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Transcriptome profiling of the whitefly *Bemisia tabaci* reveals stage-specific gene expression signatures for thiamethoxam resistance

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Abstract

Bemisia tabaci **has developed high levels of resistance to many insecticides including the neonicotinoids and there is strong evidence that for some compounds resistance is stage-specific. To investigate the molecular basis of *B. tabaci* resistance to the neonicotinoid thiamethoxam we used a custom whitefly microarray to compare gene expression in the egg, nymph and adult stages of a thiamethoxam-resistant strain (TH-R) with a susceptible strain (TH-S). Gene ontology and bioinformatic analyses revealed that in all life stages many of the differentially expressed transcripts encoded enzymes involved in metabolic processes and/or metabolism of xenobiotics. Several of these are candidate resistance genes and include the cytochrome P450 *CYP6CM1*, which has been shown to confer resistance to several neonicotinoids previously, a P450 belonging to the Cytochrome P450s 4 family and a glutathione S-transferase (GST) belonging to the sigma class. Finally several ATP-binding cassette transporters of the ABCG subfamily were highly over-expressed in the adult stage of the TH-R strain and may play a role in resistance by active efflux. Here, we evaluated both common and stage-specific gene expression signatures and identified several candidate resistance genes that may underlie *B. tabaci* resistance to thiamethoxam.**

Keywords: *Bemisia tabaci*, metabolic resistance, thiamethoxam, microarray, stage specific.

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Introduction

The sweet potato whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae), is an important pest of agricultural and horticultural crops in temperate and tropical regions worldwide. *B. tabaci* is a species complex, which is generally categorized into a series of species based on distinct biological and genetic differences (Dinsdale *et al*., 2010; De Barro *et al*., 2011; Sun *et al*., 2011). The so-called B- and Q-types are by far the most economically damaging and invasive. In China, the B-type was first detected in the mid-1990s (Luo *et al*., 2002), whereas the Q-type was first reported in 2003 (Chu *et al*., 2006). The distributions of both types have extended rapidly throughout the country, although the Q-type is currently supplanting the B-type in many areas (Zhang *et al*., 2005; Luo *et al*., 2010).

Over the years *B. tabaci* has demonstrated a remarkable ability to develop resistance to many insecticides including organophosphates, carbamates, pyrethroids, neonicotinoids and juvenile hormone mimics (Cahill *et al*., 1996; Horowitz *et al*., 2004; Nauen & Denholm, 2005). The neonicotinoids (eg imidacloprid, thiamethoxam and acetamaprid) are key compounds for whitefly control because of their excellent efficacy, versatility of application and long residual activity (Nauen *et al*., 2008). The emergence of resistance to neonicotinoids has been a consistent characteristic of Q-type whiteflies (Nauen & Denholm, 2005), whereas in the B-type resistance generally takes longer to develop. This pattern is evident in China where B-type strains collected in 2007–2008 were far more

susceptible than Q-type counterparts (Luo *et al*., 2010). More recently however, cross-resistance to imidacloprid and thiamethoxam has been reported from field populations of both B- and Q-types (Z.Y. Wang *et al*., 2010).

There is strong evidence that neonicotinoid resistance is mediated in part by cytochrome p450 monooxygenases (Nauen *et al*., 2002; Rauch & Nauen, 2003; Feng *et al*., 2009). In whiteflies, resistance to imidacloprid is strongly correlated with the expression of a specific p450, *CYP6CM1*, which can metabolize this insecticide *in vitro* (Karunker *et al*., 2008, 2009). The finding that imidacloprid resistance is age-specific in *B. tabaci* (Nauen *et al*., 2008) was shown to correlate with *CYP6CM1* mRNA levels (Jones *et al*., 2011). To date, no target-site mutations in the nicotinic acetylcholine receptor have been discovered in field populations of *B. tabaci*; however, a point mutation (R81T) in the peach-potato aphid (*Myzus persicae*), in addition to elevated cytochrome p450s, confers resistance to both imidacloprid and thiamethoxam (Bass *et al*., 2011).

The recent advancement in genomic technologies offers the opportunity for a better understanding of the complex metabolic processes underlying insecticide resistance (Bass *et al*., 2012). Transcriptome profiling has been applied to several insect species as a means of analysing gene expression patterns and associating these with insecticide-resistant phenotypes (Puinean *et al*., 2010; Liu *et al*., 2011; Karatolos *et al*., 2012; Mitchell *et al*., 2012). Furthermore, transcriptional profiling of individual stages within the insect life-cycle provide evidence for key genes up- or down-regulated during the developmental process (Arbeitman *et al*., 2002; Colgan *et al*., 2011; Harker *et al*., 2012).

Some of the present authors have previously used two approaches to compare gene expression in the adult stage of a thiamethoxam resistant (TH-R) *B. tabaci* B-type laboratory strain with the parental strain from which it was selected (TH-susceptible, TH-S). In the first study quantitative real-time PCR (qPCR) was used to examine the expression of 63 candidate genes (Xie *et al*., 2012b), whereas in a second study suppression subtractive hybridization (SSH) was used to identify differentially expressed genes followed by qPCR validation (Xie *et al*., 2012a). The first study was restricted to genes encoding cytochrome P450s whereas the second study showed poor correlation between SSH and qPCR, suggesting several 'false positives' as differentially expressed using SSH (Xie *et al*., 2012a). In the current study, a global transcriptomic approach was used to analyse gene expression patterns in the egg, fourth instar nymph and adult life stages using an Agilent custom *B. tabaci* microarray containing ~15 000 probes representing 8394 expressed sequence tags (ESTs). The microarray data for selected genes were validated using quantitative reverse-transcription PCR (qRT-PCR). The differences in expression profiles between the thiamethoxam resistant and susceptible strains are discussed in the context of our current understanding of neonicotinoid resistance in *B. tabaci*.

Results

Overview of microarray results

Changes in global gene expression in different development stages of the thiamethoxam selected strain (TH-R) were compared with the equivalent life stage of an unselected culture of the same strain (TH-S) using a custom Agilent microarray. In total, 1043 ESTs were identified as significantly differentially expressed in at least one of the three life stages examined with each development stage displaying a distinct gene expression signature (Figs 1A, 2A, Table S3). Amongst these, only ~4% (37 ESTs) were differentially expressed in all three stages (Table 2) with just 10 ESTs over-expressed. Approximately 22% (230 ESTs) of the differentially expressed genes were common to two stages and ~74% (776 genes) were unique to just one stage (Fig. 2B). The expression of 434 ESTs was altered in the egg stage, amongst which ~59% were up-regulated. The nymph stage had 553 differentially expressed ESTs with ~42% of them up-regulated. Finally in the female adult stage, 360 ESTs were differentially expressed with ~61% over-expressed.

Stage-specific gene expression

434 genes were significantly differentially expressed in the egg stage between susceptible (TH-S) and resistant (TH-R) whiteflies [false discovery rate (FDR) ≤ 0.001 and $|\log_2 \text{ratio}| \geq 1$]. The majority of these transcripts (~86%, 373 genes) fell into different fold range (Fig. 1B). Gene ontology (GO) analysis of differentially expressed transcripts at the egg stage are presented in Fig. 3 with the details shown in Table S4. For molecular function ontology the majority of the ESTs were assigned to the GO terms catalytic activity (~36%, 157) and binding (~23%, 98), for biological process ontology to metabolic process (~25%, 109), and for cellular component ontology to cell (~23%, 99) and cell part (~23%, 99), (Table S4). At this stage, a high number of the most over-expressed transcripts encoded energy regulation components such as cytochrome *c* and reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase. Other highly over-expressed transcripts encode enzymes involved in metabolic pathways and metabolism of xenobiotics, including glucosyl/glucuronosyl transferases, cytochrome P450 and glutathione S-transferases (GSTs).

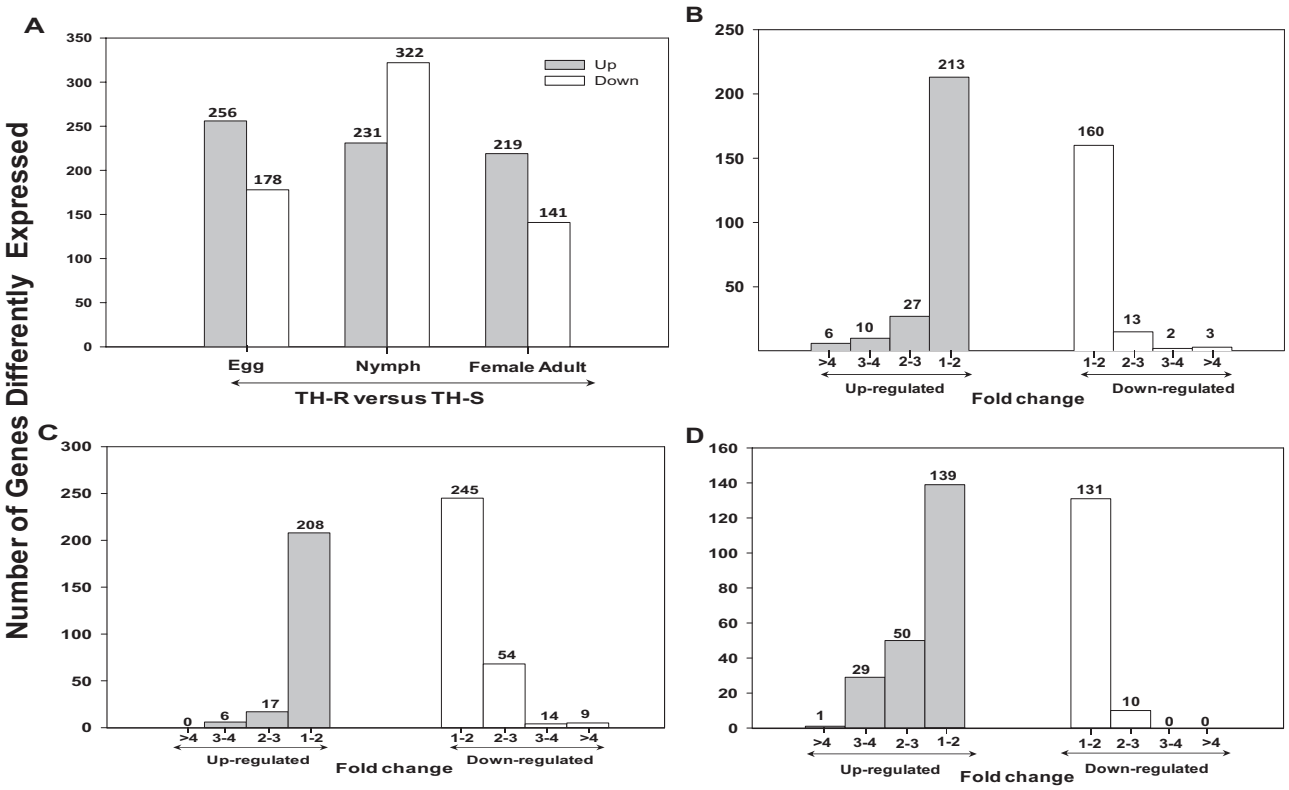

Figure 1. Stage-specific expression patterns. (A) Stage-specific expression of expressed sequence tags in microarray analysis. (B, C, D) Fold change distribution of differentially expressed genes in eggs, nymphs and female adults, respectively. The number of probes for the different classes and corresponding fold changes (fold change = 1–2, 2–3, 3–4, >4) are given. Values are given only for probes in which fold changes are statistically significant using R and SAS tests. TH-R, thiamethoxam resistant; TH-S, thiamethoxam susceptible. R, http://www.r-project.org/. SAS, Statistics Analysis System.

The nymph stage had 553 significantly differentially expressed genes (FDR ≤ 0.001 and $|\log_2 \text{ratio}| \geq 1$) and there were significantly more stage-specific ESTs at this stage than either the egg or adult stages. Data analysis showed that the majority of the transcripts (~82%, 453) fell into different fold range (Fig. 1C). In contrast to the predominance of elevated EST expression in the egg stage, genes unique to the nymph stage were more likely to be down-regulated (~58% of the 553 genes). GO analysis of differentially expressed ESTs in this stage assigned the majority of ESTs to the GO terms catalytic activity (~42%, 232), metabolic process (~24%, 134), cell part (~22%, 120) and cell (~22%, 120) for the molecular function, biological process and cellular component ontologies respectively induced (Fig. 3, Table S4). Interestingly, expression of genes encoding digestive enzymes were amongst the most highly over-expressed in the larval stage of the TH-R strain, including trehalases, sucrases, amylases, serine proteases, glucosidases and peroxidases, whereas the expression of transport genes such as ATP-binding cassette transporters (ABC transporters) was down-regulated. High numbers of ESTs encoding proteins involved in the metabolic and xenobiotic detoxification pathways were over-expressed in this stage, including two P450s, 22 GSTs and several others encoding oxidoreductase activity, such as glucose dehydrogenase, NADH dehydrogenase and 24-dehydrocholesterol reductase.

The female adult stage had 360 differentially expressed ESTs in the resistant TH-R strain compared to the equivalent stage of the TH-S strain (FDR ≤ 0.001 and $|\log_2 \text{ratio}| \geq 1$). Amongst these, 219 ESTs were up-regulated and 141 ESTs were down-regulated by at least 2.0-fold ($P < 0.05$) with ~75% (270 ESTs) in the different fold range (Fig. 1D). These included ESTs encoding proteins assigned to the GO terms catalytic activity (~43%, 154), binding (~31%, 110), metabolic process (~25%, 92), cell part (~23%, 83) and response to stimulus (~17%, 61) (Fig. 3, Table S4). High numbers of ESTs encoding heat shock proteins and detoxification enzymes such as P450s, GSTs and carboxylesterases (CEs) were amongst the most over-expressed gene in the adult stage of the TH-R strain. Finally, in contrast to the larval stage, several ESTs encoding ABC transporters were over-expressed in the adult stage in the TH-R strain.

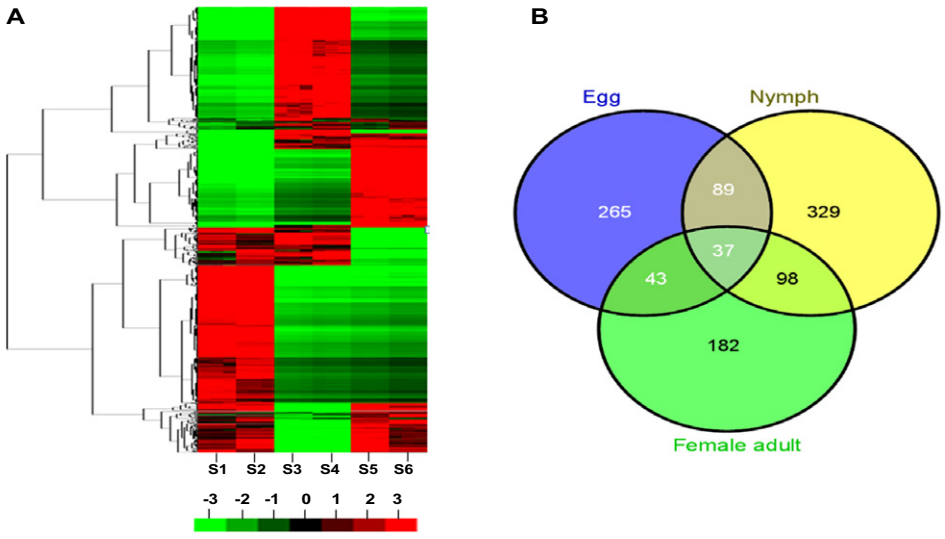

Figure 2. Stage-specific gene expression signatures in *Bemisia tabaci*. (A) Hierarchical cluster analysis of altered expressed sequence tags (ESTs) ($|\log_2$ ratio$| \geq 1$, $P < 0.05$) at the three stages in *B. tabaci* between thiamethoxam resistance and susceptible were characterized as the following: nymph stage in thiamethoxam resistant (TH-R; S1) and TH susceptible (TH-S; S2), respectively; egg stage in TH-R (S3) and TH-S (S4), respectively; female adult stage in TH-R (S5) and TH-S (S6), respectively. Green, red and black areas indicate, respectively, decreased, increased and no significant change in thiamethoxam resistance compared with susceptible. (B) Venn diagram depicting the number of ESTs with altered expression ($|\log_2$ ratio$| \geq 1$, $P < 0.05$) at three stages between thiamethoxam resistance and susceptible, respectively. The size of each region is proportional to the number of altered ESTs.

Candidate resistance/detoxification genes

In a range of insect species genes encoding members of the GST, cytochrome P450 (P450) and CE families have been most frequently associated with resistance to a range of different insecticides (Li *et al.*, 2007). The expression of ESTs encoding these potential resistance genes and those involved in excretion such as ABC transporters is illustrated in Fig. 4. An approximately equal number of ESTs encoding P450s were over-/under-expressed in the egg and adult stages of the TH-R strain compared to the TH-S strain, with a slightly higher number down-regulated in the nymph stage. Of these, only a single EST (p_00712), encoding *CYP6CM1vB*, was over-expressed in all stages in the microarray experiments (Table 2). This EST and several others encoding the same P450 (or alleles of it) were the most highly over-expressed ESTs encoding P450s in both egg and larval stages of the TH-R strain. However, in the adult stage of the TH-R strain a different P450 was the most over-expressed gene. This P450 was represented by six over-expressed ESTs (p_04027, p_07226, p_00875, p_00955, p_06100 and

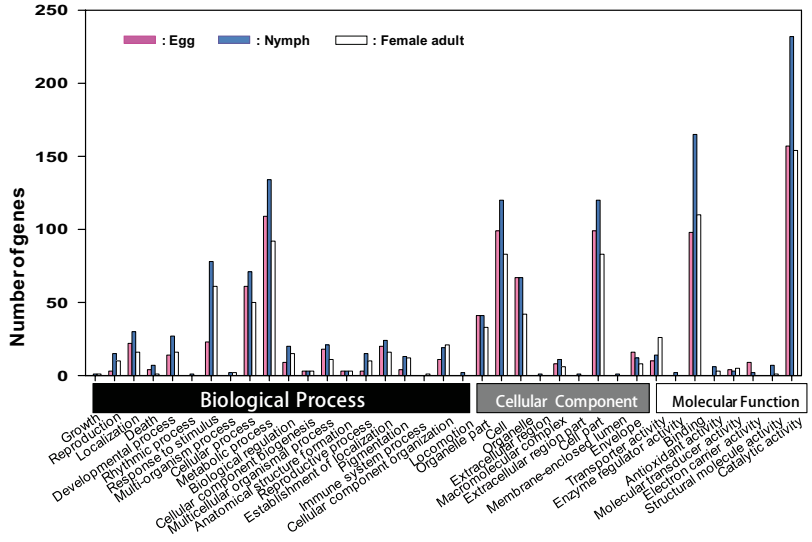

Figure 3. Functional classification of differentially expressed expressed sequence tags (ESTs) The function of differently expressed ESTs are grouped into three main ontology: biological process, cellular component and molecular function. The *y*-axis the number of genes in each GO term.

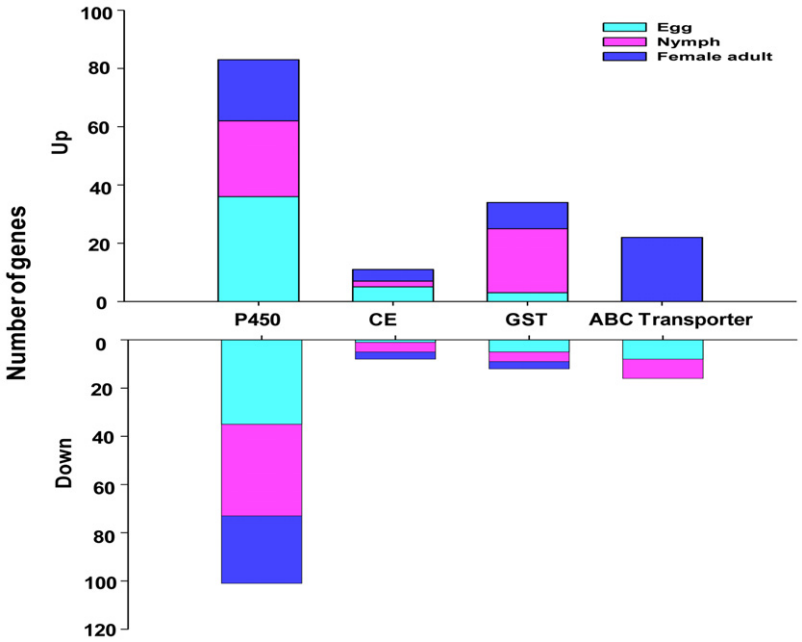

Figure 4. Expression profiles of expressed sequence tags encoding detoxification-related proteins in microarray analysis of a thiamethoxam resistant strain compared to a thiamethoxam susceptible strain. CE, carboxylesterase; GST, glutathione S-transferase; P450, cytochrome P450.

p_07903, which encode different regions of this P450) and is most similar to members of the CYP4C family of P450s. As shown in Fig. 4, overall a higher number of ESTs encoding GSTs were over-expressed in the TH-R strain than under-expressed and this was largely because a high number were over-expressed in the nymph stage (22 of 34 genes). Only a single GST (represented by ESTs p_01371, p_08162 and p_00218) was over-expressed in all three life stages and is most similar to the sigma class of GSTs. This was the most over-expressed GST in the larval and adult stages of the TH-R strain and showed the highest levels of expression at the larval stage (5.6-, 6.6- and 10.3-fold for ESTs p_08162, p_00218 and p_01371, respectively). Although over-expressed in the egg stage (2.3-fold), this GST was not the most over-expressed GST in this stage as an EST (p_00685) encoding a GST of the theta class showed slightly higher levels of over-expression (3.2-fold). As shown in Fig. 4, the greatest number of ESTs encoding CEs were overexpressed in the egg stage, including ESTs (p_00010 and p_00256) corresponding to *B. tabaci COE2* (EF675186). However, these ESTs were not over-expressed in either the nymph or adult stages. In these stages, the ESTs encoding a different CE (p_01300, p_00872 and p_06520) were over-expressed (over sixfold in the adult stage). This was most similar to the published *B. tabaci COE2* sequence but with only 58% sequence identity is unlikely to be the same gene.

Differential expression of 22 ESTs encoding ABC transporters were significantly over-expressed in the adult stage of the TH-R strain whereas eight ESTs encoding ABC transporters were down-regulated in the egg and nymph stages (Fig. 4). These ESTs encode a maximum of 12 unique ABC transporters as bioinformatic analysis revealed several ESTs encode different regions of the same ABC transporter. For example, ESTs p_01016, p_00161, p_03530, p_05190, p_04478, p_07210, p_04758 and p_00242 encode an ABC transporter most similar to the subfamily G member 4-like of *Acyrthosiphon pisum*. Other members of the ABCG subfamily were also well represented in the over-expressed ESTs (see Table S5).

We were able to assign GO terms to 572 of the differentially expressed ESTs in *B. tabaci*. One of the striking results from this analysis is that a large number of the ESTs fall under the category of terms associated with metabolic processes and catalytic activity. This is consistent with our observations that several of the up-regulated ESTs in the resistant subset are cytochrome P450s and GSTs. To investigate the biological functions further, 810 differentially expressed genes were mapped to 221 pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html). Amongst these, 16 pathways were significantly enriched (P-value ≤ 0.05) including 'Drug metabolism – cytochrome P450', and 'Metabolism of xenobiotics by cytochrome P450' (Table 3).

qRT-PCR validation

To validate the microarray data presented above, 14 ESTs significantly differentially expressed in all three life stages were selected for qRT-PCR. Three additional biological replicates per strain and life stage were used to indepen-

Table 1. Validation of microarray results by quantitative reverse-transcription PCR (qRT-PCR)

Probe ID	Gene description	Method	Fold change		
			Egg	Nymph	Female adult
p_00429	Cytochrome c oxidase subunit III	Microarray	34.655	6.142	10.222
		qRT-PCR	71.707**	2.555**	1.192
p_01080	Alpha-amylase	Microarray	2.759	6.407	3.456
		qRT-PCR	24.082*	19.383**	8.461**
p_06403	Maltase-like protein Agm2	Microarray	0.471	4.852	2.19
		qRT-PCR	4.841	19.232**	0.971
p_00337	Cytochrome P450 4C1	Microarray	3.343	0.385	0.355
		qRT-PCR	2.367**	2.183**	0.244**
p_05680	NADH dehydrogenase subunit 4	Microarray	33.39	2.12	2.823
		qRT-PCR	2.12	1.414**	0.503**
p_07435	Protease, reverse transcriptase and RNase H	Microarray	0.231	0.152	0.162
		qRT-PCR	0.070**	0.357**	7.705**
p_01780	Sucrase	Microarray	0.283	0.156	0.312
		qRT-PCR	3.158	0.495**	1.958**
p_00828	Salivary secreted protein	Microarray	12.179	0.497	5.068
		qRT-PCR	4.985**	0.896	0.171**
p_07830	Cytochrome P450 4C1	Microarray	3.759	0.43	0.419
		qRT-PCR	2.827**	1.345	1.971*
p_06169	Cytochrome P450	Microarray	3.192	0.368	0.296
		qRT-PCR	5.286**	2.197**	1.567
p_06381	Cytochrome P450 CYP6CM1vB	Microarray	3.49	3.947	2.987
		qRT-PCR	28.021**	15.597**	7.931**
p_07334	Cytochrome P450	Microarray	2.726	0.35	0.413
		qRT-PCR	4.086**	3.231**	1.351
p_01371	Glutathione S-transferase	Microarray	2.375	5.654	2.668
		qRT-PCR	15.378**	19.205**	2.038
p_06019	Cytochrome P450	Microarray	2.898	0.358	0.401
		qRT-PCR	1.559	4.41**	2.817**

Asterisks denote a significant difference in gene expression between resistant and susceptible *Bemisia tabaci*, as determined by paired *t*-tests (*$P < 0.05$, **$P < 0.001$, fold change ≥ 2 or fold change ≤ 0.5).
NADH, reduced nicotinamide adenine dinucleotide.

dently validate the results. The genes selected covered a range of different expression levels. The expression pattern revealed by qRT-PCR was consistent with that from the microarrays and a good correlation ($r = 0.75$) was observed between the two methods (Table 1). Of the P450s tested by qRT-PCR, CYP6CM1vB showed by far the highest levels of over-expression (7.9–28-fold) in all three stages of the TH-R strain. The level of over-expression of the sigma-type GST encoded by EST p_01371 was also high in qPCR, with the highest level seen in the nymph stage (~18-fold), confirming the up-regulation of this gene in the microarray.

Discussion

Biological and biochemical characterization of the TH-R strain has suggested that enhanced production of metabolic enzymes underlie its resistance to thiamethoxam (Feng *et al*., 2009). However, to date, the genes encoding the specific enzymes involved in the resistance of this strain have not been identified. The aim of this study was to identify candidate resistance genes that are differentially expressed in the TH-R strain compared to the parental strain from which it was selected (TH-S). As previous studies on *B. tabaci* have demonstrated that resistance to the neonicotinoid imidacloprid is stage-specific (Nauen *et al*., 2008), we examined gene expression in three different life stages of the TH-R strain.

Microarray analysis revealed clear, stage-specific transcriptional signatures with a remarkably small cohort of genes significantly differentially expressed in all stages of the TH-R strain compared to TH-S. GO analysis revealed that in all life stages a majority percentage of the differentially expressed transcripts were assigned to the catalytic activity and metabolic process ontologies. Overall, the most highly over-expressed transcripts in each life stage encoded enzymes involved in metabolic processes and/or metabolism of xenobiotics. Several of these over-expressed transcripts encode proteins belonging to the P450, GST and COE families, the three main superfamilies of detoxification enzymes that have frequently been associated with resistance to insecticides in a range of different arthropod species (Li *et al*., 2007).

Exposure of the TH-R strain to the metabolic enzyme inhibitor piperonyl butoxide markedly reduced the resistance of this strain to thiamethoxam, suggesting

Table 2. Expressed sequence tags differentially expressed in microarray experiments in all three life stages of the *Bemisia tabaci* thiamethoxam-resistant strain when compared to the thiamethoxam-susceptible strain

Probe ID	Gene description	Fold change*		
		Egg	Nymph	Female adult
p_00270	Cytochrome P450 4C1	3.347	0.423	0.342
p_00337	Cytochrome P450 4C1	3.343	0.385	0.355
p_00429	Cytochrome c oxidase subunit III	34.655	6.142	10.223
p_00430	Thymus-specific serine protease	0.446	0.414	0.257
p_00712	Cytochrome P450 4C1	3.014	0.449	0.364
p_00828	Salivary secreted protein	12.179	0.497	5.068
p_01080	Alpha-amylase	2.759	6.407	3.456
p_01371	Glutathione S-transferase (GST class-sigma)	2.375	5.654	2.668
p_01426	Heat shock protein 70	0.496	0.277	7.521
p_01769	Protease	0.028	0.2	0.158
p_01780	Sucrase	0.283	0.156	0.315
p_02142	Alpha-amylase, partial	0.445	0.31	0.446
p_02191	Iron dehydrogenase	0.388	0.361	0.441
p_03634	Cytochrome P450	0.425	0.495	0.484
p_04998	Glucose dehydrogenase	2.114	0.471	3.942
p_05497	Cysteine protease	0.427	0.121	0.36
p_05608	Protease	0.023	0.269	0.186
p_05647	Cytochrome *c* oxidase subunit II	14.571	2.158	2.125
p_05670	Cytochrome *c* oxidase subunit 7C	28.279	2.312	4.357
p_05680	NADH dehydrogenase subunit 4	33.389	2.12	2.823
p_05809	NADH dehydrogenase subunit 1	29.665	2.072	9.797
p_06019	Cytochrome P450	2.898	0.358	0.401
p_06169	Cytochrome P450	3.192	0.368	0.296
p_06381	Cytochrome P450 CYP6CM1vB	3.49	3.947	2.987
p_06403	Maltase-like protein Agm2	0.471	4.852	2.189
p_06428	Heat shock protein (hsp70)	0.433	0.229	11.715
p_06906	Heat shock protein 70	0.393	0.203	13.779
p_06918	Alpha-glucosidase	0.419	0.398	0.37
p_07002	Chromosome segregation ATPases-like protein	5.157	4.699	4.809
p_07176	Restnol dehydrogenase	2.431	2.039	2.454
p_07334	Cytochrome P450	2.726	0.35	0.413
p_07336	Protease	0.073	0.155	0.161
p_07435	Protease	0.231	0.152	0.162
p_07830	Cytochrome P450 4C1	3.759	0.43	0.419
p_08139	Alpha glucosidase	0.409	0.317	0.296
p_08232	Protease	0.219	0.198	0.277
p_08377	Heat shock protein 70	0.453	0.232	11.89

*Significant difference ($P < 0.05$, fold change ≥ 2or fold change ≤ 0.5).
NADH, reduced nicotinamide adenine dinucleotide.

involvement of P450s in resistance (Feng *et al*., 2009). The same authors also implicated this enzyme system in resistance by biochemical assay using P450 model substrates. In our study, two cytochrome P450s were amongst the most highly over-expressed genes in different life stages of the TH-R strain. The first of these, *CYP6CM1vB*, was the only P450 highly over-expressed in all life stages of TH-R in both microarray and qPCR experiments. This P450 has been strongly correlated with resistance to imidacloprid in both B and Q biotypes of *B. tabaci* and functionally expressed *CYP6CM1* is able to metabolize the neonicotinoids imidacloprid, clothianidin and thiacloprid (Karunker *et al*., 2008, 2009; Roditakis *et al*., 2011). Interestingly, *CYP6CM1* showed no metabolic activity against acetamiprid or thiamethoxam when functionally expressed in *Escherichia coli* (Roditakis *et al*., 2011). Despite this finding, our expression results, and the fact that the TH-R strain shows strong (47-fold) cross-resistance to imidacloprid (Feng *et al*., 2008), suggest that *CYP6CM1* also plays a role in the resistance of *B. tabaci* to thiamethoxam. One explanation for this apparent paradox is that although *CYP6CM1* may not be able to metabolize thiamethoxam directly, other studies have shown that in insects and plants thiamethoxam is rapidly metabolized to clothianidin, indicating that thiamethoxam is essentially a neonicotinoid precursor for clothianidin, which in turn can be readily metabolized by *CYP6CM1* (Nauen & Elbert, 2003).

Recently, studies of the expression of *CYP6CM1* in the nymph, pupal and adult stages of an imidacloprid-resistant *B. tabaci* strain showed marked age-specific expression of this gene, with *CYP6CM1* mRNA levels highest in the pupal stage (200-fold), also high in the adult stage (24-fold) and low in the nymph stage (~onefold) when compared to a reference susceptible strain (Jones *et al*., 2011). The authors also showed that a reduction in

Table 3. Significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways determined from differential expression associated with thiamethoxam resistance in *Bemisia tabaci*

Pathway	Differentially expressed*	Expressed†	*P*-value	KO
Starch and sucrose metabolism	127	393	0	ko00500
Metabolism of xenobiotics by cytochrome P450	64	378	0.022171	ko00980
Drug metabolism – cytochrome P450	64	376	0.019929	ko00982
Limonene and pinene degradation	61	362	0.027095	ko00903
Galactose metabolism	87	304	3.85E-13	ko00052
Protein processing in endoplasmic reticulum	90	259	0	ko04141
Antigen processing and presentation	82	218	0	ko04612
Endocytosis	60	175	3.09E-13	ko04144
Toxoplasmosis	66	171	0	ko05145
MAPK signalling pathway	64	152	0	ko04010
Spliceosome	61	146	0	ko03040
Carbohydrate digestion and absorption	24	88	0.000176	ko04973
Progesterone-mediated oocyte maturation	14	57	0.007399	ko04914
NOD-like receptor signalling pathway	11	43	0.009771	ko04621
Nicotinate and nicotinamide metabolism	7	16	0.000518	ko00760
Nonhomologous end-joining	1	2	0.018358	ko03450

*The number of differentially expressed genes that belong to each KEGG pathway.
†The number of expressed genes that belong to each KEGG pathway.
KO, KEGG orthology; MAPK, mitogen-activated protein kinase; NOD, nucleotide-binding oligomerization domain.

CYP6CM1 expression in nymphs in this strain was consistent with a concomitant fall in imidacloprid resistance levels between nymph and adult stages. Interestingly, in our study a similar pattern was not observed for the expression of this gene in the TH-R strain, with expression levels highest in the egg stage (28-fold), followed by the nymph stage (16-fold) and then the adult stage (eightfold) when compared to the TH-S strain. It would be interesting in future to compare the resistance of each life stage of the TH-R strain and the TH-S strain to see if stage-specific changes in *CYP6CM1* expression relate to changes in the susceptibility of the two strains to thiamethoxam. An alternative explanation is that these changes are related to the involvement of this gene in a developmental process (eg as the insect undergoes physiological and morphological changes from egg to nymph) in addition to its role in resistance to neonicotinoids.

In the adult stage of the TH-R strain a different P450 was the most over-expressed gene in the microarray analysis. This gene was highly over-expressed (up to 19-fold) and encodes a P450 belonging to the CYP4C family. CYP4-type P450s have been associated with resistance to insecticides previously (Karatolos *et al*., 2012), but to date have not been shown to confer resistance to neonicotinoids. However, a member of this family, *CYP4G36*, is induced in *Aedes aegypti* after treatment with imidacloprid (Riaz *et al*., 2009). It is possible that this P450 is acting in tandem with *CYP6CM1* to confer neonicotinoid resistance and the functional expression of this P450. Its ability to metabolize thiamethoxam and clothianidin (the latter for the reasons detailed above) should be examined in future to investigate this further.

A single gene encoding a GST was over-expressed in all three life stages of the TH-R strain. This GST shared greatest sequence similarity with members of the sigma class of GSTs and qPCR revealed that it was particularly highly over-expressed (>20-fold) in the larval stage of the TH-R strain. Although enhanced expression of GSTs has been shown to be a mechanism of resistance to DDT and organophosphates and has also been implicated in resistance to pyrethroids in certain insects, to date, GSTs have not been associated with resistance to neonicotinoids (Huang *et al*., 1998; Ranson *et al*., 2001; Vontas *et al*., 2001). In addition, previous studies have not been able to demonstrate a synergistic effect using the GST inhibitor diethyl maleate in combination with thiamethoxam against the TH-R strain, and biochemical assessment found no difference in GST enzyme activity between the TH-R and TH-S strains (Feng *et al*., 2009). This study used adult whiteflies for both bioassays and as the enzyme source for biochemical assays; however, the qPCR analysis carried out in our study showed that this GST gene is highly over-expressed in the egg and nymph stages but only slightly over-expressed in the adult stage, so the design of the previous study may have precluded its detection. The involvement of this GST in the resistance of different life stages of the TH-R strain to thiamethoxam clearly warrants further investigation, including functional validation of the ability of this enzyme to metabolize thiamethoxam and/or its primary metabolites.

Thiamethoxam resistance in the TH-R strain is suppressed by the COE inhibitor triphenyl phosphate and total esterase activity is increased ~threefold in this strain compared to TH-S (Feng *et al*., 2009). In the adult and

nymph stages of the TH-R strain a gene encoding a COE was significantly over-expressed compared to the TH-S strain. This gene was most similar to the published *B. tabaci COE2* sequence. Organophosphate resistance in the B-biotype of *B. tabaci* is associated with a point mutation in an ace1-type acetylcholinesterase and over-expression of COEs (Alon *et al*., 2008). However, the authors of this study observed only a twofold increase in expression of *COE2* compared to a susceptible strain, whereas another COE, *COE1*, displayed a higher level (fourfold) of expression. Although the COE gene over-expressed in our study shares similarity with *COE2*, it is not the same gene and represents a novel COE that has not been previously described in *B. tabaci*. In addition to organophosphates, COEs have also been associated with resistance to carbamates and pyrethroids; however, they have never been directly shown to confer resistance to neonicotinoids. Furthermore, certain strains of insects expressing high levels of COEs are not cross-resistant to neonicotinoids, suggesting that this enzyme family may not have the capacity to break down neonicotinoids. This is consistent with the fact that neonicotinoids are not readily hydrolysed at physiological pH values (Jeschke & Nauen, 2008). Nevertheless, selection of the TH-R strain from the TH-S parental strain has clearly driven up the expression of COEs and the elevated expression of this COE2-like gene may be related to the fact that the TH-R strain shows moderate levels of cross-resistance (~fourfold) to the carbamate carbosulfan (Feng *et al*., 2009).

A particularly strong expression signature in the adult stage of the TH-R strain was the over-expression of a high number of ESTs encoding ABC transporters. ABC transporters have been implicated in insecticide resistance in several insect species but the physiological mechanism by which these transporter proteins act to cause resistance has not been clearly defined (Buss *et al*., 2002; Porretta *et al*., 2008; Aurade *et al*., 2010; Gahan *et al*., 2010; Bariami *et al*., 2012). In contrast, in humans ABC transporters have been well studied because of their role in conferring drug resistance in cancer cells (Glavinas *et al*., 2004). The human ABC transporter ABCG2 is thought to play a role in multidrug resistance to chemotherapeutic agents and is expressed at high levels in the intestinal epithelia, in cells of the blood–brain barrier and the placenta, suggesting that its physiological role is to protect cells from potentially toxic substances and to prevent absorption of xenobiotics ingested in the diet by actively transporting compounds from cells (Ejendal & Hrycyna, 2002). In this regard it is noteworthy that members of subfamily G were particularly well represented in the ABC transformers over-expressed in the TH-R strain and the potential role of these transporters in resistance clearly warrants more detailed future investigation.

Summary and conclusions

In this study we have shown that selection of the TH-R strain with thiamethoxam over many generations has induced significant changes in gene expression and that these changes exhibit clear stage-specific signatures. Several putative candidate resistance genes have been identified as over-expressed in the resistant strains. These include two genes encoding P450s, one of which has been shown to confer resistance to other neonicotinoids previously, and genes encoding a GST, a COE and several ABC transporters. The consistency of the over-expression of these genes with thiamethoxam resistance in other *B. tabaci* strains should now be examined. This, combined with functional expression and analysis of the ability of these enzymes to detoxify thiamethoxam and/or other neonicotinoids, will help to identify precisely which confer resistance. Ultimately, the characterization of the precise molecular mechanisms underlying insecticide resistance in different life stages of thiamethoxam-resistant *B. tabaci* will facilitate the development of rational approaches to improve the management of this pest and the development of new insecticides for control.

Experimental procedures

Insect material and sample preparation

Two B-type *B. tabaci* strains, TH-S and TH-R, were used in the microarray experiments and reared as previous described (Feng *et al*., 2009; Xie *et al*., 2012a). The TH-R strain exhibited over 70-fold resistance to thiamethoxam in comparison with the TH-S strain (Xie *et al*., 2012a). Insects were collected from both strains at the following life stages: (1) eggs; (2) fourth instar nymphs; (3) and one-day-old unmated adult females. Insects were frozen at −80 °C and stored for downstream RNA work.

Microarray design

A new *B. tabaci* 8 × 15k Agilent microarray (Agilent Technologies, Palo Alto, CA, USA) was designed for this experiment. The array was designed using the eArray platform (https://earray.chem.agilent.com/earray/) and full details of the array can be found in ArrayExpress (accession number GSE42337). The array design is based on 8394 *B. tabaci* ESTs from gene families with putative association to insecticide resistance. These ESTs are presented in Table S1 and include 2014 ESTs generated from the transcriptome of the imidacloprid-resistant B biotype (Y. Wu *et al*., unpubl. data), 6293 from the *B. tabaci* B and Q biotype transcriptome (X.W. Wang *et al*., 2010; Xie *et al*., 2012b) and 87 from the National Center for Biotechnology Information (NCBI) dbEST database (http://www.ncbi.nlm.nih.gov/dbEST/). In total, the array contains 237 probes replicated 10-fold, 1142 probes in triplicate, 2079 probes as duplicate and 4936 as singular probes.

Microarray labelling and hybridization

Total RNA was isolated from three biological replicates for each life stage of TH-S and TH-R whitefly samples using TRIzol

(Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For replicates of the egg and nymph preparations around 10 mg of fresh material was extracted. For adults around 450 adults were extracted for each replicate. RNA was purified using the QIAGEN RNeasy Kit (Qiagen, CA, USA) according to the manufacturer's instructions. RNA integrity was analysed using gel electrophoresis and the yield determined using a Nanodrop ND1000 (Nanodrop, Thermo Scientific, Wilmington, DE, USA). Double-stranded cDNA was synthesized using a T7 promoter primer and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Agilent Technologies). The synthesized products were used as templates to produce cRNA using the T7 RNA polymerase and then labelled with Cy3 [Cy3 N-hydroxysuccinimide (NHS) ester, GE Healthcare, Pittsburgh, PA, USA]. Amplified cRNAs were purified by using a RNeasy Mini Kit (Qiagen) and resuspended in diethylpyrocarbonate-treated water. cDNA quantity and incorporation of the Cy3 label were confirmed by using the Nanodrop ND1000. In each array comparison, the three biological replicates for each treatment (strain and life stage) were compared using the TH-S samples as the reference. Array hybridization, slide washing and scanning were performed according to the manufacturer's instructions (Agilent Technologies).

Microarray data analysis

Spot finding, signal quantification and spot superimposition were performed using the Agilent FEATURE EXTRACTION software (Agilent Technologies). Normalization and statistical analyses of the data were performed using the Limma test (Smyth, 2004). For each array, the spot replicates of each gene were merged and expressed as median ratios ±SD before ratios were log-transformed. Mean expression ratios were then subjected to a one-sample Student's *t*-test against the baseline value of 1 (equal gene expression in both samples) with multiple testing correction (Benjamini and Hochberg FDR). Statistical significance was assigned to each pairwise comparison between thiamethoxam susceptible and resistant samples within each developmental stage. A FDR of <0.001 and an expression ratio of > twofold or <0.5-fold in either direction (up- and down-regulation) were used as criteria to consider genes differentially expressed between the two strains. The microarray data were deposited into the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE42337.

Functional annotation of identified transcripts was performed using the GO Annotation Database (http://www.ebi.ac.uk/GOA/). GO terms assigned to each gene were categorized by molecular function, biological process and cellular component, and all identified transcripts were mapped to the KEGG database. A hypergeometic test was applied to KEGG orthology (KO) terms to find significantly enriched pathways associated with differentially expressed transcripts from *B. tabaci*. The test was applied against the *B. tabaci* transcriptomic background. The calculating formula for GO and KEGG pathway enrichment analysis was

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

In this formula, N is the number of all transcripts with GO/KO annotation; n is the number of differentially expressed transcripts in N; M is the number of all transcripts that are annotated to the certain GO/KO terms; m is the number of differentially expressed transcripts in M. Pathways were considered significant if $P < 0.05$ after Bonferroni multiple testing correction.

qPCR

An additional extraction of total RNA was performed from all samples for qPCR analysis according to the protocol above. This ensured that the microarray results could be validated on independent biological material. cDNA was synthesized using the SYBR PrimeScript reverse transcription-PCR kit (Takara, Kyoto, Japan). qPCRs were carried out on the ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR green detection following a cycling regime of 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 35 s. A selection of target genes were chosen for validation according to the patterns of stage-specific expression. The primers used for real-time PCR are listed in Table S2. Three biological replicates for each sample were used for qPCR analysis, after which the average threshold cycle (Ct) was calculated for each sample. The relative expression levels between the susceptible and resistance strains at different life stages were calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001) using *NADPH* and *EF-1a* as endogenous control genes (Li *et al*., 2012). The expression stability of these two reference genes was confirmed with *BestKeeper* (Pfaffl *et al*., 2004). Statistical analysis was performed on ΔCt values using a paired Student's t-test.

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Supporting Information

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

Table S1. Gene classification in microarray design.

Table S2. Primers used for qRT-PCR analyses.

Table S3. All of the differentially expressed genes in three developmental stages.

Table S4. Gene ontology analysis of *Bemisia tabaci* in three different developmental stages.

Table S5. Differentially expressed genes related to metabolic detoxification in three developmental stages.