Insect Molecular Biology

Insect Molecular Biology (2017) **26**(2), 152–163 doi: 10.1111/imb.12276

Use of the synergist piperonyl butoxide can slow the development of *alpha*-cypermethrin resistance in the whitefly *Bemisia tabaci*

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Abstract

The development of insecticide resistance in insect pests of crops is a growing threat to sustainable food production, and strategies that slow the development of resistance are therefore urgently required. The insecticide synergist piperonyl butoxide (PBO) inhibits certain insect detoxification systems and so may delay the evolution of metabolic resistance. In the current study we characterized resistance development in the silverleaf whitefly, *Bemisia tabaci*, after selection with either a neonicotinoid (thiacloprid) or pyrethroid (*alpha*-cypermethrin) insecticide alone or in combination with PBO. Resistance development was significantly suppressed (> 60%) in the line selected with *alpha*-cypermethrin + PBO compared to the line selected with *alpha*-cypermethrin alone. RNA sequencing (RNAseq) analyses revealed an increase in frequency of a knock-down resistance mutation but no differentially expressed genes were identified that could explain the sensitivity shift. No significant difference was observed in the level of resistance between the thiacloprid and thiacloprid + PBO selected lines, and RNA sequencing (RNAseq) analyses revealed that the cytochrome P450 monooxygenase CYP6CM1, known to metabolize neonicotinoids, was significantly upregulated (>10-fold) in both lines. The findings of this study demonstrate that PBO used in combination with certain insecticides can suppress the development of resistance in a laboratory setting; however, the mechanism by which PBO supresses resistance development remains unclear.

Keywords: insecticide resistance, synergism, PBO, knock-down resistance, *Bemisia tabaci*.

First published online 21 November 2016.

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Introduction

The development of resistance to synthetic insecticides represents a significant threat to the present and future control of many insect pests. As a result strategies that slow, prevent or overcome resistance are urgently required.

One strategy that has been shown to restore the efficacy of insecticides compromised by the development of certain forms of resistance is the use of insecticide synergists (Bernard & Philogène, 1993). Synergists are compounds that usually have no or low intrinsic toxicity to insects but act by inhibiting one or more of the enzyme defence systems that insects use to detoxify insecticides. As a result synergists are particularly effective against a form of resistance mediated by the enhanced production of detoxification enzymes, termed 'metabolic resistance' (Scott *et al.*, 1990; Farnham, 1999; Feyereisen, 2015). Using a combination of insecticide and synergist may also have potential in slowing the evolution of metabolic resistance by negating the fitness advantage associated with overproducing detoxification enzymes in the presence of insecticide.

The most widely used and commercially significant insecticide synergist is piperonyl butoxide (PBO), a

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Table 1. Baseline sensitivity of a field strain of *Bemisia tabaci* to *alpha*-cypermethrin, *alpha*-cypermethrin + piperonyl butoxide (PBO), thiacloprid, and thiacloprid + PBO prior to selection

Bioassay treatment	LC_{25}	95% CL	LC_{50}	95% CL	Slope ± SE
alpha-cypermethrin	1.81	(1.42–2.23)	4.37	(3.61–5.26)	1.761 ± 0.107
alpha-cypermethrin + PBO	0.564	(0.451–0.683)	1.15	(0.964–1.369)	2.179 ± 0.144
Thiacloprid	22.95	(14.61–32.7)	63.6	(45.93–86.4)	1.524 ± 0.138
Thiacloprid + PBO	14.97	(9.63–21)	43.11	(31.53–57.6)	1.467 ± 0.127

CL, confidence limits; LC_{25}, Lethal Concentration, 25%; LC_{50}, Lethal Concentration, 50%.

broad-spectrum inhibitor of insect cytochrome P450 monooxygenases (P450s) and certain insect esterases (Young *et al.*, 2006, 2005; Feyereisen, 2015). Two studies on mosquitoes have demonstrated that PBO applied in combination with the pyrethroid deltamethrin can slow the development of resistance. In the first study, selection of southern house mosquito, *Culex quinquefasciatus* Say, larvae with deltamethrin and PBO in the ratio of 1:5 resulted in 63% suppression of deltamethrin resistance after 20 generations compared to selection with deltamethrin alone (Thomas *et al.*, 1991). In a second study on the yellow fever mosquito, *Aedes aegypti* L., larval selection of a susceptible strain with deltamethrin and PBO for 20 consecutive generations suppressed the resistance levels by 60% (Kumar *et al.*, 2002).

Although these studies have shown clear suppression of deltamethrin resistance evolution in mosquitoes, no similar studies have been performed to look at the efficacy of this approach in other insect species or using other insecticides. Furthermore, although the basis of resistance suppression by PBO has been suggested to result from inhibition of P450s, the molecular mechanisms involved remain unclear.

The silverleaf whitefly, *Bemisia tabaci* Gennadius, is a globally distributed insect pest that causes damage via direct feeding on the plant phloem, the transmission of plant pathogenic viruses and the production of honeydew that supports the growth of sooty mould. The reliance on insecticides for the control of *B. tabaci* has led to widespread and multiple resistance with both target-site and metabolic mechanisms involved (Morin *et al.*, 2002; Karunker *et al.*, 2008). A well-characterized form of target-site resistance in this species is knock-down resistance (*kdr*) to pyrethroid insecticides. This is mediated by point mutations in the pyrethroid target site, the voltage-gated sodium channel (VGSC), with three amino acid substitutions, M918V, L925I and T929V, described (Morin *et al.*, 2002; Alon *et al.*, 2006). In contrast, neonicotinoid resistance in this species appears to be exclusively associated with metabolic resistance, and principally results from overexpression of the cytochrome P450 CYP6CM1 (Karunker *et al.*, 2009, 2008; Roditakis *et al.*, 2011).

In the present study we examined the ability of PBO to supress the development of resistance in *B. tabaci* to two insecticides, the pyrethroid *alpha*-cypermethrin and the neonicotinoid thiacloprid. To explore the molecular basis of resistance evolution we then examined changes in (1) global gene expression levels, and (2) the frequency of target-site mutations in insecticide selected and unselected lines.

Results

Response to selection regimes

The baseline sensitivity of a mixed *B. tabaci* field strain from Crete (see Experimental procedures for details) to *alpha*-cypermethrin and thiacloprid +/– PBO (see Table 1) was used to determine the appropriate Lethal Concentration, 25% (LC_{25}) doses for subsequent selection. The culture was then split into five lines for selection under the different treatment regimes detailed in Table 2. The selection was maintained from the third to the eighth generation, followed by final bioassays with both the insecticide alone or in combination with PBO, to detect possible changes in the resistance levels of each line.

All four insecticide +/– PBO selection regimes resulted in a significant decrease in sensitivity compared to the unselected strain of the same generation (see Table 3) with higher resistance developing to thiacloprid than *alpha*-cypermethrin regardless of whether PBO was present. The unselected strain exhibited an unchanged sensitivity to *alpha*-cypermethrin at the start and end of the experiment, but gained sensitivity (lost resistance) to thiacloprid over the same time period (Tables 1, 3).

Table 2. Details of the selection regimes used on a field culture of *Bemisia tabaci* in this study

Strain name	Selection regime	mg/l	
B-NS	Unselected		
B1	*alpha*-cypermethrin	2	
B2	*alpha*-cypermethrin + PBO	0.5	+ 100 mg/l PBO
B3	Thiacloprid	22.5	
B4	Thiacloprid + PBO	15	+ 100 mg/l PBO

B-NS, *B. tabaci* nonselected; B1, *B. tabaci alpha*-cypermethrin selected; B2, *B. tabaci alpha*-cypermethrin + piperonyl butoxide (PBO) selected; B3, *B. tabaci* thiacloprid selected; B4, *B. tabaci* thiacloprid + PBO selected.

Table 3. Lethal Concentration, 50% values and resistance ratios of the eighth generation of *Bemisia tabaci* strains after different selection scenarios

Selection	Bioassay treatment	LC_{50}	(95% CL)	Slope ± SE	Resistance ratio	Synergism ratio
Unselected (B-NS)	alpha-cyp.	3.72	(2.4–5.82)	1.244 ± 0.117	–	
	alpha-cyp. + PBO	3.44	(2–5.8)	1.181 ± 0.149	–	1.08
alpha-cypermethrin (B1)	alpha-cyp.	103.1	(75.1–141)	1.392 ± 0.112	28	
	alpha-cyp. + PBO	64.9	(48.6–86.3)	1.36 ± 0.101	19	1.59
alpha-cypermethrin + PBO (B2)	alpha-cyp.	39.8	(25.6–61.8)	1.225 ± 0.127	11	
	alpha-cyp. + PBO	24	(15.02–38.2)	1.336 ± 0.16	7	1.65
Unselected (B-NS)	Thiacloprid	8.58	(5.93–12.2)	1.234 ± 0.112	–	
	Thiacloprid + PBO	8.37	(5.84–11.9)	1.152 ± 0.092	–	1.02
Thiacloprid (B3)	Thiacloprid	1466	(1250–1706)	2.367 ± 0.162	171	
	Thiacloprid + PBO	718	(527–992)	1.866 ± 0.183	86	2.04
Thiacloprid + PBO (B4)	Thiacloprid	1060	(746–1507)	2.014 ± 0.245	124	
	Thiacloprid + PBO	1015	(810–1274)	1.872 ± 0.143	121	1.05

alpha-cyp., *alpha*-cypermethrin; PBO, piperonyl butoxide.

Interestingly, both the lines selected with thiacloprid + PBO (B4) and *alpha*-cypermethrin + PBO (B2) exhibited lower resistance factors than the lines selected with the corresponding insecticide alone treatments. In the case of the *alpha*-cypermethrin + PBO selected line (B2) the level of resistance was 61% lower than the *alpha*-cypermethrin selected line (B1) and the difference was statistically significant (ie the 95% confidence limits of the two LC_{50} values did not overlap). In the case of the thiacloprid + PBO selected line (B4) the level of resistance was 28% lower than the thiacloprid selected line (B3); however, this difference was not significant (the 95% confidence limits of the two LC_{50} values overlapped).

Transcriptome profiling

RNA sequencing was carried out on all five lines at completion of the selection experiment in order to identify changes in gene expression associated with changes in the phenotypic level of resistance. RNAseq reads were mapped against the reference genome of *B. tabaci* and a number of comparisons between replicated sample sets was made to examine the effects of insecticide selection (with and without PBO) on gene expression.

The statistically significantly differentially expressed genes identified between the unselected strain and each insecticide (+/– PBO) selected line are summarized in Fig. 1.

In the first comparison [nonselected (B-NS) vs. *alpha*-cypermethrin selected (B1)], a relatively low number (33) of differentially expressed genes (corrected *P*-value <0.05) was observed, of which 23 returned BLAST annotation (Supporting Information Table S1). Of these, just two genes were upregulated more than twofold (Table 4) and three genes were downregulated by the same margin. None of these genes encoded detoxification enzymes commonly involved in insecticide resistance such as P450s, glutathione S-transferases (GSTs) or carboxyl/choline esterases.

A greater number of genes were identified as differentially expressed in the nonselected (B-NS) vs. *alpha*-cypermethrin + PBO selected (B2) comparison (82; see Table S1), of which 65 returned a BLAST hit. However, of these only three genes were upregulated more than

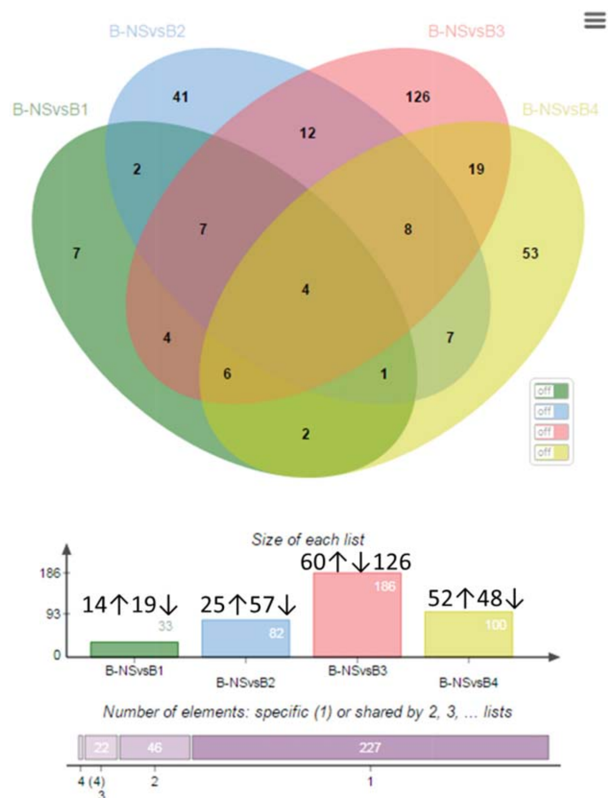

Figure 1. Top: Venn diagram showing numbers of differentially expressed genes in multiple RNA sequencing (RNAseq) comparisons of *Bemisia tabaci*. Bottom: numbers of differentially expressed genes (and direction of expression) in each treatment comparison. B-NS, *B. tabaci* nonselected; B1, *alpha*-cypermethrin selected; B2, *alpha*-cypermethrin + piperonyl butoxide (PBO) selected; B3, thiacloprid selected; B4, thiacloprid + PBO selected. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 4. Differentially expressed genes that were overexpressed more than twofold in the insecticide [+/– piperonyl butoxide (PBO)] selected strains compared to the nonselected strain

Comparison	Gene	Value B-NS	Value selected strain	Fold change	*q*-value	BLAST hit description
B-NS vs. B1	*Bta15751*	0.585	2.778	4.747	0.011	gi|500183388| cellulose biosynthesis protein [*Burkholderia pseudomallei*]
B-NS vs. B1	*Bta10848*	2.639	5.970	2.262	0.020	gi|742092993| uncharacterized protein LOC105028535 isoform X1 [*Esox lucius*]
B-NS vs. B2	*Bta09578*	1.722	0.000	nd	0.006	gi|662224075| cuticle protein 7-like, partial [*Diaphorina citri*]
B-NS vs. B2	*Bta07221*	29.361	156.393	5.327	0.006	gi|182894140| cytochrome P450 CYP6CM1vQ [*Bemisia tabaci*]
B-NS vs. B2	*Bta05068*	2.413	5.107	2.117	0.006	gi|170040533| conserved hypothetical protein [*Culex quinquefasciatus*]
B-NS vs. B3	*Bta06205*	0	1.913	nd	0.006	gi|768439871| fatty acyl-coenzyme A reductase 1-like [*Plutella xylostella*]
B-NS vs. B3	*Bta06442*	0	1.440	nd	0.006	gi|640475602| isomerase [*Pseudomonas aeruginosa*]
B-NS vs. B3	*Bta07221*	29.361	311.243	10.601	0.006	gi|182894140| cytochrome P450 CYP6CM1vQ [*Bemisia tabaci*]
B-NS vs. B3	*Bta15751*	0.585	3.773	6.447	0.006	gi|500183388| cellulose biosynthesis protein [*Burkholderia pseudomallei*]
B-NS vs. B3	*Bta05068*	2.413	8.060	3.340	0.006	gi|642916760| SCY1-like protein 2 [*Tribolium castaneum*]
B-NS vs. B3	*Bta11414*	2.031	4.260	2.098	0.044	gi|780705582| UPF0585 protein C16orf13 homolog A isoform X2 [*Wasmannia auropunctata*]
B-NS vs. B3	*Bta01982*	0.871	1.788	2.052	0.006	gi|490546258| hypothetical protein [*Vibrio orientalis*]
B-NS vs. B3	*Bta06951*	9.999	20.345	2.035	0.006	gi|47605414| endocuticle structural glycoprotein SgAbd−1 [*Schistocerca gregaria*]
B-NS vs. B4	*Bta07221*	29.361	334.569	11.395	0.006	gi|182894140| cytochrome P450 CYP6CM1vQ [*Bemisia tabaci*]
B-NS vs. B4	*Bta15751*	0.585	4.056	6.931	0.006	gi|500183388| cellulose biosynthesis protein [*Burkholderia pseudomallei*]
B-NS vs. B4	*Bta06951*	9.999	35.296	3.530	0.006	gi|47605414| endocuticle structural glycoprotein SgAbd-1 [*Schistocerca gregaria*]
B-NS vs. B4	*Bta14876*	6.058	21.302	3.516	0.006	gi|524911932| nuclear anchorage protein 1-like [*Aplysia californica*]
B-NS vs. B4	*Bta05068*	2.413	8.178	3.389	0.006	gi|642916760| SCY1-like protein 2 [*Tribolium castaneum*]
B-NS vs. B4	*Bta06952*	3.478	11.381	3.272	0.016	gi|795084641| endocuticle structural glycoprotein SgAbd-2-like [*Vollenhovia emeryi*]
B-NS vs. B4	*Bta10046*	1.106	3.415	3.088	0.006	gi|766920998| uncharacterized protein LOC105367862 [*Ceratosolen solmsi marchali*]
B-NS vs. B4	*Bta02924*	3.997	12.174	3.046	0.006	gi|749161035| hypothetical protein GNI_130520 [*Gregarina niphandrodes*]
B-NS vs. B4	*Bta07056*	4.890	13.531	2.767	0.006	gi|195109328| GI24397 [*Drosophila mojavensis*]
B-NS vs. B4	*Bta00448*	17.601	41.859	2.378	0.006	gi|391347995| uncharacterized protein LOC100900284 [*Metaseiulus occidentalis*]
B-NS vs. B4	*Bta07060*	5.306	11.636	2.193	0.006	gi|801384584| uncharacterized protein LOC105618592 [*Atta cephalotes*]
B-NS vs. B4	*Bta11106*	2.765	5.875	2.125	0.006	gi|124487754| hypothetical protein [*Maconellicoccus hirsutus*]
B-NS vs. B4	*Bta08044*	67.270	134.806	2.004	0.020	gi|190702384| transposase-like protein [*Glyptapanteles flavicoxis*]

B-NS, *B. tabaci* nonselected; B1, *B. tabaci alpha*-cypermethrin selected; B2, *B. tabaci alpha*-cypermethrin + PBO selected; B3, *B. tabaci* thiacloprid selected; B4, *B. tabaci* thiacloprid + PBO selected.

twofold (Table 4) with 17 genes downregulated by this margin. This subset of differentially expressed genes included a single detoxification gene (*Bta07221*) encoding the P450 CYP6CM1, which was overexpressed fivefold in the *alpha*-cypermethrin + PBO selected line. Additional candidate resistance genes were overexpressed, but at lower (< twofold) levels, including three genes, *Bta03784* (1.7-fold), *Bta00064* (1.5-fold), *Bta12079* (1.4-fold), encoding additional P450s, a single gene, *Bta06821* (1.4-fold), encoding an esterase with similarity to *Myzus persicae* FE4 esterase, and a gene, *Bta03180* (1.3-fold), encoding a GST (Table S1).

For the nonselected (B-NS) vs. thiacloprid selected (B3) comparison 186 genes were identified as differentially expressed, 144 of which returned annotation (Table S1). A total of eight of these was upregulated more than twofold (Table 4) with 14 annotated genes downregulated by the same margin (Table S1). Of these the gene encoding the P450 *CYP6CM1* (*Bta07221*) was the only overexpressed candidate detoxification gene and was upregulated 10-fold in the thiacloprid selected line, although a gene, *Bta06951*, encoding a cuticular protein was also overexpressed (twofold).

In the case of the nonselected (B-NS) vs. thiacloprid + PBO selected (B4) comparison 100 genes were identified as differentially expressed, 67 of which returned annotation (Table S1). Of these 13 genes were upregulated more than twofold (Table 4) and five downregulated by the same margin. A similar pattern was observed to the thiacloprid selected regime with *CYP6CM1* the primary overexpressed candidate gene (11-fold). Two other genes (*Bta06951* and *Bta06952*)

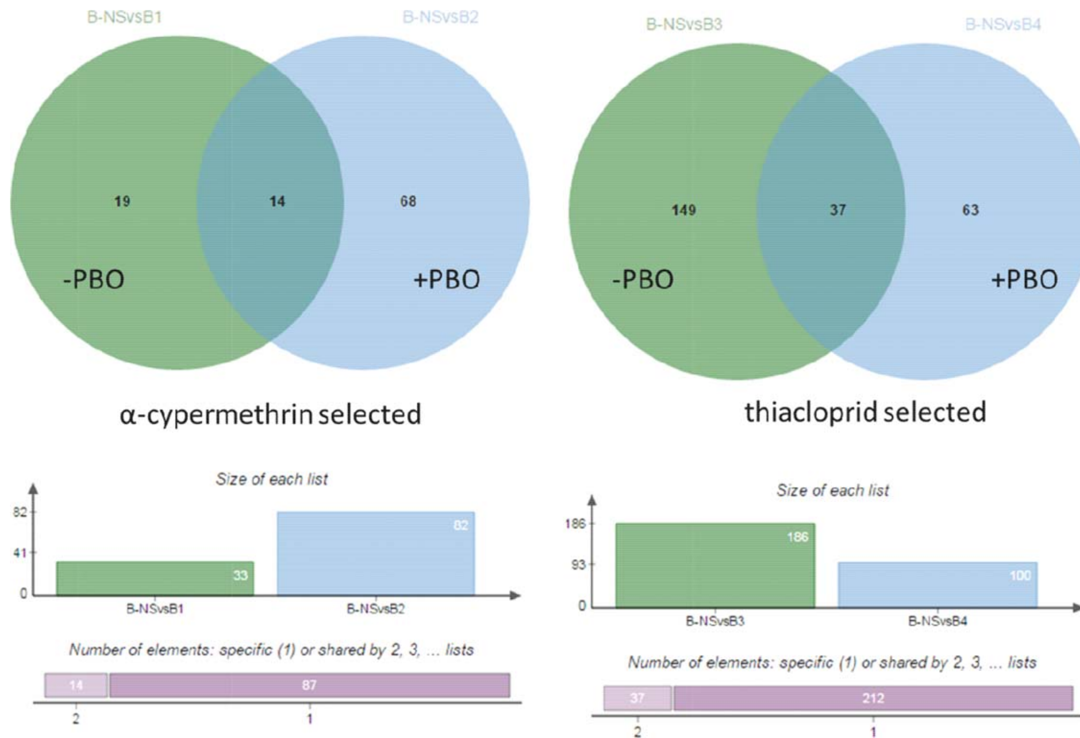

Figure 2. Venn diagram highlighting unique and common differentially expressed genes after selection with insecticide alone or insecticide + piperonyl butoxide (PBO). B-NS, *B. tabaci* nonselected; B1, *alpha*-cypermethrin selected; B2, *alpha*-cypermethrin + piperonyl butoxide (PBO) selected; B3, thiacloprid selected; B4, thiacloprid + PBO selected. [Colour figure can be viewed at wileyonlinelibrary.com]

encoding cuticular proteins were also overexpressed > twofold. Finally, two additional genes encoding a second P450 (*Bta06382*) and a GST (*Bta03181*) were overexpressed 1.4- and 1.3-fold, respectively.

Common differentially expressed genes after selection with insecticide alone or insecticide + PBO are illustrated in Fig. 2 and shown in full in Table S2. Very few common genes were identified (see Table S2) between the *alpha*-cypermethrin selected line (B1) and the *alpha*-cypermethrin + PBO selected line (B2) and few if any of these were obvious candidates to explain the resistance phenotype of the *alpha*-cypermethrin selected lines. A greater number of common genes was observed between the thiacloprid (B3) and thiacloprid + PBO (B4) selected lines (see Table S2), with *CYP6CM1* being the most common overexpressed candidate gene.

To identify genes differentially expressed between lines selected with insecticide alone and those selected with insecticide + PBO we called genes differentially expressed between the *alpha*-cypermethrin selected line (B1) and the *alpha*-cypermethrin + PBO selected line (B2), and the thiacloprid selected line (B3) and thiacloprid + PBO selected line (B4). A total of 177 genes was differentially expressed between the line treated with *alpha*-cypermethrin (B1) and the line treated with *alpha*-cypermethrin + PBO (B2), of which 141 returned a BLAST hit (Table S1). Of these, five were upregulated more than twofold in the line treated with *alpha*-cypermethrin + PBO (B2) and 11 downregulated by the same margin (Table 5). Of these, *CYP6CM1* was the only detoxification gene (overexpressed fivefold), although a single cuticular protein was downregulated 3.2-fold. Additional genes encoding P450s were overexpressed at lower levels, *Bta00064* (1.5-fold), *Bta12079* (1.5-fold), *Bta06378* (1.5-fold), *Bta14906* (1.4-fold), *Bta09536* (1.4-fold), along with three genes encoding GSTs, *Bta03180* (1.5-fold), *Bta03176* (1.4-fold) and *Bta14179* (1.3-fold), a gene encoding Uridine 5′-diphospho(UDP)-glucuronosyltransferase, *Bta13757* (1.4-fold) and an ATP-binding cassette (ABC) transporter, *Bta07767* (1.4-fold).

A total of 242 genes was identified as differentially expressed between the line selected with thiacloprid (B3) and the line selected with thiacloprid + PBO (B4) (Table S1), of which 187 returned a BLAST hit. Of these, 16 genes were upregulated in the line selected with thiacloprid + PBO (B4) > twofold and two were downregulated by the same margin (Table 6). None of these included genes encoded detoxification enzymes; however, several detoxification genes were moderately upregulated below this cut-off in the thiacloprid + PBO selected line (B4), including nine genes encoding P450s *Bta08018* (1.5-fold), *Bta06382* (1.5-fold), *Bta10847* (1.4-fold), *Bta02884*

Table 5. Genes that were differentially expressed > twofold between strains selected with *alpha*-cypermethrin alone compared to *alpha*-cypermethrin + piperonyl butoxide (PBO)

Comparison	Gene	Value B1	Value B2	Fold change	*q*-value	LAST hit description
B1 vs. B2	*Bta07221*	28.558	156.393	5.476332	0.006312	gil339896279l cytochrome P450 [*Bemisia tabaci*]
B1 vs. B2	*Bta07218*	1.36789	5.31237	3.883614	0.046972	gil158292776l AGAP005207-PA [*Anopheles gambiae str.* PEST]
B1 vs. B2	*Bta09936*	1.47055	3.57918	2.4339	0.044209	gil Krueppel-like factor 7 [*Ceratosolen solmsi marchali*]
B1 vs. B2	*Bta05068*	2.22195	5.10747	2.298655	0.006312	gil hypothetical protein YQE_06445, partial [*Dendroctonus ponderosae*]
B1 vs. B2	*Bta06985*	0.655341	1.40815	2.148739	0.006312	gil AGAP007069-PA [*Anopheles gambiae str.* PEST]
B1 vs. B2	*Bta11420*	4.65529	2.3025	0.494599	0.006312	gil662196044l cathepsin B-like cysteine proteinase 4 isoform X2 [*Diaphorina citri*]
B1 vs. B2	*Bta01459*	2.26356	1.05798	0.467397	0.006312	gil645029947l uncharacterized protein LOC103316462 [*Nasonia vitripennis*]
B1 vs. B2	*Bta02143*	26.2236	12.244	0.466908	0.006312	gil567857698l hypothetical protein CICLE_v10005342mg [*Citrus clementina*]
B1 vs. B2	*Bta14718*	26.3228	11.4882	0.436435	0.006312	gil662196046l cathepsin B-like cysteine proteinase 4 isoform X3 [*Diaphorina citri*]
B1 vs. B2	*Bta10057*	7.21768	3.01002	0.417035	0.030051	gil762091652l matrilin-2-like [*Crassostrea gigas*]
B1 vs. B2	*Bta10056*	3.35317	1.30059	0.387869	0.006312	gil761908560l uncharacterized protein LOC100635860 [*Amphimedon queenslandica*]
B1 vs. B2	*Bta10848*	5.96986	2.05184	0.3437	0.006312	gil742092993l uncharacterized protein LOC105028535 isoform X1 [*Esox lucius*]
B1 vs. B2	*Bta13784*	16.6896	5.27742	0.316211	0.006312	gil662184445l uncharacterized protein LOC103509026 [*Diaphorina citri*]
B1 vs. B2	*Bta10046*	1.55634	0.489802	0.314713	0.006312	gil769838863l uncharacterized protein LOC105422781 [*Pogonomyrmex barbatus*]
B1 vs. B2	*Bta06951*	11.8695	3.65648	0.308055	0.006312	gil751206626l endocuticle structural glycoprotein SgAbd-2-like [*Solenopsis invicta*]
B1 vs. B2	*Bta07059*	2.9297	0.887313	0.302868	0.038927	gil91091224l uncharacterized protein LOC655551 [*Tribolium castaneum*]

B1, *Bemisia tabaci alpha*-cypermethrin selected; B2, *B. tabaci alpha*-cypermethrin + PBO selected.

(1.4-fold), *Bta11922* (1.4-fold), *Bta04552* (1.3-fold), *Bta09536* (1.3-fold), *Bta06705* (1.3-fold) and *Bta01329* (1.3-fold), one encoding a GST *Bta12670* (1.9-fold), one encoding a carboxyl-esterase (CE), *Bta04439* (1.5-fold), and one encoding an ABC transporter, *Bta11838* (1.5-fold) (Table S1). No genes encoding detoxification enzymes were downregulated in this line compared to the line selected with thiacloprid (B3) alone.

Gene ontology (GO)-term enrichment analysis was conducted for all RNAseq comparisons (see Table S3), revealing a relatively limited number of over- and under-represented terms. The term most commonly significantly enriched across comparisons was GO:0016491 'oxidoreductase activity', which was significantly over-represented in the nonselected (B-NS) vs. *alpha*-cypermethrin + PBO (B2), the nonselected (B-NS) vs. thiacloprid (B3) and the thiacloprid (B3) vs. thiacloprid + PBO (B4) comparisons, and significantly under-represented in the *alpha*-cypermethrin (B1) vs. *alpha*-cypermethrin + PBO (B2) comparison.

Quantitative PCR (qPCR) was used to validate a subset of the differentially expressed genes identified by

Table 6. Genes that were differentially expressed > twofold between strains selected with thiacloprid alone compared to thiacloprid + piperonyl butoxide (PBO)

Comparison	Gene	Value B3	Value B4	Fold change	*q*-value	Blast.Hit.Description.HSP.
B3 vs. B4	*Bta00545*	3.70799	0	nd	0.036158	gil742099885l ribosomal protein S6 kinase alpha-4-like [Esox lucius]
B3 vs. B4	*Bta06707*	0.334182	1.44385	4.320535	0.049382	gil556767641l uncharacterized protein LOC102329352 [Pantholops hodgsonii]
B3 vs. B4	*Bta05340*	0.658979	2.37466	3.603553	0.006312	gil302676748l glycoside hydrolase family 13 protein [Schizophyllum commune H4-8]
B3 vs. B4	*Bta05776*	0.449326	1.45159	3.230595	0.006312	gil571574131l flocculation protein FLO11 isoform X2 [Apis mellifera]
B3 vs. B4	*Bta13784*	2.21973	7.15115	3.221628	0.019606	gil662184445l uncharacterized protein LOC103509026 [Diaphorina citri]
B3 vs. B4	*Bta02924*	4.02079	12.1738	3.027695	0.006312	gil257205780l Histidine-rich glycoprotein precursor [Schistosoma japonicum]
B3 vs. B4	*Bta05558*	2.49454	7.4322	2.979376	0.006312	gil729697196l hypothetical protein RMCBS344292_19220 [Rhizopus microsporus]
B3 vs. B4	*Bta02847*	4.88005	12.1228	2.484163	0.036158	gil225709586l Sulfotransferase 1A1 [Caligus rogercresseyi]
B3 vs. B4	*Bta08782*	0.804249	1.83004	2.275463	0.019606	gil194762118l GF14059 [Drosophila ananassae]
B3 vs. B4	*Bta14011*	4.81407	10.8393	2.251599	0.006312	gil654420047l 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline decarboxylase [Meiothermus chliarophilus]
B3 vs. B4	*Bta14666*	0.542804	1.21182	2.232515	0.049382	gil572271649l arylsulfatase B-like [Apis dorsata]
B3 vs. B4	*Bta07248*	1.57544	3.36857	2.138175	0.006312	gil662199030l uncharacterized protein LOC103509808 [Diaphorina citri]
B3 vs. B4	*Bta06637*	0.577278	1.19551	2.070946	0.046972	gil752894974l peroxidase isoform X1 [Camponotus floridanus]
B3 vs. B4	*Bta00958*	1.80491	3.66762	2.032014	0.006312	gil282165806l C-type lectin precursor [Tribolium castaneum]
B3 vs. B4	*Bta04682*	0.806266	1.6295	2.021043	0.006312	gil358339385l Golgi-associated plant pathogenesis-related protein 1 [Clonorchis sinensis]
B3 vs. B4	*Bta14876*	10.5414	21.3021	2.020805	0.006312	gil531728358l hypothetical protein QUI_2362 [Clostridium difficile P59]
B3 vs. B4	*Bta10530*	4.8498	2.12683	0.43854	0.006312	gil805804725l uncharacterized protein LOC100878566 [Megachile rotundata]
B3 vs. B4	*Bta01459*	2.34917	0.957852	0.40774	0.006312	gil645038086l uncharacterized protein LOC103318060 [Nasonia vitripennis]

B3, *Bemisia tabaci* thiacloprid selected; B4, *B. tabaci* thiacloprid + PBO selected.

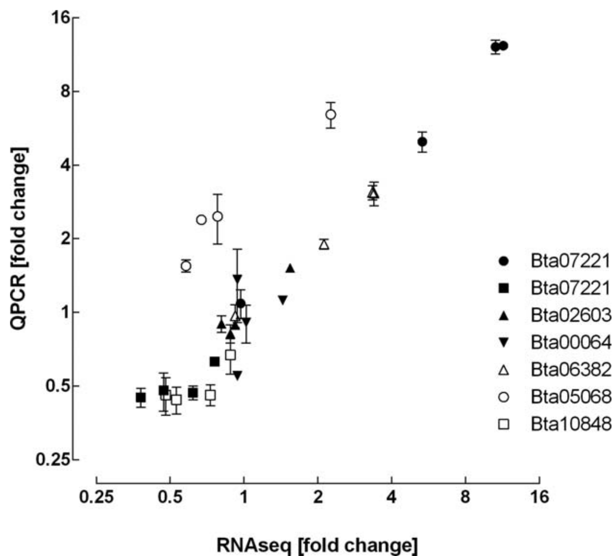

Figure 3. Quantitative PCR (qPCR) validation of RNAseq illustrated in a XY plot. RNA sequencing (RNAseq) gene expression estimates (log_2) (*x*-axis) vs. fold change (log^2) obtained from qPCR experiments (*y*-axis); error bars represent the 95% confidence limits. Key lists Gene ID; for gene description please refer to Table S6.

RNAseq, including *CYP6CM1*. Excellent concordance (Pearson's $r = 0.9532$, $P = < 0.00001$) was seen between gene expression estimates obtained by RNA-seq and qPCR analyses (Fig. 3, Table S6) with the pronounced overexpression of *CYP6CM1* confirmed in the thiacloprid selected lines.

The overexpression of several P450s has been implicated in neonicotinoid resistance in *B. tabaci* in previous studies (Karunker *et al.*, 2008; Wang *et al.*, 2009; Yang *et al.*, 2013; Ilias *et al.*, 2015); however, limited work has been done to explore if qualitative changes are also involved in resistance. To explore if qualitative changes in candidate P450s underlie the resistance in the lines under thiacloprid (+/– PBO) selection a Single Nucleotide Polymorphism (SNP) analysis was carried out for *CYP6CM1*, *CYP6CX1*, *CYP6CX3*, *CYP6CX4*, *CYP6DZ7* and *CYP4C64* (Table S7). Several synonymous and some nonsynonymous SNPs were identified in all six candidate P450s when compared to reference sequences obtained from GenBank. However, no differentially selected SNPs could be identified between the selection regimes for five of the candidate P450s and only a weak footprint of selection could be identified for an allelic variant of *CYP6CM1* increasing in frequency from ~0.6 in the nonselected strain to ~0.75 in the lines selected with thiacloprid (+/– PBO).

Target-site genotyping

To identify mutations in the pyrethroid target-site, the raw RNAseq reads obtained from each *B. tabaci* line were mapped against the previously published cDNA sequence of the VGSC to examine the nucleotide sequence at known resistance 'hot-spots' (Alon *et al.*, 2006; Morin *et al.*, 2002). Variation was found at two *kdr* sites that resulted in the L925I (tta to ata) and T929V (act to gtt) amino acid substitutions (Table 7). In the nonselected strain the frequency of these two mutations was 0.63 and 0.24 with the two mutations never observed on the same allele (ie sequenced read). Interestingly, in the lines selected with *alpha*-cypermethrin or *alpha*-cypermethrin + PBO the frequency of the L925I mutation was close to fixation whereas T929V was observed at a frequency below 0.05. In contrast no significant change in frequency of either mutation was observed in lines selected with thiacloprid or thiacloprid + PBO compared to the unselected strain (Table 7). No additional mutations were observed at other previously reported *kdr* mutation sites (including L1014 and M918) in any of the lines.

As the RNAseq coverage of the *kdr* sites was below 30 reads for each sample, PCR amplification of the VGSC and sequencing of individual insects were carried out for the nonselected as well as the pyrethroid selected lines. This approach also allowed us to determine whether individuals were heterozygous or homozygous for the *kdr* mutations. DNA was isolated from individuals, PCR amplified and sequenced around the *kdr* region of the VGSC. In generation 3 the allele frequencies for the L925I and the T929V mutations were 0.56 and 0.28, respectively, in the nonselected strain (B-NS). In generation 8 the frequencies were 0.5 and 0.31, respectively (Table 8). Hence without selection the frequencies of all three alleles (wildtype L925/T929, L925I/T929 and L925/T929V) remained broadly similar. Whilst the L925I

Table 7. Frequency of the L925I and T929V knock-down resistance (*kdr*) mutations in RNA sequencing (RNAseq) data of *Bemisia tabaci* strains in generation 8

Strain	Total reads	TTA reads	ATA read	L925I frequency	Total reads	ACT	GTT	T929V frequency	Cumulative *kdr* frequency
B-NS	16	6	10	0.63	25	19	6	0.24	0.87
B1	19	1	18	0.95	23	22	1	0.04	0.99
B2	25	1	24	0.96	36	35	1	0.03	0.99
B3	14	5	9	0.64	17	11	6	0.35	0.99
B4	20	6	14	0.70	22	18	4	0.18	0.88

B-NS, *B. tabaci* nonselected; B1, *B. tabaci alpha*-cypermethrin selected; B2, *B. tabaci alpha*-cypermethrin + piperonyl butoxide (PBO) selected; B3, *B. tabaci* thiacloprid selected; B4, *B. tabaci* thiacloprid + PBO selected.

Table 8. Frequency of the L925I and T929V knock-down resistance (*kdr*) mutations in *Bemisia tabaci* strains obtained by DNA sequencing of individual specimens

Strain	Generation	# individuals sequenced	TTA	TTA/ATA	ATA	L925I frequency	ACT	ACT/GTT	GTT	T929V frequency	Cumulative *kdr* frequency
B-NS	3	16	3	8	5	0.56	7	9	0	0.28	0.84
B-NS	8	16	4	8	4	0.50	7	8	1	0.31	0.81
B1	8	32	2	9	21	0.80	23	8	1	0.16	0.95
B2	8	32	1	1	30	0.95	30	1	1	0.05	1

B-NS, B. *tabaci* nonselected; B1, *B. tabaci alpha*-cypermethrin selected; B2, *B. tabaci alpha*-cypermethrin + piperonyl butoxide selected.

mutation was found in both the homozygous and heterozygous state at high frequency, the L929V mutation occurred almost exclusively in the heterozygous form with only one out of 32 sequenced nonselected specimens being homozygous for this mutation. As suggested by the RNAseq data, under selection with *alpha*-cypermethrin (+/– PBO) the wildtype allele frequency decreased and the L925I allele increased. Interestingly the L925I mutation was nearly fixed under *alpha*-cypermethrin + PBO selection, with 31 specimens homozygous for this mutation and one individual heterozygous for both L925I and T929V. By contrast, under selection with *alpha*-cypermethrin without PBO the frequency of the L925I mutation increased (allele frequency 0.8) but the T929V mutation was still observed at moderate frequency (allele frequency 0.16). Again the T929V allele was mostly present in individuals in the heterozygous form.

Discussion

Previous studies have demonstrated that the use of PBO in combination with insecticide can suppress the development of pyrethroid resistance in mosquitoes (Thomas *et al.*, 1991; Kumar *et al.*, 2002). Here we show that the same is true for the crop pest *B. tabaci*. Although all four treatment regimes used in this study resulted in a significant increase in resistance, higher selection for resistance was observed for thiacloprid than *alpha*-cypermethrin and, in both cases insecticide treatment alone gave higher selection of resistance than did the insecticide in combination with PBO. The higher levels of resistance observed in the thiacloprid treated lines are to a large degree because of a loss of resistance to thiacloprid in the unselected line used as a comparator. Interestingly, the unselected line exhibited no change in sensitivity to *alpha*-cypermethrin during the experiment suggesting that although no fitness penalty is incurred in the case of *alpha*-cypermethrin resistance in the absence of selection, a fitness cost is associated with thiacloprid resistance. This may result from differences in the primary underlying mechanisms of resistance to these two compounds (discussed below).

Although resistance developed in both the thiacloprid + PBO and *alpha*-cypermethrin + PBO treated lines, the final resistance level was lower (significantly in the case of *alpha*-cypermethrin + PBO) than for the lines selected with the corresponding insecticide alone. Resistance was suppressed by 60% in the case of *alpha*-cypermethrin + PBO and 28% in the case of thiacloprid + PBO. The former is consistent with the levels of resistance suppression seen in previous studies on mosquitoes after selection with deltamethrin with and without PBO (see Introduction). The obvious caveat to our study and those conducted previously is that they have been carried out in the laboratory and are unlikely to fully reflect the evolution of resistance in the field. Validation of these results in a realistic environment and at an appropriate spatial scale is therefore required. This is likely to be easier in the case of *B. tabaci*, than for mosquitoes, as it is primarily a glasshouse pest in Europe, and this would allow pest populations to be repeatedly sampled during treatment regimes.

Global transcriptome profiling revealed measurable changes in gene expression in the insecticide (+/– PBO) selected lines. Interestingly, and perhaps counterintuitively, a greater number of detoxification genes were upregulated in the lines selected with both insecticide and PBO, even though resistance was slower to develop. This finding is actually consistent with previous research that has shown that PBO actively induces the expression of both P450s and GSTs in *Drosophila melanogaster* Meigen (Willoughby *et al.*, 2007), and the expression of detoxification gene families in mammals (Škrinjarić-Špoljar *et al.*, 1971; Watanabe *et al.*, 1998). Because of this finding, it has been suggested that the increased production of detoxification enzymes upon PBO exposure may actually increase insecticide tolerance if these enzymes are capable of insecticide metabolism (Willoughby *et al.*, 2007). Our findings reveal that PBO suppresses the development of resistance despite the induction of detoxification genes, suggesting that either PBO does not induce resistance-conferring enzymes, or may slow resistance development by some other means.

In the case of *alpha*-cypermethrin selection no candidate genes encoding enzymes previously shown to detoxify this compound were upregulated > twofold in lines selected with this compound (+/– PBO), suggesting that metabolic mechanisms play a secondary role in the resistance of these lines (see below). In contrast,

with thiacloprid (+/– PBO) a substantial increase was observed in the expression of the P450 *CYP6CM1* (11-fold in both lines). Overexpression of *CYP6CM1* has been previously associated with neonicotinoid resistance in a number of Mediterranean (MED) and MEAM1 (Middle East-Asia Minor I, formerly referred to as biotype B) biotypes of *B. tabaci* laboratory and field strains from several geographical origins (Karunker *et al*., 2008; Roditakis *et al*., 2011). Functional expression of recombinant CYP6CM1 has also demonstrated that this enzyme has the capacity to detoxify several neonicotinoids including thiacloprid (Karunker *et al*., 2009; Roditakis *et al*., 2011). The overexpression of this P450 therefore probably explains a significant part of the resistance seen in the thiacloprid selected lines in our study. Interestingly, the level of expression of this gene was the same in both the line selected with thiacloprid alone and the line selected with thiacloprid + PBO. This suggests that *CYP6CM1* does not explain the difference in resistance levels of these two lines, and that other genes may be involved. Beyond *CYP6CM1* other candidate P450 genes have been implicated in neonicotinoid resistance in *B. tabaci* in previous studies, ie *CYP6CX1*, *CYP6CX3*, *CYP6CX4*, *CYP6DZ7* and *CYP4C64* (Yang *et al*., 2013; Ilias *et al*., 2015); however, none of these was upregulated by thiacloprid selection in our study. No other obvious candidate genes were identified as downregulated in the thiacloprid + PBO line compared to the thiacloprid selected line that might explain the greater level of resistance observed in the latter. A SNP analysis to evaluate qualitative changes in P450s that have been implicated in neonicotinoid resistance in previous studies in *B. tabaci* (*CYP6CM1*, *CYP6CX1*, *CYP6CX3*, *CYP6CX4*, *CYP6DZ7* and *CYP4C64*) failed to identify major differentially selected alleles amongst the treatment regimes in this study. The unselected strain gained sensitivity (lost resistance) to thiacloprid over the time period of the selection experiment. qPCR analysis of *CYP6CM1* expression in this strain sampled before and at the end of the selection period revealed no significant change in the expression of the gene (Table S6), indicating that this P450 does not explain the difference in phenotype of this strain at the two time points and providing additional evidence of alternative mechanisms of resistance.

Although target-site resistance has never been described for neonicotinoid resistance in *B. tabaci* it has been reported for pyrethroid resistance (see Introduction). Our RNAseq analyses revealed the presence of two *kdr* mutations, L925I and T929V, in the unselected line and all insecticide selected lines. Both of these mutations have been described previously; however, their relative importance in resistance has not been determined (Morin *et al*., 2002; Alon *et al*., 2006). The L925I mutation increased in frequency in the *alpha*-cypermethrin + PBO selected line near fixation, whereas the T929V mutation decreased in frequency (Table 8). The *alpha*-cypermethrin selected line showed a similar trend, albeit the shift in frequency was less profound. In contrast, no change in the frequency of either mutation was observed in the thiacloprid (+/– PBO) lines as was expected because neonicotinoids do not target the VGSC. The increase in frequency of these *kdr* mutations in the *alpha*-cypermethrin selected lines probably explains a significant proportion of the resistance exhibited by these lines. Furthermore, it also demonstrates that both selecting with insecticide plus synergist and insecticide minus synergist will result in increased frequency of target-site mutations present in the population as standing genetic variation. Despite the fact that increased frequencies of the L925I were observed in the *alpha*-cypermethrin and *alpha*-cypermethrin + PBO selected lines, significant differences were observed in the level of resistance between these two lines suggesting that additional, nontarget site, mechanisms contribute to the resistance phenotype exhibited by the *alpha*-cypermethrin selected line. This finding was also supported by PBO/insecticide bioassays of the *alpha*-cypermethrin selected line where, in contrast to the unselected line, resistance could be synergized (resistance reduced by 1.6-fold; Table 3). However, interrogation of genes differentially expressed between these two lines failed to identify any known candidate resistance genes that were upregulated in the *alpha*-cypermethrin selected lines.

The finding of this study and those reported previously reveal that PBO used in combination with an insecticide can suppress the development of resistance in a laboratory setting. Further work is required to validate this in the field and explore the potential benefits in terms of the extended duration of product efficacy provided by this approach.

The molecular basis of resistance suppression by PBO has been suggested to result from inhibition of metabolic resistance; however, we were not able to provide evidence to support this by transcriptome profiling of selected lines. The mode of action of PBO in delaying resistance therefore requires further characterization. Finally, our results demonstrate that insecticide selection with or without PBO is unlikely to affect the speed of resistance development mediated by target-site mechanisms.

Experimental procedures

Insect strain and selection regime

Two field strains of *B. tabaci* MEAM1 collected in southern Crete, one collected from the Tympaki area from aubergine, *Solanum melongena* L., and the other one from the Ampelouzos area from European black nightshade, *Solanum nigrum* L.,

were combined to a mixed field strain and used in this study. The biotype of the *B. tabaci* strain used in this study was determined by mitochondrial *cytochrome oxidase I* gene sequencing using primers C1-J-2195 (5′-TTGATTTTTTGGTCATCCA-GAAGT-3′) and L2-N-3014 (5′-TCCAATGCACTAATCTGCCA-TATTA-3′) as described by (Frohlich *et al.*, 1999). The strain was maintained at Rothamsted Research on 4–5-week-old cotton plants (*Gossypium hirsutum* L. cv. Vicky) at 26°C and a 16:8 h day : night cycle. The population reached a stable population size from the second generation and its sensitivity to *alpha*-cypermethrin and thiacloprid with and without PBO was tested by bioassay when it reached the third generation according to Insecticide Resistance Action Committees (IRAC's) susceptibility test method 015 (IRAC, 2016). Both insecticides were used in the form of their commercial formulations: Fastac EC10 (10 g *alpha*-cypermethrin per litre) (BASF, Limburgerhof, Germany) and Calypso 480 SC (480 g thiacloprid per litre) (Bayer CropScience AG, Monheim, Germany). The concentration range tested for *alpha*-cypermethrin and thiacloprid was 0.96–600 and 4.8–3000 mg active ingredient/l, respectively. All bioassays comprised three biological replicates. Mortality was assessed after 72 h and lethal concentrations (LC_{25} and LC_{50}) were calculated by probit analysis. The LC_{25} of the initial bioassay on the third generation was subsequently used to select the strain for insecticide resistance with the insecticides alone or in combination with PBO at a final concentration of 100 mg/l. PBO used was technical grade, and was dissolved in acetone before adding it to the spraying solution and the final concentration of acetone in the spraying solution was 1% volume : volume. The PBO concentration selected was based on values recorded in the literature as it has been reported that PBO itself can cause mortality in *B. tabaci* at higher concentrations (Devine & Denholm, 1998). From generation 3 to generation 8 the whitefly strain was split into five lines and reared in separate cages. Fresh cotton plants were supplied every 4 weeks when adults of the next generation appeared in the cages. Untreated cotton plants were used to maintain the unselected reference strain and pretreated plants (spray application) were used for different selection scenarios using the insecticides and PBO at the concentrations detailed above. The experimental lines comprised B-NS, *B. tabaci* nonselected; B1, *B. tabaci alpha*-cypermethrin selected; B2, *B. tabaci alpha*-cypermethrin + PBO selected; B3, *B. tabaci* thiacloprid selected; B4, *B. tabaci* thiacloprid + PBO selected. In order to determine shifts in susceptibility towards insecticides and insecticide-PBO mixtures the final generation (generation 8) of each population was tested by bioassay at the end of the selection experiment.

RNAseq

RNAseq was used to analyse global gene expression levels in selected/unselected *B. tabaci* lines and to determine the frequency of known target-site resistance mechanisms. Total RNA was extracted from three biological replicates (pooled homogenates of 30 insects) of each line detailed above using the Bioline Isolate RNA Mini Kit (Bioline, London, UK) according to the manufacturer's instructions. Prior to RNAseq experiments the quality and quantity of RNA were checked using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and by running an aliquot on a 1.5% agarose gel. For the latter, RNA was mixed with 1 × loading buffer (95% formamide; 0.025% xylene cyanol; 0.025% bromophenol blue; 18 mM ethylenediaminetetraacetic acid; 0.025% sodium dodecyl sulphate), heated for 5 min at 65°C and briefly chilled on ice prior to loading. Total RNA was used as a template for the generation of barcoded libraries (TrueSeq RNA library preparation, Illumina, San Diego, California, USA). Libraries were sequenced by The Genome Analysis Centre (TGAC, Norwich, UK) with replicates multiplexed for sequencing on an Illumina HiSeq2500 flowcell (100 bp paired end reads) to generate at least 15 000 000 reads per biological replicate.

FastQC (v. 0.11.1) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to check the quality of the raw reads obtained and data obtained was deposited in ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-4601. To identify changes in gene expression between lines that had undergone different selection regimes the open source Tuxedo (Trapnell *et al.*, 2012), workflow was used to map with TopHat (http://tophat.cbcb.umd.edu/) (Trapnell *et al.*, 2009) against the annotated reference genome to which access was provided by The International *Bemisia* Genome Initiative. Gene expression was estimated with Cufflinks and differential expression was tested with Cuffdiff (Trapnell *et al.*, 2012).

GO enrichment analysis of the differentially expressed genes was implemented by the GOseq R package, based on the Wallenius noncentral hypergeometric distribution (Young *et al.*, 2010), which takes into account variation in gene length and expression bias in RNAseq. GO terms were regarded enriched when the corrected P-value was < 0.05. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed by the standard function of Blast2GO (Conesa *et al.*, 2005) software. We searched all the genes against the KEGG database to further generate a list of pathways associated with individual gene categories. An enrichment analysis (Fisher's exact test) of GO-terms for differentially expressed transcripts (up- and downregulated genes tested separately) was performed against the reference gene set in Blast2GO at an False Discovery Rate < 0.05. Venn diagrams were generated using jvenn (Bardou *et al.*, 2014).

qPCR

Primers for qPCR were designed to amplify a fragment of ~100 bp using the Primer3 Geneious plugin (Untergasser *et al.*, 2012) (Table S4). 1.5 μg of RNA was used for reverse transcription using a Maxima H Minus First Strand cDNA Synthesis Kit from Thermo Fisher Scientific (Waltham, MA, USA), adding both random hexamer and oligo (dT) primers. Each PCR reaction consisted of 5 μl cDNA (3.125 ng), 7.5 μl SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma Aldrich, St Louis, MO, USA) and 0.5 μl of each forward and reverse primer (0.25 μM). PCRs were run on a Stratagene Mx3000P™ Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) with cycling conditions: 2 min at 95°C followed by 40 cycles of 95°C for 30 s, 57°C for 20 s and 72°C for 25 s. A final melt-curve step was included post-PCR (ramping from 72 to 95°C by 1°C every 5 s) to confirm the absence of any nonspecific amplification. The efficiency of PCR for each primer pair was assessed using a serial dilution from 100 to 0.01 ng of cDNA. Each

qPCR experiment consisted of at least three independent biological replicates with two technical replicates. Data were analysed according to the delta delta Cycle Threshold (ΔΔCT) method (Pfaffl, 2001), using the geometric mean of two selected housekeeping genes (*elongation factor 1-alpha* and *tubulin 1a*) for normalization according to the strategy described previously (Vandesompele *et al.*, 2002).

SNP analysis of P450s

To evaluate whether qualitative changes may underlie the resistance in thiacloprid (+/– PBO) selected lines a SNP analysis was carried out for *CYP6CM1* (GenBank accession number GQ214539), *CYP6CX1* (GenBank accession number GQ292715), *CYP6CX3* (GenBank accession number JN165264), *CYP6CX4* (GenBank accession number JN165265), *CYP6DZ7* (GenBank accession number JX1443650) and *CYP4C64* (GenBank accession number JX144366). The RNAseq raw reads from each treatment were merged into individual files (B-NS and B1 to B4) and mapped against the P450 sequences mentioned above using Burrows-Wheeler Aligner (Li & Durbin, 2009). The resulting Binary Alignment/Map Format (BAM) files from individual samples (B-NS, B1, B2, B3 and B4) were then analysed in Geneious (v. R8; Biomatters, Auckland, New Zealand) and the frequency of SNPs was determined at a minimum variant frequency of 0.1 and maximum variant *P*-value of 10^{-6}.

kdr mutation genotyping

To identify mutations associated with target-site resistance to pyrethroid insecticides, partial mRNA sequence of the voltage-gated sodium channel of *B. tabaci* (GenBank accession number AJ440727) was used in a BLAST search against the whole transcriptome raw read library. For BLAST search we used a locally (Rothamsted Research Ltd) installed DeCypher® BLAST server (TimeLogic DeCypher, Active Motif, Carlsbad, CA, USA). The returned hits were extracted and the associated mate sequences were fetched from the whole transcriptome raw read library. The extracted sequences were then mapped against the mRNA reference mentioned above. The generated BAM files from individual samples (B-NS, B1, B2, B3 and B4) were then manually analysed in Geneious (v. R8) and the frequency of target-site mutations at known resistance 'hot-spots' was examined (Morin *et al.*, 2002; Alon *et al.*, 2006).

To estimate the frequency of the *kdr* mutations identified by RNAseq during selection more precisely, a fragment of the VGSC was PCR amplified from genomic DNA and sequenced directly from samples derived from generations 3 and 8. Individual specimens were incubated with Microlysis plus extraction buffer (Microzone Ltd, Haywards Heath, Sussex, UK) following the manufacturer's recommended protocol for tough cells. Using primers that have been reported previously (Morin *et al.*, 2002) a ~900-bp fragment of the VGSC was amplified in a single round of PCR. A typical PCR (25 μl) contained 0.5 μM of each primer (Table S2), 2 μl extracted DNA, 12.5 μl DreamTaq (Thermo Fisher Scientific, Waltham, MA, USA) containing Taq polymerase, 2 × PCR buffer and 4 mM $MgCl_2$ (2 mM final concentration). Cycling conditions were 94°C for 2 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min, and a final elongation at 72°C for 5 min. PCR products were verified by agarose gel electrophoresis prior to PCR clean-up and sequencing (sequencing primer 'kdr-seq', Table S5), which was carried out by Eurofins Genomics (Ebersberg, Germany). Sequence analysis was carried out with Geneious R8.

Acknowledgements

The work leading to this paper has received funding from the European Union Seventh Framework Program (FP7/2007-2013) under grant agreement no. 605740 (October 2013–December 2015). We thank the International *Bemisia* Genome Initiative for the prepublication use of the *B. tabaci* MEAM1 genome sequence to map RNAseq data.

References

Alon, M., Benting, J., Lueke, B., Ponge, T., Alon, F. and Morin, S. (2006) Multiple origins of pyrethroid resistance in sympatric biotypes of *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Insect Biochem Mol Biol* **36**: 71–79. doi:10.1016/j.ibmb.2005.10.007

Bardou, P., Mariette, J., Escudié, F., Djemiel, C. and Klopp, C. (2014) jvenn: an interactive Venn diagram viewer. *BMC Bioinformatics* **15**: 293. doi:10.1186/1471-2105-15-293

Bernard, C.B. and Philogène, B.J.R. (1993) Insecticide synergists: role, importance, and perspectives. *J Toxicol Environ Health* **38**: 199–223. doi:10.1080/15287399309531712

Conesa, A., Götz, S., Garcia-Gomez, J.M., Terol, J., Talon, M. and Robles, M. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**: 3674–3676. doi:10.1093/bioinformatics/bti610

Devine, G. and Denholm, I. (1998) Potential of piperonyl butoxide for the management of the cotton whitefly, *Bemisia tabaci*. In *Piperonyl Butoxide: The Insecticide Synergist* (Jones, D.G., ed.), pp. 227–237. Academic Press, San Diego.

Farnham, A.W. (1999) The mode of action of piperonyl butoxide with reference to studying pesticide resistance. In *Piperonyl Butoxide* (Jones, D.G., ed.), pp. 199–213. Academic Press, San Diego.

Feyereisen, R. (2015) Insect P450 inhibitors and insecticides: challenges and opportunities. *Pest Manag Sci* **71**: 793–800. doi:10.1002/ps.3895

Frohlich, D.R., Torres-Jerez, I.I. Bedford, I.D., Markham, P.G. and Brown, J.K. (1999) A phylogeographical analysis of the *Bemisia tabaci* species complex based on mitochondrial DNA markers. *Mol Ecol* **8**: 1683–1691.

Ilias, A., Lagnel, J., Kapantaidaki, D.E., Roditakis, E., Tsigenopoulos, C.S., Vontas, J. *et al.* (2015) Transcription analysis of neonicotinoid resistance in Mediterranean (MED) populations of *B. tabaci* reveal novel cytochrome P450s, but no nAChR mutations associated with the phenotype. *BMC Genomics* **16**: 939. doi:10.1186/s12864-015-2161-5

IRAC (2016) *IRAC's susceptibility test method 015*. http://www.irac-online.org/methods/trialeurodes-vaporariorum-bemisia-tabaci-adult/. Accessed on 4 April 2016.

Karunker, I., Benting, J., Lueke, B., Ponge, T., Nauen, R., Roditakis, E. *et al.* (2008) Over-expression of cytochrome P450 CYP6CM1 is associated with high resistance to imidacloprid in the B and Q biotypes of *Bemisia tabaci* (Hemiptera:

Aleyrodidae). *Insect Biochem Mol Biol* **38**: 634–644. doi: 10.1016/j.ibmb.2008.03.008

Karunker, I., Morou, E., Nikou, D., Nauen, R., Sertchook, R., Stevenson, B.J. *et al*. (2009) Structural model and functional characterization of the *Bemisia tabaci* CYP6CM1vQ, a cytochrome P450 associated with high levels of imidacloprid resistance. *Insect Biochem Mol Biol* **39**: 697–706. doi: 10.1016/j.ibmb.2009.08.006

Kumar, S., Thomas, A., Sahgal, A., Verma, A., Samuel, T. and Pillai, M.K.K. (2002) Effect of the synergist, piperonyl butoxide, on the development of deltamethrin resistance in yellow fever mosquito, *Aedes aegypti* L. (Diptera: Culicidae). *Arch Insect Biochem Physiol* **50**: 1–8. doi:10.1002/arch.10021

Li, H. and Durbin, R. (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**: 1754–1760. doi:10.1093/bioinformatics/btp324

Morin, S., Williamson, M.S., Goodson, S.J., Brown, J.K., Tabashnik, B.E. and Dennehy, T.J. (2002) Mutations in the *Bemisia tabaci* para sodium channel gene associated with resistance to a pyrethroid plus organophosphate mixture. *Insect Biochem Mol Biol* **32**: 1781–1791. doi:10.1016/S0965-1748(02)00137-6

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.

Roditakis, E., Morou, E., Tsagkarakou, A., Riga, M., Nauen, R., Paine, M.J.I. *et al*. (2011) Assessment of the *Bemisia tabaci* CYP6CM1vQ transcript and protein levels in laboratory and field-derived imidacloprid-resistant insects and cross-metabolism potential of the recombinant enzyme. *Insect Sci* **18**: 23–29. doi:10.1111/j.1744-7917.2010.01384.x

Scott, J.G., Cochran, D.G. and Siegfried, B.D. (1990) Insecticide toxicity, synergism and resistance in the German cockroach (Dictyoptera: Blattellidae). *J Econ Entomol* **83**: 1698–1703.

Škrinjarić-Špoljar, M., Matthews, H.B., Engel, J.L. and Casida, J.E. (1971) Response of hepatic microsomal mixed-function oxidases to various types of insecticide chemical synergists administered to mice. *Biochem Pharmacol* **20**: 1607–1618. doi:10.1016/0006-2952(71)90289-9

Thomas, A., Kumar, S. and Pillai, M.K.K. (1991) Piperonyl butoxide as a countermeasure for deltamethrin-resistance in *Culex quinquefasciatus* Say. *Entomon* **16**: 1–10.

Trapnell, C., Pachter, L. and Salzberg, S.L. (2009) TopHat: discovering splice junctions with RNA-seq. *Bioinformatics* **25**: 1105–1111.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R. *et al*. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* **7**: 562–578. doi:10.1038/nprot.2012.016

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., *et al*. (2012) Primer3—new capabilities and interfaces. *Nucleic Acids Res* **40**: Pp. e115.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**: RESEARCH0034. doi: 10.1186/gb-2002-3-7-research0034

Wang, Z., Yao, M. and Wu, Y. (2009) Cross-resistance, inheritance and biochemical mechanisms of imidacloprid resistance in B-biotype *Bemisia tabaci*. *Pest Manag Sci* **65**: 1189–1194. doi:10.1002/ps.1808

Watanabe, T., Manabe, S., Ohashi, Y., Okamiya, H., Onodera, H. and Mitsumori, K. (1998) Comparison of the induction profile of hepatic drug-metabolizing enzymes between piperonyl butoxide and phenobarbital in rats. *J Toxicol Pathol* **11**: 1–10.

Willoughby, L., Batterham, P. and Daborn, P.J. (2007) Piperonyl butoxide induces the expression of cytochrome P450 and glutathione S-transferase genes in *Drosophila melanogaster*. *Pest Manag Sci* **63**: 803–808. doi:10.1002/ps.1391

Yang, X., Xie, W., Wang, S.I., Wu, Q.J., Pan, H.P., Li, R.M. *et al*. (2013) Two cytochrome P450 genes are involved in imidacloprid resistance in field populations of the whitefly, *Bemisia tabaci*, in China. *Pestic Biochem Physiol* **107**: 343–350. doi: 10.1016/j.pestbp.2013.10.002

Young, M.D., Wakefield, M.J., Smyth, G.K. and Oshlack, A. (2010) Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol* **11**: R14. doi:10.1186/gb-2010-11-2-r14

Young, S.J., Gunning, R.V. and Moores, G.D. (2005) The effect of piperonyl butoxide on pyrethroid-resistance-associated esterases in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *Pest Manag Sci* **61**: 397–401. doi:10.1002/ps.996

Young, S.J., Gunning, R.V. and Moores, G.D. (2006) Effect of pretreatment with piperonyl butoxide on pyrethroid efficacy against insecticide-resistant *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Bemisia tabaci* (Sternorrhyncha: Aleyrodidae). *Pest Manag Sci* **62**: 114–119. doi:10.1002/ps.1127

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. List of differentially expressed genes.

Table S2. Common differentially expressed genes after selection with insecticide alone or insecticide and PBO.

Table S3. Gene ontology term enrichment analysis.

Table S4. Quantitative PCR validation.

Table S5. Primer sequences for knock-down resistance (*kdr*) analysis on genomic DNA.

Table S6. Quantitative PCR validation of RNA sequencing (RNAseq) gene expression estimates.

Table S7. SNP analysis of CYP6CM1, CYP6CX1, CYP6CX3, CYP6CX4, CYP6DZ7 and CYP4C64.