Insect science

Insect Science (2015) 22, 150–156, DOI 10.1111/1744-7917.12106

SHORT COMMUNICATION

Over-transcription of genes in a parathion-resistant strain of mosquito *Culex pipiens quinquefasciatus*

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Abstract Insecticide resistance is an evolutionary adaptation that develops quite quickly in mosquitoes because of the high selection pressure of chemical insecticides, rapid generation time and large population size. Identification of genes associated with insecticide resistance is fundamental to understand the complex processes responsible for resistance. We compared the gene transcriptional profiles of parathion-resistant and -susceptible *Culex pipiens quinquefasciatus* using a combination of suppression subtractive hybridization and complementary DNA (cDNA) microarray techniques. A total of 278 colonies were selected from the resistant-susceptible mosquito subtractive library, 38 of which showed more than two fold stronger immunoblotting signals in the resistant strain than in the susceptible strain using cDNA microarray selection. The sequencing results showed that the 38 colonies can be matched to 12 genes of *C. p. quinquefasciatus*. Eight genes were confirmed to be overexpressed by more than two fold in the resistant strain. These genes encode chymotrypsin-1, theta glutathione S-transferase, lipase 3, larval serum protein 1 β chain, cytochrome *b*, mitochondrial ribosomal large subunit, 28S rRNA, and a protein with unknown function. This study serves as a preliminary attempt to identify new genes associated with organophosphate resistance in this mosquito species and provides insights into the complicated physiological phenomenon of insecticide resistance.

Key words cDNA microarray; chymotrypsin; gene up-regulation; insecticide resistance; suppression subtractive hybridization; theta glutathione S-transferase

Introduction

The *Culex pipiens* complex is comprised of four subspecies: *C. p. pipiens*, *C. p. pallens*, *C. p. quinquefasciatus* and *C. p. molestus*. They are the most geographically widespread and medically important vectors of human disease (Hickner *et al*., 2013). Mosquito-borne tropical diseases, such as malaria, dengue and filariasis, continue to be significant causes of morbidity and mortality in several countries (Shetty *et al*., 2013). Chemical control is the main method to control mosquitoes, and previous resistance investigations showed that mosquitoes in China had become resistant to organophosphates (OP), organochlorines and pyrethroids (Cui *et al*., 2006a; Liu *et al*., 2006). Emergence and severity of resistance problems shorten the lifespan of currently available insecticides and undermine the efficacy of newly developed insecticides because of cross-resistance and multiple resistance mechanisms.

Understanding the molecular basis of resistance mechanisms is crucial to develop new insecticidal compounds and to design novel resistance management strategies. Resistance mechanisms in mosquitoes can be divided

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into two groups: metabolic resistance (degradation of the active ingredient by detoxification enzymes) and target-site resistance (mutations in the protein targets of insecticides). The well-known detoxification enzymes include cytochrome P450 monooxygenases (oxidative metabolism), glutathione S-transferases (GSTs; conjugations and dehydrochlorination) and carboxylesterases (CCEs; ester bond hydrolysis and sequestration) (Hemingway *et al*., 2004). Insecticide targets are composed of the synaptic acetylcholinesterase (AChE) encoded by ace gene, the γ-aminobutyric acid (GABA) receptor encoded by *Rdl* and the voltage-dependent sodium channel encoded by *kdr* (Tantely *et al*., 2010). The known OP resistance mechanisms in *C. pipiens* complex include 13 resistance alleles of CCEs that amplify on the genome (Raymond *et al*., 1998; Raymond *et al*., 2001; Cui *et al*. 2006b, 2007a; Ben Cheikh *et al*., 2008, 2009) and the insensitive AChE resulting from the G119S mutation (Weill *et al*., 2003).

Overexpression is the main mechanism by which detoxification enzymes confer insecticide resistance in mosquitoes (Raymond *et al*., 1998; Ranson *et al*., 2001; David *et al*., 2005; Cui *et al*., 2007b). Large-scale screening of differentially expressed genes between a resistant and a susceptible strain by microarray (David *et al*., 2005; Zhu *et al*., 2010), transcriptome sequencing (David *et al*., 2010; Yan *et al*., 2012) or suppression subtractive hybridization (SSH) (Wu *et al*., 2004; Liu *et al*., 2007) has become popular and indispensable for the identification of new genes involved in insecticide resistance. In this study, a suppression subtractive library was constructed to screen and compare the up-regulated genes in parathion-resistant and -susceptible strains of *C. p. quinquefasciatus.* Eight genes were identified to be overexpressed by at least two fold in the resistant strain after sequential verifications of SSH by complementary DNA (cDNA) microarray and real-time polymerase chain reaction (PCR). Most of these eight genes have not previously been implicated in insecticide resistance. The present results provide insights into the new molecular mechanisms of insecticide resistance in mosquitoes.

Materials and methods

Mosquito strains

Mosquito strains of *C. p. quinquefasciatus* included S-lab and R-SG. The former is an OP-susceptible strain that was reared at the laboratory without any insecticide contact for many years (Georghiou *et al*., 1966). The latter, a field population collected in Foshan, Guangdong Province in 2007, was treated at the fourth instar of every generation with parathion at the laboratory (Institute of Zoology, Chinese Academy of Sciences, Beijing, China) for 2 years. A parathion-resistance of R-SG was 770-fold that of S-lab before use in this study determined by bioassays on fourth-instar larvae and the G119S mutation in the *ace-1* gene was detected in the R-SG by sequencing. The mosquitoes were maintained at 26 $\pm$ 1 °C and a long-day photoperiod (14 h light/10 h dark cycle).

SSH

Total RNA of 300 fourth-instar larvae from each mosquito strain was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with TURBO DNA-free kit (Ambion, Austin, TX, USA) to remove genomic DNA contamination. After assessing RNA quality with a Nanodrop spectrophotometer, high-quality cDNA was synthesized using the Super SMART^{TM} PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA) following the manufacturer's protocol. After cDNA purification with NucleoSpin Extract II Kit (Clontech, Palo Alto, CA, USA), SSH was performed using cDNA synthesized from the resistant strain (R-SG) as the tester and cDNA from the susceptible strain (S-lab) as the driver. The tester and driver cDNA fragments were hybridized with the PCR-Select^{TM} cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA). The cDNA fragments that were more abundant in the tester were selectively amplified according to the manufacturer's directions. To estimate the efficiency of subtraction, the abundance of β-actin cDNA fragments was compared before and after subtraction by PCR amplification for 15, 18, 21, 24 and 27 cycles with primers β-actin-For and β-actin-Rev (Table 1). The PCR products were examined on a 2% agarose/ethidium bromide gel.

cDNA microarray analysis

The final PCR products from the subtractive library were inserted into the pMD19-T Simple vector (Takara, Otsu, Shiga, Japan) and used to transform the Top 10 chemically competent cells (TIANGEN, Beijing, China). Positive clones were identified by PCR amplification using Nested PCR Primer 1 and Nested PCR Primer 2R provided by the PCR-Select^{TM} cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA). The PCR conditions were as follows: initial denaturation at 94 °C for 8 min; 30 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min; and final extension at 72 °C for 5 min. The PCR products were denatured for 10 min at 95 °C and then cooled on ice. We spotted 1 μL of denatured

Table 1 Primer sequences and anticipated product sizes in real-time polymerase chain reaction.

Clone no.	Sequence	Product size (bp)
10	For: AGCAGGAATACCACGACGAT	156
	Rev: GGGAATGAAGGTGTTCGTTG	
24	For: ACAATCGACCTGCAATAC	138
	Rev: GAAACCCTGGCATAAACT	
37	For: ATAAACTCCTCGTCCTACAT	145
	Rev: TACAGCAGACATTGAAACAG	
59	For: CCATAGAGGGTGATAGGC	127
	Rev: GTCTCGTGGTGATATTTAGC	
118	For: CCTTAGGGATAACAGCGTA	174
	Rev: GAAACCAACCTGGCTTAC	
125	For: TTCCCGCACAATTAGAGG	113
	Rev: CTACAAAATTCGGCGTCC	
175	For: GCTGACCAGGGTATCGTT	119
	Rev: GTTGGTGCCGAATAGTGC	
178	For: ATGGCAAGGTCGTCAAGAAC	195
	Rev: GCAGGAAGATCAGCTCAACC	
210	For: GTCGTTCGTGCTGTGGTA	232
	Rev: CAACTCGCAAGAGGTCAA	
251	For: ATGGGACATCGGTAGGAG	137
	Rev: CGATTGGAGCAAGGTTAG	
252	For: ACTGCTCACCTCCCTTGT	165
	Rev: TGTTCTGTTCCGTGTTGC	
272	For: CTTTGTGCTGCACTATCAA	110
	Rev: TCAACTAGAATGTGGCTTTT	
β-actin	For: CGGGTATTGTGCTGGACTC	214
	Rev: GCGACGTAGCACAGCTTCT	

PCR product of each positive clone on 10 cm × 10 cm Hybond-N + nylon membranes (Amersham Pharmacia, Piscataway, NJ, USA) at three different positions as technical replicates. The PCR product of β-actin was spotted as a positive control and the PCR mixture without templates was used as negative control.

cDNA fragments from S-lab and R-SG strains were labeled with Digoxigenin-11-dUTP using DIG High Prime DNA Labeling and Detection Starter Kit (Roche, Basel, Switzerland) and hybridized at a final concentration of 25 ng/mL with the nylon membranes spotted with positive clones of subtractive genes at 42 °C overnight, respectively, according to the manufacturer's instructions. The membranes were photographed after immunological detection. The clone spots were quantified with ImageJ (http://rsb.info.nih.gov/ij) and normalized by background subtraction chosen from areas where no PCR product was spotted. The candidate clones with a differential expression greater than two fold between the resistant and susceptible strains were sequenced and further verified by real-time quantitative reverse transcription (RT)-PCR. Three biological replicates were carried out. Sequencing results were blasted against a non-redundant protein database and nucleotide database in NCBI (http://www.ncbi.nlm.nih.gov).

Real-time quantitative RT-PCR

Total RNA of 50 fourth-instar larvae from S-lab or R-SG strain was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and was reverse-transcribed to cDNA using Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA) following the manufacturer's protocol. The transcriptional levels of 12 genes verified by cDNA microarrays were compared in the two strains by real-time quantitative PCR. PCR was carried out in 20 μL of reaction agent composed of 1 μL of template cDNA, 10 μL of 2× SYBR Green PCR Master Mix (Takara, Otsu, Shiga, Japan), and 0.25 μmol/L of each primer (Table 1) on a Stratagene Mx3000P thermal cycler. The thermal cycling conditions were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 20 s, 58 °C for 20 s, and 68 °C for 40 s. To exclude the presence of unspecific products, a melting curve analysis of products was performed routinely after amplification by high-resolution data collection during an incremental temperature change from 55 °C to 95 °C with a ramp rate of 0.2 °C/s. β-actin was chosen as an internal control to normalize cDNA samples. Three biological replicates and three technical replicates for each biological replication were analyzed for each gene. Fold change between the two strains was analyzed with the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

Results

Suppression subtractive library

A total of 278 colonies were obtained using the cDNA of a parathion-resistant strain as the tester and that of the susceptible strain as the driver, among which 213 colonies showed a single band after PCR amplification. The lengths of the PCR products ranged from 200 bp to 750 bp. As shown in Figure 1, SSH was efficient. The β-actin PCR product was discernible after 27 amplification cycles with the subtractive template compared with 18 cycles with the non-subtractive template.

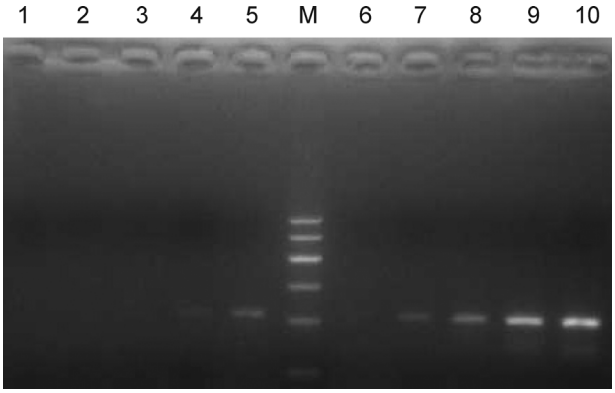

Fig. 1 Reduction of β-actin abundance by polymerase chain reaction (PCR)-select subtraction to show the subtraction efficiency. PCR was performed on the subtracted (Lanes 1–5) or unsubtracted (Lanes 6–10) secondary PCR product with β-actin-For and β-actin-Rev primers (Table 1). Lanes 1 and 6, 15 cycles; Lanes 2 and 7, 18 cycles; Lanes 3 and 8, 21 cycles; Lanes 4 and 9, 24 cycles; Lanes 5 and 10, 27 cycles. M, marker.

cDNA microarray selection

The PCR products of the 213 positive colonies from SSH were hybridized with cDNA probes prepared from the resistant and susceptible strains, respectively (Fig. 2). The immunoblotting signals from 38 colonies were more than two fold stronger in the resistant strain than in the susceptible strain. The sequencing results showed that the 38 colonies can be matched to 12 genes of *C. p. quinquefasciatus* in NCBI, encoding chymotrypsin-1, HzC4 chymotrypsin, phosphorylase, theta GST, lipase 3, sterol carrier protein 2, larval serum protein 1 β chain, cytochrome *c* oxidase subunit I, cytochrome *b*, mitochondrial ribosomal large subunit, 28S rRNA and a protein with unknown function. Out of the 38 colonies, 23 were the transcripts of the gene with unknown function (Table 2).

Real-time PCR confirmation

The transcriptional levels of the 12 differentially expressed genes between parathion-resistant and susceptible strains were verified by real-time PCR. The results showed that eight genes were up-regulated by more than two fold in the resistant strain: genes of chymotrypsin-1, theta GST, lipase 3, larval serum protein 1 β chain, cytochrome *b*, mitochondrial ribosomal large subunit, 28S rRNA and a protein with unknown function. The highest up-regulation was observed in the gene of chymotrypsin-1, which was overexpressed by approximately 53-fold in the resistant strain, followed by the gene of theta GST with a 19-fold over-expression in the resistant strain (Table 2).

Discussion

Insecticide resistance is a complicated physiological phenomenon and multiple genes or pathways are involved. This study serves as the first attempt to identify the genes

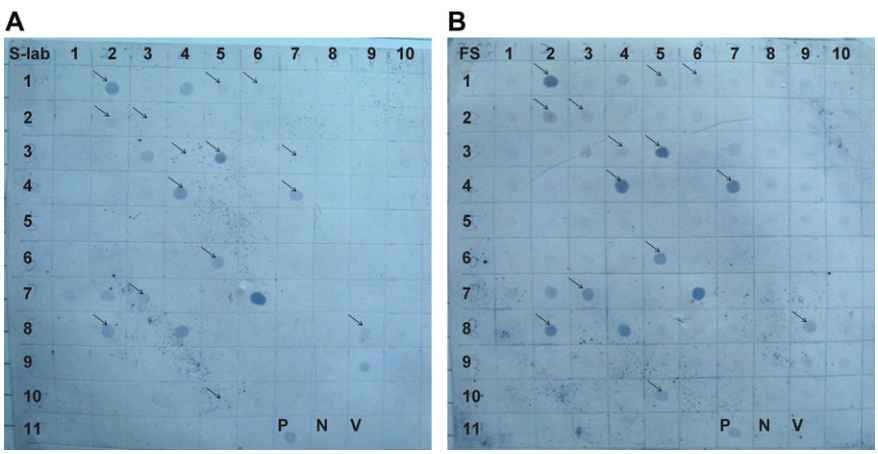

Fig. 2 Complementary DNA microarray for the susceptible strain S-lab and the parathion-resistant strain FS. The polymerase chain reaction (PCR) products of the 213 positive colonies from suppression subtractive hybridization (SSH) (only 100 colonies are shown) were hybridized with the cDNA probes prepared from the susceptible (A) and resistant (B) strains, respectively. Arrows indicate the colonies in which immunoblotting signals were more than two fold stronger in the resistant strain than in the susceptible strain. The PCR product of β-actin was spotted as positive control (P), and the PCR mixture without templates was used as negative control (N). The area where no PCR product was spotted was chosen for signal normalization (V).

Table 2 Genes up-regulated in a parathion-resistant strain of *Culex pipiens quinquefasciatus*.

Clone no.	Size of PCR product (bp)	No. of identical clones	Putative product	Accession no. in GenBank	Fold change in real-time PCR
10	630	1	Cytochrome *c* oxidase subunit I	ACY69607	1.9
24	603	1	Chymotrypsin-1	XP001843394	53.6**
37	492	2	Cytochrome *b*	YP001649173	2.1*
59	439	23	Unknown protein	XP001865960	3.2**
118	251	2	Mitochondrial ribosomal large subunit	DQ401444	3.0**
125	546	1	Phosphorylase	XM001846334	1.3
175	513	1	Theta glutathione S-transferase	XM001868740	19.1**
178	541	1	Sterol carrier protein 2	XP001862602	1.4
210	629	1	Lipase 3	XP001844309	2.9*
251	300	3	Hzc4 chymotrypsin	XP001843407	1.0
252	418	1	Larval serum protein 1 β chain	XP001869047	2.3
272	233	1	28S rRNA	X93384	3.6**

*$P \leq 0.05$; **$P \leq 0.01$. PCR, polymerase chain reaction.

up-regulated in parathion-resistant mosquitoes using a combination of SSH and cDNA microarray techniques. Twelve genes were screened, among which eight were overexpressed by at least two fold in the resistant strain compared with the susceptible strain by real-time PCR. These genes encode chymotrypsin-1, theta GST, lipase 3, larval serum protein 1 β chain, cytochrome *b*, mitochondrial ribosomal large subunit, 28S rRNA and a protein with unknown function. Apart from the function of GST in insecticide resistance, the function of most of these genes in insecticide resistance has not yet been reported.

The gene, chymotrypsin-1 (XP001843394), is the most significantly over-transcribed gene in a parathion-resistant strain detected by SSH. Chymotrypsin is a proteolytic enzyme (serine protease) activated in the presence of trypsin (Wilcox, 1970). Chymotrypsin functions in the digestive systems of many organisms, such as in female adults of *Aedes aegypti* (Jiang *et al*., 1997), and metabolizes endogenous and xenobiotic peptide compounds. Blood feeding and pathogen infection can stimulate chymotrypsin expression in insects (Bao *et al*., 2008; Bian *et al*., 2008; Zhang *et al*., 2009). Chymotrypsin overexpression was also detected in pyrethroid-resistant mosquitoes, such as deltamethrin-resistant strains of *Culex pipiens pallens* using SSH (Wu *et al*., 2004; Gong *et al*., 2005). One possible hypothesis for the role of chymotrypsin is that chymotrypsin can sequester or degrade insecticide molecules in a similar or different way from cleaving peptide amide bonds. Further biochemical tests are required to verify this hypothesis.

GST is one of three detoxification families implicated in insecticide metabolism in insects. Elevated GST activity has been associated with resistance to all the major classes of insecticides (Enayati *et al*., 2005). The most common resistance mediated by GSTs in mosquitoes is to dichlorodiphenyltrichloroethane through increased rates of dehydrochlorination as found in *Ae. aegypti*, *Anopheles gambiae* and *An. dirus* (Grant *et al*., 1991; Prapanthadara *et al*., 1993; Prapanthadara *et al*., 1996). A total of 35 cytosolic GST genes are present in *C. p. quinquefasciatus* (Yan *et al*., 2012). The insect-specific Delta- and Epsilon-class GSTs contain the majority of GSTs associated with insecticide detoxification (Ranson *et al*., 2001). In this study, a theta GST was found up-regulated by 19-fold in a parathion-resistant strain compared with the susceptible strain of *Culex* mosquitoes. This paper is the first to report on theta GSTs putatively involved in resistance to OP insecticides in insects. However, their physiological functions *in vivo* remain unclear. In addition, the detoxification of parathion and methyl parathion by GSTs in the diamondback moth *Plutella xylostella* was described through dearylation of these two OPs instead of dealkylation (Chiang & Sun, 1993). Whether the metabolic mechanism of this theta GST toward parathion is similar to that of the diamondback moth needs further investigation.

Mitochondria and ribosomes react to insecticide selection. Cytochrome *b* is a component of respiratory chain complex III, also known as bc1 complex or ubiquinol-cytochrome-c reductase in the mitochondria of eukaryotes, participating in electron transport and adenosine triphosphate generation (Esposti *et al*., 1993). Although cytochrome *b* is thought not to be directly involved in insecticide resistance, cytochrome *b* overexpression in parathion-resistant mosquitoes indicates that mitochondrial respiration increases when resistant mosquitoes

metabolize more insecticide molecules, which consume more energy compared with susceptible mosquitoes. Apart from the influence on mitochondria, insecticides also affect the ribosome, an organelle where proteins are synthesized. In a parathion-resistant strain of *C. p. quinquefasciatus*, mitochondrial ribosomal large subunit and 28S rRNA are over-transcribed by approximately 3- and 3.6-fold respectively. In the pyrethroid (deltamethrin and permethrin)-resistant strains of *C. p. pallens* and *C. p. quinquefasciatus*, mitochondrial ribosomal large subunit, 16S rRNA, and many ribosomal proteins such as L19, L22, L38 and L39 are also up-regulated (Wu *et al*., 2004; Liu *et al*., 2007). This ribosomal reaction suggests that mosquitoes could promote metabolism and protein synthesis to minimize the damage from insecticides.

The absence of esterase A and esterase B, which are two well-known detoxification genes for OP resistance in the *C. pipiens* complex (Raymond *et al*., 1998; Cui *et al*., 2006b), from the double screening of SSH and cDNA microarray, indicates that the selective system is defective. This defect may be attributed to the polymorphism among the alleles of these two genes, which can be as low as 89% (Cui *et al*., 2007a), thereby impairing the hybridization efficiency.

Conclusions

In conclusion, this study reveals the complexity of insecticide resistance and provides insights into the molecular mechanisms governing the development of insecticide resistance. The relationship of these genes with insecticide resistance must be verified to exclude the noise from the difference of genetic backgrounds between the two strains and to characterize their functions in future.

Acknowledgments

This study was financially supported by the National Natural Science Foundation of China (Grant No. 31272364) and the Important National Science & Technology Specific Project of China (Grant No. 2012ZX10004219).

Disclosure

This manuscript and its authors are not involved in any potential conflicts of interest, including financial interests and relationships and affiliations.

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Accepted December 10, 2013