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Transcriptome Analysis of the Differentially Expressed Heat-resistant Genes between *Calliptamus italicus* and *Gomphocerus sibiricus*

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Abstract

Calliptamus italicus and *Gomphocerus sibiricus* are indicator species in Xinjiang's low-altitude (700–1,900 m) and high-altitude (2,000–3,400 m) grasslands, respectively. *C. italicus* is tolerant to high-temperature stress, with its semilethal temperature (LT_{50}) being 10.5°C higher than that of *G. sibiricus*. The two locust species were subjected to high-temperature stress to explore the molecular mechanisms and differences in high temperature tolerance between the two locust species. Next, the next generation sequencing (NGS) data were mapped to reference transcripts obtained using single molecule realTime (SMRT) sequencing to construct a nonparameter transcriptome. The transcriptomic response of these two locust species displayed different patterns. *C. italicus* had 126 differentially expressed genes (DEGs), with 59 and 67 being significantly up-regulated and down-regulated, respectively. The heat shock protein (*Hsp*) genes were highly expressed upon two locust species exposure to high-temperature stress, with *Hsp70* being expressed the most. *G. sibiricus* had 86 DEGs, of which 45 were significantly up-regulated and 41 significantly down-regulated. In addition, the expression of the key enzyme encoding gene Myo-inositol oxygenase (*MIOX*) in inositol degradation was the highest in *G. sibiricus*. In the KEGG pathway, the biological processes and metabolic pathways were the most enriched pathways in *C. italicus* and *G. sibiricus*, respectively. Moreover, the quantitative fluorescence results were consistent with the transcriptome results, implying that the transcriptome results were accurate. The findings in this study provide valuable information for future research exploring the evolution mechanisms of heat resistance in *C. italicus* and *G. sibiricus*.

Key words: *Calliptamus italicus*, *Gomphocerus sibiricus*, high-temperature stress, transcriptome, heat-resistant gene

Temperature is crucial to the survival of insects since they are poikilotherms (Neven 2000, Xiong et al. 2019). An implicit assumption made when evaluating the impact of temperature changes on insect survival is that the thermal environment affects insects' resistance to extreme temperatures, subsequently determining their spatial distribution range (Kellermann et al. 2012). The global average and extreme temperatures are expected to continue increasing (Barnett et al. 2005, Milly et al. 2005, IPCC 2007, Diffenbaugh and Field 2013). Consequently, the adaption of animals to the thermal environment is currently attracting increased attention (Jesus et al. 2016), with the evolutionary mechanism of the adaptation being a research hotspot (Gunderson and Stillman 2015).

The adaptability of insects to temperature is an important factor determining their survivability and evolution in a given ecological environment (Xiong et al. 2019). Enhanced understanding of the physiological and molecular mechanisms of biological, thermal adaptability is key to predicting the impact of climate change on the organisms (Dahlhoff and Rank 2007, Walters et al. 2009, Marshall and Sinclair 2010, Gleason et al. 2015, Cui et al. 2018). In insects, exposure to external environmental stimuli activates numerous genes in their bodies during their response to external stresses (Yu and Fang 2009). Studies have shown that *Hsps* expression is induced upon insects' exposure to high temperatures, increasing the insects' resistance to temperature stress (Wallace et al. 2015).

Bactrocera dorsalis has higher heat resistance, a wider distribution range, and higher expression of *Hsp23* compared to *B. correcta* (Gu et al. 2019). In addition, the different geographical distributions of *Liriomyza huidobrensis* and *L. sativae* are related to the *Hsps* expression levels under different temperature conditions (Huang and Kang 2007). For example, the expression levels of *Hsp70* in *Chrysomela aeneicollis*, a species inhabiting the southern end of the Sierra Nevada Mountains in eastern California, decreases with an increase in elevation (Dahlhoff and Rank 2007). Similarly, the expression levels of *Hsp70* and *Hsp90* in *Locusta migratoria* living at different latitudes are different (Wang and Kang 2005), with a higher heat resistance being recorded in the low-latitude region.

C. italicus and *G. sibiricus* are the dominant locust species in Xinjiang, China, which cause severe economic, social, and ecological losses annually. *C. italicus* is an indicator species for desert grasslands mainly distributed in the low-altitude areas (700–1,900 m), while *G. sibiricus* is an indicator species in the alpine grassland area, mainly in the high-altitude areas (2,000–3,400 m) (Chen 1981). The *C. italicus* is more tolerant to high temperatures than *G. sibiricus*. It can tolerate temperatures as high as 42°C, and its semilethal temperature range is 47–49°C. On the contrary, *G. sibiricus* tolerates temperatures not higher than 33°C, and their semilethal temperature range is 36–39°C (Li et al. 2014, Li et al. 2015a, Qian et al. 2017). However, the molecular mechanisms of temperature resistance in *C. italicus* and *G. sibiricus* at different temperatures have not yet been established.

Therefore, in the present study, *C. italicus* and *G. sibiricus* locusts were subjected to high-temperature stress, followed by a transcriptome analysis using high-throughput RNA sequencing (RNA-seq) before and after the stress. The roles of DEGs under a high temperature are discussed in ecological and evolutionary adaptations. The findings contribute to future research and in controlling the locusts.

Materials and Methods

Locusts Collecting and Breeding

Adult *C. italicus* and *G. sibiricus* were collected from Manas, Changji, Xinjiang Uygur Autonomous Region (43° 54′ N and 86° 21′ E; altitude: 1,310 m) and the Balikun, Hami, Xinjiang Uygur Autonomous Region (43° 24′ N, 93° 31′E; altitude: 2,220 m), respectively. After being brought back to the laboratory, the insects were kept in cages in the rearing room at the appropriate temperature of 27 and 24°C, respectively. The size of the cages was 40 cm × 40 cm × 50 cm, the rearing density in the cages was 150 heads on average. The locusts were fed with fresh wheat seedlings and maintained at an relative humidity (RH) of 25% ± 5%, and the light period was set as 16 L:8 D.

Temperature Treatment Test

Adult *C. italicus* and *G. sibiricus* were kept for one week at appropriate temperatures and then subjected to short term heat stress experiments. Fifty healthy male and female Italian locusts and Siberian locusts of similar size were starved for more than 24 h to empty their digestive tract. In the experimental group, they were placed in an artificial climate chamber at 37 and 45°C, respectively, and the survival rate of both males and females was about 50% after 4 h of high temperature stress, and the individuals that could crawl normally were selected. The locusts in the two groups were then treated with liquid nitrogen to freeze them to death and stored in a refrigerator at −80°C awaiting further analysis. Three biological replicate groups were set in the control groups and the treatment groups.

Illumina cDNA Library Construction and Next Generation Sequencing

Total RNA from a total of 24 samples (three biological replicates per group) of male and female *C. italicus* and *G. sibiricus* under appropriate temperature and high temperature stress treatments was extracted using Trizol Reagent (Invitrogen) reagents in Nanodrop 2000 (Thermo Fisher Scientific) and Agilent 2100 (Agilent Technologies, CA) were evaluated for the number and completeness of RNA samples. Qualified RNA samples were used to construct cDNA libraries. The cDNA libraries were constructed using the NEBNext Ultra RNA Library Prep Kit (NEB, MA), the libraries passed the library inspection, and the second-generation transcriptome sequencing analysis was completed using a two-end sequencing (Pair-End) approach based on the illumina technology sequencing platform.

Table 1. Quality of sequencing

Sample name	Raw reads	Clean reads	Clean bases (G)	Q30 (%)	GC content (%)
Cinf	162,647,290	160,256,552	24.03	94.03	47.10
Cinm	145,916,884	143,951,454	21.60	94.17	47.67
Gsnf	168,531,604	164,574,092	24.69	93.94	49.55
Gsnm	162,392,424	159,497,070	23.94	93.10	47.97
Cihf	154,265,296	152,127,068	22.83	93.80	47.26
Cihm	161,990,752	159,578,930	23.94	95.15	48.94
Gshf	150,624,176	147,036,460	22.05	92.96	48.17
Gshm	152,602,346	149,161,678	22.38	95.06	47.39

Cinf: *C. italicus* females at normal temperature; Cinm: *C. italicus* males at normal temperature; Gsnf: *G. sibiricus* females at normal temperature; Gsnm: *G. sibiricus* males at normal temperature; Cihf: *C. italicus* females under heat stress; Cihm: *C. italicus* males under heat stress; Gshf: *G. sibiricus* females under heat stress; Gshm: *G. sibiricus* males under heat stress.

Table 2. Summary of assembled RNA-seq results

	Transcripts		Unigenes	
Type	*C. italicus*	*G. sibiricus*	*C. italicus*	*G. sibiricus*
Total number	22,848	22,707	14,293	14,485
Total length	59,605,647	70,229,804	38,094,025	44,136,206
N50 length	3,058	3,552	3,161	2,012
Mean length	2,609	3,093	2,666	3,048

Table 3. Summary of database search results

	C. italicus		G. sibiricus	
Database	Transcripts	Percentage of transcripts (%)	Transcripts	Percentage of transcripts (%)
Nr	12,804	56.04	13,061	57.52
Nt	5,359	23.46	5,356	23.59
SwissProt	11,353	49.69	11,878	52.31
GO	10,093	44.17	10,533	46.39
KOG	10,099	44.20	10,505	46.26
Pfam	10,093	44.17	10,533	46.39
KEGG	12,607	55.18	12,766	56.22

PacBio Library Construction and SMRT Sequencing

Twelve RNAs of male and female locusts under suitable temperature and high temperature stress were selected and mixed in equal amounts to form two sets of RNA mixed samples. mRNA was reverse transcribed into cDNA using SMARTer PCR cDNA Synthesis Kit (TaKaRa), and some cDNAs were taken for fragment screening using BluePippin to enrich for fragments larger than 4 Kb, and the screened fragments were subjected to large-scale PCR to obtain sufficient total cDNA. The library was qualified and triple sequencing was performed on a Pacific Biosciences RS sequencer. All sequencing was performed at Novogene (Tianjin, China).

The next generation sequencing reads for each group were mapped to the reference sequence (full-length transcripts obtained by third generation sequencing) using Bowtie 2. Next, the read count statistical analysis was performed on the mapping results using recursive simultaneous equations model (RSEM), aiming to provide full-length sequences for *C. italicus* and *G. sibiricus*.

Gene Function Annotation and Differential Gene Expression Analysis

Gene function annotation was performed using seven different databases (Nr, Nt, Swiss-Prot, GO, KOG, Pfam, and KEGG) using the BLAST software. To ensure experimental reliability and reasonable sample selection, we tested the correlation of gene expression levels between samples, and calculated Pearson's correlation coefficient (r) between the three biological replicates(https://www.chiplot.online, accessed on 23 October 2022). DEGs between the two groups were further analyzed using the DESeq software. The criterion used for screening DEGs was $\log_2(\text{FoldChange})| > 1$ and $p < 0.05$. The identified DEGs were mapped to each term in the gene ontology (GO) database (http://www.geneontology.org). GO enrichment analysis was performed using the GOseq software. The Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis of the DEGs was performed using the KOBAS software. The p-value calculating formula in the hypergeometric test is:

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

In this equation, N and n indicate the number of genes with GO/KEGG annotations and the number of DEGs in N, respectively. The variables M and m represent the numbers of genes and DEGs, respectively, in each GO/KEGG term.

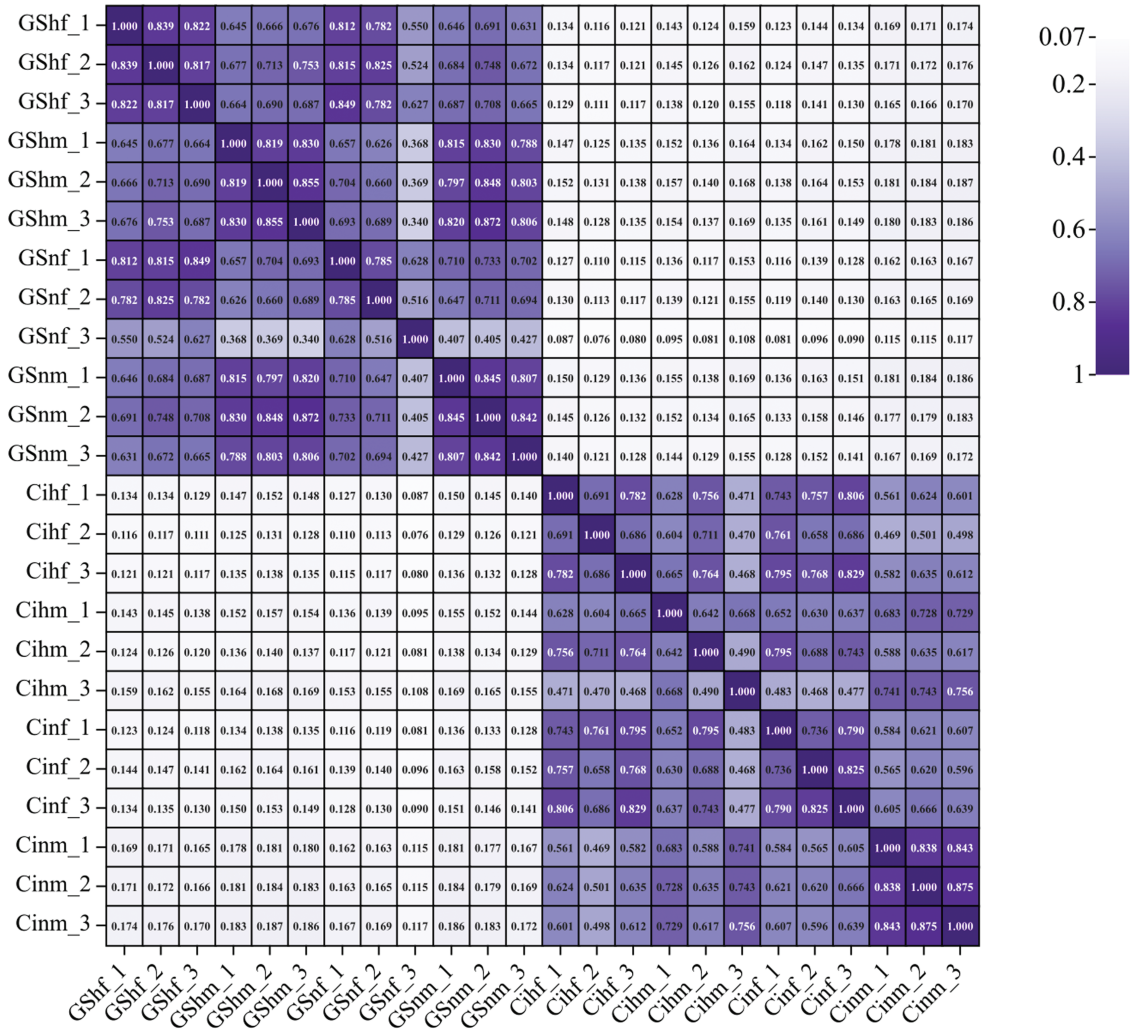

Fig. 1. Gene expression correlation analysis graph.

Real-Time Quantitative PCR(qPCR) Verification

In total, six DEGs were selected from the KEGG enrichment pathway ($p < 0.05$) to verify the reliability of the qPCR data. The qPCR was performed on a LightCycler 480 real-time PCR system. The reaction system consisted of 2 μL cDNA, 10 μL TB Green Premix Ex Taq II (Tli RNaseH Plus), 0.8 μL forward and reverse primers, and 6.4 μL sterile water. The primers used were designed using the Primer Premier 6.0 software (Supp Table 1 [online only]). Each reaction was repeated thrice. *β-actin* served as the internal control, and the $2^{-\triangle\triangle CT}$ method was employed to calculate the relative expression level of the target genes.

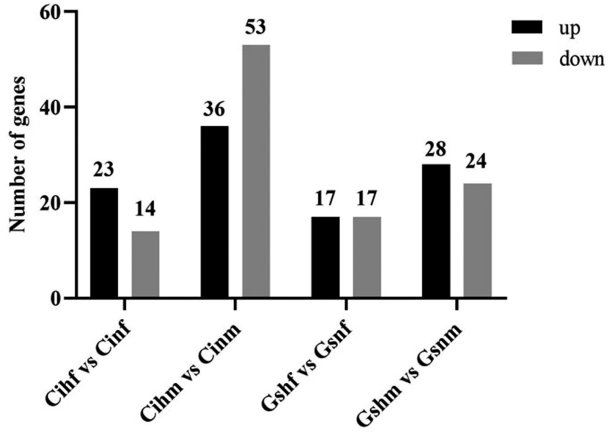

Fig. 2. Different expression genes of statistic. Number of up-regulated and down-regulated expression genes between Cihf vs Cinf, Cihm vs Cinm, Gshf vs Gsnf and Gshm vs Gsnm. The x-axis represents the comparison scheme between the indicated different temperature. The y-axis represents the corresponding number of DEGs. The black columns represent up-regulated expression genes and gray columns represent down-regulated expression genes.

Results

Full-Length Transcriptome Sequencing and Functional Annotation

After sequencing, a total of 1,258,970,772 raw reads were obtained (Table 1). After quality control and raw data correction, 14,293 and 14,485 unigenes were obtained, with an N50 of 3,161 bp and 2,012 bp, respectively (Table 2). Seven databases were used to annotate 22,848 and 22,707 transcripts from *C. italicus* and *G. sibiricus*, with a high overall annotation rate. *C. italicus* and *G. sibiricus* recorded the highest annotation rates of 56.04% and 57.52%, respectively, with the Nr database. The Nt database gave the lowest annotation rates of 23.46% and 23.59%, respectively (Table 3).

A heat map analysis of the Pearson's correlation coefficient of all RNA-seq samples from *C. italicus* and *G. sibiricus* found that the mRNA-level expression patterns of different biological replicates of the same treatment and between different treatments were relatively similar. It has good biological replicates ($R^2 > 0.8$) (Fig. 1).

Following exposure to high-temperature stress, 37 and 89 DEGs were identified in the male and female *C. italicus* with 23 and 36

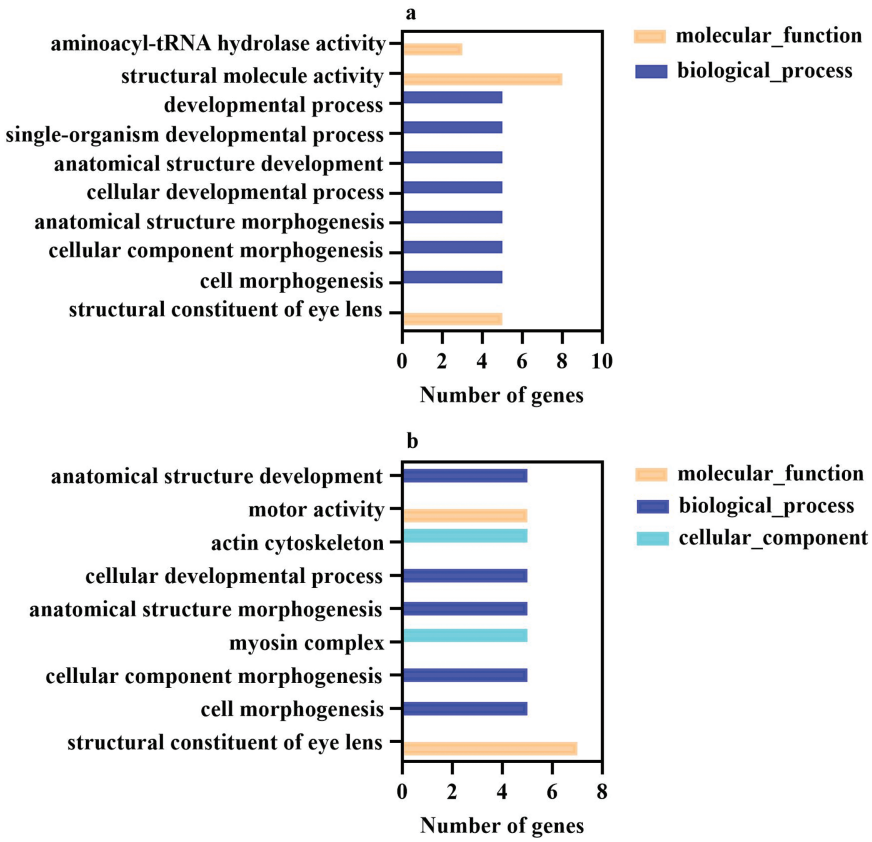

Fig. 3. The result of GO enrichment in C. italicus. GO enrichment analysis revealed the biological processes most associated with detected DEGs. Based on the GO results, cellular progress, metabolic progress, binding, and catalytic activity were the most enriched GO terms under heat stress. a: GO enrichment of DEGs in Cihf vs Cinf; b: GO enrichment of DEGs in Cihm vs Cinm.

genes up-regulated and 14 and 53 genes down-regulated, respectively. In *G. sibiricus*, 34 and 52 DEGs were identified in female and male locusts with 17 and 28 genes up-regulated and 17 and 24 genes down-regulated, respectively (Fig. 2). The shared DEGs between the male and female *C. italicus* were *Hsp70*, *CRYAB*, and *PDPK1*, and between the male and female *G. sibiricus* were *MIOX*, *PAPSS*, and *TPM1*.

GO and KEGG Enrichment of DEGs

Based on the GO enrichment analysis, the most enriched DEGs term in *C. italicus* exposed to high-temperature stress was molecular function with structural molecular activity as the most significant functional term in females and components of structural constituent eye lens in males. In addition, their biological process had the most enriched functional terms, with most of them being related to cell morphogenesis and development (Fig. 3). On the contrary, there were no significantly enriched GO pathways in the *G. sibiricus* exposed to high-temperature stress.

There were 50 and 57 KEGG pathways enriched in male and female *C. italicus*, respectively, with seven significantly enriched pathways (corrected $p < 0.05$) in the biological processes and human disease pathways being shared between the two. Among the seven, the three most significantly enriched KEGG pathways were the Longevity regulating pathway-multiple species, protein processing in endoplasmic reticulum, and antigen processing and presentation (Fig. 4A and B). There were 52 and 35 pathways enriched in the female and male *G. sibiricus*, respectively. Both were significantly enriched in the biosynthesis of monobactam biosynthesis in the metabolic pathways ($p < 0.05$; Fig. 4C and D, Supp Table 2 [online only]).

Under high-temperature stress, the *Hsp* were highly expressed in the seven shared pathways *C. italicus* (Fig. 5A and B). In addition, the *Hsp110*, *Hsp70,* and *sHsp* were significantly up-regulated ($|\log_2(\text{FoldChange})| > 1$, corrected $p < 0.05$), with *Hsp70* being the most up-regulated. In *G. sibiricus*, most enriched genes were related to metabolism pathways, such as *MIOX* and *PAPSS*. However, no

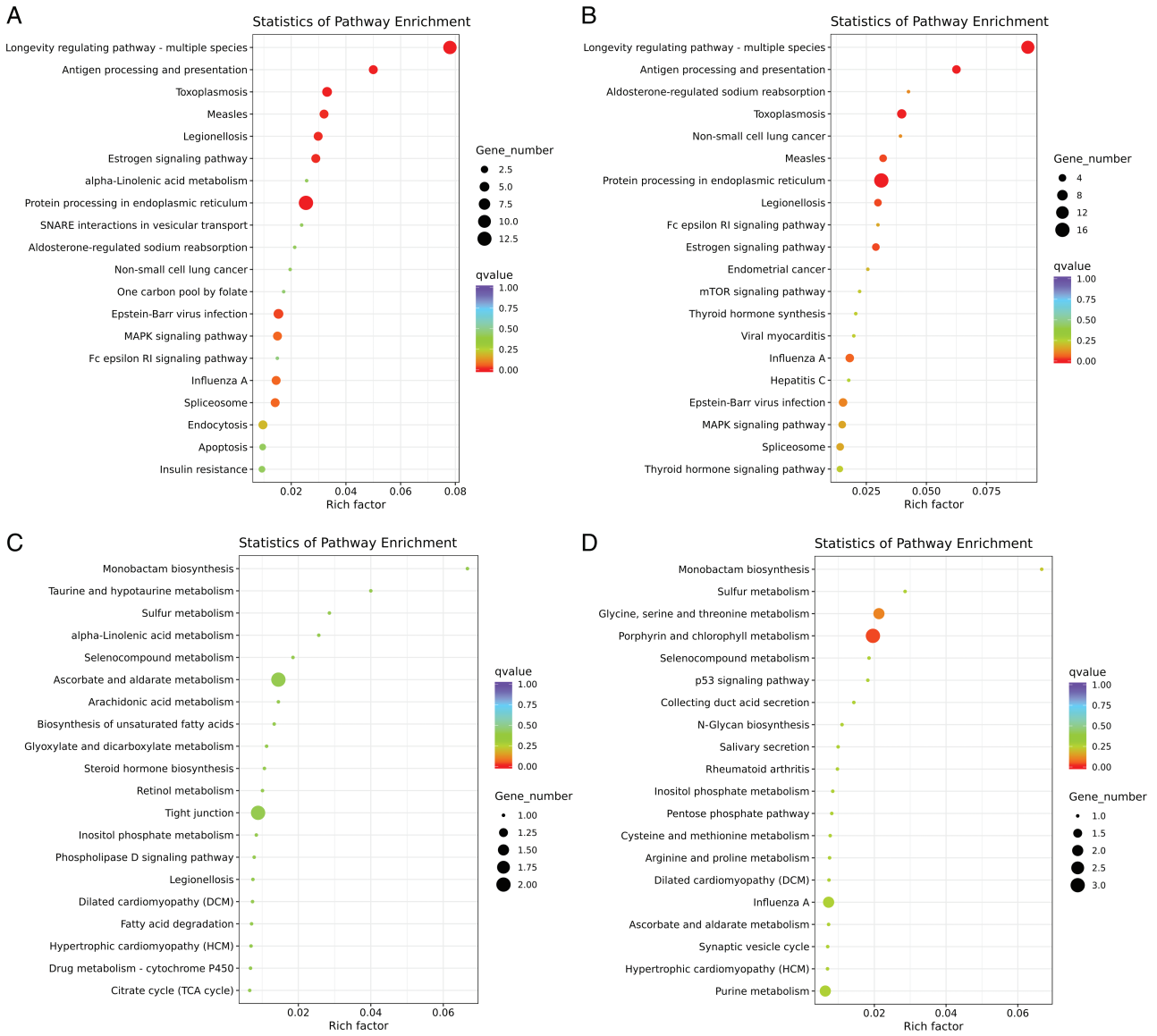

Fig. 4. Top 20 KEGG enrichment pathways of DEGs. The x-axis represents rich factor and the y-axis represents the KEGG pathways. a: KEGG enrichment of DEGs in Cihf vs Cinf; b: KEGG enrichment of DEGs in Cihm vs Cinm; c: KEGG enrichment of DEGs in Gshf vs Gsnf; d: KEGG enrichment of DEGs in Gshm vs Gsnm.

gene in the regulatory pathways related to *Hsp*s was enriched (Fig. 5C and D, Supp Table 3 [online only]).

Data Verification by Real-Time Fluorescent qPCR

The expression levels of selected genes in *C. italicus* and *G. sibiricus* locusts were quantified using qPCR to verify the accuracy of the transcriptome data. There was a high correlation between the DEGs expression profile analyzed by qPCR and the RNA-seq results (R^2 = 0.7808). In addition, based on the qPCR quantitative results, the expression levels of *Hsp70*, *CRYAB*, and *PDPK1* were increased, with Hsp70 having the highest expression level in the *C. italicus MIOX* and in the *G. sibiricus* (Figs. 6 and 7).

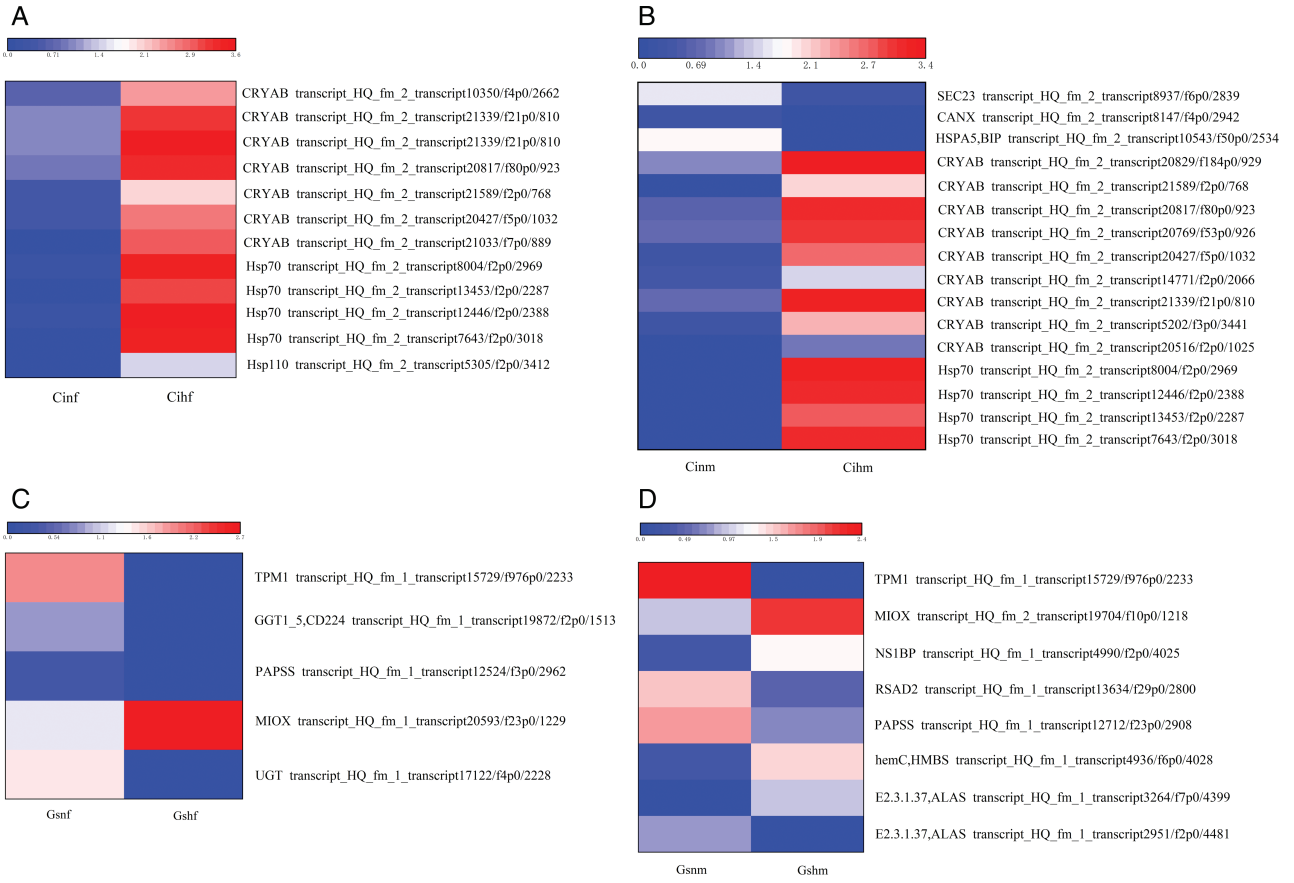

Fig. 5. Heatmap of the expression level of different pathway. a-b shows the expression levels of some genes in the female and male C. italicus; c-d shows the expression levels of some genes in the female and male G. sibiricus. The color scale is shown at the upper left, which encompasses from the lowest (blue) to the highest (red) FPKM value.

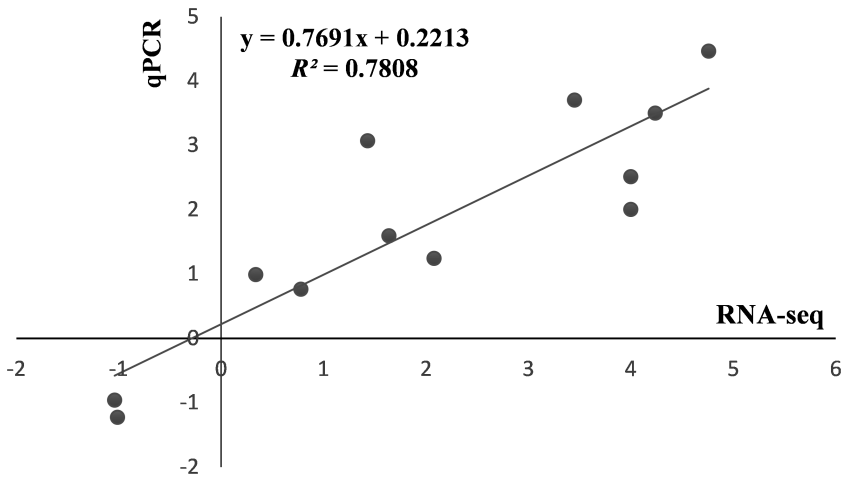

Fig. 6. Verification of DEGs. R2 analysis of the differential multiplicative relationship yielded good compatibility between the x-axis RNA-seq assay results and the y-axis qPCR quantification results.

Discussion and Conclusion

The Molecular Mechanisms of Adaptation of *C. italicus* and *G. sibiricus* locusts to High Temperature at Different Altitudes

With continuing changes in climate conditions, insects inhabiting different altitudes have evolved various adaptation mechanisms related to their body color, morphology, physiology, and molecular characteristics to survive under high temperatures (Peng et al. 2016, Cui 2018). This study used transcriptome sequencing to explore the differential molecular mechanisms between *C. italicus* and *G. sibiricus* locusts under high temperatures. Our findings revealed a larger number of DEGs between *C. italicus* and *G. sibiricus* locusts exposed to high-temperature stress. In *C. italicus*, the up-regulated and down-regulated genes played critical roles in the processes gurning against the effects of external high-temperature stress. Besides, most GO terms in *C. italicus* were enriched in one biological process, namely the cell morphogenesis and development process, which indicates that most cells need to maintain cell homeostasis, repairs, and replacement of damaged cells to cope and survive at high-temperature conditions (Howard et al. 2011, Li et al. 2015b, Liu et al. 2017). However, no significantly enriched GO pathway was identified in *G. sibiricus* under high-temperature conditions. Longevity regulating pathway-multiple species was the most significantly enriched KEGG pathway in *C. italicus* under high-temperature stress, consistent with what was established in *Monochamus alternatus* (Li et al. 2020). Under high-temperature stress, the changes in protein processing in the endoplasmic reticulum are related to the stabilization, denaturation, and properties of aggregated proteins, which fold the endoplasmic reticulum into its original form, thus playing an important role in protein-related homeostasis (Day et al. 2002). Since insects lack acquired immunity, their defense system is entirely controlled by innate immune responses. Thus, under high-temperature stress, the immune-related pathways are enriched, including antigen processing and presentation. The induction of many *Hsps* in the immune-related pathway also proves that heat stress may directly induce the immune response (Li et al. 2020), where the pathways related to *Hsps* are highly enriched under high-temperature stress (Chen et al., 2018).

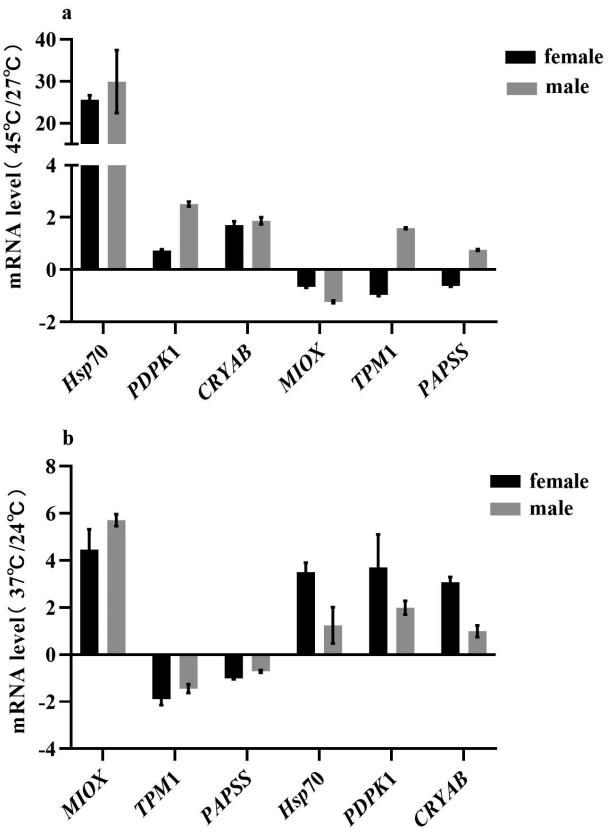

Fig. 7. The relative expression of key genes of *C. italicus* and *G. sibiricus* female and male under high temperature stress. a: *C. italicus*.b: *G. sibiricus*. Expression ratios of 6 candidate genes in heat shock compared with control. The fold changes of the genes were calculated as the log 2 value of each heat shock/control comparison and are shown on the y-axis. Black and gray bars represent gene. expression data ($2^{-\triangle\triangle Ct}$) that was obtained by qPCR analysis. Each error bar indicates the standard error.

However, *G. sibiricus* was not enriched, and it may be that *Hsps* is not the key gene for its resistance to high temperature under this temperature stress. A large number of studies have proved that the response of *Hsps* to heat stress does not always exist. As the temperature increases, its expression level shows a trend of increasing first and then decreasing. When the temperature is too high, *Hsps* is no longer a key gene for resistance to high temperature stress, so its expression is no longer elevated. The thermal protection of *Hsps* can only work within a certain temperature range. Species may synthesize other genes to cope with extreme heat the body can't handle (Zheng et al. 2010, Xiang et al. 2017). *L. huidobrensis* and *L. sativae* showed a trend of increasing and then decreasing expression of *Hsp90*, *70*, and *20* in the range of 25–45°C. The temperatures for maximal (T_{max}) induction of *Hsp* expression in the more thermotolerant *L. sativae* was higher than that of *L. huidobrensis*. Therefore, *L. huidobrensis* is more susceptible to heat than *L. sativae*. This is similar to the results of this paper (Huang and Kang 2007). Instead, the biosynthesis of a single bacteriocin mostly described in the detoxification and antibiotic studies has been identified as the main pathway activated in *G. sibiricus* under heat stress (Liu et al. 2017, Yu et al. 2018). In the present study, *PAPSS* was the main DEG in this pathway. However, since this pathway and *PAPSS* have not been reported in the heat resistance studies of insects, their specific roles in high-temperature resistance need to be further studied.

The *Hsp70* was the most significantly expressed in *C. italicus* under heat stress, and its expression level was dominant in all *Hsps*. These findings are consistent with previous studies on *Grapholita molesta* and *Helicoverpa zea* (Zhang and Denlinger 2010, Chen et al. 2014). Besides, under high-temperature conditions (38°C), the expression of the *Hsp23* in *B. dorsalis*, an insect species with high heat resistance and wider distribution, is more than nine times that of *B. correcta* (Gu et al. 2019). Our findings also revealed that the *Hsp70* expression level in the more heat-resistant *C. italicus* is 17 times higher than that of *G. sibiricus*. Therefore, it can be concluded that insects with a higher temperature tolerance have a highly expressed *Hsps*. Under high-temperature stress, the expression of the *sHsp* is mainly involved in aging, the immune system, endocrine system, folding classification degradation, and signal transduction pathways. In addition, the up-regulation of the *sHsp* prevents the aggregation of intracellular proteins. The interaction between *sHsp* and *Hsp*70 creates the foundation for the *Hsps* network (King and Macrae 2015), confirmed in the heat-resistant adaptation mechanisms of insects (Gu et al. 2019).

The *MIOX* was the most expressed in *G. sibiricus* exposed to high-temperature treatment. It regulates the sugar metabolism and pentose pathways by breaking down inositol, enhancing its environmental adaptation (Cui 2015). Although the role of *MIOX* in regulating plant stress resistance has been elucidated (Yang et al. 2017), its role in insects

coping with adversity warrants further research. Besides, the *PDPK1* induces the phosphorylation and activity of *MIOX* (Baibaswata et al. 2011). It is also key in diapause regulation in *Dalia antiqua* and *Culex pipiens* (Sim and Denlinger 2008, Shen et al. 2014) and conferring resistance to adversity stress in *B. dorsalis* (Xu 2014). Therefore, the synergistic up-regulation of *PDPK1* and *MIOX* plays an important role in conferring resistance to high-temperature stress in *G. sibiricus*.

Overall, the key heat-resistant genes identified in this study were *Hsp70*, *CRYAB*, *MIOX*, and *PDPK1*, which need to be verified further using RNA interference or knockout techniques.

Changes in Species Distribution and Prevention and Control Strategies Concerning Climate Change

Based on the findings in this study, locust species inhabiting different altitudes have different responsive mechanisms to high-temperature stress. *C. italicus* with a wider distribution range had more DEGs and enriched regulatory pathways, hence having stronger adaptability to high-temperature stress. The prediction of the future suitable areas for the two species using the CLIMEX software further revealed that the range of suitable areas for *C. italicus* would significantly increase compared to *G. sibiricus* due to their stronger adaptability (Luo 2021). Besides, the different distribution ranges phenomenon in insects caused by the differences in high-temperature adaptability has been confirmed (Bahrndorff et al. 2010, Willot et al. 2017, Gu et al. 2019).

Acknowledgments

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Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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