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Transcriptome Sequencing and Analysis of Changes Associated with Insecticide Resistance in the Dengue Mosquito (*Aedes aegypti*) in Vietnam

Nguyen Thi Kim Lien,[1]* Nguyen Thi Hong Ngoc,[2] Nguyen Ngoc Lan,[1] Nguyen Thu Hien,[1,3] Nguyen Van Tung,[1,3] Nguyen Thi Thanh Ngan,[1] Nguyen Huy Hoang,[1,3] and Nguyen Thi Huong Binh[2]

[1]*Institute of Genome Research, Vietnam Academy of Science and Technology, Hanoi, Vietnam;* [2]*National Institute of Malariology Parasitology and Entomology, Hanoi, Vietnam;* [3]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam*

Abstract. The mosquito *Aedes aegypti* is a transmission vector for dangerous epidemic diseases in humans. Insecticides have been used as the most general vector control method in the world. However, *Ae. aegypti* have developed many resistant mechanisms such as reduced neuronal sensitivity to insecticides (target-site resistance), enhanced insecticide metabolism (metabolic resistance), altered transport, sequestration, and other mechanisms. It has become a major problem for vector control programs. Transcriptome sequencing and bioinformatic analysis were used to compare transcription levels between a susceptible strain (Bora7) and a resistant strain (KhanhHoa7) collected from the field. A total of 161 million Illumina reads, including 66,076,678 reads from the Bora7 strain and 69,606,654 reads from the KhanhHoa7 strain, were generated and assembled into 11,174 genes. A comparison of the KhanhHoa7 transcriptome to that of Bora7 showed 672 upregulated genes and 488 downregulated genes. We identified the highly upregulated genes: cytochrome P450 *4C1*, *4C3*, *4C21*, *4D1*, *4D1* isoform X2, *4D2*, *4D2* isoform X2, *4G15*, *6A2*, *6A8*, *6D3*, and *9E2*; Glutathione S transferase (GST1), UGT1-3, 1-7, 2B15, and 2B37; binding cassette transporter (ABC) transporter F family member 4 and ABC transporter G family member 20. Interestingly, there was a significant increase in the expression of the genes such as *CYP9E2* (8.3-fold), *CYP6A8* (5.9-fold), *CYP6D3* (5.4-fold), *CYP4C21* (5.4-fold), *CYP4G15* (5.2-fold), *GST1* (3.5-fold), and ABC transporter 4 (2.1-fold). Our results suggested a potential relationship between the expression of the genes in metabolic processes and insecticide resistance in the studied strain. These results may contribute to the understanding of the mechanisms of insecticide resistance in *Ae. aegypti*.

INTRODUCTION

The mosquito *Aedes aegypti* is a transmission vector for dangerous epidemic diseases in humans, such as dengue, yellow fever, and chikungunya.[1] In particular, dengue is the most dangerous disease, affecting 50 million people per year, and the number of affected people has increased 30-fold in the last 50 years.[1,2] Insecticides have been used as the most general vector control method in the world. However, excessive insecticide use has led to the development of resistance in mosquitoes and has become a major problem for vector control programs.

Two major mechanisms of resistance are reduced neuronal sensitivity to insecticides (target-site resistance) and enhanced insecticide metabolism (metabolic resistance). Target-site resistance caused by mutation in voltage-gated sodium channels (VGSC) is known as knockdown resistance.[3] Metabolic resistance involves detoxification enzymes such as cytochrome P450 monooxygenases (P450s or CYPs for genes), carboxy/cholinesterases, glutathione S-transferases (GSTs), and UDP-glucosyltransferases.[4–7] Many studies showed that resistance of mosquitoes to pyrethroids is linked to the induction of detoxification genes.[8–10]

Monooxygenase-mediated metabolism (by activity of cytochrome P450 monooxygenases—P450s) is the most common mechanism in insects to resist insecticides.[11] Cytochrome P450s are known to play an important role in detoxifying exogenous compounds such as insecticides.[11,12] In insects, increased metabolic resistance is a result of enhanced levels of P450 proteins and P450 activity because of the overexpression of P450 genes.[5,13–16]

There are many reports that have demonstrated a relationship between resistance and elevated P450 activity in *Ae. aegypti*. Diversity of resistance is conferred by the existence of multiple isoforms and differential expression of cytochrome P450s.[17] To date, more than 600 P450 genes have been identified in insects. Some genes belonging to the families *CYP4*, *CYP6*, *CYP9*, and *CYP12* are associated with insecticide resistance.[6,18] Whole-genome sequencing has revealed that *Ae. aegypti* has 160 P450-coding genes, and 44 of these belong to the *CYP6* family.[19] *CYP6Z9* was found to be 4-fold overexpressed in a permethrin-resistant strain collected in northern Thailand.[19] *CYP6M6*, *CYP6Z8*, and *CYP6M11* have also been identified as inducible by permethrin and pollutants.[20] The overexpression of *CYP6M6* and *CYP6Z6* was found in the Vauclin strain in Martinique, indicating the involvement of *CYP6* in pyrethroid resistance in *Ae. aegypti*.[20] Bariami et al.[21] detected differences in the expression of cytochrome genes such as *CYP6BB2*, *CYP9J9*, *CYP9J10*, *CYP9J26*, *CYP9J27*, and *CYP9J28* in *Ae. aegypti* pyrethroid-resistant populations from the Cayman Islands and Cuba.

High GST activity related to insecticide resistance has also been reported in many species of insects.[19,22–28] For pyrethroid detoxification, GSTs have no direct role in the metabolism of pyrethroids, but they have the capacity to reduce the peroxidative damage caused by pyrethroids by detoxifying lipid peroxidation products.[29] Glutathione S-transferases can also bind pyrethroid insecticides in a sequestering mechanism as a passive way of detoxification.[30] In mosquitoes, approximately 30 *GST* genes have been identified within different subfamilies.[19,31] Members of the epsilon *GST* subfamily (GSTe7 and GSTe2) have been found in the dehydrochlorination of dichlorodiphenyltrichloroethane (DDT)

*Address correspondence to Nguyen Thi Kim Lien, Institute of Genome Research, Vietnam Academy of Science and Technology 18 Hoang Quoc Viet Rd., Hanoi, Vietnam. E-mails: ntkimlienibt@gmail.com or nklien@igr.ac.vn

TABLE 1

Percentage mortality in field samples of *Aedes aegypti* 24 hours after exposure to insecticide-impregnated papers and resistance status using the WHO test (WHO, 2016)

	Bora strain		KhanhHoa strain	
Insecticides (diagnostic dose)	Mortality (%)	Resistance status	Mortality (%)	Resistance status
Dichlorodiphenyltrichloroethane (4%)	100	S	0	R
Propoxur (1%)	100	S	42	R
Malathion (5%)	100	S	100	S
Deltamethrin (0.05%)	100	S	37	R
Lambda-cyhalothrin (0.05%)	100	S	21	R
Permethrin (0.75%)	100	S	7	R

R = resistant; S = susceptible.

and pyrethroid deltamethrin in *Ae. aegypti*.[24,32,33] However, there are a large number of genes encoding detoxification enzymes,[34–36] so determining the exact enzymes that play the main role in resistance in each mosquito species is a great challenge.[37]

In addition, many studies have indicated that there are potential polymorphisms affecting the function of detoxification enzymes and provided evidence for the roles of these enzymes in insecticide resistance in mosquitoes.[38] Recently, polymorphisms of the P450 gene *CYP9M10* have been demonstrated to be involved in pyrethroid resistance in *Culex pipiens*.[39] This suggested that a deep analysis of the polymorphisms associated with resistance can improve our knowledge of the mechanisms developed by mosquitoes to resist insecticides. Today, thanks to the development of next-generation sequencing technologies such as mRNA sequencing (RNA-seq), the expression and polymorphism of genes involved in insecticide resistance have been evaluated.[40–42]

Vietnam is one of the countries that have the most dengue cases in Southeast Asia with 50,000 cases reported in 2016.[43] To control transmission vectors, Vietnam used a large amount of insecticide with 24 tons of DDT (in 1993 and 1994) and 21,000 L of photo-stable pyrethroid formulations.[44] Every year, this figure is estimated to be 4,000 tons of chemicals belonging to the pyrethroid group, leading to the development of resistance in many mosquito species, including the *Ae. aegypti* mosquito. In the past few years, many reports on the resistance in *Ae. aegypti* have showed that there was a reduction in susceptibility, and many populations were found to develop high resistance to pyrethroid and DDT.[44–50] The aim of this study was to use RNA-seq to investigate transcription levels and analysis of the changes associated with resistance in *Ae. aegypti* in Vietnam.

METHODS

Sample collection and resistance testing. Larvae were collected from natural habitats in Khanh Hoa Province of Vietnam between August and September 2016 and reared into adults under rearing conditions in the insectary. The samples were collected in accordance with the standard procedure of the Ministry of Health, Vietnam. The larvae were collected from clean water containers (rain water or domestic water) of households, 100 households for each sampling location and two locations for each province. Specimens were morphologically identified to species and used in the study.[51] Adult *Ae. aegypti* mosquitoes were reared in standard insectary conditions (27°C ± 2°C, 12 hours/12 hours light/dark, and 70% ± 10% relative humidity) in net cages and fed with sugar water and then starved for 3–4 hours before insecticide tests. Laboratory strain, Bora Bora 52 (originating from Laboratori se Lutte contre les Insectes nuisibles, Montpallier, France) (denoted by Bora7 in this study) was used as a control strain to compare with a resistant insecticide strain (KhanhHoa7).

To determine the resistance level, bioassays were carried out according to the WHO procedure[52] using 1,500 adult females of 2–5-day-old F1 generation with four replicates of 25 mosquitoes per tube. The insecticides tested were 0.75% permethrin (type I of pyrethroid), 0.05% deltamethrin (type II of pyrethroid), 0.05% lambda-cyhalothrin (pyrethroid), 4% DDT (organochlorine), 1% propoxur (carbamate), and 5% malathion (organophosphate). Resistance status was classified according to WHO[52] criteria: resistance for < 90% mortality, probable resistance for 90–98% mortality, and susceptible for > 98% mortality. The resistant mosquitoes were used for transcriptome analysis.

RNA extraction and preparation of cDNA libraries. For each sample, total RNA was extracted from 10 adult mosquitoes using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA quality and quantity were assessed with a NanoDrop instrument (NanoPhotometer – IMPLEN P3000, Munchen, Germany). Ten samples from each strain, representing a total of 100 mosquitoes, were pooled and then used for preparing cDNA libraries using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB#E7490, Illumina, Ipswich, MA) and the NEBNext Ultra DNA Library Prep Kit (NEB#E7370, Illumina, Ipswich, MA). Two replicates of cDNA libraries were prepared for each strain.

Sequencing, read mapping, and gene expression analysis. Sequence reads (100-bp paired end) from each cDNA library were generated in the Illumina HiSeq 2000 instrument. The reads were then mapped to the *Ae. aegypti* genome sequence (AaegL5.0

TABLE 2

Filtered data statistics in studied strains

Sample	Read length	Number of reads	Total read mapped (%)	Number of bases	Q20 (%)	Q30 (%)	GC (%)	*N* (ppm)
Bora	147.89	77,385,050	66,076,678 (85.38)	11,444,233,590	95.55	89.13	34.57	4.64
KhanhHoa	147.66	83,870,558	69,606,654 (82.99)	12,384,682,936	95.14	88.21	40.02	4.44

GC% = percentage of G + C in the read; *N* (ppm) = number of base "*N*" per million bases; Q20, Q30 = percentage of bases with quality scores (Qphred) higher than 20 or 30, respectively.

TABLE 3
Gene expression level in studied strains

Sample	The number of gene expression in different levels (reads per kilobase of exon model per million reads)					
	0–0.1	0.1–1	1–3	3–15	15–60	> 60
Bora7	561 (4.78%)	2,603 (22.58%)	2,403 (20.84%)	3,814 (33,08%)	1,486 (12.89%)	661 (5.73%)
KhanhHoa7	574 (4.72%)	2,031 (16.71%)	1,762 (14.49%)	4,644 (38.20%)	2,257 (18.56%)	890 (7.32%)

gene set, http://vectorbase.org) using the TopHat algorithm with default parameters (http://tophat.cbcb.umd.edu, v1.0.14),[53] leading to an average mapping rate of 80%. The reads were first filtered based on the sequence quality (mean read quality ≥ 30 and with Ns < 10%) and mapping quality (alignment score ≥ 98). For the gene ontology (GO) annotation of the unigenes, the BLAST2GO program (https://www.blast2go.com/) was used.[54]

Transcript abundance of the unigenes in each sample was calculated and normalized using the fragments per kilobase million (FPKM) method.[55] Differentially expressed gene (DEG) analysis was performed using the DEGseq R package with a threshold of |log2 (fold change)| > 1 and corrected *P*-value < 0.05.[56] Gene ontology enrichment analysis was performed by mapping the DEGs to the GO database, and the gene numbers from each GO term were calculated as compared with the genomic background.[57] Visualization of the clusters of the DEG expression pattern was performed using the MultiExperiment Viewer (MEV; ver. 4.9).

Single nucleotide polymorphism/indel analysis. The mpileup function in SAMtools (v0.1.18)[58] was used to compare each sample with the reference genome for single nucleotide polymorphism (SNP) detection. The correlation between mutation and gene information can be derived based on the annotated gene information in the database, enabling annotation of the mutation site. Single nucleotide polymorphism alleles were computed between the insecticide-resistant and susceptible strains.

RESULTS

Insecticide resistance levels. The insecticide resistance levels of the Bora strain and the mosquito strain collected from Khanh Hoa are shown in Table 1. The results indicated that the Bora strain was sensitive to all types of insecticide and the KhanhHoa strain was sensitive to organophosphate but strongly resistant to organochlorine (DDT), carbamates, and pyrethroids. Twenty-four–hour mortalities were 37% for deltamethrin, 21% for lambda-cyhalothrin, and 7% for permethrin. These results suggested that insecticide resistance was present in the KhanhHoa population at high levels, and the KhanhHoa7 strain was hence used for transcriptome analysis.

Identification of DEGs in strains. More than 161 million 150-bp cDNA reads were sequenced across two samples (77,385,050 in Bora7 and 83,870,558 in KhanhHoa7). More than 80% of the reads were successfully mapped to the *Ae. aegypti* genome, including 11,444,233,590 bases from Bora7 and 12,384,682,936 bases from KhanhHoa7. Filtering for sequence quality and mapping score (Table 2) revealed the percentage of bases with quality scores (Qphred) higher than 20 or 30. Mapped reads were assigned to genomic features—exons, introns, and intergenic regions. For the KhanhHoa7 strain, the percentages of reads mapped to exonic, intergenic, and intronic regions were 52.08%, 14.84%, and 33.08%, respectively. These percentages were 41.17% (exonic region), 24.77% (intergenic region), and 34.06% (intronic region) in the Bora7 strain. These reads were generated and assembled into 11,174 genes. We also identified 3,440 novel transcripts, which may indicate that novel genes are specifically expressed in resistant strains. RNA-seq sequence data have been deposited at NCBI under the accession number PRJNA429544.

Analysis of differences in transcription was performed on 11,528 and 12,158 transcripts showing a transcription signal

TABLE 4
Annotation for the genes involved in insecticide resistance

Gene name	AAEL annotation	Physical positions of the genes	Fragments per kilobase million ($P < 0.001$)
Cytochrome P450 *4C1* (*CYP4C38*)	AAEL012266	2.384053732–384080949	18.85
Cytochrome P450 *4C3*	AAEL008017	1.74332275–74367682	2.39
Cytochrome P450 *4C21* (*CYP325V1*)	AAEL017136	3.112513177–112534429	15.60
Cytochrome P450 *4D1* (*CYP4K3*)	AAEL007798	3.152871519–152883632	8.64
Cytochrome P450 *4D1* isoform X2	AAEL007807	3.152793074–152802023	5.22
Cytochrome P450 *4D2* (*CYP4AR2*)	AAEL010154	2.380179249–380180990	5.11
Cytochrome P450 *4D2* isoform X2	AAEL007816	3.152716768–152729259	15.79
Cytochrome P450 *4G15*	AAEL006824	1.82804948–82813910	91.34
Cytochrome P450 *6A2* (*CYP6BY1*)	AAEL017539	3.399253843–399255488	4.59
Cytochrome P450 *6A8*	AAEL017061	1.271375696–271377569	23.83
Cytochrome P450 *6D3* (*CYP6Z6*)	AAEL009123	1.2418888478–418890194	27.20
Cytochrome P450 *9E2* (*CYP9J28*)	AAEL014617	3.368627096–368628939	83.21
GST1 (GST3)	AAEL007947	2.351708207–351709186	70.93
UGT1-3	AAEL000687	2.458603992–458605906	15.35
UGT1-7	AAEL010366	2.182796211–182798339	57.27
UGT2B15	AAEL003091	2.213572075–213585955	11.92
UGT2B37	AAEL002688	2.464868089–464912877	23.88
ABC transporter F family member 4	AAEL016973	2.294820170–294833814	31.28
ABC transporter G family member 20	AAEL014428	1.159149149–159331370	6.13

AAEL = *Aedes aegypti* Liverpool strain; GST = Glutathione S-transferase; UGT = UDP-glucosyltransferases.

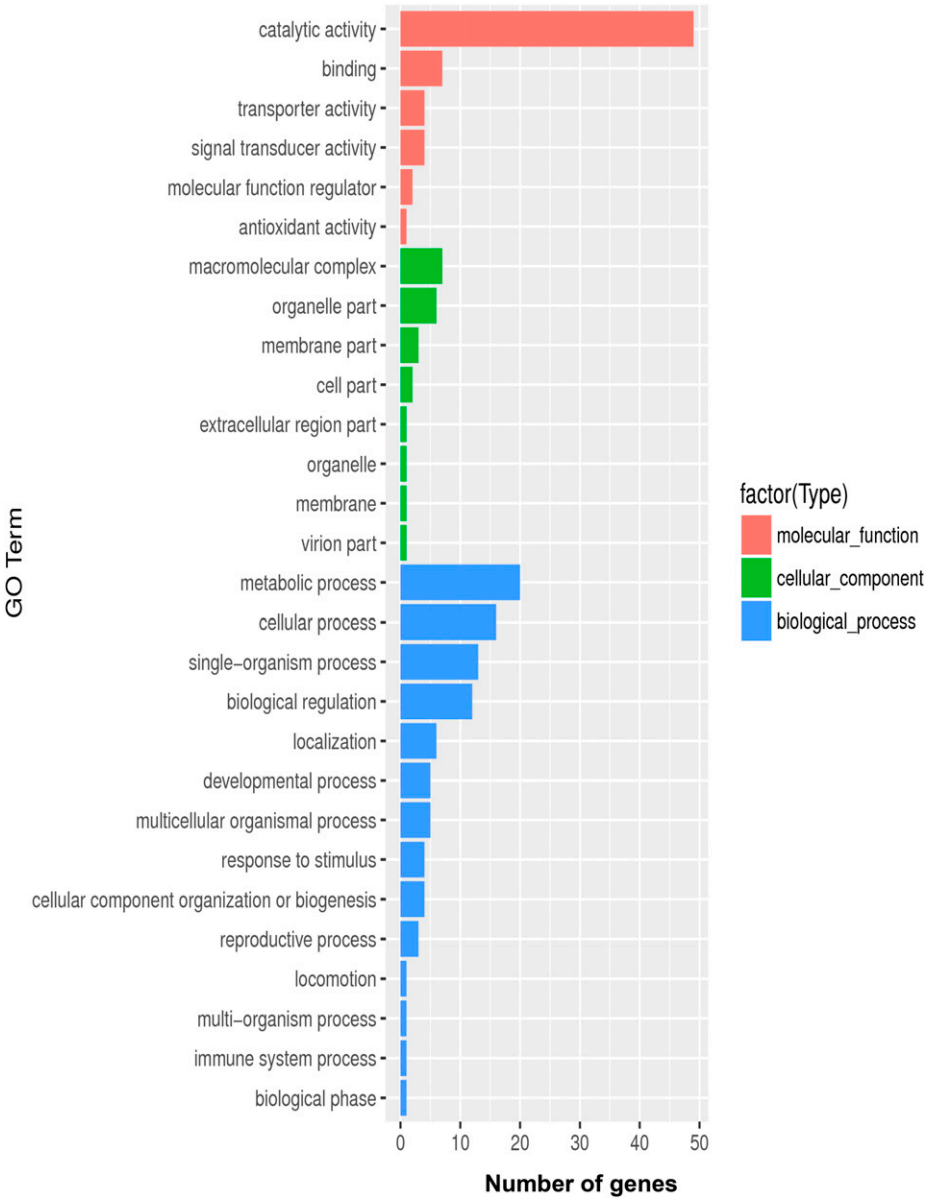

FIGURE 1. Histogram presentation of gene ontology (GO) classification. The results are summarized as three main categories: biological processes, cellular components, and molecular functions. The *x* axis shows the number of the matched genes and the *y* axis shows subgroups of molecular functions from GO classification. This figure appears in color at www.ajtmh.org.

higher than 0.1 Reads per kilobase of exon model per million reads (RPKM) in Bora7 and KhanhHoa7 strains, respectively (Table 3). A total of 1,762 (14.49%) transcripts had RPKM > 1, and approximately 4,644 (38.20%) transcripts (which had RPKM values from 3 to 15), 2,257 (18.56%) transcripts (RPKM values from 15 to 60), and 890 (7.32%) transcripts (RPKM values > 60) were identified in the KhanhHoa7 strain.

A transcriptome comparison between KhanhHoa7 and Bora7 showed 672 upregulated genes and 488 downregulated genes. We identified a large number of genes involved in resistance, including carboxylesterase for three, cuticle protein for 100, esterase for 22, endocuticle structural glycoprotein for 14, cytochrome P450 for 72, GST for 35, larval/pupal cuticle protein for four, larval cuticle protein for 42, probable cytochrome P450 for 69, and UDP-glucuronosyltransferase for 30. These genes were carboxylesterase 1F with an FPKM value of 2.32; esterase B1 with an FPKM value of 28.20; cytochrome P450 *4C1*, *4C3*, *4C21*, *4D1*, *4D1* isoform X2, *4D2*, *4D2* isoform X2, *4G15*, *6A2*, *6A8*, *6D3*, and *9E2* with FPKM values ranging from 2.39 to 147.66; GST 1 with an FPKM value of 70.93; and UDP-glucuronosyltransferase 1-3, 1-7, 2B15, and 2B37 with FPKM values of 15.35, 57.27, 11.92, and 23.88 (P-value < 0.001), respectively (Table 4). In addition, there was increased expression of genes, including ABC transporter F family member 4 and ABC transporter F family member 20, with FPKM values of 31.28 and 6.13 (P-value < 0.001), respectively. However, the cuticle protein genes (including cuticle protein, endocuticle structural glycoprotein, and larval cuticle protein genes) were downregulated in KhanhHoa7 as compared with the susceptible strain Bora7.

These genes exhibited an increase in expression from 1.5- to 8.3-fold (P-value < 0.01) in the resistant strain compared with the control strain. Interestingly, there were significant

TABLE 5
Single nucleotide polymorphism/indel genomic distribution in the studied strains

Region	Number of single nucleotide polymorphisms/indels		Number of genes
	Bora7 strain (%)	KhanhHoa7 strain (%)	
Exonic	93,079 (70.00%)	126,381 (71.01%)	10,781
Intergenic	19,065 (14.34%)	24,044 (13.51%)	–
Intronic	17,609 (13.24%)	23,247 (13.06%)	–
Splicing	41	100 (0.06%)	92
Exonic splicing	17	46	–
Upstream	325 (0.24%)	575 (0.32%)	412
Downstream	2,807 (2.11%)	3,539 (1.98%)	1,685
Up/downstream	22	36	–
Total	132,965	177,968	–

increase in the expression of the genes such as *CYP9E2* (*CYP9J28*) (8.3-fold), *CYP6A8* (5.9-fold), *CYP6D3* (*CYP6Z6*) (5.4-fold), *CYP4C21* (5.4-fold), *CYP4G15* (5.2-fold), *GST1* (3.5-fold), and ABC transporter 4 (2.1-fold). These results suggested that these genes may be related to insecticide resistance in the studied strain. To validate our transcriptome data, three genes, including *CYP4C1*, *CYP6Z6*, *CYP9J28*, were selected randomly for quantitative real time-PCR (qRT-PCR) analysis, using the same RNA samples as for the transcriptome sequencing. The qRT-PCR results showed similar upregulated results based on transcriptome sequencing analysis. The results were 6.60-, 6.72-, and 18.00-fold, respectively, in qRT-PCR.[59]

Functional annotation and classification of the DEGs. Gene ontology functional enrichment analyses can provide information about how DEGs are related to certain biological functions and hence were used to classify genes into the predicted functional groups. In this analysis, ~50,000 transcripts were categorized into 28 functional groups, including biological processes, cellular components, and molecular functions (Figure 1). Among the biological processes, the most abundant groups included metabolic process, cellular process, single-organism process, biological regulation, localization, and development process; within this category, the dominant subcategories were metabolic process, cellular process, single-organism process, and biological regulation. The next most enriched category was molecular function, with the following subcategories: catalytic activity, binding, transporter, signal transducer, molecular function regulator, and antioxidant activity. Last was the group of cellular component functions.

Identification of SNPs/indels and alternative splicing in strains. A total of 117,968 SNPs/indels loci were identified between the KhanhHoa7 strain and the *Ae. aegypti* reference genome. Distribution of these polymorphisms in the genome is shown in Table 5. These variations were distributed with ~70% of them located in exonic, ~13% in intergenic (within 1 kb of gene boundaries), ~13% in intronic, ~0.32% in upstream, and ~2% in downstream regions of the insecticide-resistant strain. More than 164,328 SNPs and 4,517 indels were detected within the exonic region of 7,341 genes; another 4,824 SNPs and 399 indels were detected within the downstream regions of 1,685 genes; 802 SNPs and 29 indels were found in the upstream regions of 412 genes; and 126 SNPs/indels were found in alternative spliced sequences in these strains.

Among them, 1,010 SNPs were found in genes (113 genes) related to resistance, including ABC transporter, acetylcholinesterase, carboxylesterase, esterase, GST, cytochrome P450, and cuticle protein. These data will be useful in understanding the effect of SNPs on resistance in *Ae. aegypti*. However, the role of these SNPs in the genes has not been studied in this study. These results were the same as in the report of David et al.[12] with 220,449 SNPs that were detected in *Ae. aegypti* pyrethroid-resistant strains. In this study, 60%

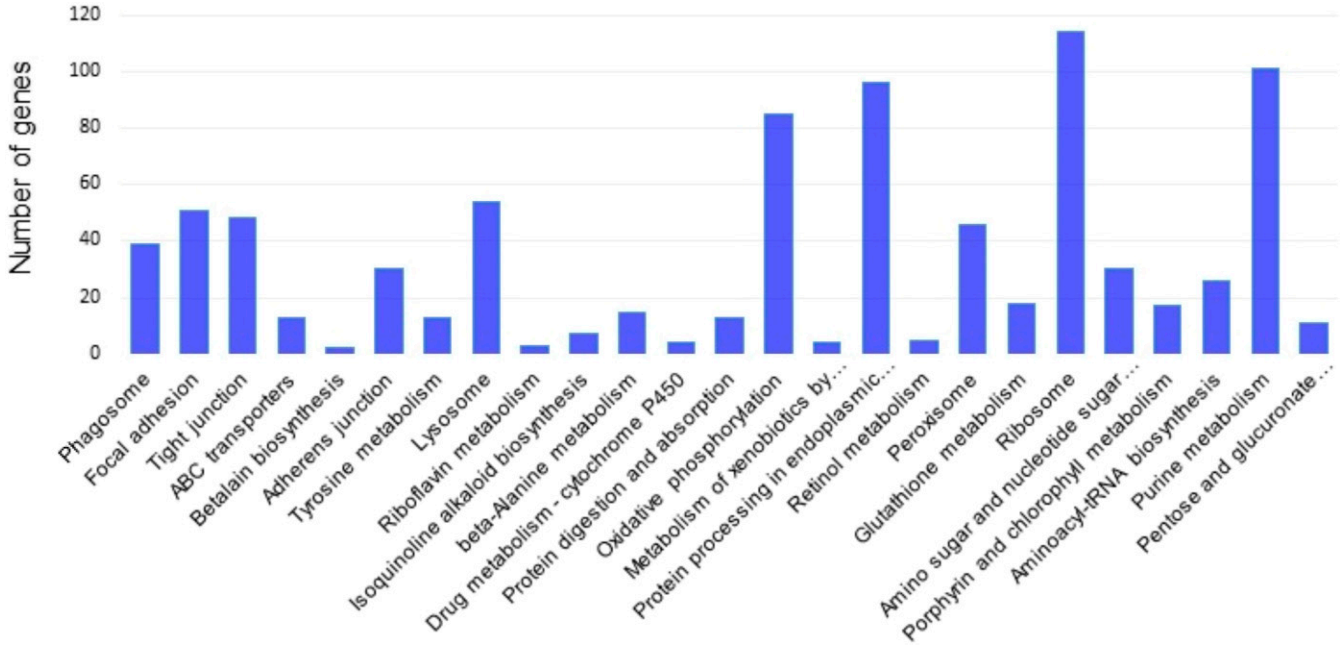

FIGURE 2. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the top 25 pathways is shown. The *x* axis shows pathways from KEGG classification and the *y* axis shows the number of genes. This figure appears in color at www.ajtmh.org.

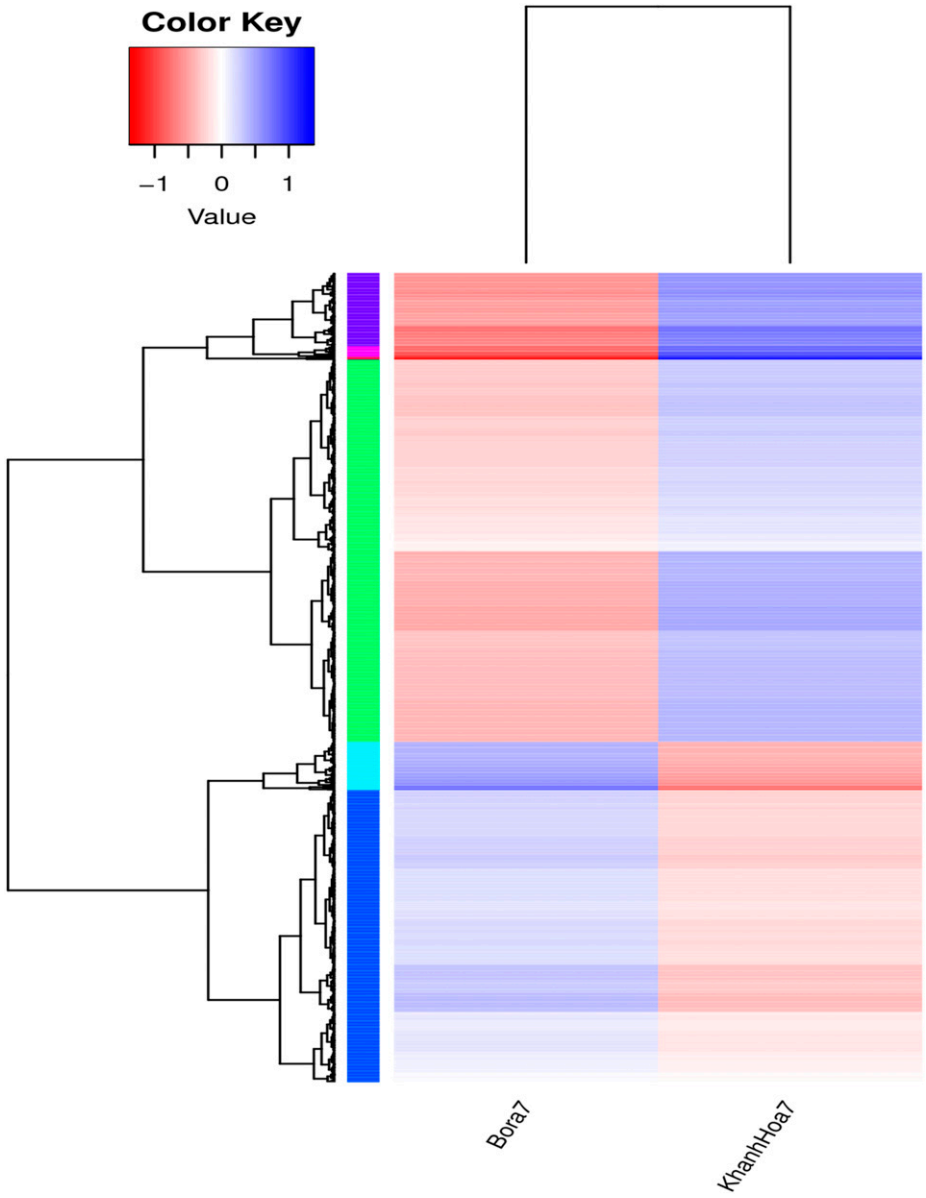

FIGURE 3. Heatmap analysis showing the expression pattern of differentially expressed genes in different strains. Log10 (reads per kilobase of exon model per million reads + 1) values are used for clustering. Intensity of color indicates expression levels, with genes of high expression in blue and low expression in red.This figure appears in color at www.ajtmh.org.

of SNPs were located in coding regions, 20% in gene boundaries, 2.5% in 5′UTR (5′ untranslated region), and 9% in 3′UTR (3′ untranslated region).[12]

DISCUSSION

Studying resistance levels and mechanisms related to resistance in vectors is interesting. In this study, the test results showed that the KhanhHoa7 strain had strong resistance to DDT, carbamates, and pyrethroids. These results were perfectly consistent with those of the previous studies.[46,48,50] In addition, studies have also shown that *A. aegypti* mosquitoes from Khanh Hoa carried mutations (Ile1011Val, Val1016Ile, Val1016Gly, Ala1007Gly, and Phe1558Cys) in the *VGSC* gene. To better understand the mechanisms that lead to the development of resistance in *A. aegypti* mosquitoes, we conducted transcriptome sequencing of susceptible and resistant strains.

High-throughput sequencing approaches such as RNAseq have provided information on gene expression and nucleotide variations over the whole transcriptome of a given sample.[60,61] Transcriptome analysis allows the simultaneous study of the effects of multiple genes and can explain the mechanisms of gene action in different processes, including the enhancement of resistance in mosquitoes. Thus, the metabolic resistance mechanisms are known to enhance the expression levels and activities of enzymes to detoxify and decompose insecticide molecules.[62] Resistance can result in the overexpression of a single enzyme or an active combination of different enzymes.

In mosquitoes, the *CYP6Z* subfamily was previously associated with the response to pyrethroids, carbamates, and organochlorines. Enhanced expression of *Ae. aegypti CYP6Z9*

was found to be 4-fold in a permethrin-resistant strain collected in northern Thailand.[19] *CYP9J23* is also identified as having an increase in expression of 5.3-fold in *Ae. aegypti* larvae due to inducible by permethrin and pollutants.[20] Bariami et al.[21] detected differences in the expression of cytochrome genes such as *CYP6BB2*, *CYP9J9*, *CYP9J10*, *CYP9J19*, *CYP9J26*, *CYP9J27*, and *CYP9J28* (with 2.5 to 14.21-fold change) in *Ae. aegypti* pyrethroid-resistant populations from the Cayman Islands and Cuba. Cytochrome P450 *CYP6Z9* has also been reported to show an increase in expression of more than 20% in *Anophenles gambiae* by Stevenson et al.[63] In addition, elevated levels of *GSTs* have been found to be involved in insecticide resistance, as they are overexpressed in pyrethroid-resistant populations.[12,19,22,23,25–28] Lima et al.[64] reported the expression of *GSTE7* and *CYP6N12* in resistant *Ae. aegypti* from central Amazonia. The roles of the *Ae. aegypti GSTE7* and *GSTE2* genes in increased susceptibility to the pyrethroid deltamethrin were studied recently using RNA interference–mediated knockdown of the genes.[33] Overexpression of ABC transporter 4 in *Ae. aegypti* was reported by Bariami et al.[21] In our study, we also obtained the same results as those in the previous studies with an increase in the expression of the genes involved in the metabolisms.

Remarkably, in our study, the results indicated that there was downregulation of genes encoding cuticle protein in resistant compared with susceptible strains, although cuticular resistance was characterized recently by thickening of the cuticle layer, leading to slower penetration of the insecticide and, thus, reduction in the amount of insecticide within the insect. A similar resistance mechanism has been demonstrated in the cotton bollworm *Helicoverpa armigera* for pyrethroids.[65] Cuticle thickening linked to pyrethroid resistance has also been identified in the oriental fruit fly *Bactrocera dorsalis*.[66] Cuticular resistance has been mentioned often recently in *Anopheles* mosquitoes.[67–71] In *Ae. aegypti*, differential expression of genes encoding cuticle proteins has been reported in mosquito larvae.[60,72] The overexpression of multiple genes encoding cuticle proteins has been found in mosquito larvae that were selected with imidacloprid for several generations in the laboratory.[72] However, Kasai et al.[71] showed that there was no significant difference in the rate of permethrin reduction by the cuticle between resistant and susceptible strains. However, more detailed studies on the role of genes and resistance mechanisms in *Ae. aegypti* mosquitoes are needed.

To analyze the interplay between the up- and downregulated genes, all DEGs were mapped to the referential canonical pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using a cutoff E value of 10^{-5}. The 25 pathways are presented in Figure 2. Distribution analysis of genes defined by KEGG revealed clustering of the DEGs in protein synthesis pathways and metabolism pathways. The six largest pathways were ribosome (ko03010), purine metabolism (ko00230), protein processing in endoplastic reticulum (ko04141), oxidative phosphorylation (ko00190), lysosome (ko04142), and peroxisome (ko04146). These were the main pathways involved in metabolism and detoxification processes. Significant differences in expression patterns between Bora7 and KhanhHoa7 strains were clearly observed in the heatmaps (Figure 3). The cluster analysis of these DEGs between the two strains revealed that the gene expression pattern in the resistant strain was distinctly different from that in the susceptible strain, suggesting that the transcriptional regulations of DEGs for their functions were unique in the resistant strain.

CONCLUSION

In this study, a sample collected from the field was used to compare transcriptional differences with the susceptible strain (Bora7). Using RNA-Seq and DEG analysis, we identified the molecular diversity of genes involved in biological pathways or different expression of genes involved in metabolism and other biological processes. These changes likely resulted from SNPs/indels or differential expression of metabolic pathways. Some candidate genes were revealed, such as cytochrome P450, GST, UDP-glucuronosyltransferase, and ABC transporters. These results suggested a potential relationship between these genes and metabolic processes. However, in our study, there was a downregulation of genes that encoded cuticle proteins.

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Authors' addresses: Nguyen Thi Kim Lien, Nguyen Ngoc Lan, Nguyen Thu Hien, Nguyen Van Tung, Nguyen Thi Thanh Ngan, and Nguyen Huy Hoang, Institute of Genome Research, Vietnam Academy of Science and Technology, Hanoi, Vietnam, E-mails: ntkimlienibt@gmail.com or nklien@igr.ac.vn, ngoclana11108@yahoo.com, thuhienth87@gmail.com, tungnv53@gmail.com, nganthanh27@yahoo.com, and nhhoang@igr.ac.vn. Nguyen Thi Hong Ngoc and Nguyen Thi Huong Binh, National Institute of Malariology Parasitology and Entomology, Hanoi, Vietnam, E-mails: ngocnimpe@gmail.com and nguyenhuongbinh74@gmail.com.

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