Transcriptome Analysis of *Aedes Albopictus* Midguts Infected by Dengue Virus Identifies a Gene Network Module Highly Associated with Temperature

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Abstract

Background: Dengue is prevalent worldwide and transmitted by *Aedes* mosquitoes. Temperature is a strong driver of dengue transmission. However, little is known about the underlying mechanisms.

Methods: *Aedes albopictus* mosquitoes exposed or not to dengue virus (DENV)-2 were reared at 23°C, 28°C and 32°C, and midguts and residual tissues were evaluated at 7 days after infection. RNA sequencing of midgut “pools” from the control group (CT), midgut breakthrough group (MB) and midgut nonbreakthrough group (MNB) at different temperatures was performed. The transcriptomic profiles were analyzed using the R package, followed by weighted gene correlation network analysis (WGCNA) and KEGG analysis to identify the important molecular mechanisms regulated by temperature.

Results: The midgut infection rate (MIR) and midgut breakthrough rate (MBR) at 28°C and 32°C were significantly higher than those at 23°C, which indicates that high temperature facilitates DENV-2 breakthrough in the *Ae. albopictus* midgut. Transcriptome sequencing was performed to investigate the antiviral mechanism in the midgut. The results show that the midgut gene expression datasets clustered regarding temperature, blood feeding and midgut breakthrough. Over 1500 differentially expressed genes (DEGs) were identified by pairwise comparisons of midguts at different temperatures. To assess key molecules regulated by temperature, we used WGCNA to identify 28 modules of co-expressed genes, and the ME3 module correlated with temperature. KEGG analysis indicates that RNA degradation, Toll and IMD signaling and other pathways are regulated by temperature.

Conclusions: Temperature affects the infection and breakthrough of *Ae. albopictus* midguts invaded by DENV-2, and *Ae. albopictus* midgut transcriptomes change with temperature. The candidate genes and key pathways regulated by temperature provide targets for the prevention and control of dengue.

Background

Dengue fever is an arthropod-borne disease caused by the dengue virus (DENV) [1]. In general, clinical manifestations vary after infection with DENV. The most people are asymptomatic or have only minor symptoms, including fever, rash, and body pain. However, a proportion of patients develop severe dengue, which manifests as severe bleeding, shock, and even death [2]. Although dengue is epidemic worldwide, it is mainly distributed in tropical and subtropical regions. It is estimated that approximately 400 million people are infected with DENV globally every year, with 25% of the population presenting clinical symptoms [3]. In recent years, dengue outbreaks have appeared in the Americas, Bangladesh, the Philippines, and Nepal [4–7]. In China, dengue frequently occurs in Guangdong, Guangxi, and Yunnan provinces. In 2014, the largest outbreak was reported in Guangdong Province, with 47 056 cases and 6 deaths [8, 9]. The epidemic area of dengue is constantly expanding, and the number of people at risk increases yearly.

Dengue is transmitted by mosquitoes of the genus *Aedes*, such as *Aedes aegypti* and *Aedes albopictus*. *Ae. aegypti* is internationally recognized as the principal vector of dengue; *Ae. albopictus* is the secondary

vector [10]. In China, the distribution of *Ae. aegypti* is limited to only a few areas of Guangdong, Hainan and Yunnan, and *Ae. albopictus* is the dominant mosquito species that causes dengue outbreaks in China; *Ae. albopictus* is distributed in perennial warm provinces and the north, southwest and southeast coastal areas [11]. Moreover, the geographical distribution of *Ae. albopictus* is continuing to expand with the acceleration of global warming, urbanization and trade, with an increased risk of dengue spread.

Temperature affects the prevalence of dengue [12, 13], and in the absence of measures to mitigate climate change, an additional 7.5 million dengue cases per year can occur. If the increase in temperature is maintained within 2°C, the annual increase in dengue cases can be reduced by 1 to 3 million; if it is controlled within 1.5°C, the number of cases will be further reduced [14]. In general, the risk of dengue increases with temperature change, which is primarily associated with improving the vector competence of mosquitoes to transmit DENV. In one study, when the temperature was higher than 26°C, the extrinsic incubation period (EIP) of DENV in *Ae. aegypti* was approximately 1 week, but the EIP was prolonged when the temperature was lower than 21°C; if the temperature was lower than 18°C, *Ae. aegypti* could not transmit DENV [15]. Similar results were obtained for *Aedes albopictus* [16]. Nevertheless, the mechanism by which temperature affects the transmission of DENV by *Aedes* remains unclear.

Mosquito-borne viruses must overcome the mosquito's midgut and salivary gland barrier to replicate in mosquitoes; indeed, the midgut is the first barrier against a virus. Thus, the balance of the midgut environment affects the virus replication [17, 18]. For example, in *Ae. aegypti* infected with Zika virus (ZIKV) and cultured at 20°C, 28°C, and 36°C, the gene expression profiles of the midgut changed with temperature, especially under low-temperature conditions (20°C), which shows significant alterations in gene expression related to blood digestion, active oxygen metabolism and innate immunity [19].

Our previous research showed that DENV-2 was confined to the midgut of *Ae. albopictus* and slowly proliferated at 18°C; when the temperature was 23–32°C, DENV-2 broke through the midgut barrier of *Ae. albopictus* and invaded the salivary glands [16]. To better understand the interaction between *Ae. albopictus* and DENV-2 under different temperatures, the infection status in the midgut and remaining tissues of *Ae. albopictus* at 23°C, 28°C and 32°C was investigated. Transcriptome sequences proceeded from the midgut of *Ae. albopictus*. In addition, gene network modules highly related to temperature were identified using a weighted gene correlation network analysis (WGCNA).

Methods

Mosquitoes

Ae. albopictus was collected in Foshan City, Guangdong Province, China, and bred in a standardized room (constant 27±1°C, 70–80% relative humidity and a 16 h:8 h light-dark photoperiod). The eggs hatched into larvae in dechlorinated water, and the larvae were fed turtle food until the pupal stage. The pupae were transferred into a cup and placed in a mosquito cage. Adults emerged during 2–3 d and were fed 10% glucose. The mosquitoes were fed defibrinated sheep blood for egg production.

The proliferation of dengue virus

Experimental operation of DENV-2 was performed in a biological safety cabinet. DENV-2 (New Guinea C) proliferated in C6/36 cells. C6/36 cells cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) were inoculated with DENV-2 at a multiplicity of infection (MOI) of 1; the culture flask was gently shaken for 15 min at room temperature and subsequently incubated at 37°C and 5% CO₂ for 2 days. The supernatant was harvested after centrifugation at 1 500×*g* for 5 min. The DENV-2 titer was determined based on the 50% tissue culture infective dose (TCID₅₀)[20].

Mosquito infection with DENV-2

The mosquito infection experiment was performed in a biosafety secondary laboratory (BSL-2). Five- to 7-day-old female *Ae. albopictus* mosquitoes were starved for 16–20 h. Fresh DENV-2 (8.625 log₁₀TCID₅₀/mL) was mixed with defibrinated sheep blood at a ratio of 2:1. After incubating at 37°C for 30 min, the blood meal was transferred into a Hemotek blood reservoir unit (Discovery Workshops, Lancashire, UK). *Ae. albopictus* mosquitoes were allowed to feed on the blood meal for 30 min. The engorged mosquitoes were anesthetized with CO₂, placed in 250 mL paper cups covered with gauze (10 mosquitoes/cup), and maintained in different incubators precisely set at 23°C, 28°C and 32°C, 80% relative humidity and a 16 h:8 h (light:dark) photoperiod. For the control group, DENV-2 was replaced with RPMI-1640/2% FBS. All mosquitoes were fed 10% glucose solution.

DENV-2 detection

The midgut and residual tissues of each mosquito were dissected and independently detected at 7 days postinfection (dpi). Total RNA was extracted according to the manufacturer's protocol. cDNA was synthesized using 4 µL RNA and random primers following the recommendations of the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA). DENV-2 positivity was determined by polymerase chain reaction (PCR) using the previously described primers (forward primer: 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'; reverse primer: 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3')[21]. PCR was performed according to the protocol of Maxima Hot Start Green PCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA). The 511-bp target fragment was identified by 1% agarose gel electrophoresis. The residual RNA was stored at -80°C.

Determination of the midgut infection, midgut breakthrough and midgut nonbreakthrough rates of *Ae. albopictus*

If DENV-2 was simultaneously detected in the midgut and the residual tissue of *Ae. albopictus*, it was considered that DENV-2 had broken through the midgut barrier of the mosquitoes and constituted the midgut breakthrough group (MB). If DENV-2 was detected only in the midgut and not in residual tissue, it was considered that the virus had not broken through the midgut barrier, which was termed the midgut nonbreakthrough group (MNB). The midgut infection rate (MIR), midgut breakthrough rate (MBR), and midgut nonbreakthrough rate (MNBR) of *Ae. albopictus* were determined using the following formulas.

MIR =number of positive midguts/total number of midguts tested × 100%

MBR =number of mosquitoes with a positive midgut and residual tissue/the number of positive midguts × 100%

MNBR =number of mosquitoes with a positive midgut but negative residual tissue/the number of positive midguts × 100%

Library preparation and RNA sequencing

Midguts from the CT, MB and MNB groups at each temperature (23°C, 28°C and 32°C) were selected and divided into 9 groups according to the results of the above experiment. The quality and integrity of a single midgut were tested using a 2100 bioanalyzer (Agilent, Santa Clara, USA); midgut RNA concentrations were detected using a Nanodrop 2000. Three midgut samples in each group were mixed as a "pool", where each group had 3 biologically repeated "pools". In total, 27 pools were sent to Wuhan Huada Medical Laboratory for RNA sequencing. Total RNA was purified with the mRNA enrichment method or rRNA removal method, and a cDNA library was constructed. The prepared libraries were sequenced with DNaseqPE150 from BGI (China).

RNA sequence analysis in response to temperature and DENV-2 exposure

The raw data obtained by RNA sequencing were filtered to remove low-quality data, contaminating linkers, and a high N content of unknown bases. The filtered data, as clean reads, were compared with the genome of the Foshan strain of *Ae. albopictus* (AaloF1, VectorBase, <https://www.vectorbase.org>), and novel transcripts were analyzed. Principal component analysis (PCA) was used to evaluate the sample repeatability and overall differences between samples. The gene length and total reads were normalized. Relative expression levels were assessed as fragments per kilobase of transcript per million fragments mapped (FPKM). Intragroup and intergroup Pearson correlation coefficients were calculated according to FPKM values, and a heatmap was drawn.

Identification of differentially expressed genes and co-expression network modules

In the R (version 4.0.5) environment, Ballgown package version 2.16.0 was used to analyze the differentially expressed genes (DEGs)[22]. The DEGs of MB and MNB at each temperature and those between two adjacent temperatures in MB or MNB are illustrated by a Venn diagram. DEGs were identified as genes with 2-fold or more changes between samples and a false discovery rate (FDR) <0.05. The genes were also analyzed using WGCNA packages[23]. Genes were clustered and divided into different modules; the correlation between the module and the temperature was analyzed, and the module with the highest correlation with temperature was determined. A topological overlap matrix (TOM) was employed to visualize the network of the most relevant modular genes. The R package

ClusterProfiler version 3.12.0 was applied for gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses[24].

Quantitative real-time PCR (qRT–PCR)

The genes regulated by temperature were validated by qRT–PCR. The levels of 18 genes were detected in the midguts at different temperatures. RNA and cDNA were synthesized using the above-described protocol. qRT–PCR was performed in triplicate for each sample with SYBR Green Mix (Yeasen, Shanghai, China). The gene expression was normalized to rpS7. The primer sequences are listed in Additional file 2: Table S1. The program was 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 10 s.

Data analysis

All statistical analyses were performed with SPSS 20.0 (IBM, Chicago, IL, USA). MIR, MBR, and MNBR for *Ae. albopictus* infected with DENV-2 were separately compared at different temperatures using chi-square (and Fisher's exact) tests. *P* values were corrected by Bonferroni adjustment. The expression levels of genes were analyzed using Kruskal–Wallis H test. *P*<0.05 was considered statistically significant.

Results

Temperature affects the DENV-2 infection and midgut breakthrough in *Ae. albopictus*.

The DENV-2 virus titer (log₁₀) determined by TCID₅₀ was 8.625. In total, 466 of *Ae. albopictus* mosquitoes that ingested blood meals containing DENV-2 were used to analyze the midgut infection and breakthrough rates at 23°C, 28°C and 32°C. The MIR was significantly higher at 28°C and 32°C than at 23°C (*P*<0.01, *P*<0.001) (Fig. 1A). Although the MIR was higher at 32°C than at 28°C, there was no significant difference between the two groups (*P*=0.107) (Fig. 1A). The MBR of *Ae. albopictus* infected by DENV-2 was closely related to temperature and gradually increased when the temperature increased, although MNBR gradually decreased. The MBR was significantly higher at 28°C and 32°C than at 23°C (*P*<0.01, *P*<0.001), whereas the MNBR had the opposite trend (Fig. 1B). The MNBR was significantly higher than the MBR at 23°C, but the MBR was significantly higher than the MNBR at 28°C and 32°C (Fig. 1B). These results indicate that the DENV-2 infection and midgut breakthrough in *Ae. albopictus* are affected by temperature.

Characterization of *Ae. albopictus* transcriptomes at different temperatures.

Transcriptome sequencing was performed on 27 samples from the CT, MB, and MNB groups at different temperatures (23°C, 28°C and 32°C). Clean reads were obtained after removing low-quality data, and trimmed reads were obtained. Nearly 80% of the reads were mapped to the *Ae. albopictus* Foshan strain genome (Table 1).

Table 1
Overview of the mapping of RNA-seq reads

Sample name	Accession Number	Total reads	Trimmed Reads in Pairs	Mapped Trimmed Reads to the Genome	Mapping Rate(%)
CT23-1	SRR16503765	54772829	43027438	44830001	81.85
CT23-2	SRR16503764	53395633	42115248	43759069	81.95
CT23-3	SRR16503753	54865514	43008208	45189103	82.36
CT28-1	SRR16503745	55807603	42799344	45686153	81.86
CT28-2	SRR16503744	54595956	42754238	44446199	81.41
CT28-3	SRR16503743	55657054	43167914	45847667	82.38
CT32-1	SRR16503742	54596339	43143450	44902572	82.24
CT32-2	SRR16503741	54575842	43233636	44911335	82.29
CT32-3	SRR16503740	53828160	43116042	44519031	82.71
MB23-1	SRR16503739	55680172	43272084	45751800	82.17
MB23-2	SRR16503763	55593162	43166298	46254004	83.20
MB23-3	SRR16503762	53854014	43234376	43751782	81.24
MB28-1	SRR16503761	54532721	42916424	44240737	81.13
MB28-2	SRR16503760	55082520	43313808	45303233	82.25
MB28-3	SRR16503759	53442771	42876264	43282494	80.99
MB32-1	SRR16503758	54496832	43216764	44790010	82.19
MB32-2	SRR16503757	54009938	43101818	44081104	81.62
MB32-3	SRR16503756	53385010	42789656	43476908	81.44
MNB23-1	SRR16503755	55517642	42595030	46663792	84.05
MNB23-2	SRR16503754	54594901	42346994	44982402	82.39
MNB23-3	SRR16503752	55500160	42492630	46458788	83.71
MNB28-1	SRR16503751	56410592	43144664	47226360	83.72
MNB28-2	SRR16503750	54893018	43252930	45876636	83.57

Sample name	Accession Number	Total reads	Trimmed Reads in Pairs	Mapped Trimmed Reads to the Genome	Mapping Rate(%)
MNB28-3	SRR16503749	54651677	42863124	45496019	83.25
MNB32-1	SRR16503748	55653684	43066758	45287828	81.37
MNB32-2	SRR16503747	53792991	42047606	45040839	83.73
MNB32-3	SRR16503746	53760318	43236118	43159101	80.28

PCA was used to analyze the variance of in-group samples and gene expression patterns associated with temperature, infection and breakthrough of the midgut. The PCA plot showed a high degree of reproducibility among replicate samples (Fig. 2A), and gene expression datasets of midguts clustered at different temperatures (Fig. 2B). Overall, the expression profiles of midgut transcripts changed in response to blood feeding between the control group and the infection group (Fig. 2C), and the clustering of transcript profiles was affected by midgut breakthrough (Fig. 2D). Furthermore, we analyzed Pearson correlation coefficients among all samples, and a heatmap showed correlation values of paired samples between 0.75 and 1 (Additional file 1: Figure S1).

Temperature alters *Ae. albopictus* transcriptomes during DENV-2 infection.

DEGs between CT and MB were analyzed at each temperature. There were 469 DEGs at 23°C, 139 at 28°C and 156 at 32°C, which included 3 upregulated and 10 downregulated genes at two temperatures (Fig. 3A). Compared to DEGs between CT and MB, the transcriptomes between CT and MNB were altered, with 180 DEGs at 23°C, 90 at 28°C and 140 at 32°C, which included 3 upregulated genes and 4 downregulated genes at two temperatures (Fig. 3B). Since temperature is an important factor that affected the midgut barrier, we analyzed MB and MNB DEGs at different temperatures. Most DEGs were observed at 23°C, followed by 28°C, with the smallest number at 32°C. There were no overlapping genes among the groups (Fig. 3C). The data show that the *Ae. albopictus* midgut transcriptomes change in response to temperature.

In addition, DEGs were different between MB and MNB compared to CT at the same temperature. At 23°C, 231 upregulated and 241 downregulated genes sharply changed between CT and MB, which was far more than those between CT and MNB (Fig. 3D). At 28°C, the numbers of DEGs between CT and MB were almost identical to those between CT and MNB (Fig. 3E). At 32°C, there were 45 upregulated and 82 downregulated genes between CT and MB and 33 upregulated and 46 downregulated genes between CT and MNB (Fig. 3F). These results demonstrate that the *Ae. albopictus* midgut transcriptome is affected by the midgut barrier.

Key gene network associated with the DENV-2 infection identified by WGCNA.

To construct a WGCNA network, we first calculated the thresholding power to perform network topology analysis. The thresholding power was set at 4 in WGCNA because the scale independence reached 0.9 and presented suitable mean connectivity (Fig. 4A). Modules were determined using the WGCNA R package. The hierarchical cluster tree showed 28 modules of co-expressed genes (Fig. 4B), and the eigengene adjacency heatmap indicated a relationship between pairwise gene modules (Fig. 4C). Correlations between modules and temperature and ingestion DENV-2 blood meal were analyzed by WGCNA. According to the results, the ME3 module had the highest correlation with temperature, and the ME12 module was highly associated with the ingestion of a viral blood meal (Fig. 4D).

Functional analysis of the key module associated with temperature.

In this study, we focused on the pathway by which temperature affected the transmission of DENV-2 by *Ae. albopictus*. The ME3 module correlated with temperature was evaluated by GO and KEGG analyses. Unfortunately, no significant GO terms were enriched in this module (Additional file 1: Figure S2). In the KEGG analysis, RNA degradation, Toll and IMD signaling pathways and other pathways were enriched. Hence, these pathways are regulated by temperature (Fig. 5A). The genes involved in RNA degradation, Toll and IMD signaling pathways, nitrogen metabolism, fatty acid degradation and riboflavin metabolism are listed in Table 2 (Fig. 5B).

Table 2
Module genes regulated by temperature

Module-regulated pathway	Gene number	Protein
RNA degradation	109401683	m7GpppN-mRNA hydrolase
	109420650	U6 snRNA-associated Sm-like protein LSm4
	109399943	CCR4-NOT transcription complex subunit 6-like
	109418111	protein PAT1 homolog 1
	109401240	heat shock 70-kDa protein cognate 5
Toll and Imd pathway	109404401	modular serine protease-like
	115262585	uncharacterized LOC115262585
	109422736	peptidoglycan-recognition protein LB-like
	109426919	uncharacterized LOC109426919
	115266575	myeloid differentiation primary response protein MyD88-like
Nitrogen metabolism	109399946	glutamine synthetase 2 cytoplasmic
	115256594	carbonic anhydrase 1-like
	109419671	carbonic anhydrase 1-like
Fatty acid degradation	109400327	very long-chain specific acyl-CoA dehydrogenase, mitochondrial-like
	109416406	acetyl-CoA acetyltransferase, cytosolic-like
	109407255	carnitine O-palmitoyltransferase 2, mitochondrial-like
Riboflavin metabolism	115255392	testicular acid phosphatase
	109405306	flavin reductase (NADPH)

Validation of hub genes regulated by temperature

According to references, we selected eight genes related to the antiviral immunity of mosquitoes for validation. The expression levels of these genes were analyzed from RNA-seq datasets (Fig. 6) and detected by qRT-PCR (Fig. 7). The expression trends of most samples showed similar results between RNA-seq and qRT-PCR. Kruskal–Wallis H test demonstrated that “LOC109399943” annotated as “CCR4-NOT transcription complex subunit 6-like”, “LOC109418111” annotated as “protein PAT1 homolog 1” and “LOC109401240” annotated as “heat shock 70-kDa protein cognate 5” were significantly different in the overall samples. These hub genes regulated by temperature will provide targets for dengue prevention.

Discussion

Model predictions and laboratory studies have shown that temperature affects the vector competence of *Ae. albopictus* for transmitting DENV [16, 25]. However, little is known about the underlying mechanisms. In this study, RNA sequencing of the midguts of *Ae. albopictus* in the CT, MB and MNB groups was conducted, and the results reveal different transcriptional variations in response to DENV infection and temperature. WGCNA was used to identify key gene networks regulated by temperature. Then, we determined hub pathways associated with temperature.

In this research, we collected *Ae. albopictus* mosquitoes with different infection statuses. To simultaneously collect mosquitoes with and without midgut breakthrough at 23°C, 28°C and 32°C, we determined the optical concentration of DENV-2 to be 8.625 log₁₀ TCID₅₀/mL based on preliminary experimental results, with a time to harvest of 7 days after infection. Under this condition, the MIR, MBR and MNBR of *Ae. albopictus* were detected. The trend of the results was consistent with previous experimental research and model predictions [16, 25, 26].

The transcriptome of the midgut of *Aedes* mosquitoes is altered by the DENV infection. Indeed, expression profiles of the *Ae. aegypti* midgut responding to DENV infection were changed. For example, antimicrobial peptides (AMPs) induced by the Toll immune pathway reportedly increased at 7 days post-infection [27], and *Ae. albopictus* midgut genes were modulated by DENV exposure in a time- and site-specific manner [28]. In our study, the expression profiles of the *Ae. albopictus* midgut clustered after the ingestion of a blood meal containing DENV-2. Furthermore, we analyzed *Ae. albopictus* transcriptomes of the MB and MNB groups at different temperatures and found that temperature affected the midgut transcriptome clustering: low temperature resulted in more DEGs. Similar to our findings, previous research has demonstrated that temperature shapes *Ae. aegypti* gene expression after ZIKV infection [19].

To better understand the relationship between temperature and infection in *Ae. albopictus*, we used WGCNA, which is a method for gene co-expression networks [29, 30]. In 2008, the WGCNA software package was developed using R; it is mainly used to analyze large sample expression data, such as gene expression microarray or RNA sequencing data [23]. WGCNA has been proven to be an effective method to detect co-expressed modules and hub genes in tumors, plants, parasites, etc [31-33]. In this study, the highest correlation with temperature was observed for the ME3 module.

The mosquito transcriptome changed in response to DENV, which might be related to the mosquito's antiviral system. In contrast to the innate and adaptive immunity of humans to resist the invasion of pathogens, mosquitoes lack adaptive immunity and mainly rely on innate immunity to suppress virus proliferation. RNA interference (RNAi), Toll, immunodeficiency factor (IMD), Janus kinase pathway signal transduction and activation (JAK-STAT) and other pathways play an important role in the mosquito antiviral immunity [34]. The gene expression profiles of mosquitoes infected with viruses were transformed following temperature change. In a previous study, *Ae. aegypti* infected with chikungunya

virus (CHIKV) were cultured at 18°C, 28°C and 32°C; the Toll, IMD and JAK-STAT pathways were upregulated at 28°C, and high temperature appeared to damage the immune defenses [35]. In our study, the pathways of the ME3 module regulated by temperature included RNA degradation, Toll pathway and IMD pathway.

DENV is an RNA virus, and the RNA degradation pathway is closely related to the proliferation of DENV in mosquitoes [36]. Many eukaryotic proteins that interact with the 5'm7GpppN cap affect the metabolism and translation of mRNA [37]. We speculate that m7GpppN-mRNA hydrolase is involved in the replication of DENV in *Ae. albopictus*. Sm-like (Lsm) proteins participate in pre-mRNA splicing, nuclear RNA processing and miRNA biogenesis and mediate the antiviral immunity through the RNAi pathway [38]. PAT1 is an RNA-binding protein that plays a role in mRNA decay by physically linking de-adenylation with de-capping and by acting as a translation repressor to regulate the gene expression [39]. The Lsm1–7-Pat1 complex binds to cis-acting regulatory sequences of viral positive-strand RNA genomes, which promotes the transcription, translation and replication of the virus [40]. heat shock cognate 70 protein interacts with CHIKV to promote its entry into C6/36 cells [41], and 70-kDa heat shock cognate proteins have been identified as the most critical components in DENV-4 binding and entry into C6/36 cells [42]. Additionally, the CCR4-NOT transcription complex subunit 6-like belongs to the CCR4-NOT complex family, which is involved in the transcription, translation and mRNA decay [43]. The expression level of CCR4-Not complex genes was up-regulated in DENV-infected cells, which is conducive to the proliferation of DENV [44]. In this study, it was highly expressed at 32°C, which suggests that DENV-2 proliferated more quickly at 32°C, which helped DENV-2 break through the midgut barrier.

Regarding Toll pathways, pattern recognition receptors (PRRs) recognize mosquito-borne viruses, which promotes the maturation of Spätzle (Spz). The Spz interaction with Toll-like receptors (TLRs) involves myeloid differentiation gene 88 (MyD88), followed by the activation of the nuclear transcription factor (NF-κB), which induces the release of nuclear antimicrobial peptides (AMPs) and other antiviral molecules [45]. Three proteins are regulated by temperature: modular serine protease, peptidoglycan-recognition protein (PGRP) and MyD88. Modular serine protease is recruited to the lysine-type peptidoglycan recognition complex, which activates the serine protease cascade [46]. It serves as a crucial enzyme to elicit insect immune responses, especially for Toll pathway activation [47]. PGRP is the most important PRR in insects; it can bind to and hydrolyze bacterial peptidoglycan to activate innate immunity [48,49]. MyD88 serves as the key mediator of Toll signaling. The inhibition of MyD88 significantly enhances the replication of DENV-2 in *Ae. Aegypti* [50]. MyD88 is also involved in the antiviral immunity of *Ae. aegypti* against Japanese encephalitis virus (JEV) [51].

In this study, functional verification of these genes regulated by temperature was absent. We will design the siRNA and dsRNA of these genes to detect the proliferation ability of DENV-2 at the cell level and analyze the vector competence of *Ae. albopictus* to transmit DENV-2 at different temperatures.

Conclusion

To explore the mechanism by which temperature affects the transmission of DENV-2 by *Ae. albopictus*, we examined variational transcriptomes of *Ae. albopictus* midguts at different temperatures using RNA sequencing. The altered genes identified may be involved in virus resistance or viral infection, and *Ae. albopictus* may be more susceptible to DENV-2 when these genes are altered. Some important pathways, including the RNA degradation, Toll and IMD pathways, were identified by WGCNA and KEGG analysis as being regulated by temperature. This study provides experimental evidence to prevent and control dengue.

Abbreviations

DENV

dengue virus

CT

The control group

MB

The midgut breakthrough group

MNB

The midgut nonbreakthrough group

MIR

Midgut infection rate

MBR

Midgut breakthrough rate

MNBR

Midgut nonbreakthrough rate

DEGs

Differentially expressed genes

WGCNA

Weighted gene correlation network analysis

PCA

Principal component analysis

TCID₅₀

50%tissue culture infective dose

GO

Gene ontology

KEGG

Kyoto encyclopedia of genes and genomes

qRT-PCR

Quantitative real-time PCR.

Declarations

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Not applicable.

Authors' contributions

ZZL and YX designed the study. ZZL wrote the manuscript. ZZL and YDL collected the mosquito samples. YX, SHX, YJL and CH analyzed the data. LX, XGC and KYZ contributed reagents and revised the initial manuscript draft. All authors approved the final manuscript.

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Availability of data and materials

Publicly available datasets were analyzed in this study. Sequencing data were deposited at the NCBI Sequence Read Archive (SRA) under the accession number SRP342340.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Analysis of the midgut of *Ae. albopictus* infected with DENV-2 at different temperatures. *Ae. albopictus* orally infected with DENV-2 were reared at 23°C, 28°C and 32°C. The midguts and residual tissue were dissected at 7 d, and DENV-2 was detected by PCR. (a) Midgut infection rate (MIR). (b) Analysis of midgut breakthrough in *Ae. albopictus* at different temperatures. The error bars represent the 95% confidence interval (CI).

Figure 2

PCA of the general transcriptome characteristics of *Ae. albopictus*. PCA1 and PCA2 accounted for 10.61% and 7.15% of the total variance in the dataset, respectively. Cluster analysis of transcriptome data from a single sample(a), samples at different temperatures (b), midgut datasets associated with viral treatment(c) and midgut breakthrough status(d).

Figure 3

Venn diagram representation of the number of DEGs identified by the RNA-seq analysis in the midguts of *Ae. albopictus*. Number of DEGs between CT and MB(a), CT and MNB(b), MB and MNB(c) at different temperatures; (d) MB and MNB compared to CT at 23°C; (e) MB and MNB compared to CT at 28°C; (f) MB and MNB compared to CT at 32°C.

Figure 4

WGCNA revealed gene co-expression networks. (a) Analysis of network topology for various soft-thresholding powers. (b) The hierarchical cluster tree shows 28 modules of co-expressed genes. (c) The eigengene adjacency heatmap indicates the relationship between the pairwise gene modules. (d) Correlations between modules and temperature. Each unit contains the weight correlation coefficients and *P* values.

Figure 5

Functional analysis of the key module associated with temperature. (a) KEGG enrichment analysis of DEGs from the ME3 module. (b) Genes corresponding to the partial pathway regulated by temperature.

Figure 6

Analysis of the expression of hub genes regulated by temperature by RNA-seq.

Figure 7

Validation of the expression of hub genes regulated by temperature by qRT-PCR.

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