenthrin 100 g L^{-1} EC (Gyro®; CERTIS) was commercially obtained and diluted to the required concentrations in distilled water containing 0.1 g L^{-1} of the non-ionic wetter Agral® (Syngenta). A stock solution of 10,000 mg L^{-1} DDT (technical grade, BDH Chemicals Ltd) in acetone was further diluted to the required concentrations using 10 % acetone in distilled water containing 0.1 g L^{-1} of the non-ionic wetter Agral®.

Responses of adults to bifenthrin and DDT were quantified using leaf-dip bioassays. Leaf discs were cut from bean plants and dipped for 20 s into the required concentrations of insecticide or into 0.1 g L^{-1} Agral® for controls. Treated leaf discs were placed on 1 % agar inside petri-dishes and allowed to air-dry. At each concentration, three replicates of 20-30 adult females of the required strain were placed in each petri-dish and confined with an absorbent cellulose pad and lid in order to minimise static and humidity. All bioassays were maintained at 24 °C and mortality was scored after 48 h. For bifenthrin, each concentration consisted of three

replicates and concentration-mortality relationships were fitted by probit analysis, using GenStat 12th edition software (VSN International Ltd, Hertfordshire, UK). Resistance factors were calculated by dividing estimated LC_{50} values for field-collected strains by those for the susceptible reference strain (TV1). Lack of overlap of 95 % confidence limits on estimated LC_{50} values denoted significant differences between responses of strains. For DDT, assays used five replicates at each of two diagnostic concentrations (30 and 100 mg L^{-1}). A two-sample unpaired t-test was used to investigate the differences in the responses of the four strains to DDT.

Total esterase activity was detected using 1-naphthyl acetate as a substrate. In each well of a 96-well plate, a single female whitefly was homogenised in 200 µl sodium phosphate buffer (0.1 M, pH 7.5, 0.1 % Triton X-100). 200 µl substrate solution (0.06 % Fast Blue RR in sodium phosphate buffer, 0.2 M, pH 6.0) and 1 mM 1-naphthyl acetate as substrate were added to 100 µl aliquots of homogenate (equivalent to 0.5 whiteflies). Microplates were then read kinetically at 450 nm and intervals of 10 sec for 20 min at room temperature, using a Vmax kinetic microplate reader (Molecular Devices). The amount of protein in the enzyme source was determined using Bradford reagent and bovine serum albumin as a standard. Cytochrome P450 monooxygenase activity was determined by O-deethylation of 7-ethoxycoumarin, following a method already described for tobacco whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Rauch & Nauen, 2003).

4.2.3 RNA, cDNA reverse transcription and gDNAextraction

Total RNA was extracted from fresh samples of the four strains (TV1, TV3, TV6 and TV8). Approximately 100 adult whiteflies from each population were flash frozen in liquid nitrogen and total RNA extracted using the Isolate RNA Mini Kit (Bioline)

according to the manufacturer's protocol. First strand cDNA was synthesised from 1 µg total RNA by reverse transcriptase (Superscript II®, Invitrogen) using the manufacturer's protocol. cDNA was stored at -20 ºC for further use. Genomic DNA (gDNA) was extracted from individual adult whiteflies of the strain TV8 and homogenised in 100 µl of DNAZOL® (Invitrogen) at a tenth scale of the manufacturer's protocol. The gDNA was resuspended in 20 µl of nuclease-free water.

4.2.4 RT-PCR and voltage-gated sodium channel sequencing

Recently, the transcriptome of *T. vaporariorum* was published (Karatolos *et al.*, 2011a), where 3 contigs (Tv22691, Tv37637 and Tv21272) and 18 reads corresponding to *para*-type sodium channel partial sequences were identified. The contigs and reads were assembled and manually curated using Geneious software (Biomatters Ltd, Auckland, New Zealand), resulting in a total of 8 partial sequences of the sodium channel (Additional file 4.1). These sequences were used to design primers for PCR amplification and sequencing of a fragment of the VGSC gene that contained the IIS4-6 region (Table 4.2). PCR reactions (25 µl) contained 1 µl cDNA or approximately 40 ng gDNA, 12.5 µl DreamTaq® Green DNA Polymerase (Fermentas), 15 pmol of each primer and RNase free water. The cycling conditions were 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s for cDNA PCR or 30 s for gDNA PCR. A final extension of 72 °C for 5 min was included at the end of the cycles. The PCR fragments were purified using the Wizard® SV Gel and PCR Clean-up System (Promega) according to the manufacturer's protocol and sent to Eurofins MWG (Germany) for direct sequencing.

Table 4.2 Primer sequences for PCR amplification and sequencing of domain II S4-6 of the *para*-type voltage-gated sodium channel (VGSC) of *Trialeurodes vaporariorum*.

Primer name	Sequence	Purpose
vgsc-f1	ATCTTCTGCGTTTGGGATTG	PCR amplification of domain IIS4-6 region (1189 bp)
vgsc-r1	CATCAAATGGCCTGTGTTTG	PCR amplification of domain IIS4-6 region (1189 bp)
vgsc-f2	GACCCTTTCGTCGAGTTGTT	sequencing of PCR fragment
vgsc-r2	TGTTTGTGACTGCCGTAGGA	sequencing of PCR fragment
vgsc-f3	TAGCAAAATCATGGCCGACA	gDNA PCR amplification of a region in domain IIS4-6 (129 bp)
vgsc-r3	AGTTGCATGCCCATGACG	gDNA PCR amplification of a region in domain IIS4-6 (129 bp)
vgsc-r4	CCATGACGGCAAAGATGAA	Sequencing of gDNA fragments

4.3 RESULTS AND DISCUSSION

4.3.1 Bioassays and biochemical assays

The LC_{50} value for the susceptible strain (TV1) against bifenthrin was 0.95 mg L^{-1}. All three field strains were significantly resistant compared to TV1, based upon overlapping 95 % CL of LC_{50} values. The highest resistance factor recorded was 662-fold for the German strain TV8, followed by 471-fold for TV6 and 263-fold for TV3 (Table 4.3). These results are in accordance with previous reports of pyrethroid resistance in *T. vaporariorum* (Wardlow *et al*., 1976; Wardlow, 1985).

There was no evidence for elevated cytochrome P450 activity in any of the bifenthrin-resistant strains compared to TV1 (Table 4.3). Total esterase activity with 1-naphthyl acetate as a substrate did not differ significantly between the strains TV1, TV3, and TV6 but it was elevated (1.6-fold compared to TV1) in the most resistant

strain TV8 (Table 4.3). This difference in total esterase activity is unlikely to be solely responsible for the high resistance of TV8.

Table 4.3 Responses of *Trialeurodes vaporariorum* adults to the pyrethroid bifenthrin, enzyme (esterase and cytochrome P450) activities, and amino-acid substitutions disclosed in the sodium channel gene. Resistance factors (RFs) relative to TV1 are given for all the field strains.

Strain	LC_{50} (mg L^{-1}) (95% CL)	Slope (±s.e.)	RF	Enzyme activity (±s.e.)		*kdr* mutations
				Esterase[a]	P450[b]	
TV1	0.95 (0.71-1.22) a	1.64 (±0.17)	1	0.83±0.10 a	76.5±4.62 a	none
TV3	249 (175-357) b	1.02 (±0.09)	263	1.01±0.05 a	86.2±3.20 a	T929I
TV6	447 (336-576) bc	2.29 (±0.29)	471	0.86±0.12 a	86.6±6.89 a	L925I
TV8	628 (486-805) c	2.51 (±0.29)	662	1.39±0.11 b	79.4±6.71 a	M918L + L925I

[a]Esterase activity (mOD/min/mg protein), [b]Cytochrome P450-dependent monooxygenase activity (pmol/30min/mg protein) (7-ethoxycoumarin O-deethylase). Different letters in the same column indicate significant differences between strains.

The mortality of strain TV3 to DDT (Figure 4.1) was significantly reduced compared to the strain TV1 after treatments with a diagnostic dose of 30 mg L^{-1} (t = 42.5, df = 8, $P < 0.001$) and 100 mg L^{-1} (t = 50.7, df = 8, $P < 0.001$). The responses of the strains TV6 and TV8 to the 30 mg L^{-1} dose of DDT, were significantly different to that of TV1 (t = 4.71, df = 8, P = 0.002 and t = 7.33, df =8, $P < 0.001$ respectively). However, there was no difference in the responses of TV1, TV6 and TV8 to the 100 mg L^{-1} dose, which killed almost all the tested whiteflies of those strains. These data confirm the presence of DDT resistance in *T. vaporariorum*, with TV3 being the most resistant strain.

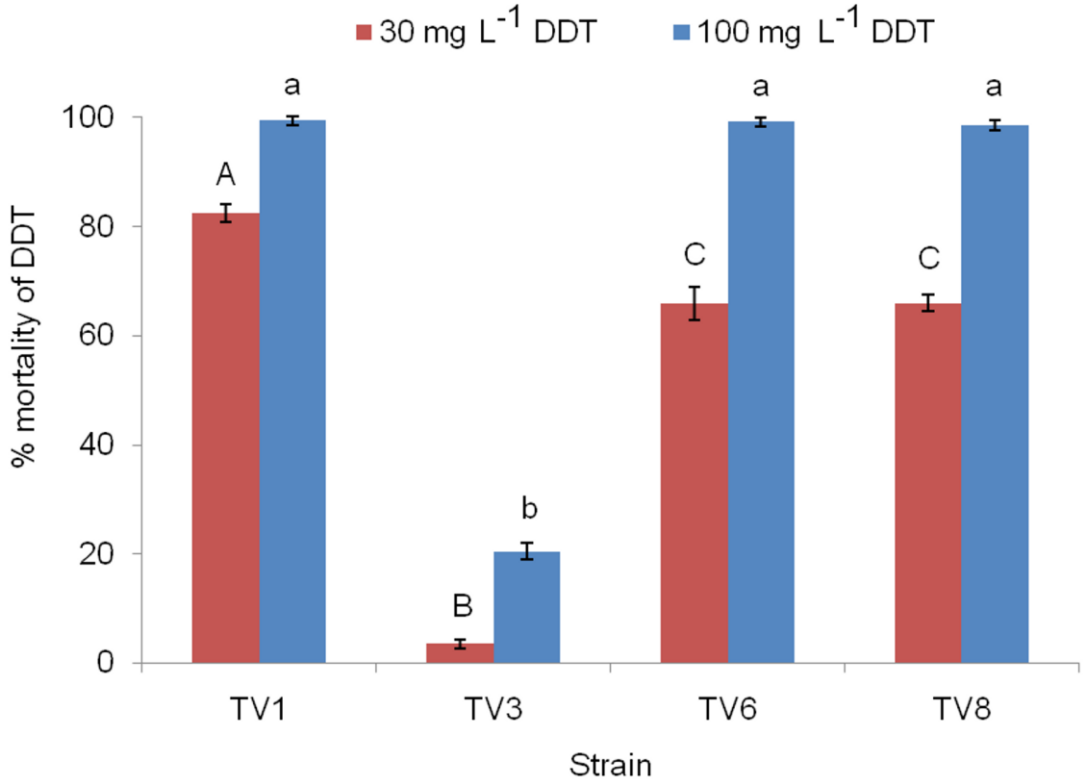

Figure 4.1 Effect (percentage mortality) of 30 and 100 mg L^{-1} doses of DDT against adults of *Trialeurodes vaporariorum* for the strains TV1, TV3, TV6 and TV8. Different letters (upper case for 30 mg L^{-1} and lower case for 100 mg L^{-1}) indicate significant differences between strains, based on a two sample unpaired t-test.

4.3.2 Sodium channel gene sequencing

Direct sequencing of a fragment (1,189 bp) that includes domain II S4-6 of the sodium channel (Additional file 4.2), revealed three non-synonymous SNPs potentially associated with *kdr*-type pyrethroid resistance. These caused the following amino acid substitutions, methionine 918 to leucine (M918L), leucine 925 to isoleucine (L925I) and threonine 929 to isoleucine (T929I) (Table 4.3; Figure 4.2; numbering according to the housefly sodium channel (Williamson *et al.*, 1996), Genbank accession X96668). Mutations at these same positions have been implicated in pyrethroid resistance for several other closely related hemipteran species, including the tobacco whitefly *B. tabaci* (mutations M918V, L925I and T929V) (Morin *et al.*, 2002; Alon *et al.*, 2006; Roditakis *et al.*, 2006), the peach potato aphid, *Myzus*

persicae Sulzer (Hemiptera: Aphididae) (M918T, L1014F) (Martinez-Torres *et al*., 1999; Eleftherianos *et al*., 2008) and the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) (M918L) (Yang & Williamson, 2001). Interestingly, in the two whitefly species *T. vaporariorum* and *B. tabaci*, none of the identified mutations in domain IIS4-6 were accompanied by the L1014F mutation, as has been found in *M. persicae* and other pest species (Davies *et al*., 2007; Soderlund, 2008). However, recent studies evaluating the functional properties of individual sodium channel mutations by their *in vitro* expression in *Xenopus* oocytes (Usherwood *et al*., 2007) have shown that mutations at positions M918, L925 and T929 alone are all able to significantly reduce the pyrethroid sensitivity of the channel and give rise to the 'super-*kdr*' phenotypes shown by TV3, TV6 and TV8 (all > 250 fold resistance to bifenthrin).

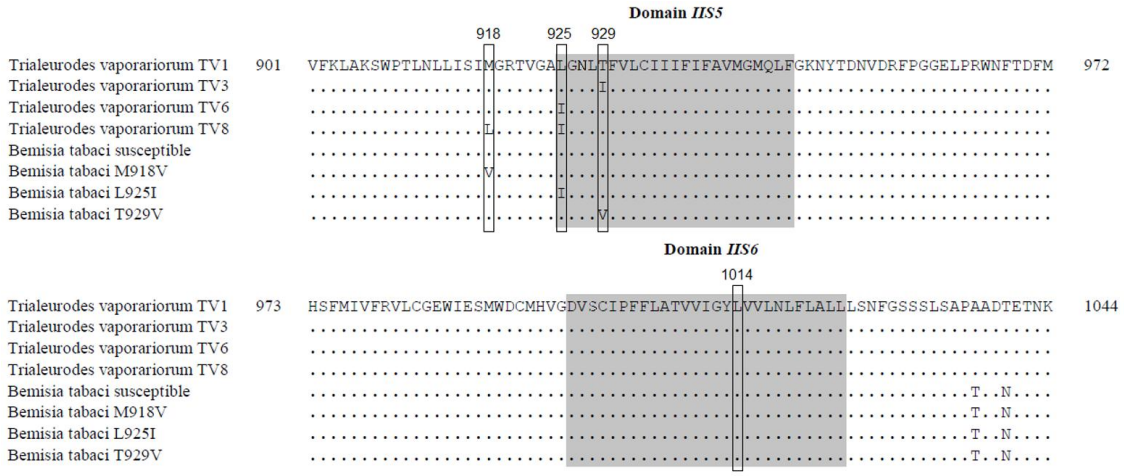

Figure 4.2 Amino-acid alignment of the *para*-type sodium channel of *Trialeurodes vaporariorum* with that of the related species *Bemisia tabaci* (Morin *et al*., 2002; Alon *et al*., 2006; Roditakis *et al*., 2006). Dots represent identical residues to TV1. Domain II S5 and domain II S6 are indicated by grey shading. The positions of mutation sites associated with pyrethroid resistance in *Bemisia tabaci* (M918V, L925I and T929V), as well as the position of the L1014F mutation found in other insect pests but not in whiteflies are boxed.

Direct sequencing of mass homogenates of approximately 100 adults of each strain was used to investigate the distribution and relative frequencies of the three mutations between strains. As expected, for the susceptible strain, TV1, there was no evidence of any of the mutations within the sequencing trace chromatograms. In the case of TV3 only the T929I mutation was identified, while the more resistant strain TV6 possessed only the L925I mutation. Direct sequencing of the TV8 strain, with the highest levels of bifenthrin resistance, revealed that both the M918L and L925I substitutions were present. The sequencing traces indicated that both TV3 and TV6 appeared to be homozygous for the T929I and the L925I mutations respectively, while TV8 was heterozygous for the L925I and M918L mutations. Subsequent sequencing of 20 individual TV8 adult males (haploid) revealed that all males had either M918L (8 of 20) or L925I (12 of 20), but that these mutations never occurred together within the same sodium channel allele.

The strain TV3, which carried only the T929I mutation (Table 4.3), was the most resistant strain to DDT (Figure 4.1). This result suggests that the T929I mutation present in TV3 has a more dramatic effect on DDT binding, while the M918L and L925I mutations (present in the less resistant TV6 and TV8 strains) have less impact on DDT binding. This is consistent with modelling predictions for the pyrethroid/DDT binding site, which suggest that DDT overlays the acid group of the pyrethroid molecule and occupies the upper part of the binding cavity, away from the M918 and L925 residues (O'Reilly *et al*., 2006), and also experimental evidence from the oocyte expression assays that confirm channels carrying resistance mutations from the lower part of the cavity (M918T, L925I) do indeed retain high sensitivity to DDT (Usherwood *et al*., 2007).

Previous studies have highlighted similarities in the phenotypic characteristics of insecticide resistance between *T. vaporariorum* and *B. tabaci* (Nauen *et al*., 2008; Gorman *et al*., 2010; Karatolos *et al*., 2010). The fact that both of these whitefly species have similar/identical amino-acid substitutions at the same positions of the sodium channel provides further support that these residues are indeed important for pyrethroid/DDT binding and resistance.

4.4 CONCLUSION

In this study, significant reductions in bifenthrin sensitivity were found in European strains of *T. vaporariorum* confirming the presence of pyrethroid resistance. Direct sequencing of these strains revealed three amino-acid substitutions in the domain II S4-S5 and S5 regions of the *para* sodium channel gene. These mutations (M918L, L925I and T929I) were located at the same positions as those of pyrethroid resistant *B. tabaci*, indicating strong parallels in the phenotypic expression and underlying mechanisms of pyrethroid resistance in these closely-related species.

4.5 ADDITIONAL FILES

Additional file 4.1 Partial voltage-gated sodium channel nucleotide sequences identified in the *Trialeurodes vaporariorum* transcriptome (Karatolos *et al*., 2011a) (Additional file 4.1.txt)

Additional file 4.2 Partial nucleotide sequence of domain IIS4-6 of the *para*-type voltage-gated sodium channel of *Trialeurodes vaporariorum* (Additional file 4.2.txt)

Chapter 5

Resistance to spiromesifen in *Trialeurodes vaporariorum* is associated with a single amino acid replacement in its target enzyme acetyl-CoA carboxylase

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Abstract

BACKGROUND: Spiromesifen is a novel insecticide and is classed as a tetronic acid derivative. It targets the insects' acetyl-CoA carboxylase (ACCase) enzyme, causing a reduction in lipid biosynthesis. At the time of this publication, there are no reports of resistance to this class of insecticides in insects although resistance has been observed in several mite species. The greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) is a serious pest of protected vegetable and ornamental crops in temperate regions of the world and spiromesifen is widely used in its control. **RESULTS:** Mortality rates of UK and European populations of *T. vaporariorum* to spiromesifen were calculated and up to 26-fold resistance was found. We therefore sought to examine the molecular mechanism underlying spiromesifen resistance in this important pest. Pre-treatment with piperonyl butoxide did not synergise spiromesifen, suggesting a target-site resistance mechanism. The full length ACCase

gene was sequenced for a range of *T. vaporariorum* strains and a strong association was found between spiromesifen resistance and a glutamic acid substitution with lysine in position 645 (E645K) of this gene. A TaqMan allelic discrimination assay confirmed these findings.

CONCLUSION: Although this resistance is not considered sufficient to compromise the field performance of spiromesifen, this association of E645K with resistance is the first report of a potential target site mechanism affecting an ACCase inhibitor in an arthropod species.

5.1 INTRODUCTION

The spirocyclic tetronic and tetramic acid derivatives (spirodiclofen, spiromesifen and spirotetramat) are a new class of effective insecticides and acaricides (Nauen *et al*., 2003; Nauen, 2005; Nauen *et al*., 2005; Brück *et al*., 2009). Spirodiclofen is one of the most widely used agents for controlling mites, while spiromesifen is also used to control whiteflies (Nauen *et al*., 2005; Kontsedalov *et al*., 2009). They act as inhibitors of acetyl-CoA carboxylase (ACCase) and cause significant reduction in total lipid biosynthesis (Nauen *et al*., 2003; Nauen, 2005; Nauen *et al*., 2005). Spirotetramat is the newest representative of the class and extends the spectrum of control to include aphids (Brück *et al*., 2009). Recently, resistance was found to affect spirodiclofen effectiveness against a laboratory selected strain of the mite *Tetranychus urticae* Koch (Acari: Tetranychidae) (van Pottelberge *et al*., 2009a; van Pottelberge *et al*., 2009b), as well as field and laboratory selected populations of *Panonychus citri* McGregor (Hu *et al*., 2010) and *Panonychus ulmi* Koch (both Acari: Tetranychidae) (Kramer & Nauen, 2011). However, there are no documented cases of resistance to this class of insecticides in any insect.

The greenhouse or glasshouse whitefly *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae), is an increasingly important pest of protected vegetable and ornamental crops in temperate regions of the world (Byrne *et al*., 1990; Brødsgaard & Albajes, 1999). All life stages other than eggs cause damage by phloem feeding or by excretion of honeydew, and large infestations can result in stunted plants and leaf necrosis (Brødsgaard & Albajes, 1999). *T. vaporariorum* also transmits numerous plant viruses belonging to the Closteroviridae family (Jones, 2003; Khurana & Garg, 2004). It is the target of many insecticides and has developed resistance to numerous chemical classes including pyrethroids (Wardlow, 1985) and neonicotinoid insecticides (Bi & Toscano, 2007; Gorman *et al*., 2007; Karatolos *et al*., 2010). As spiromesifen is now extensively used against *T. vaporariorum* we wanted to examine if this species can also evolve resistance to this novel insecticide.

ACCase, the target of spiromesifen, is a key enzyme for the metabolism of fatty acid and it catalyses the production of malonyl-CoA from acetyl-CoA and CO_2, a reaction which requires the hydrolysis of ATP (Cronan & Waldrop, 2002). Interestingly, ACCase is also the target of two chemical classes of herbicides (aryloxyphenoxypropionates and cyclohexanediones) and target site resistance mutations have already been reported in several weed species (Délye *et al*., 2002a; Délye *et al*., 2002b). Here, the effect of spiromesifen was evaluated on field populations of *T. vaporariorum* and moderate resistance was found in some populations. Sequencing technology was used to search for point mutations in the ACCase gene that are associated with spiromesifen resistance.

5.2 MATERIALS AND METHODS

5.2.1 Insect strains

Four strains of *T. vaporariorum* including a laboratory susceptible reference strain were used in this study (Table 5.1). These encompassed three countries of origin and both edible and ornamental plant hosts. All strains were reared at Rothamsted Research without exposure to insecticides on French bean plants, *Phaseolus vulgaris* L., cv. "Canadian Wonder" (Fabaceae), under a 16 h photoperiod at 24 °C.

Table 5.1 *Trialeurodes vaporariorum* strains, origins, year of collection and original host

Strain	Country of origin	Year of collection	Original host
TV1	UK	1971	French bean
TV3	UK	2008	Ornamentals
TV6	Turkey	2006	Vegetables
TV8	Germany	2008	Ornamentals

5.2.2 Insecticides and bioassays

Spiromesifen (Oberon®, 24 % SC, Bayer CropScience) was used as a commercially-available formulation. Spiromesifen was diluted to the required concentrations in distilled water containing 0.1 g L^{-1} of the non-ionic wetter Agral® (Syngenta). Spirotetramat was received as a technical grade solution (Bayer CropScience). A stock solution in acetone of a 1,000 mg L^{-1} spirotetramat was further diluted to the required concentration in 10 % acetone solution in distilled water containing 0.1 g L^{-1} of the non-ionic wetter Agral®. Technical piperonyl butoxide (PBO) (PCP 'Ultra') was provided by Dr. Graham Moores (Rothamsted Research).

Since spiromesifen is particularly active against juvenile stages of whiteflies (Nauen *et al.*, 2003), responses of second instar nymphs were quantified using leaf-dip bioassays. Nymphs were tested by trimming leaves on intact bean plants into

rectangles of approximately 40 mm x 50 mm. The trimmed plants were placed in cages with at least 200 adult whiteflies for 24 h to obtain a synchronised cohort of eggs, following which the adults were removed. Leaves were dipped after 11 days (when the majority of immature whiteflies had reached second instar) for 15 seconds in the required concentration of insecticide or into 0.1 g L^{-1} Agral® for controls. Bioassays were maintained at 24 °C and mortality was scored after 22-25 days after counting dead immature stages and pupae from which adults had emerged. Concentration-mortality relationships were fitted by probit analysis, using the software GenStat 12th edition (VSN International Ltd, Hertfordshire, UK). Resistance factors were calculated by dividing LC_{50} values for field-collected strains by those for the susceptible standard (TV1). Lack of overlap of 95 % confidence limits on fitted LC_{50} values denoted significant differences between responses of strains.

Egg hatch suppression after application of a diagnostic concentration of 1000 mg L^{-1} spiromesifen on the strains TV1 and TV6 (pre- and post-selection) was tested using a modified leaf dip method whereby 40 mm x 50 mm rectangular trimmed leaves infested with one day old whitefly eggs (100-300 eggs) were dipped for 15 seconds in 1000 mg L^{-1} spiromesifen or into 0.1 g L^{-1} Agral® for controls. Egg hatch suppression was scored after 11 days. A two sample unpaired t-test was used to investigate the difference in the responses (percentage egg hatch suppression) between the strains, using GenStat 12th edition (VSN International Ltd, Hertfordshire, UK).

5.2.3 Selection experiments and PBO synergism

Insecticide selections were done using the nymphal leaf dip method for the field strains TV3, TV6 and TV8. Initially a concentration of 10 mg L^{-1} spiromesifen was used for two successive generations, followed by a single selection with 15 mg L^{-1},

resulting in the final selected strains TV3SpSel, TV6SpSel and TV8SpSel. The mortality of the selected and unselected strains was investigated using a diagnostic concentration of 10 mg L^{-1} spiromesifen and spirotetramat. For synergism bioassays, second instar nymphs from the selected strain TV6SpSel and the unselected TV6 as a control, were initially dosed with 0.1 % PBO solution in acetone followed 5 h later by a diagnostic concentration of 10 mg L^{-1} of spiromesifen. A two sample unpaired t-test was used to investigate the difference in the responses (percentage mortality) between the strains, using GenStat 12th edition.

5.2.4 RNA extraction and cDNA reverse transcription

Total RNA was extracted from fresh samples of the four populations (TV1, TV3, TV6 and TV8) as well from the spiromesifen selected populations. Approximately 100 adult whiteflies from each population were flash frozen and total RNA was extracted using the Isolate RNA Mini Kit (Bioline) according to the manufacturer's protocol. First strand cDNA was synthesised from 1000 ng total RNA by reverse transcriptase (Superscript II®, Invitrogen) using the manufacturer's protocol. cDNA was stored at -20 °C for further use.

5.2.5 RT-PCR and 5' RACE-PCR of acetyl-CoA carboxylase

Recently, the transcriptome of *T. vaporariorum* was published and four partial sequences of the target of spiromesifen ACCase were identified (Karatolos *et al*., 2011a). These sequences were used to design primers (Additional file 5.1) for PCR amplification and sequencing of the full length ACCase gene. PCR reactions (25 µl) contained 1 µl cDNA, 12.5 µl DreamTaq® Green DNA Polymerase (Fermentas), 15 pmol of each primer and RNase free water. The cycling conditions were 95 °C for 2

min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for the appropriate time, depending on the size of the template (Additional file 5.1). A final extension of 72 °C for 5 min was included at the end of the cycles. RACE PCR was used to amplify the 5' end of ACCase. The FirstChoice® RLM-RACE Kit (Ambion) was used according to the manufacturer's protocol. The PCR and RACE PCR fragments were purified using the Wizard® SV Gel and PCR Clean-up System (Promega) according to the manufacturer's protocol and sent to Eurofins MWG (Germany) for direct sequencing.

5.2.6 DNA extractions and TaqMan® allelic discrimination assay

Direct sequencing revealed a single nucleotide polymorphism (SNP) with a potential role in spiromesifen resistance. This was a guanine (G) to adenosine (A) change that causes a glutamic acid to lysine substitution. This target SNP was used to design a TaqMan® SNP genotyping assay. Genomic DNA (gDNA) for SNP genotyping was extracted from individual adult whiteflies homogenised in 100 µl of DNAZOL® (Invitrogen) at the tenth scale of the manufacturer's protocol. The DNA was resuspended in 20 µl of RNase free water. In order to identify potential introns on the gDNA sequence of ACCase, a fragment that included the identified mutation was PCR amplified with the primers mut-f''' and mut-r''' (Additional file 5.1) using gDNA extracted from the strain TV1 as a template. This PCR fragment (1,255 bp) was purified and sent to Eurofins MWG for direct sequencing. Two introns of 665 and 286 bp were identified in this fragment (Additional file 5.2) and this genomic sequence was used to design primers and probes of the TaqMan® SNP genotyping assay.

Two primers and two fluorescent dye-labelled probes were designed and manufactured by Applied Biosystems (Foster City, CA, USA). The forward primer ACC-mut_F (CATAGCTGATCGTAAAGTAACTGATGCT) and the reverse primer ACC-mut_R (AAACTGATGAGAAATGACAGAAAAATTATAAAATTCAAAA) were unmodified PCR primers. The probe ACC-mut_V (CCTCTCTTGAAAGGTG) was labelled with the reporter dye VIC at the 5' end for detection of individuals with the G allele, and the probe ACC-mut_M (CCTCTCTTAAAAGGTG) was labelled with 6-FAM at the 5' end specific for individuals with the A allele. Both probes contained a non-fluorescent quencher dye (NFQ) and minor groove binding (MGB) groups at the 3' ends. The NFQ dye suppresses VIC or 6-FAM fluorescence until the probe is broken down during PCR, while the MGB groups increase the stability of matched duplexes, thereby resulting in a more accurate allelic discrimination.

PCR reactions (20 µl) contained 2 µl gDNA, 10 µl SensiMix DNA Kit (Quantace Ltd, Neutral Bay, Australia), 900 nM of each primer and 200 nM of each probe, and the total volume (20 µl) was made up with RNase free water. Real-time PCR was performed on a Rotor-Gene 6000® (Corbett Research). The cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. The increase in VIC and 6-FAM reporter dyes, representing individuals with the G and A allele respectively, was monitored in real time using the Rotor-Gene software. For each assay, fluorescence values of the negative controls were averaged and subtracted from the raw data to correct for background fluorescence. The endpoint values of fluorescence for each dye were then plotted against each other in bivariate scatter plots that gave a clear clustering of the samples and enabled easy scoring of individuals with the G or A alleles. 10 haploid males and 10 diploid females were

randomly chosen to investigate the frequency of the mutated (nucleotide A) allele for each field or selected population.

5.3 RESULTS AND DISCUSSION

5.3.1 Responses of nymphs to spiromesifen and selection experiments

The LC_{50} value for spiromesifen for the susceptible strain TV1 was 0.61 mg L^{-1}. The susceptibilities of three field strains were significantly lower compared to TV1, based upon overlapping 95 % CL of LC_{50} values. The highest resistance factor recorded was 26, for TV8 (a German strain), followed by 6.87 for TV3 (from the UK) and 4.49 for TV6 (from Turkey) (Table 5.2). This is the first documented case of spiromesifen resistance in whiteflies. However, all the LC_{50} values reported here considerably lower than the field application rate of spiromesifen, which is approximately 144 mg L^{-1}.

Table 5.2 Responses of *Trialeurodes vaporariorum* nymphs to spiromesifen. Resistance factors (RFs) relative to TV1 are given for all the field strains.

Strain	LC_{50} (mg L^{-1})* (95% CL)	Slope	RF
TV1	0.61 (0.48-0.76) a	1.76 (± 0.15)	1
TV3	4.20 (3.62-4.82) b	1.63 (± 0.79)	6.87
TV6	2.75 (2.48-3.04) c	1.68 (± 0.06)	4.49
TV8	15.7 (14.4-17.2) d	2.40 (± 0.11)	25.7

*Different letters indicate significant difference between strains, based on overlapping 95% CL of LC_{50} values.

Bioassays showed that a concentration of 10 mg L^{-1} spiromesifen usually causes 100 % mortality of susceptible TV1 insects (Figure 5.1). This concentration was used to select all three field strains for two successive generations, followed by one generation of selection with 15 mg L^{-1}. Selection of nymphs with spiromesifen

significantly increased survival of TV3 (t = 11.74, df = 8, P < 0.001) and TV6 (t = 10.43, df = 8, P < 0.001) at a diagnostic concentration of 10 mg L^{-1} (Figure 5.1). However, selection had no effect on the response of TV8 (t = 0.34, df = 8, P = 0.743) (Figure 5.1). These results suggest that TV8 was largely homozygous for spiromesifen resistance whereas TV3 and TV6 were heterozygous prior to selection. Selection of nymphs with spiromesifen also increased resistance of TV3 (t = 12.31, df = 6, P < 0.001) and TV6 (t = 9.10, df = 6, P < 0.001) to spirotetramat (Figure 5.1), supporting a hypothesis of cross-resistance between these two compounds.

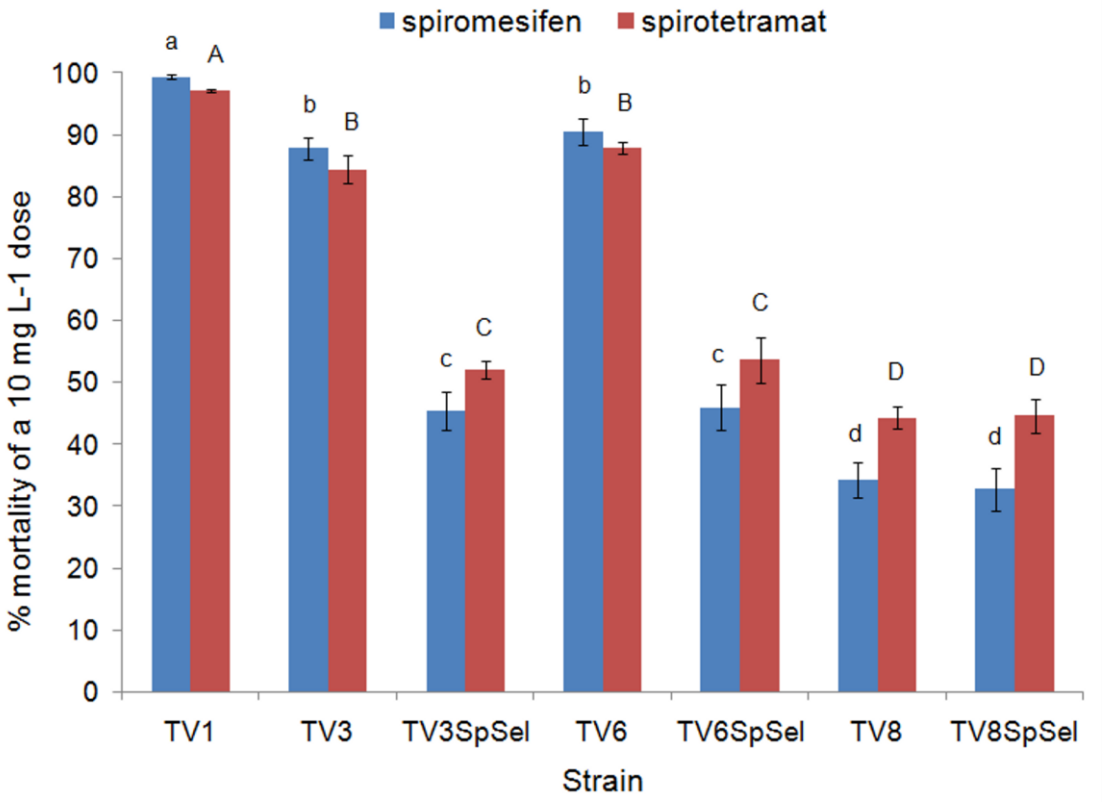

Figure 5.1 Percentage mortality of a 10 mg L^{-1} dose of spiromesifen (n = 5) or spirotetramat (n = 4) to all the field strains of *Trialeurodes vaporariorum* pre- and post-selections with spiromesifen. Different letters (lower-case for spiromesifen and upper-case for spirotetramat) indicate significant differences in the responses between the strains, based upon a two sample unpaired t-test.

Resistance to spirodiclofen has been reported in several mite species (van Pottelberge *et al*., 2009a; van Pottelberge *et al*., 2009b; Hu *et al*., 2010; Kramer & Nauen, 2011), with evidence for a role of cytochrome P450 monooxygenases in spirodiclofen detoxification (van Pottelberge *et al*., 2009b; Kramer & Nauen, 2011). Resistance was less potent in mite eggs than nymphs, and such age-specificity provides further evidence for a detoxification resistance mechanism (van Pottelberge *et al*., 2009a; van Pottelberge *et al*., 2009b; Hu *et al*., 2010; Kramer & Nauen, 2011). However, in *T. vaporariorum* the egg stage of strain TV6 was significantly less susceptible after spiromesifen selection, compared to the unselected control ($t = 3.08$, $df = 6$, $P = 0.02$) (Figure 5.2). Furthermore, pre-treatment of this TV6 with the detoxification enzyme inhibitor piperonyl butoxide (PBO) did not synergise the effect of spiromesifen (Table 5.3), with only a slight but not significant decrease in mortality in both the pre- and post-selection strains ($t = 0.60$, $df = 8$, $P = 0.564$ and $t = 0.95$, $df = 8$, $P = 0.368$ respectively). Lack of synergism by PBO indicates that cytochrome P450s and carboxylesterases are unlikely to be involved in spiromesifen resistance, providing indirect evidence of a target site mechanism.

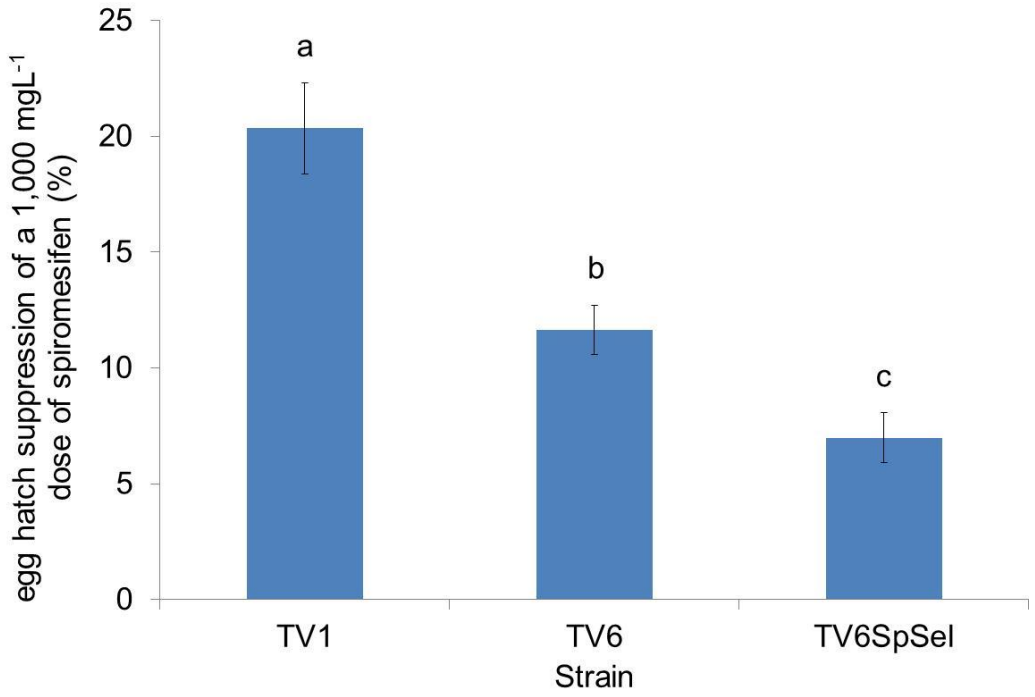

Figure 5.2 Percentage egg hatch suppression of a 1,000 mg L^{-1} dose of spiromesifen to the susceptible strain TV1 of *Trialeurodes vaporariorum* and to TV6 pre- and post-selections with spiromesifen. Different letters indicate significant differences in the responses between the strains, based upon a two sample unpaired t-test.

Table 5.3 Percentage mortality of a 10 mg L^{-1} dose of spiromesifen before and after pre-treatment with PBO to the *Trialeurodes vaporariorum* strain TV6, pre- and post-selection.

Insecticide	Strain	% mortality of 10 mg L^{-1} (± SE)[a]
Spiromesifen	TV6	92.1 (± 2.22) a
Spiromesifen + PBO	TV6	90.2 (± 2.17) a
Spiromesifen	TV6SpSel	46.0 (± 3.67) b
Spiromesifen + PBO	TV6SpSel	41.6 (± 2.73) b

[a]Same letters indicate no significant differences in the responses of the strains before and after pre-treatment with PBO, based upon a two sample unpaired t-test.

5.3.2 Acetyl-CoA carboxylase sequencing

Direct sequencing and RACE PCR generated the full length coding sequence of the wild-type ACCase for *T. vaporariorum* (Additional file 5.3). The mRNA includes a 5' UTR of 65 bp and a 3' UTR of 711 bp. The cDNA contains a 7,035 bp open reading frame encoding 2,345 amino acid residues, with a calculated molecular mass of 264.886 kDa and a predicted isoelectric point of 5.97 (CLC Main Workbench 6.1). The amino-acid sequence of ACCase from *T. vaporariorum* showed 79 %, 78 % and 78 % identity with the ACCase sequences from the pea aphid, *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae), the red flour beetle, *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) and the body louse, *Pediculus humanus corporis* Linnaeus (Phthiraptera: Pediculidae), respectively. The organisation of the *T. vaporariorum* ACCase protein was the same as those of other known eukaryotic-type ACCases (Wakil *et al*., 1983; Cronan & Waldrop, 2002). The three most conserved domains among the insect ACCases, the biotin-carboxylase (BC) domain, the biotin-carboxyl carrier (BCC) domain and the carboxyl-transferase (CT) domain (Wakil *et al*., 1983; Cronan & Waldrop, 2002), were located at amino-acid positions 111 to 609, 743 to 807 and 1660 to 2209 respectively (Additional file 5.4).

ACCase is a key enzyme in lipid biosynthesis and catalyses the production of malonyl-CoA from acetyl-CoA and CO_2. This carboxylation of the acetyl-CoA is a two-step reaction. The first reaction is an ATP-dependend carboxylation of a biotin group (carboxybiotin), catalysed by the BC domain and the second reaction is the transfer of this activated carboxyl group to acetyl-CoA, catalysed by the CT domain (Zhang *et al*., 2004; Arabolaza *et al*., 2010).

5.3.3 Point mutation within *Trialeurodes vaporariorum* ACCase

ACCase is also the target of aryloxyphenoxypropionates (FOP) and cyclohexanediones (DIM) herbicides. Resistance to FOP and DIM herbicides in green foxtail, *Setaria viridis* (L.) Beauv. (Cyperales: Poaceae), and black-grass, *Alopecurus myosuroides* Huds (Cyperales: Poaceae) is caused by a point mutation at the first nucleotide of an isoleucine codon that causes an isoleucine-leucine substitution within the chloroplastic ACCase CT domain (Délye *et al*., 2002a; Délye *et al*., 2002b), which is the site of action of these herbicides (Zhang *et al*., 2004). Since the chemical structure of these herbicides is close to that of spiromesifen and its relatives, it seems justified to assume that all these chemicals bind to the same site (Bretschneider *et al*., 2005).

An initial investigation of the CT domain sequence of the *T. vaporariorum* ACCase did not reveal any single nucleotide polymorphism (SNP) associated with spiromesifen resistance. Full length sequencing revealed that ACCase was extremely conserved between *T. vaporariorum* strains and only one non-synonymous SNP was identified with a potential association to spiromesifen resistance. This was a guanine (G) to adenosine (A) change that causes a substitution of the negative charged glutamic acid (E) to the positive charged lysine (K) in amino-acid position 645 (E645K) of ACCase. The E645K substitution is very drastic as it increases the logarithmic constant pKa value of the amino acid side chain from 4.3 to 10.5. Figure 5.3 shows an amino-acid alignment of a fragment that surrounds the E645K position of the sequenced strains of *T. vaporariorum*, including ACCase sequences of other insect and mite species. This mutation was found between the BC and BCC domains of the ACCase and amino-acid E in position 645 of the gene appears to be highly conserved among insect and mite species (Figure 5.3). Although this mutation is in a

different domain than the CT domain (the most likely binding site for spiromesifen) it is possible that spiromesifen binding is modulated by allosteric interactions with other regions of the gene as shown for human ACCase beta type (Madauss *et al*., 2009).

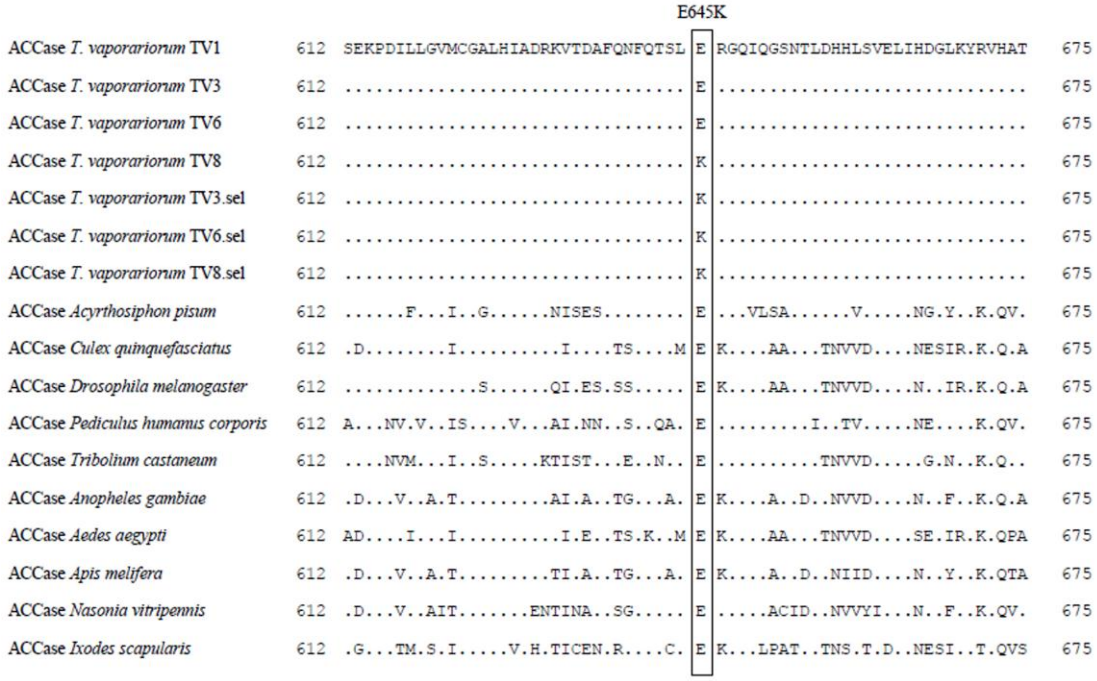

Figure 5.3 Amino-acid alignment of a partial sequence of ACCase, surrounding the E645K SNP position in field and spiromesifen selected strains of *Trialeurodes vaporariorum*, including partial ACCase sequences of *Acyrthosiphon pisum* (XP_003245354), the southern house mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae) (XP_001847001), the common fruit fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (NP_724636), *Tribolium castaneum* (XP_969851), *Pediculus humanus corporis* (XP_002429216), the malaria mosquito *Anopheles gambiae* Giles (Diptera: Culicidae) (XP_001688518), the yellow fever mosquito *Aedes aegypti* Linnaeus (Diptera: Culicidae) (XP_001651879), the western honey bee *Apis melifera* Linnaeus (Hymenoptera: Apidae) (XP_003250600), the jewel wasp *Nasonia vitripennis* Ashmead (Hymenoptera: Pteromalidae) (XP_001606974), and the deer tick *Ixodes scapularis* Say (Acari: Ixodidae) (XP_002408386). The dots denote amino-acid identity. The position of the E645K mutation is clearly marked in the figure. Note that the residue in this position represents the consensus for each strain after a sequencing of a mass homogenate of whiteflies.

The E645K mutation introduces the protein kinase C (PKC) phosphorylation site (SLK motif) of serine in position 643. In the past it was suggested that reversible phosphorylation of ACCase by PKC plays a role in concert with allosteric activation by citrate to regulate activity of the enzyme in vitro (Vaartjes *et al*., 1987; Kim *et al*., 1989). The introduced phosphorylation site in position 643 of E645K mutants of *T. vaporariorum* could play an important role in spiromesifen resistance, as the phosphorylated enzyme could be less sensitive to spiromesifen inhibition.

Sequencing traces did not reveal any peak corresponding to the mutant allele in the susceptible strain, TV1. In the field strains TV3 and TV6, there was a peak related to the mutation E645K (the A nucleotide instead of the wild-type G), but the consensus sequences were not different from the TV1 ACCase sequence. However, these strains after selection with spiromesifen showed an increased proportion of the E645K mutation (Figure 5.3). Direct sequencing of the most resistant strain TV8 revealed a high proportion of the E645K substitution. Its presence at lower frequencies in TV3 and TV6 is consistent with the change in response of these strains observed after selection with spiromesifen.

5.3.4 TaqMan allelic discrimination assay for the E645K mutation in ACCase

The identified sequences were used to develop an ACCase assay to diagnose the E645K mutation. Initially, 10 haploid males and 10 diploid females of TV1 and all the field strains (TV3, TV6 and TV8) were tested individually for the frequency of the SNP (A allele instead of G) causing the amino acid substitution. The frequency of the mutant allele was 0 in the susceptible strain TV1, 0.93 for TV8 (the most resistant strain), 0.2 for TV3 and 0.17 for TV6 (Figure 5.4). There was a significant correlation

(R^2 = 0.997, P < 0.001) between LC_{50} values for spiromesifen of the four strains and the frequency of the mutant allele (Figure 5.4).

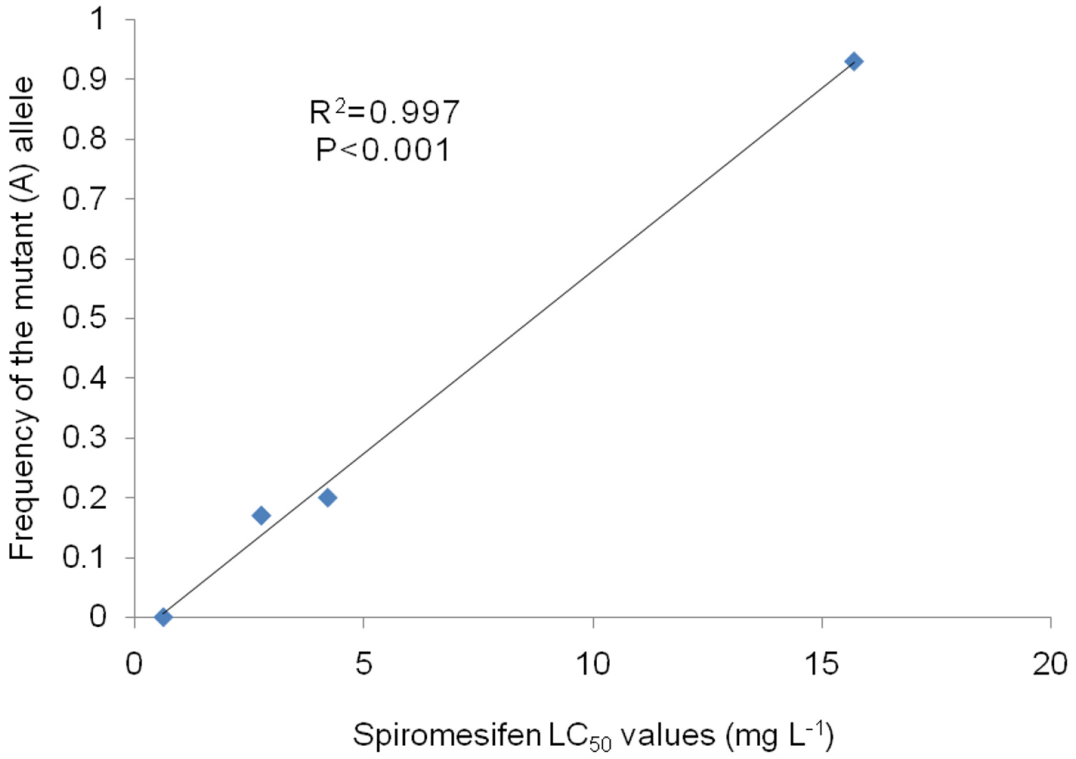

Figure 5.4 Correlation between the responses of all the strains of *Trialeurodes vaporariorum* (TV1, TV3, TV6 and TV8) to spiromesifen and the frequency of the mutant allele (nucleotide A instead of G) in position E645K of the spiromesifen's target ACCase. The frequency of the mutant allele was calculated from the TaqMan allelic discrimination assay. n = 20 for each tested strain (10 haploid males and 10 diploid females). Summary results of correlation analysis are given.

Selection of strain TV6 with 10 mg L^{-1} spiromesifen substantially increased the frequency of the mutant allele (Table 5.4). In contrast, selection of strain TV8 had little effect on the mutant frequency. These results mirror closely the observed changes in response to spiromesifen, further strengthening an association between the mutation and the resistance phenotype.

Table 5.4 Percentage survival (± SE) after application of a diagnostic concentration of 10 mg L^{-1} of spiromesifen and frequency of the mutant allele in position E645K of the strains TV6 and TV8 pre- and post-selection.

Strain	% survival of a dose of 10 mg L^{-1} spiromesifen (± SE)[a]	Mutation frequency[b]
TV6 unselected	9.57 (± 2.18) a	0.17
TV6 spiromesifen selected	54.04 (± 3.67) b	0.73
TV8 unselected	65.72 (± 2.87) c	0.93
TV8 spiromesifen selected	67.26 (± 3.51) c	0.97

[a]Different letters indicate significant differences between the responses of the strains based on a two sample unpaired t-test; [b]The frequency of the mutation was calculated from the TaqMan allelic discrimination test of a population of 10 haploid males and 10 diploid females for each tested strain.

Unfortunately, ACCase activity measurement in small sucking insect pests, such as whiteflies and aphids, is extremely difficult. This, along with functional expression for the ACCase, would help to characterise the ACCase enzyme of *T. vaporariorum* in order to confirm that the E645K mutation is the direct cause of spiromesifen resistance. Based on our findings, it will be important to investigate this region of the ACCase for potential point mutations in other arthropod species that develop resistance to tetronic or tetramic acid derivatives.

5.4 ADDITIONAL FILES

Additional file 5.1. Sequences of primers used in this study. Primer sequences are listed along with the purpose for which they were designed (Additional file 5.1.xls)

Additional file 5.2. Partial genomic sequence of the ACCase gene including two introns around the position of the E645K mutation (Additional file 5.2.txt)

Additional file 5.3. Full length nucleotide and amino acid sequences of the wild-type ACCase gene of *Trialeurodes vaporariorum* (Additional file 5.3.txt)

Additional file 5.4. Conserved domains (identified on the NCBI conserved domains database: http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) of the ACCase of *Trialeurodes vaporariorum* including the position of the E645K mutation (Additional file 5.4.tif)

Chapter 6

Over-expression of a cytochrome P450 is associated with resistance to pyriproxyfen in the greenhouse whitefly *Trialeurodes vaporariorum*

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Abstract

BACKGROUND: The juvenile hormone mimic, pyriproxyfen is a suppressor of insect embryogenesis and development, and is effective at controlling pests such as the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) which are resistant to other chemical classes of insecticides. Although there are reports of insects evolving resistance to pyriproxyfen, the underlying resistance mechanism(s) are poorly understood.

RESULTS: Bioassays against eggs of a German (TV8) population of *T. vaporariorum* revealed a moderate level (21-fold) of resistance to pyriproxyfen. This is the first time that pyriproxyfen resistance has been confirmed in this species. Sequential selection of TV8 rapidly generated a strain (TV8pyrsel) displaying a much higher resistance ratio (>4000-fold). The enzyme inhibitor piperonyl butoxide (PBO) suppressed this increased resistance, indicating that it was primarily mediated via metabolic detoxification. Microarray analysis identified a number of significantly over-expressed genes in TV8pyrsel as candidates for a role in resistance including cytochrome-P450 dependent monooxygenases (P450s). Quantitative PCR highlighted

a single P450 gene (*CYP4G61*) that was highly over-expressed (81.7-fold) in TV8pyrsel.

CONCLUSION: Over-expression of a single cytochrome P450 gene (*CYP4G61*) has emerged as a strong candidate for causing the enhanced resistance phenotype. Further work is needed to confirm the role of the encoded P450 enzyme CYP4G61 in detoxifying pyriproxyfen.

6.1 INTRODUCTION

Insecticide resistance in crop pests usually arises via one of two types of mechanisms: either reduced binding of the insecticide to its target through mutation of the target site (e.g. acetylcholinesterase for organophosphates/carbamates, the voltage-gated sodium channel for pyrethroids and the nicotinic acetylcholine receptor for neonicotinoid insecticides) (Pittendrigh *et al*., 2008), or increased detoxification or sequestration of insecticides (Ranson *et al*., 2002; Pittendrigh *et al*., 2008) by enzymes such as carboxylesterases (CEs) (Oakeshott *et al*., 2005), glutathione-S-transferases (GSTs) (Ranson & Hemingway, 2005) and cytochrome P450-dependent monooxygenases (Feyereisen, 2005).

Pyriproxyfen (2-[1-methyl-2-(4-phenoxyphenoxy)-ethoxy] pyridine) is a juvenile hormone analogue (JHA) effective against some arthropod pests including the greenhouse whitefly *Trialeurodes vaporariorum* Westwood and the sweet potato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). Pyriproxyfen is a potent suppressor of embryogenesis and later development that competes for juvenile hormone receptor binding sites and regulates the transition from one developmental stage to another (Ishaaya & Horowitz, 1992; Ishaaya *et al*., 1995; Ishaaya & Horowitz, 1995). The mode of action of pyriproxyfen is not fully understood due to

the lack of a known signalling pathway and/or a receptor molecule. However, the *Methoprene-tolerant* gene (*Met*) (also known as *Resistance to juvenile hormone*) has been proposed as a possible candidate for the juvenile hormone (JH) receptor as it has been shown to confer resistance to toxic doses of JH when mutated (Shemshedini & Wilson, 1990; Dubrovsky, 2005).

Resistance to pyriproxyfen was first documented in *B. tabaci* from Israel in 1998 (Denholm *et al*., 1998; Horowitz *et al*., 1999) and early studies suggested that P450s were not involved in the catabolism of pyriproxyfen (Devine *et al*., 1999). However, more recent biochemical work on laboratory selected strains from Arizona indicated that P450s and GSTs were involved in pyriproxyfen detoxification (Ma *et al*., 2010). To date, resistance to this compound has not been described in *T. vaporariorum,* an important virus vector and pest of protected vegetable and ornamental crops in temperate regions of the world (Byrne *et al*., 1990; Jones, 2003) that has developed resistance to numerous other chemical classes including pyrethroids and neonicotinoids (Wardlow, 1985; Bi & Toscano, 2007; Gorman *et al*., 2007; Karatolos *et al*., 2010).

The aim of the present study was to investigate potential mechanisms of pyriproxyfen resistance in a laboratory selected strain of *T. vaporariorum* exhibiting over 4000-fold resistance to pyriproxyfen. 454-based pyrosequencing has recently been used to provide a substantial expressed sequence tag (EST) data-set containing over 50,000 sequence contigs for *T. vaporariorum* (Karatolos *et al*., 2011a). We have used this as a reference transcriptome for cDNA microarray design and then to identify candidate genes that are associated with the resistance phenotype.

6.2 MATERIALS AND METHODS

6.2.1 Insect strains

Three strains of *T. vaporariorum* including an insecticide susceptible reference strain (TV1) were used in this study (Table 6.1). All were reared at Rothamsted Research without exposure to insecticides on French bean plants, *Phaseolus vulgaris* L., cv. "Canadian Wonder" (Fabaceae), under a 16 h photoperiod at 24 ºC.

Table 6.1 *Trialeurodes vaporariorum* strains, origins, year of collection and original host.

Strain	Country of origin	Year of collection	Original host
TV1	UK	1971	French bean
TV3	UK	2008	Ornamentals
TV8	Germany	2008	Ornamentals

6.2.2 Insecticides and bioassays

Pyriproxyfen 0.5 G was obtained as a commercial formulation (Sumilarv®; Sumitomo Chemical Corporation) and diluted to the required concentrations in distilled water containing 0.1 g L^{-1} of the non-ionic wetter Agral® (Syngenta). Technical piperonyl butoxide (PBO) (PCP 'Ultra') was provided by Dr. Graham Moores (Rothamsted Research).

The responses of strains TV1 (susceptible standard strain), TV3 and TV8 to pyriproxyfen were determined using a leaf-dip bioassay method modified to measure egg-hatch suppression. Leaves on intact bean plants were cut into rectangles of approximately 40 mm x 50 mm. These plants were placed in cages with at least 200 adult whiteflies for 24 h to obtain a synchronised cohort of eggs, after which the adults were removed. Egg infested leaves were dipped for 15 seconds in the required concentration of insecticide or into 0.1 g L^{-1} Agral® as a control. Treated plants were

maintained at 24 °C and mortality was scored after 11 days by counting un-hatched eggs and live nymphs. Concentration-mortality relationships were fitted by probit analysis, using the software GenStat 12th edition (VSN International Ltd, Hertfordshire, UK). Resistance factors were calculated by dividing LC_{50} values for field strains by that for the susceptible standard (TV1). Lack of overlap of 95 % confidence limits on fitted LC_{50} values denoted significant differences in response. For the synergism bioassays, whitefly eggs were initially dipped into a 0.1 % PBO solution in acetone followed 5 h later by insecticide as described above.

Insects of TV8 were selected for resistance by treating eggs for three successive generations with 3 mg L^{-1}, 5 mg L^{-1}, and 10 mg L^{-1} pyriproxyfen, respectively, to generate a selected strain denoted TV8PyrSel. In order to investigate for patterns of cross-resistance, the pyriproxyfen selected and the unselected parental strain were tested with diagnostic doses of the neonicotinoid imidacloprid 200 g L^{-1} SL (Confidor®; Bayer CropScience), the pyrethroid bifenthrin 100 g L^{-1} EC (Gyro®, CERTIS) and the tetronic acid derivative spiromesifen 240 g L^{-1} SC (Oberon®; Bayer CropScience).

6.2.3 Microarray design

A SurePrint G3 (8 x 60k) expression array was designed using Agilent's eArray platform. The base composition and the best probe methodologies were selected to design sense orientation 60-mer probes with a 3′ bias. The recently published *T. vaporariorum* EST assembly (54,751 contigs) (Karatolos *et al*., 2011a) was used as the reference transcriptome. 60-mer probes were designed for all 54,751 assembled contigs, including contigs encoding detoxification enzymes (P450s, GSTs and CEs). Additional probe groups for 15 plant genes of *Phaseolus vulgaris* Linnaeus (Fabales:

Fabaceae) for negative controls and a default set of Agilent controls were also included. The array was filled to capacity using alternate 60-mer probes for a selection of the *T. vaporariorum* contigs that returned a blast result in the nr database (Karatolos *et al*., 2011a). The final slide layout consists of 8 arrays of 62,976 elements. This array design can be made available (and ordered) by third parties on request through a shared work space set up on eArray. Additional file 6.1 provides information about probes and corresponding contigs, as well as a description of the top BLAST hit in the NCBI nr database for each contig (note that only descriptions of contigs with a BLAST result are shown in this file).

This microarray was used to compare gene expression in the highly selected pyriproxyfen resistance strain TV8PyrSel with the susceptible standard strain TV1. Total RNA was extracted from four pools of approximately 500 whitefly eggs, using the Isolate RNA Mini Kit (Bioline) according to the manufacturer's protocol. 830 ng of each total RNA was used to generate labelled cRNA, which was hybridized to arrays and these were washed and scanned as described in Agilent's Quick Amp Labelling Protocol (Version 6.5). The microarray experiment consisted of four biological replicates and for each of these, two hybridisations were done in which the Cy3 and Cy5 labels were swapped between samples for a total of eight hybridisations between resistant and susceptible strains.

Microarrays were scanned with an Agilent G2505C US10020348 scanner, and fluorescent intensities of individual spots were obtained using the Agilent Feature Extraction software with default Agilent parameters. Data normalization, filtering, dye flipping and statistical analysis were performed using the GeneSpring GX suit. For statistical analysis, a t-test against zero using the Benjamini-Hochberg false

discovery rate (FDR) method for multiple testing corrections was used to detect significantly differentially expressed genes. Genes meeting a p value cut-off of 0.01 and showing a transcription ratio > 2 fold in either direction were considered to be differentially transcribed between the two strains. All microarray data were MIAME compliant and they were submitted to the Gene Expression Omnibus (GEO) database with accession number GSE31316.

6.2.4 Quantitative RT–PCR

Quantitative RT-PCR was used to validate microarray data by examining the expression profile of 14 genes (primarily ones encoding P450s) chosen on the basis of their likelihood as candidates for causing resistance. Primers were designed to amplify a fragment of 90–150 bp in size and are listed in Additional file 6.2. Total RNA was prepared as described before and four micrograms was used for cDNA synthesis using Superscript III and random hexamers (Invitrogen) according to the manufacturer's instructions. PCR reactions (20 µl) contained 4 µl of cDNA (10 ng), 10 µl of SensiMix SYBR Kit (Bioline), and 0.25 mM of each primer. Samples were run on a Rotor-Gene 6000 (Corbett Research) using the temperature cycling conditions of: 10 min at 95°C followed by 40 cycles of 95 °C for 15 s, 57 °C for 15 s and 72 °C for 20 s. A final melt-curve step was included post-PCR (ramping from 72 °C–95 °C by 1°C every 5 s) to confirm the absence of any non-specific amplification. The efficiency of PCR for each primer pair was assessed using a serial dilution of 100 ng to 0.01 ng of cDNA. Each qRT-PCR experiment consisted of three independent biological replicates with three technical replicates for each. Data were analysed according to the ΔΔCT method (Pfaffl, 2001), using the geometric mean of two selected housekeeping genes (*para* which encodes the voltage gated sodium channel,

and *EF1a* which encodes the elongation factor 1-alpha) for normalisation according to the strategy described previously (Vandesompele *et al*., 2002).

6.2.5 Determination of P450 gene copy number by quantitative PCR

Quantitative PCR was used to determine *CYP4G61* gene copy number as described above but using genomic DNA (from strains TV1, TV8 and TV8pyrsel) as the template. For this, DNA from individual adult haploid male whiteflies was extracted using DNAZOL® (Invitrogen) at one tenth scale of the manufacturer's protocol and using RNase A to remove contaminating RNA. The DNA was then diluted to 2.5 ng/µl and 4 µl used in RT-PCR as detailed above. Data were analysed according to the ΔΔCT method (Pfaffl, 2001) and normalised independently using two housekeeping genes, *para* (present in a single copy in insects as revealed by several genome sequencing projects (Karunker *et al*., 2008)), and elongation factor 1-alpha (present in two copies in Hymenoptera and Diptera but in a single copy in most other insects (Danforth & Ji, 1998)).

6.2.6 Amplification of full length cDNA from *CYP4G61*

To verify the assembly, the full length coding sequence of *CYP4G61* was amplified by nested PCR using primers cyp4g61-f1 and cyp4g61-r1, followed by cyp4g61-f2 and cyp4g61-r2. Sequencing was performed using primers cyp4g61-f2, cyp4g61-f3, cyp4g61-f4, cyp4g61-f5 and cyp4g61-r2, cyp4g61-r3, cyp4g61-r4, cyp4g61-r5 (Additional file 6.2). PCR reactions (20 µl) contained 4 µl of cDNA (10 ng), 12.5 µl DreamTaq® Green DNA Polymerase (Fermentas), 15 pmol of each primer, and RNase free water. The cycling conditions were 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 4 min with a final extension of

72 °C for 5 min. PCR fragments were purified using the Wizard® SV Gel and PCR Clean-up System (Promega) according to the manufacturer's protocol and sent to Eurofins MWG (Germany) for direct sequencing.

6.2.7 Sequence analysis

Molecular mass and isoelectric point were predicted by Compute pI/Mw tool (http://us.expasy.org/tools/pi_tool.html). The N-terminal transmembrane anchor of the CYP4G61 protein was predicted by the TNHMM Server v.1.0 (http://www.cbs.dtu.dk/services/TMHMM/). DNA and predicted protein sequences were assembled, analysed, and aligned using the Vector NTI Advance 10 package (Invitrogen). The full length sequence of the *CYP4G61* gene in this study was identified and manually curated in the recent 454-based transcriptome study of *T. vaporariorum* and it was named by David Nelson (Department of Molecular Science, University of Tennessee, Memphis) in accordance with the P450 nomenclature committee convention (Nelson, 2009; Karatolos *et al*., 2011a). Substrate recognition sites (SRS) were predicted by aligning the CYP4G61 protein with other P450 proteins where SRS positions were known (Gotoh, 1992).

6.3 RESULTS AND DISCUSSION

6.3.1 Bioassays

The UK strain TV3 and the German strain TV8 showed 5- and 21-fold resistance to pyriproxyfen, respectively (Table 6.2). Selection of TV8 with pyriproxyfen increased resistance to 4,574-fold compared to TV1 and 223-fold compared to the unselected TV8. These findings provide the first confirmation of pyriproxyfen resistance in *T. vaporariorum*. The response of TV8pyrsel after only three generations of selection

demonstrated a very potent resistance to this insecticide. Interestingly, pyriproxyfen is not registered for use in Germany. The moderate resistance found in TV8 could be due to either cross-resistance between pyriproxyfen and a different class of insecticides or transfer of whitefly infested plant materials from regions were pyriproxyfen is used for whitefly control. However, selection of TV8 with pyriproxyfen did not result in enhanced resistance to other compounds belonging to major insecticides classes used for whitefly control, such as neonicotinoids, tetronic acid derivatives and pyrethroids (Figure 6.1). This leads to the conclusion that the resistance identified in this strain is due to European or global plant trade.

Table 6.2 Responses of *Trialeurodes vaporariorum* eggs to pyriproxyfen and synergism effect of pyriproxyfen after pre-treatment with PBO to the susceptible TV1 and the pyriproxyfen selected strain TV8pyrsel. Resistance factors (RFs) relative to TV1 are given for all the field strains.

Insecticide	Strain	LC_{50} (mg L^{-1}) (95% CL)*	Slope	RF
pyriproxyfen	TV1	0.014 (0.012-0.016) a	2.05 (±0.11)	1
pyriproxyfen + PBO	TV1	0.010 (0.009-0.011) a	2.15 (±0.10)	1
pyriproxyfen	TV3	0.05 (0.04-0.06) b	1.21 (±0.06)	5
pyriproxyfen	TV8	0.29 (0.26-0.31) c	1.89 (±0.08)	21
pyriproxyfen	TV8PyrSel	63.9 (58.3-70.2) d	2.96 (±0.13)	4574
pyriproxyfen + PBO	TV8PyrSel	0.22 (0.18-0.27) c	1.94 (±0.11)	21

*Different letters indicate significant difference between strains, based on overlapping 95% CL of LC_{50} values.

Pre-treatment of TV8pyrsel with the enzyme inhibitor piperonyl butoxide (PBO) reduced resistance to the level found in the pre-selected strain (TV8). There was no equivalent synergism of pyriproxyfen by PBO in the susceptible strain TV1 (Table 6.2). This result provided strong evidence that pyriproxyfen resistance in TV8pyrsel is primarily due to enhanced detoxification by either cytochrome P450

monooxygenases or CEs (both enzyme families are inhibited by PBO). This hypothesis was investigated further using microarrays and quantitative PCR.

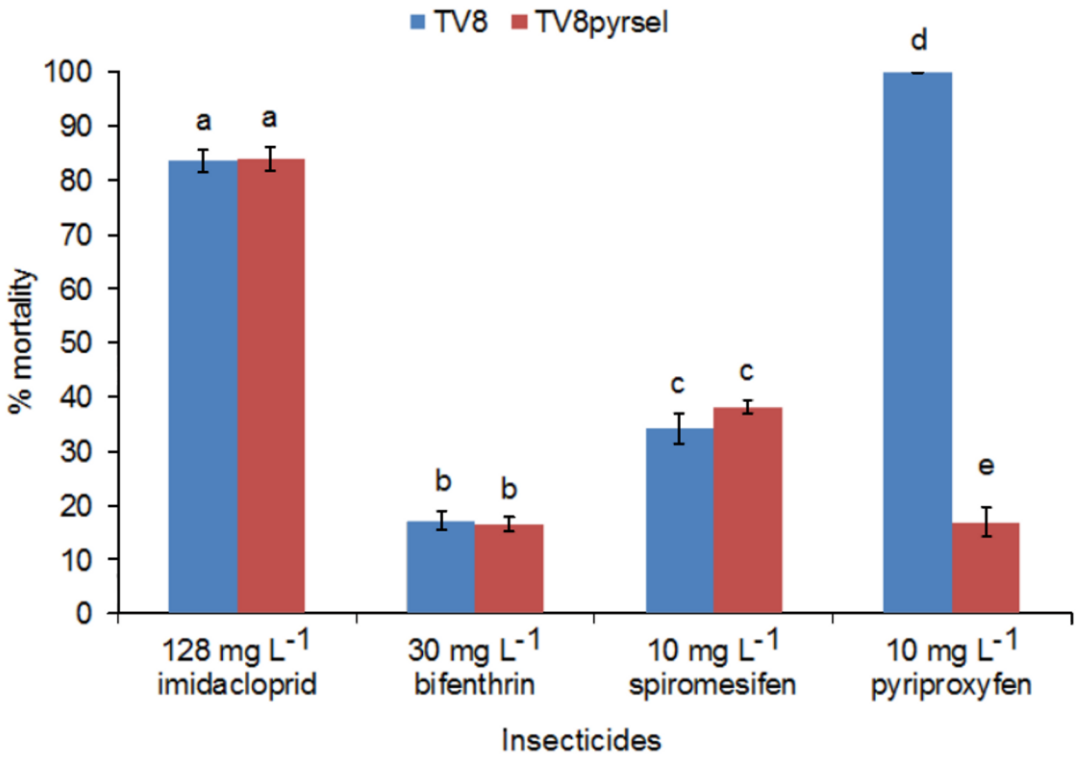

Figure 6.1 Percentage mortality of diagnostic doses of imidacloprid (neonicotinoid), bifenthrin (pyrethroid), spiromesifen (tetronic acid derivative) and pyriproxyfen in the selected strain TV8pyrsel and the unselected parental strain TV8. Different letters indicate significant differences between strains based on a two-sample unpaired t-test.

6.3.2 Microarray and quantitative real-time PCR analyses

Microarray analysis identified 3,474 probes (5.5 % of the probes which corresponded to 3,227 unique contigs) as significantly differentially transcribed between the pyriproxyfen selected strain TV8pyrsel and the susceptible standard TV1 (Additional file 6.3). These genes along with Log2, calculated fold-change values and closest BLAST hits are listed in Additional file 6.4. 1,865 probes (1,032 corresponding to genes with unknown function) had elevated expression in TV8pyrsel and 1,609 (1,105 of unknown function) were down-regulated relative to TV1. Of the 833 over-

expressed probes with a known function, 25 were identified as potential candidates for causing insecticide resistance (Table 6.3). These included probes corresponding to genes encoding cytochrome P450s (19), CEs (3), GSTs (3), enzymes that have been implicated in insecticide resistance in many arthropod species (Feyereisen, 2005; Oakeshott *et al.*, 2005; Ranson & Hemingway, 2005).

Table 6.3 Selected metabolic genes identified by microarray as differentially transcribed between the pyriproxyfen resistant *Trialeurodes vaporariorum* strain TV8pyrsel and the susceptible TV1. The family/gene names of known genes, as well as accession of the top blast hits are given (Karatolos *et al*., 2011a).

Contig number	Family/gene name [1]	Probe Name	Fold change	Log 2	Hit accession
8639	P450 *CYP6DT4*	CUST_4671_PI425265390	7.53	2.91	ACT68012
31414	P450 *CYP6DS1*	CUST_4667_PI425265390	6.58	2.72	ACT68012
5194	P450 *CYP6CM4*	CUST_4669_PI425265390	6.40	2.68	ACD84797
4648	P450 *CYP4G61*	CUST_1785_PI425308678	6.00	2.59	XP_001944205
21292	P450 *CYP4G61*	CUST_7415_PI425265390	5.59	2.48	XP_001944205
13018	P450 *CYP18A1*	CUST_4064_PI425308678	5.36	2.42	XP_002427451
13018	P450 *CYP18A1*	CUST_6265_PI425265390	5.29	2.40	XP_002427451
41451	P450 *CYP6DT6*	CUST_50026_PI425265390	5.16	2.37	ACT68012
41451	P450 *CYP6DT6*	CUST_3539_PI425308678	5.04	2.33	ACT68012
16136	P450 *CYP314A1*	CUST_5821_PI425308678	5.05	2.34	XP_001948607
16136	P450 *CYP314A1*	CUST_10486_PI425265390	4.98	2.32	XP_001948607
5866	P450 *CYP4G60*	CUST_7412_PI425265390	4.25	2.09	XP_001944205
9000	P450 *CYP6DT7*	CUST_49998_PI425265390	3.91	1.97	CAZ65617
45973	P450 *CYP6DT5*	CUST_4692_PI425265390	3.17	1.67	ACT68012
50476	P450 *CYP6DP2*	CUST_50025_PI425265390	2.53	1.34	CAH65682
41100	P450 *CYP6DP2*	CUST_4690_PI425265390	2.48	1.31	CAH65682
50476	P450 *CYP6DP2*	CUST_3538_PI425308678	2.44	1.29	CAH65682
4672	P450 *CYP353C1*	CUST_2054_PI425308678	2.11	1.08	EFA01331
4672	P450 *CYP353C1*	CUST_49588_PI425265390	2.08	1.06	EFA01331
5401	CE clade A	CUST_51868_PI425265390	3.26	1.71	ABV45410
11569	CE clade A	CUST_15720_PI425265390	2.98	1.58	XP_001663733
4777	CE clade A	CUST_51886_PI425265390	2.53	1.34	XP_392698
11236	microsomal gst	CUST_54255_PI425265390	4.05	2.02	XP_002428068
263	microsomal gst	CUST_54254_PI425265390	2.02	1.01	XP_002428068
7168	delta gst	CUST_13266_PI425265390	2.49	1.32	EFA01955
17998	CE clade A	CUST_52068_PI425265390	-2.79	-1.48	EFA06762
42539	P450 *CYP306A1*	CUST_7409_PI425265390	-2.74	-1.46	XP_001600763

[1] cytochrome P450 names were given by Dr David Nelson (Nelson, 2009)

Twelve gene sequences (Table 6.3) encoding cytochrome P450s (19 probes) were elevated in TV8pyrsel (2.08-7.53 fold). In six cases duplicate probes (generated either for the same contig or for allelic variant of the same contig) corresponding to the same P450 gene (*CYP4G61* of the CYP4 family, *CYP6DT6* and *CYP6DP2* of the CYP3, *CYP18A1* of the CYP2, and the mitochondrial P450s *CYP314A1* and *CYP353C1*) were over-expressed in TV8pyrsel (2.08-6 fold). In other insect pests, members of the CYP2, CYP3 and CYP4 microsomal P450 families are most commonly implicated in the metabolism of synthetic insecticides (Feyereisen, 2005). Microsomal P450s have been implicated in pyriproxyfen resistance in the house fly, *Musca domestica* L. (Diptera: Muscidae) (Bull & Meola, 1994; Zhang *et al*., 1998), the yellow fever mosquito, *Aedes aegypti* L. (Diptera: Culicidae) (Andrighetti *et al*., 2008) and the whitefly, *B. tabaci* (Ma *et al*., 2010). In the house fly, P450s were shown to metabolise pyriproxyfen into two major metabolites; 4'-OH-pyr and 5''-OH-pyr (Zhang *et al*., 1998). In the TV8pyrsel strain of *T. vaporariorum*, a total of seven genes belonging to the CYP3 family, two to the CYP4 family, and one to the CYP2 family were over-expressed. In *B. tabaci*, it was shown that pyriproxyfen treatment in a resistant strain induces expression of the cytochrome P450 *CYP9F2* gene (Ghanim & Kontsedalov, 2007). However, no close ortholog of this gene was over-expressed in *T. vaporariorum.*

Three sequences encoding CEs (contigs 5401, 11569 and 4777), all of them belonging to clade A (Karatolos *et al*., 2011a) were identified as being over-expressed in the resistant strain (Table 6.3). The level of expression of these sequences was moderate (2.53–3.26-fold) and the proteins these genes encode are therefore unlikely to be playing a significant role in resistance to pyriproxyfen. Three sequences encoding GSTs (contigs 7168, 11236 and 263) were elevated in the resistant strain

(Table 6.3). Of these contig 7168 belongs to the delta class, members of which have been shown to be associated with insecticide resistance. However, this sequence was elevated by only 2.49-fold in the resistant strain.

Of the 504 probes with a known function that were down-regulated in TV8pyrsel, only two detoxification genes were identified (Additional file 6.4; Table 6.3). These included one contig (42539) encoding a cytochrome P450 (*CYP306A1*) with a negative fold change of -2.74 (0.36-fold) and a single sequence encoding a CE (belonging to clade A) with a fold change of -2.79 (0.36-fold).

Real-time quantitative PCR was used to validate the microarray results by examining the expression profile of nine selected P450 genes (8 over-expressed and 1 down-regulated in the resistant strain), one CE that was found to be down-regulated in TV8pyrsel and finally two housekeeping genes (*EF1a* and *para*). For each housekeeping gene, data were normalised using the other as a reference. In all cases, the over- or under-transcription of the genes was confirmed (Table 6.4), although expression ratios obtained from RT-PCR were frequently different from those generated by microarray. Discrepancies in the data obtained from microarray experiments using the Agilent array platform and real-time quantitative RT-PCR have been described previously (Poupardin *et al*., 2008; Marcombe *et al*., 2009; Puinean *et al*., 2010) and our results again highlight the importance of RT-PCR validation of array results.

Table 6.4 Fold change in expression of selected metabolic enzymes (P450s and a carboxylesterase (CE)), *EF1a*, and *para* in the pyriproxyfen resistant *Trialeurodes vaporariorum* strain TV8pyrsel (compared to the standard susceptible strain TV1) determined by quantitative PCR and microarray technology.

Gene name	contig number	Fold change - microarray	Fold change compared to TV1 - q pcr (95 % CL)	
			TV8	TV8pyrsel
CYP6DT4	8639	7.53	1.19 (0.96-1.42)	1.20 (1.07-1.33)
CYP6DS1	31414	6.58	1.45 (1.43-1.46)	1.29 (0.98-1.59)
CYP6CM4	5194	6.40	0.99 (0.97-1.02)	1.15 (0.97-1.33)
CYP4G61	21292, 4648	5.59-6.00	1.42 (0.69-2.15)	81.7 (81.6-81.9)
CYP4G60	5866	4.25	1.24 (0.91-1.57)	1.14 (0.98-1.30)
CYP18A1	13018	5.29-5.36	1.09 (0.90-1.29)	2.54 (2.29-2.78)
CYP6DT6	41451	5.04-5.16	1.13 (0.98-1.28)	1.43 (1.32-1.55)
CYP6DT7	9000	3.91	1.59 (0.86-2.32)	1.12 (1.09-1.14)
CYP6DT5	45973	3.17	1.54 (1.13-1.95)	1.15 (0.93-1.37)
CE Tv17998	17998	0.36	0.51 (0.33-0.69)	0.75 (0.68-0.81)
CYP306A1	42539	0.36	0.31 (0.12-0.51)	0.44 (0.13-0.74)
EF1a[1]	1983	0.93-1.17	0.81 (0.53-1.08)	0.94 (0.32-1.56)
para[1]	21272, 22691, 37637	1.04-1.58	1.24 (0.98-1.50)	1.06 (0.44-1.68)

[1] For each housekeeping gene, data were normalised using the other one as a reference.

Of the candidate genes encoding detoxification enzymes examined by RT-PCR only a single P450 gene (*CYP4G61*) was found to be highly over-expressed in TV8pyrsel displaying an 81.7-fold increase in transcription. The significantly lower expression level obtained from the microarray data for this gene may be partially explained by the well-known underestimation of expression ratios by microarrays compared with RT-PCR (Yuen *et al.*, 2002). The expression of the *CYP4G61* gene in the original unselected field strain TV8 was also examined by RT-PCR (Table 6.4). The expression ratio of this gene in this strain compared to TV1 was 1.41-fold (0.66-2.16)

indicating that the enhanced expression of this gene in the highly resistant strain TV8pyrsel is a result of sequential selection with pyriproxyfen.

Members of the CYP4G cytochrome P450 subfamily have been shown to be involved in insecticide detoxification in other insect species. Examples are *CYP4G8* and *CYP4G19* which are involved in pyrethroid detoxification in the cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) (Pittendrigh *et al*., 1997) and the German cockroach, *Blattella germanica* Linnaeus (Blattodea: Blattellidae) (Pridgeon *et al*., 2003) respectively. Two other genes of this family are known to be induced after treatments with insecticides; *CYP4G36*, induced by imidacloprid in *A. aegypti* (Riaz *et al*., 2009) and *CYP4G2*, induced by permethrin in *M. domestica* (Zhu *et al*., 2008). The *CYP4G61* in *T. vaporariorum* shares 66 % amino acid identity with *CYP4G8* (AAD33077), 60 % with *CYP4G36* (EAT44585), 56 % with *CYP4G19* (AAO20251), 48 % with *CYP4G2* (ABV48808).

6.3.3 *CYP4G61* copy number

It has been recently shown that the enhanced transcription of a cytochrome P450 gene (*CYP6CY3*) in a resistant clone of peach potato aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae) is due to structural amplification of the gene (Puinean *et al*., 2010). Quantitative PCR was used to determine *CYP4G61* gene copy number using genomic DNA from individual adult male whiteflies (haploids) as template. Data were normalised using two genes; *para* (present in a single copy in insects as revealed by several genome sequencing projects (Karunker *et al*., 2008)) and *EF1a* (present in two copies in Hymenoptera and Diptera, but in a single copy in most other insect species (Danforth & Ji, 1998)). The mean cycle threshold values of three biological replicates in quantitative PCR of the *CYP4G61*, *EF1a* and *para* genes were

essentially the same in all strains (for TV1 CTs of 27.5, 27.6 and 27.7 respectively, for TV8 27.3, 27.5 and 27.7, and for TV8pyrsel 27.1, 27.5 and 27.5) indicating that haploid *T. vaporariorum* males carry a single copy of the *CYP4G61* gene. Neither the field strain TV8 or the pyriproxyfen selected TV8pyrsel showed any significant fold increase compared to TV1. TV8 showed a fold change of 1.08 (0.56-1.60) and 1.14 (0.86-1.42) compared to the TV1 using *EF1a* or *para* to normalise respectively. Similarly, TV8pyrsel showed a fold increase of 1.25 (0.35-2.14) and 1.19 (0.67-1.71) compared to the TV1. These results indicate that the increased expression of the *CYP4G61* gene likely arises through mutation of *cis*-acting promoter sequences and/or *trans*-acting regulatory loci (Li *et al*., 2007) rather than gene amplification.

6.3.4 *CYP4G61* cDNA characterization

Two allelic variant contig sequences representing the *CYP4G61* gene were identified in the InsectaCentral database (http://insectacentral.org) and manually curated (Karatolos *et al*., 2011a). These were contig 21292 (partial sequence, assembled by 28 454-reads) and contig 4648 (full length sequence, assembled by 106 454-reads). These contigs were assembled from reads from two cDNA libraries, one for the susceptible strain TV1 and the other for a neonicotinoid resistant strain. These libraries were tagged prior to sequencing using molecular markers (Karatolos *et al*., 2011a). An initial analysis of these assemblies (after reassembling them from the related ESTs) revealed the presence of 10 silent single nucleotide polymorphisms (SNPs) at nucleotide positions 126, 435, 774, 867, 966, 1146, 1329, 1356, 1620 and 1653 (Additional file 6.5). There were only two substitutions, which cause an amino acid change; one was a G/C at amino acid position 282 that causes an amino acid substitution of a glycine to an alanine (G/A) and an A/T at position 395 that causes an

amino acid substitution of a serine to cysteine (S/C) (Additional file 6.5). The complete mRNA includes a 5’ UTR of 164 bp and a 3’ UTR of 270 bp.

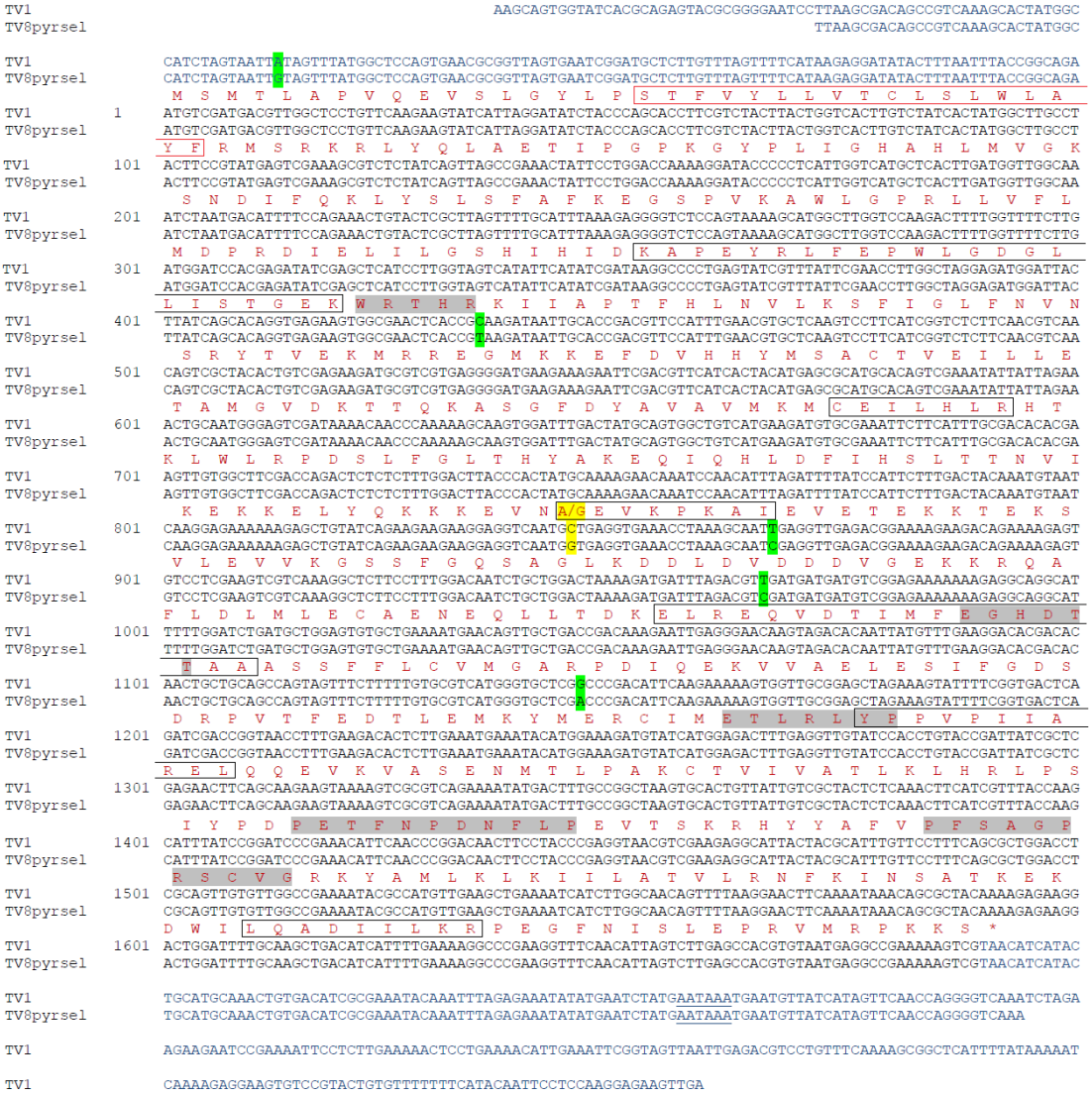

Figure 6.2 Complete cDNA sequence of the *CYP4G61* gene of *Trialeurodes vaporariorum* for the strains TV1 and TV8pyrsel. Conserved domains common to cytochrome P450s are highlighted in grey. These are the helix C motif, the helix I motif, the helix K motif, the PERF motif and the heme-binding “signature” motif. 5’ and 3’ UTR sequences are representing with light blue text. The polyadenylation signal, AATAAA is underlined. Sites of SNPs are colour marked: green for silent or UTR SNPs and yellow for amino-acid substitutions. Marked in boxes are the N-terminal transmembrane anchor (red-line box) and the SRS 1-6 (black-line box).

The cDNA contains a 1689 bp open reading frame (Figure 6.2) encoding 563 amino acid residues, with a calculated molecular mass of 64,449 kDa and a predicted isoelectric point of 8.65. The encoded protein contains conserved domains common in cytochrome P450s, such as the helix C motif (WxxxR; position 141), helix I (oxygen binding) motif ([A/G]Gx[E/D]T[T/S]; position 363), the helix K motif (ExxRxxP; position 421), the PERF motif (PxxFxP[E/D]RF; position 472) and the heme-binding "signature" motif (PFxxGxxxCxG; position 495). The polyadenylation signal AATAAA is located 73 nucleotides downstream of the 3' end coding region.

Variations in the coding sequence of *CYP4G61* in two strains of *T. vaporariorum* (TV1 and TV8pyrsel; Additional file 6.6) were investigated by either an analysis of the reassembled sequence for the strain TV1 (for which there was excellent sequence coverage) or by direct nucleotide sequencing for TV8pyrsel. In TV1, nine polymorphic sites were identified (nucleotide positions 126, 435, 774, 845, 867, 966, 1146, 1356, 1620), but only one change (C/G) at amino acid position 282 results in an amino acid substitution of an alanine to a glycine (A/G) (Figure 6.2). Only one polymorphism (C/T) for this strain appeared to occur in a conserved protein motif (helix C), but this was a synonymous substitution. For TV8pyrsel the coding sequence was much more conserved than TV1, probably as a result of selection, and only two silent polymorphisms in nucleotide positions 126 and 774 were identified. After comparing the consensus cDNA sequence of TV1 and TV8pyrsel, four synonymous SNPs at positions 145 (CGC/CGT), 289 (ATT/ATC), 322 (GTT/GTC), and 382 (CGG/CGA) and one non-synonymous SNP at position 282 (GCT/GGT) conferring an alanine to glycine (A/G) substitution were observed. Interestingly, the latter appeared to be at the beginning of the SRS 3. Finally, a single SNP (A/G) was found in the 5'UTR of the *CYP4G61* (Figure 6.2).

6.4 CONCLUSION

Based on the results of this study, *CYP4G61* emerges as the strongest candidate for further investigation into its role in conferring potent resistance to pyriproxyfen in *T. vaporariorum*. In particular, functional characterisation of this P450 to confirm its ability to detoxify pyriproxyfen is now required. We have shown that the enzyme inhibitor PBO synergises pyriproxyfen resistance and it will be interesting to examine the effect of this and other inhibitors on recombinantly-expressed *CYP4G61* as a mean to identify possible measures for overcoming resistance in the field.

6.5 ADDITIONAL FILES

Additional file 6.1 Probe IDs, corresponding contigs and closest BLAST hits in the NCBI nr database for each unique contig. (Note that only the contigs that returned a BLAST result are shown) (Additional file 6.1.xls)
http://www.editorialmanager.com/pone/download.aspx?id=1753183&guid=62692807-322d-420b-b189-f686eb237026&scheme=1

Additional file 6.2 Sequences of primers used in this study. Primer sequences are listed along with the purpose for which they were designed (Additional file 6.2.xls)
http://www.editorialmanager.com/pone/download.aspx?id=1753186&guid=2f9a24ba-3186-4efa-965d-6b6a73556c7c&scheme=1

Additional file 6.3 Volcano plot for the *Trialeurodes vaporariorum* microarray. Genes meeting a p value cut-off of 0.01 and showing a transcription ratio > 2 fold in either direction were considered to be differentially transcribed between the two strains and here are represented by dark dots (Additional file 6.3.tif)

http://www.editorialmanager.com/pone/download.aspx?id=1753132&guid=48893cbe-8339-4d1a-b135-76164daad121&scheme=1

Additional file 6.4 Genes identified by microarray analysis as significantly differentially transcribed between the pyriproxyfen resistant strain TV8pyrsel and the susceptible TV1. Here, the full list of these genes, along with probe name, p-value, fold-change and log2 fold-change, as well as a description based on the closest BLAST hit are detailed (Additional file 6.4.xls)
http://www.editorialmanager.com/pone/download.aspx?id=1753135&guid=3bc8b612-c858-456a-bd0d-cff35fba6b13&scheme=1

Additional file 6.5 Amino acid alignment of two translated contigs (4648 and 21292) that they are coding for the full length *CYP4G61* gene. Silent SNPs are marked in yellow coloured boxes and amino acid substitutions in green boxes (Additional file 6.5.tiff)
http://www.editorialmanager.com/pone/download.aspx?id=1753138&guid=ce10d539-df8e-4fac-943a-b67dcc403e58&scheme=1

Additional file 6.6 *CYP4G61* nucleotide sequences for the strains TV1 (assembled from 454 reads for this strain) and TV8pyrsel (identified by direct cDNA sequencing of this strain) (Additional file 6.6.txt)
http://www.editorialmanager.com/pone/download.aspx?id=1753140&guid=609edbde-3bde-4723-a212-8d317703737f&scheme=1

Chapter 7

Concluding discussion

7.1 NEW TECHNOLOGIES AND ADVANCES IN INSECTICIDE RESISTANCE RESEARCH

The study of the molecular mechanisms underlying insecticide resistance in insect pests has been aided greatly by recent advances in sequencing technology (Pittendrigh *et al*., 2008). The high demand for cost-effective sequencing has led to new generation, high-throughput DNA sequencing technologies that can rapidly produce large amounts of sequence information for insect species lacking a fully sequenced genome. These include technologies such as 454-based pyrosequencing of expressed sequence tags (EST) and Illumina (Solexa) sequencing that both parallelise the sequencing process and produce millions of reads at once (Schuster, 2008; Papanicolaou *et al*., 2009). These technologies were used recently to identify genes involved in insecticide resistance of several pests, such as detoxifying enzymes and insecticide target proteins (Pauchet *et al*., 2009; Pauchet *et al*., 2010; Wang *et al*., 2010).

Before this project, the lack of transcriptomic data for *Trialeurodes vaporariorum* hampered the characterisation of resistance mechanisms. However, the transcriptome generated in the present study by 454-pyrosequencing (Chapter 3; Karatolos *et al*., 2011a) not only revealed genes potentially involved in insecticide resistance (insecticide targets and detoxifying enzymes), but will also facilitate the study of several other aspects of the biology of *T. vaporariorum*. These data and the

techniques they generated aided the cost-effective characterisation of insecticide resistance to some of the major insecticides used to control this species, such as pyrethroids, tetronic acid derivatives and pyriproxyfen. Mutations in the voltage gated sodium channel, the target of pyrethroids, and the acetyl-coA carboxylase, the target of spiromesifen, were associated with insecticide resistance. Furthermore, the EST dataset generated here was used as a reference transcriptome for a cDNA microarray design to identify candidate genes associated with pyriproxyfen resistance.

7.2 PARALLEL EVOLUTION OF INSECTICIDE RESISTANCE

Trialeurodes vaporariorum and *Bemisia tabaci* are the two most important whitefly crop pests in the world. They share similar biology and life cycles, and they are both highly polyphagous (Bink-Moenen & Mound, 1990; Byrne *et al*., 1990). Their taxonomic relatedness offers the prospect of transferring information on resistance mechanisms and other biological traits between these two species. Furthermore, in warm Mediterranean areas, like Southern Spain, these two species often coexist (Arnó & Gabarra, 1994; Arnó *et al*., 2006), affecting a wide range of protected and outdoor crops. This coexistence could drive the evolution of insecticide resistance in these two species, due to exposure to the same insecticides and similar selection pressures, and raises intriguing questions about the similarity of the resulting resistance mechanisms.

Most of the recent research on mechanisms of insecticide resistance in whiteflies has focused in *B. tabaci*, including investigations of resistance to neonicotinoids (Rauch & Nauen, 2003; Karunker *et al*., 2008; Nauen *et al*., 2008; Karunker *et al*., 2009; Gorman *et al*., 2010), pyrethroids (Morin *et al*., 2002; Alon *et al*., 2006; Roditakis *et al*., 2006) and pyriproxyfen (Ghanim & Kontsedalov, 2007; Ma *et al*., 2010).

Results presented in this thesis highlight some clear parallels in the phenotypic and genetic characteristics of insecticide resistance in *T. vaporariorum* and *B. tabaci*. One example is the cross-resistance identified between neonicotinoids and pymetrozine, and their differential expression across life-stages (Nauen *et al*., 2008; Gorman *et al*., 2010; Karatolos *et al*., 2010; Chapter 2). Furthermore, mutations at the same positions of the sodium channel of the two species were found to be implicated with pyrethroid resistance (Morin *et al*., 2002; Alon *et al*., 2006; Roditakis *et al*., 2006; Karatolos *et al*., 2011b; Chapter 4). There are therefore strong parallels in the phenotypic expression and underlying mechanisms of insecticide resistance in these closely-related species.

Examples of parallel evolution of insecticide resistance are analogous mutations of insecticide targets, such as the L1014F mutation in the voltage gated sodium channel (Soderlund & Knipple, 2003) and the A302S substitution at the *Resistance to dieldrin* (*Rdl*) locus (ffrench-Constant, 1994) that have independently occurred across a range of insect species. Parallel evolution is also found in detoxifying enzymes with a primary example being DDT resistance in several *Drosophila* species (Diptera: Drosophilidae), caused by retrotransposon insertions into the *CYP6G1* gene that results in its overexpression (Schlenke & Begun, 2004). The extent of this parallelism demonstrates that the opportunities available to insects to resist insecticides without incurring severe biological costs are very limited.

7.3 ROLE OF GLOBAL TRADE AND TRANSPORT IN THE SPREAD OF RESISTANCE GENES

Mutations that confer resistance to insecticides may sometimes arise repeatedly within the same species (multiple origins of resistance alleles). Examples of

resistance genes having multiple origins in the same species are cyclodiene resistance in the red flour beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) (Andreev *et al*., 1999) and pyrethroid knock-down resistance in several insect species (Anstead *et al*., 2005; Alon *et al*., 2006; Pinto *et al*., 2007). However, insecticide resistance mutations may also appear only once (single origin) and subsequently spread through migration, global trade or transport. As already mentioned, DDT resistance in *Drosophila* species was associated with overexpression of a single P450 gene (*CYP6G1*), as shown for a range of strains collected throughout the world. The resistant *CYP6G1* alleles for these strains were highly conserved, implying a resistant allele with a single origin, which subsequently spread globally (Daborn *et al*., 2001; Daborn *et al*., 2002). In the mosquito *Culex pipiens* L. (Diptera: Culicidae), a single organophosphate resistance allele with a single origin (known as *Ester*^{2}) has a worldwide distribution. It is believed that this resistant allele has spread through passive migration on ships or airplanes (Raymond *et al*., 1991; Labbe *et al*., 2005). Similar passive spread of insecticide resistance has been identified in the aphid *Myzus persicae*, where studies of amplified esterase genes, point to a small number of isolated mutations that spread rapidly through migration, global trade or transport (Field & Devonshire, 1997; Field & Devonshire, 1998; Devonshire *et al*., 1998).

In this study, a strain from Germany showed reduced susceptibility to pyriproxyfen, although this compound is not registered for use in that country. This is most possibly caused by transfer of whitefly infested plant material from regions where pyriproxyfen is used for whitefly control. Interestingly, the same strain was found to possess the highest resistance to almost all the tested compounds (Chapter 2-6), indicating that throughout the years it has accumulated mechanisms of resistance, perhaps facilitated by being exposed to selection at different geographical localities.

These results demonstrate the importance of the expanding global trade of agricultural products, in the spread of arthropod pests and their resistant genes, as already discussed in the past (Raymond *et al*., 1991; Denholm *et al*., 2002). Thus, insecticide resistance management strategies need to take into account the trade of plant materials and be implemented on a global scale (Denholm *et al*., 2002).

7.4 STABILITY OF INSECTICIDE RESISTANCE

It is often reported or speculated that high insecticide resistance imposes a fitness cost on resistant pests, by affecting their fecundity, survival and/or behaviour (Ferrari & Georghiou, 1981; Liu & Han, 2006; Onstad & Guse, 2008) through epistatic effects of resistance mutations on other biological traits (Coustau *et al*., 2000; Hall *et al*., 2004). Such fitness costs can contribute to a decline of the frequency of resistant alleles in the absence of selection by insecticides, as demonstrated for the whitefly *B. tabaci* (Wilson *et al*., 2007) and the red spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) (Dennehy *et al*., 1990). However, there are cases were insecticide resistance carries no apparent fitness cost leading to more stable resistant phenotypes (i.e. DDT resistance in *Drosophila* (McCart *et al*., 2005) and dicofol resistance in the European red mite *Panonychus ulmi* Koch (Acari: Tetranychidae) (Dennehy *et al*., 1990)). In some cases, despite evidence for a fitness cost, resistance can be very stable in the absence of selection pressure by a specific insecticide. This can be caused by the use of other insecticides that are affected by the same mechanism and which impose similar selection pressures (ffrench-Constant *et al*., 2000; McCart *et al*., 2005).

Migration of susceptible or resistant insect pests can undoubtedly affect the stability of resistance (Denholm & Rowland, 1992). In outdoor field conditions where

susceptible individuals can regularly invade, the frequency of the resistance genes is diluted and therefore resistance is reduced. However, in the confined environment of glasshouses resistance develops faster and is much more stable (Devonshire, 1989; Denholm & Rowland, 1992).

In the past, it was suggested that without selection pressure, neonicotinoid insecticide resistance in *B. tabaci* (Nauen *et al*., 2002; Rauch & Nauen, 2003) and *T. vaporariorum* (Gorman *et al*., 2007) was unstable to some extent. In this study, neonicotinoid resistance of up to 22-fold was reported for *T. vaporariorum* (Chapter 2; Karatolos *et al*., 2010). However, this resistance has dropped very quickly in the laboratory without selection pressure and thus it was not possible to come to any conclusions on the mechanisms of neonicotinoid resistance.

7.5 FUTURE WORK

In the present work, a range of target site modifications or metabolic mechanisms were identified in insecticide resistant strains of *T. vaporariorum*. However, some require further investigation in order to functionally characterise the identified resistance mechanisms.

Chapter 2 provides evidence of cross-resistance between neonicotinoids and pymetrozine based on insecticide detoxification. Given the similarities in the phenotypic characteristics of cross-resistance in *T. vaporariorum* and *B. tabaci* (Nauen *et al*., 2008; Gorman *et al*., 2010; Karatolos *et al*., 2010; Chapter 2), there are likely to be strong parallels in the underlying mechanism(s). In *B. tabaci*, a cytochrome P450 gene (*CYP6CM1*) has been confirmed to be highly associated with neonicotinoid resistance (Karunker *et al*., 2008; Karunker *et al*., 2009). Further work

is required in order to investigate whether overexpression of a cytochrome P450 is responsible for neonicotinoid and pymetrozine resistance in *T. vaporariorum* and its similarity to *CYP6CM1*. The cDNA microarray designed in Chapter 6 could be used to identify candidate genes associated with neonicotinoid resistance in a range of field collected or laboratory selected strains of *T. vaporariorum*.

In Chapter 5, a mutation in the acetyl-coA carboxylase (ACCase) enzyme was associated with spiromesifen resistance. Functional characterisation of this enzyme in *T. vaporariorum* is now required in order to fully understand this resistant mechanism. Unfortunately, ACCase activity measurement in small sucking insect pests is extremely difficult, preventing the biochemical study of this enzyme in whiteflies (Ralf Nauen, personal communication). However, this mutation was found to introduce a protein kinase C phosphorylation site in mutant whiteflies. As ACCase is known to be regulated by phosphorylation/dephosphorylation (Vaartjes *et al*., 1987; Hardie *et al*., 1989; Kim *et al*., 1989), further work on the introduced phosphorylation site is required.

In Chapter 6, overexpression of a cytochrome P450 gene (*CYP4G61*) was found to be associated with pyriproxyfen resistance in *T. vaporariorum*. Molecular diagnostics need to be developed for screening field populations of *T. vaporariorum*, in order to investigate the consistency of this mechanism with pyriproxyfen resistance. Furthermore, functional expression of the *CYP4G61* gene could assist the characterisation of this P450, in order to confirm its ability to detoxify pyriproxyfen.

List of abbreviations

ACCase: Acetyl-CoA carboxylase, the target of tetronic and tetramic acid derivatives, as well as the target of FOP and DIM herbicides

AChE: Acetylcholinesterase, the target of organophosphate and carbamate insecticides

BC: The biotin-carboxylase domain of ACCase

BCC: The biotin-carboxyl carrier domain of ACCase

CE: Carboxylesterase

CCE: Carboxyl/cholinesterase

CT: The carboxyl/transferase domain of ACCase

DDT: Dichlorodiphenyltrichloroethane

DIM: Cyclohexanediones herbicides

EC: Enzyme Classification term

***EF1a*:** Elongation factor 1a gene

EST: Expressed Sequence Tag (a short sub-sequence of a cDNA sequence)

FOP: Aryloxyphenoxypropionate herbicides

GABA receptor: γ-aminobutyric acid receptor, the target of organochlorines and Phenylpyrazoles (Fiproles) insecticides

GEO: Gene Expression Omnibus

GluCl: Glutamate-gated chloride channel

GST: Glutathione-S transferase

GO: Gene Ontology

IGRs: Insect growth regulators

IPM: Integrated Pest Management

IRAC: Insecticide Resistance Action Committee

IRM: Insecticide Resistance Management

JH: Juvenile hormone

JHA: Juvenile hormone analogue

***kdr*; super-*kdr*:** knock-down resistance traits

LC_{50}: The dose required to kill half the members of a tested population

nAChR: Nicotinic acetylcholine receptor, the target of neonicotinoid insecticides

***Met*:** *Methoprene-tolerant* gene, also known as *Resistance to juvenile hormone*

OPs: Organophosphate insecticides

ORF: Open reading Frame; a DNA sequence that does not contain a stop codon in a given reading frame

PBO: The detoxification enzyme inhibitor piperonyl butoxide

PKC: Protein kinase C

P450: Cytochrome P450 monooxygenase

RACE PCR: Rapid Amplification of cDNA Ends

***Rdl*:** *Resistance to dieldrin* gene

RR: Resistance ratio

RS: Resistance factor

RyR: Ryanodine receptor, the target of diamides insecticides

SAGE: Serial Analysis of Gene Expression; a new generation sequencing technology

SF: Selection factor

SNP: Single nucleotide polymorphism

SRS: Substrate Recognition Site

VGSC: *para*-type voltage gated sodium channel, the target of pyrethroid insecticides, pyrethrins and DDT

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