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Transcriptomic Insights into the Molecular Response of Stingless Bees (Tetragonula pagdeni) to Low-Temperature Stress

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Short Report

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Abstract

Stingless bees are vital tropical pollinators that are increasingly threatened by climatic stressors. While heat stress has been studied, the molecular mechanisms underlying their response to low temperatures remain largely unexplored. This study firstly investigated the transcriptomic response of the stingless bee *Tetragonula pagdeni* to acute low-temperature stress. De novo transcriptome assembly was performed using Trinity based on paired-end Illumina sequencing (NovaSeq 6000 platform). Using RNA sequencing, we compared the gene expression profiles of adult bees exposed to 10°C for 2 hours with those of controls maintained at 25°C. Three biological replicates per treatment group were utilized. We identified 1, 208 significantly differentially expressed genes (DEGs), with 666 upregulated and 542 downregulated. KEGG pathway enrichment analysis revealed that these DEGs were significantly involved in fundamental metabolic pathways, ribosome biogenesis, nucleotide and sulfur metabolism, and xenobiotic detoxification via cytochrome P450. Weighted Gene Co-expression Network Analysis (WGCNA) further delineated distinct modules of co-expressed genes. Our findings provide the first comprehensive molecular insights into the cold stress response in *T. pagdeni*, highlighting key genetic mechanisms related to metabolic adjustment and cellular stress protection. This study provides a foundation for understanding the molecular basis of cold tolerance in stingless bees and developing strategies to safeguard these essential pollinators under climate change.

Introduction

Social bees provide critical ecosystem services by pollinating both wild plants and agricultural crops[1–3]. Among wild bees, stingless bees are the most abundant and diverse group of the subfamily Apinae, serving a critical pollination function in tropical ecosystems[4, 5]. Moreover, stingless bees play a vital role in social, economic, cultural, and ecological contexts[6, 7]. These bees, which are eusocial in nature, are mainly distributed across tropical and subtropical areas[8, 9]. As members of the tribe Meliponini, they represent the largest group of eusocial bees, comprising more than 600 described species[10]. Through pollination, stingless bees deliver essential ecosystem services that facilitate the reproduction of a wide variety of plants[11, 12]. The stingless bee *Tetragonula pagdeni* (Schwarz, 1939), widely distributed across Southeast Asia, is commonly employed as a commercial pollinator[13]. Among the 33 stingless bee species in Thailand, *Tetragonula pagdeni* is one of the most common used for meliponiculture[14].

Temperature is a major abiotic stress associated with climate change and is critical for the survival of eusocial bees, including honeybees and stingless bees[15–17]. Both excessive heat and cold can adversely affect bee development and survival [18–20]. With prepupae and early pupae being particularly sensitive to low-temperature stress, leading to increased brood mortality, reduced worker lifespan[21], and impaired brain development and learning[22]. Transcriptomic studies have revealed key genetic responses to temperature stress, such as heat-tolerance genes in *Apis cerana* queens[20]. Tropical stingless bees are especially vulnerable because they already operate near their upper thermal limits[17, 23, 24]. Sublethal heat stress during development causes morphological, immunological, and behavioral

deficits that may undermine colony fitness[25], and a field study confirmed that a severe heatwave directly drove colony decline, with adult population size mitigating brood mortality via thermoregulation[17]. In the cold-adapted *Apis mellifera sinisxinyuan*, short-term temperature stress was found to regulate cold tolerance through the differential expression of key genes involved in critical molecular functions[26]. The research revealed that low-temperature exposure suppresses both the glutathione metabolism and peroxisome pathways in the honeybee pupal brain, leading to increased oxidative stress and providing a molecular explanation for cold-induced neural damage[27]. Understanding the interactive effects of combined thermal, desiccation, and nutritional stress is critical to predicting organismal responses to climate change and developing effective conservation strategies. Although there is heightened variability in how bee individual organisms respond to temperature stress, this variation can have profound implications for colony-level performance[28]. These studies collectively demonstrate that low-temperature stress exerts molecular-level impacts on honeybees.

However, the impact of low temperatures on stingless bees has not yet been studied. Although often overlooked as a stressor, low temperatures can reach approximately 10°C in winter within some mountainous habitats for stingless bees. As the influence of cold stress on stingless bees is currently uncharacterized, this study applied transcriptomic profiling to characterize their molecular responses after 2 hours at 10°C. This research provides key insights into the molecular adaptations of tropical stingless bees (*Tetragonula pagdeni*) response to low-temperature stress, which will inform future strategies for their scientific breeding and protection.

Matetial and Methods

Bee samples preparation

Healthy colonies of *Tetragonula pagdeni* were used in this study. It was sourced from Menglie Town, Jiangcheng County, Pu'er City, Yunnan Province, China. The geographical coordinates of the site were 22°34'10.488"N, 101°52'20.276"E. Adult bees were collected from this colony for the experiments. Adult stingless bees were sampled from the colony to standardize for size, morphology, and age. The bees were then allocated into either a control (TC)or an experimental group (TL) and housed in separate rearing boxes. Each group comprised six replicates, each containing 20 bees. The control group was placed in a constant-temperature chamber at 25°C, while the experimental group was maintained at 10°C; both groups were held under these conditions for 2 hours. Subsequently, all samples were rapidly collected, immersed directly in liquid nitrogen for flash-freezing, and then stored at − 80°C (< 7 days). This procedure was used to immediately halt enzymatic activity for transcriptomic analysis.

Transcriptome sequencing and analysis

Transcriptome sequencing was conducted at Wuhan Metware Biotechnology Co., Ltd. The procedure included RNA extraction, RNA quality assessment, library construction, sequencing, and a de novo

transcriptome analysis. Given that no complete genome for the stingless bee is available in current databases[29], this study employed a transcriptome analysis without a reference genome using Trinity[30] for Splice clean reads. To ensure the accuracy and reliability of the de novo transcriptome assembly, we implemented a rigorous bioinformatics pipeline.

High-quality reads were obtained by filtering the raw sequencing data and were subsequently assembled into a transcriptome. These high-quality reads were then mapped back to the assembled transcriptome for gene expression quantification. Differential expression analysis was performed between sample groups, followed by functional annotation and enrichment analysis of the differentially expressed genes. The set of differentially expressed genes (DEGs) between the two biological conditions was identified by differential expression analysis using DESeq2[31], with a significance threshold of |log2FoldChange| >= 1 and FDR < 0.05.

Weighted Gene Co-expression Network Analysis (WGCNA) was performed to construct a hierarchical clustering tree based on pairwise gene expression correlations. Genes were clustered into modules with a merge cut height threshold of 0.25 and a minimum module size of 50 genes. The horizontal spacing is arbitrary and serves only for visual clarity. Modules represent groups of genes with highly correlated expression patterns across samples, suggesting potential functional relatedness or involvement in shared biological processes.

Results

Data Assessment and Analysis of Significantly Differential genes

Principal component analysis (PCA) was conducted to assess the global transcriptomic differences between the cold-treated and control groups. Based on gene expression profiles, principal components 1 and 2 explained 17.71% and 11.8% of the total variance, respectively. The clear divergence between TC and TL groups, evidenced by both PCA and clustering (Fig. 1), indicates that cold exposure triggered a significant transcriptional reprogramming in adult *Tetragonula pagdeni*.

In the Fig. 2, volcano plot visualizing differentially expressed genes under cold stress. The analysis identified 666 significantly upregulated (red) and 542 downregulated (blue) genes using a threshold of $|\log_2(\text{fold change})| > 1$ and an adjusted *p*-value < 0.05. Several differentially expressed genes warrant emphasis — specifically, the up-regulated genes Cluster-2041.22 (Probable cytochrome P450 6a14) and Cluster-40494.2 (transcription elongation regulator 1 isoform X1), whereas Cluster-23219.1 and Cluster-36629.1 were notably down-regulated. The remaining 42, 871 genes that did not meet these criteria are plotted in gray. The predominance of upregulated genes suggests an active transcriptional activation in response to cold stress.

In the Fig. 3, clustering heatmap of differentially expressed genes (DEGs). The heatmap displays the expression patterns of DEGs across all samples. Each row represents a gene, and each column represents a sample. The samples are grouped by conditions (TC vs.TL), and genes are clustered based on similar expression profiles. The color key indicates the Z-score of normalized gene expression, with red representing high expression and blue representing low expression.

Functional annotation and enrichment of differentially expressed genes

To functionally interpret the identified DEGs, we performed a KEGG enrichment analysis. The findings are summarized in the enrichment scatter plot (Fig. 4), where the Rich Factor serves as a measure of enrichment degree and the -log10(Q-value) indicates enrichment significance. Our analysis revealed a significant overrepresentation of genes in several key pathways, including metabolic pathways, ribosome, nucleotide metabolism, sulfur metabolism, drug metabolism – other enzymes, amino sugar and nucleotide sugar metabolism, and metabolism of xenobiotics by cytochrome P450. Among these, metabolic pathways was the most prominent, containing the largest number of DEGs (indicated by the point size) and showing the highest level of enrichment and significance.

To predict the functional roles of the identified differentially expressed genes (DEGs), we performed a eukaryotic Orthologous Groups (KOG) analysis. The results (Fig. 5) categorize these genes into 25 broad functional groups. The largest proportion of DEGs was assigned to [R] General function prediction, followed by [T] Signal transduction mechanisms, [S] Function unknown, and [O] Posttranslational modification, protein turnover, chaperones. This distribution suggests that the biological process under investigation predominantly affects cellular processes related to protein synthesis and signaling.

To identify co-expression modules, we performed hierarchical clustering of genes based on topological overlap, and the resulting cluster dendrogram was cut using the dynamic tree cut method. The co-expression modules were identified from the cluster dendrogram using the Dynamic Hybrid tree cut algorithm, with each module denoted by a unique color (Fig. 6). Hierarchical clustering of genes yielded a cluster dendrogram, from which 40 distinct co-expression modules (labeled with different colors, Fig. 6) were identified.

Discussion

This study presents the first transcriptomic analysis of the stingless bee *Tetragonula pagdeni* under low-temperature (10°C) stress, revealing a significant molecular reprogramming in response to an acute cold challenge. The clear separation between control and treated groups in the PCA, along with the identification of over 1,200 differentially expressed genes (DEGs), underscores the profound impact of a 2-hour exposure to 10°C on adult bees. This extensive transcriptional shift suggests that *T. pagdeni*, despite its tropical origin, mounts a robust and coordinated genetic response to counteract the

detrimental effects of cold stress. As a complete stingless bee genome is unavailable[29], a de novo transcriptome analysis was conducted in this study.

Our findings are consistent with other transcriptomic studies on bees under thermal stress. For instance, transcriptomic approach has been successfully applied to uncover the molecular mechanisms of cold resistance in *Apis cerana cerana*[32] and to diagnose multiple stressors in bumble bees[33] using landscape transcriptomics. In our study, the functional enrichment of the DEGs provides crucial insights into the potential adaptive strategies employed by these bees. The most prominent response was the significant enrichment of genes in broad metabolic pathways. This likely reflects a critical re-wiring of energy metabolism to meet the heightened energy demands of stress responses and cellular homeostasis under cold conditions. Transcriptome analysis of the cold-tolerant *Apis mellifera sinisxinyuan* revealed that short-term temperature treatment regulates cold hardiness, a process mediated by key genes such as *Abra* and *Pla1*, which are involved in signal transduction and metabolic pathways[26]. The concurrent upregulation of pathways involved in amino sugar and nucleotide sugar metabolism may indicate increased production of cryoprotectants or restructured carbohydrate metabolism, a common survival strategy in insects facing thermal stress.

Furthermore, the significant enrichment in ribosome-related pathways suggests an increased capacity for protein synthesis. This could be directed towards the production of molecular chaperones and stress-responsive proteins to mitigate cold-induced protein denaturation and maintain proteostasis. This interpretation is supported by the KOG classification, which showed a substantial number of DEGs in the category of [O] Posttranslational modification, protein turnover, chaperones. The induction of such genes is a hallmark of the cellular stress response across organisms.

The study identified a key gene module (darkorange) associated with low-temperature stress in honeybees, implicating autophagy, endocytosis, and MAPK signaling pathways in the cold response mechanism[34]. The researchers identified distinct transcriptomic signatures for various stressors and applied them to field bees, revealing heat stress during a heatwave and starvation/cold stress in cooler mornings[33]. The significant upregulation of Cluster-2041.22, a probable cytochrome P450, is of particular interest. This finding strongly suggests a crucial role for this enzyme in the physiological response to cold stress. As established in prior research, this could be due to the role of P450 enzymes in metabolizing lipids and hormones, whose homeostasis might be disrupted by cold, or in combating cold-induced oxidative stress[35]. Cold stress can indirectly lead to the accumulation of reactive oxygen species (ROS), and the upregulation of these systems may represent a crucial mechanism to alleviate oxidative damage during cold exposure and the subsequent rewarming phase[36].

In conclusion, our transcriptomic profiling reveals that the cold stress response in *Tetragonula pagdeni* is a complex, multifaceted process. It involves a concerted effort to manage energy resources through metabolic shifts, protect cellular integrity by enhancing protein turnover and chaperone systems, and potentially mitigate secondary oxidative damage. These findings lay a foundational molecular framework for understanding how tropical stingless bees, a critically important group of pollinators, may cope with

the thermal extremes associated with a changing climate. Future research can focus on validating the functions of key candidate genes identified here and exploring the long-term colony-level consequences of these molecular responses to inform effective conservation and management practices.

Declarations

Author Contributions:

Dahe Yang and Xinmeng Li: investigation, data curation, conceptualization, formal analysis, writing-original draft, writing-review and editing. Yuzhu Huo: data curation, formal analysis. Kunlin Wan and Changsheng Ma: software, investigation, writing-review and editing. Yaoting Zhang and Hui Xie; investigation, methodology. TianZheng Xu and Hua Wang: resources, supervision. Xuwei Zhu and Zhaonan Zhang: conceptualization, funding acquisition, resources, supervision, writing-review and editing.

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Figures

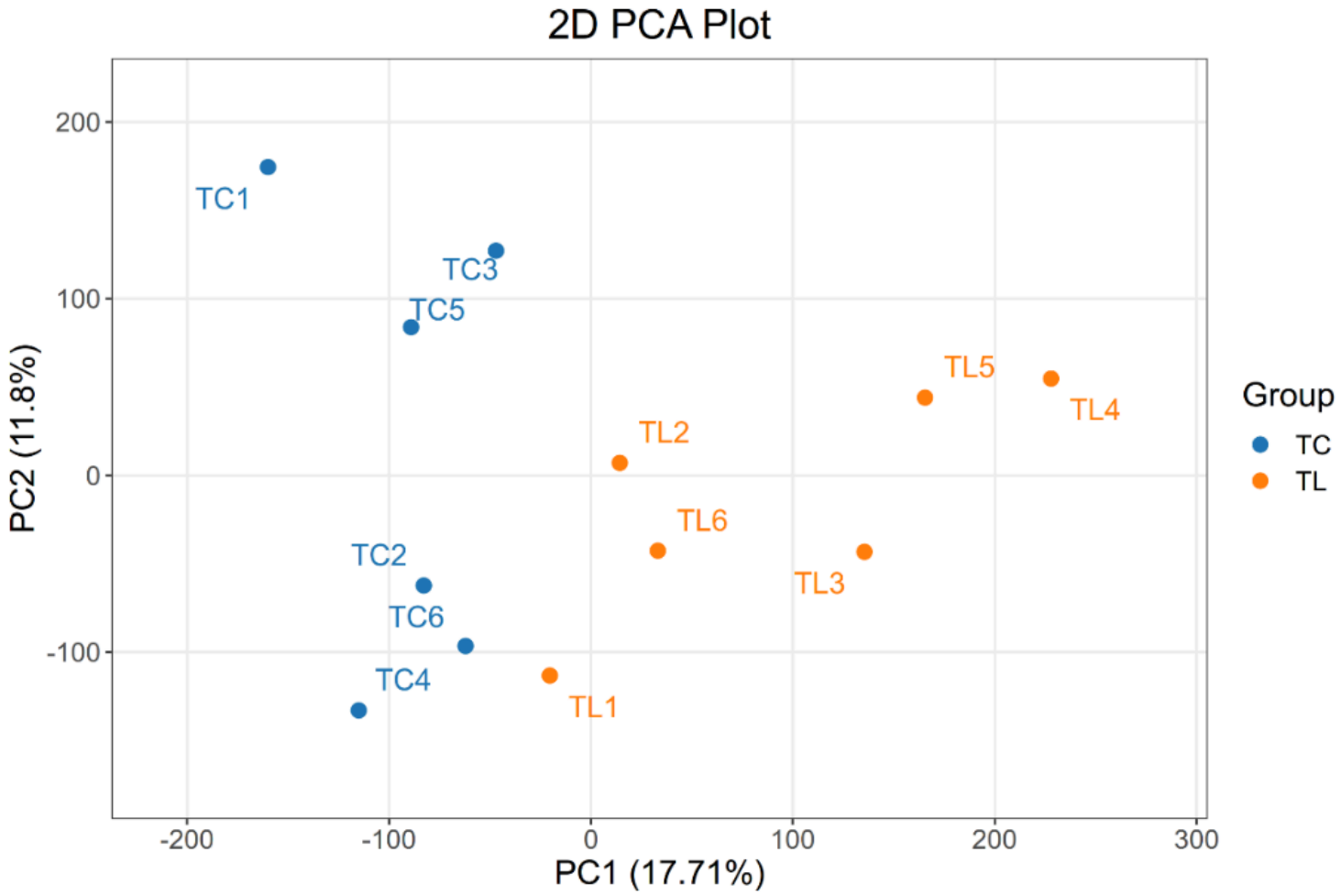


Figure 1

Principal component analysis (PCA) assessing the transcriptomic divergence between TC and TL groups. The percentages indicate the variance explained by each principal component.

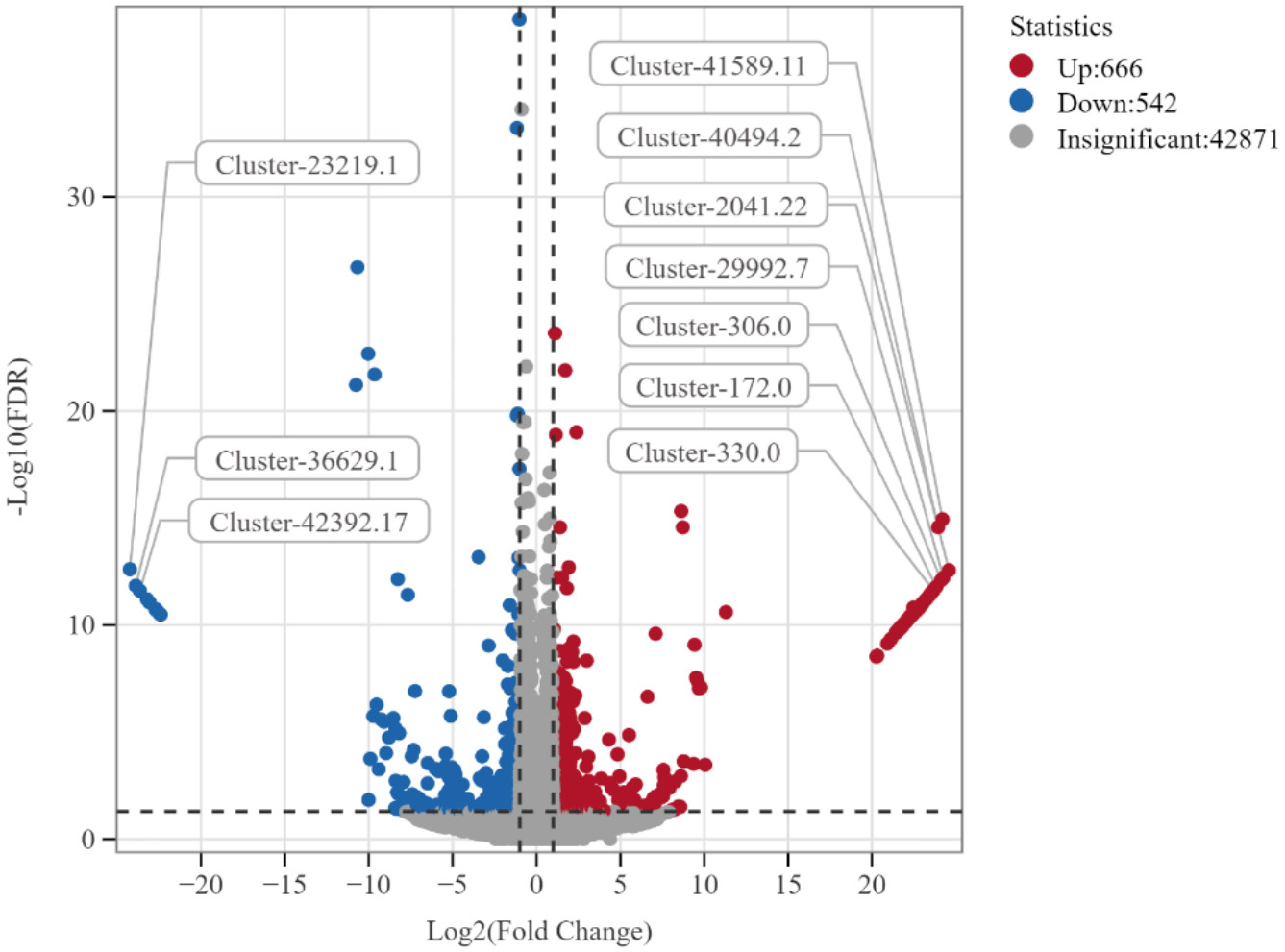


Figure 2

Volcano plot of differentially expressed genes following cold stress. The plot compares the transcriptomic profiles of the control (n=6) and cold-treated groups (n=6) of *Tetragonula pagdeni*.

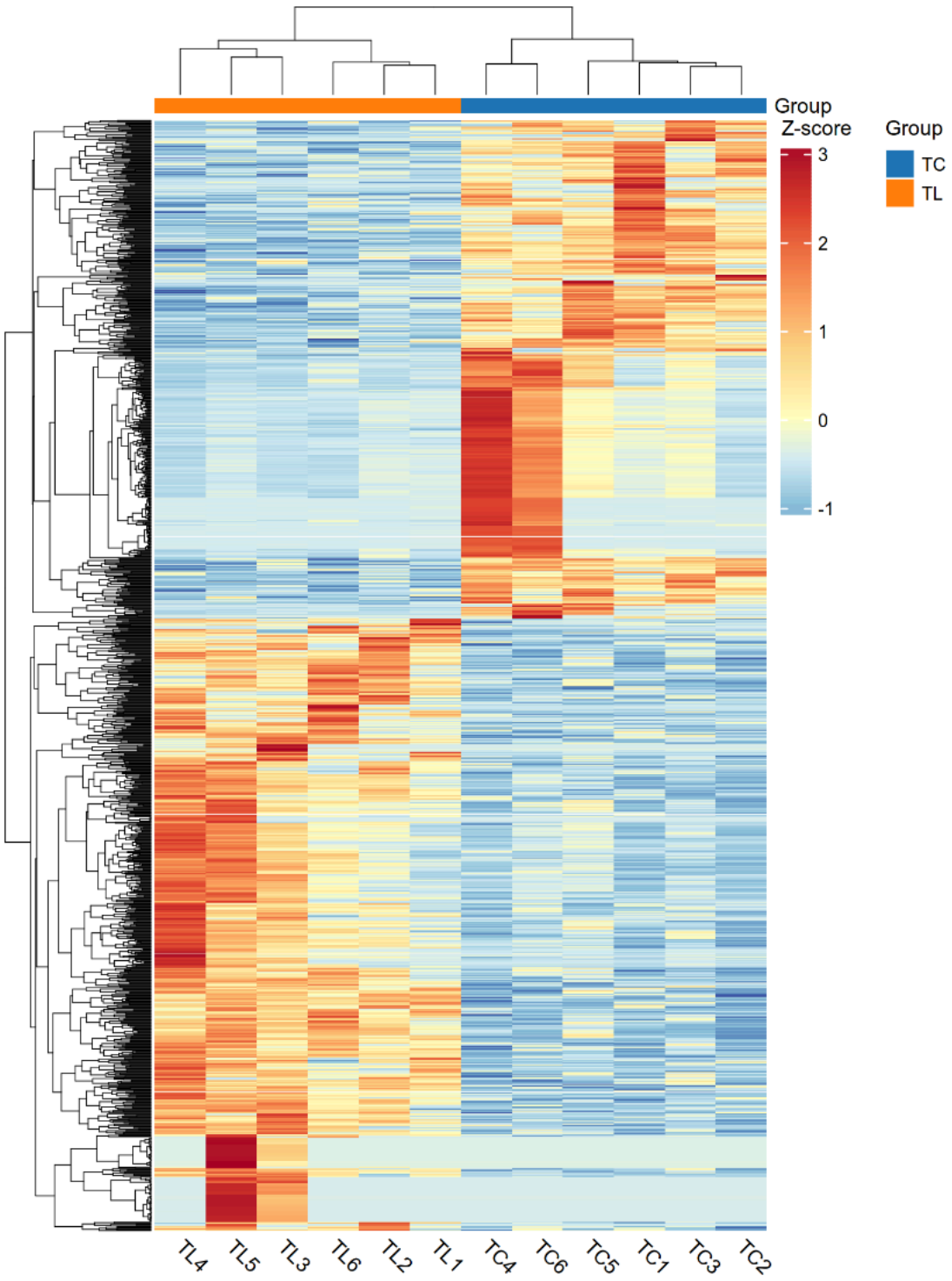


Figure 3

Heatmap of differentially expressed genes. The heatmap visualizes the expression patterns of DEGs across all samples, with colors corresponding to Z-score-scaled expression levels.

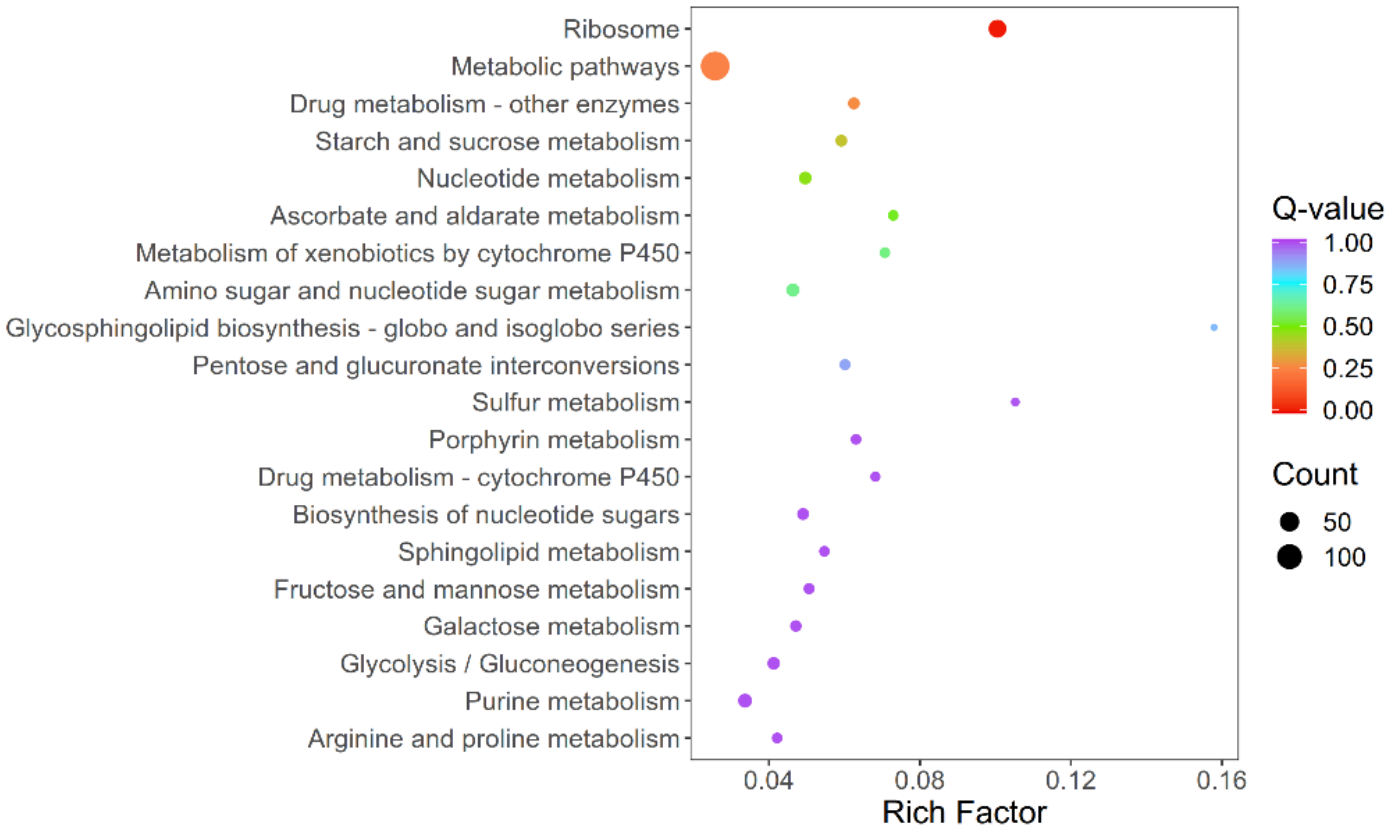


Figure 4

Scatter plot of KEGG pathway enrichment analysis for differentially expressed genes (DEGs). The X-axis indicates the Rich Factor, representing the degree of enrichment. The Y-axis shows the statistical significance of the enrichment as -log10(Q-value). A higher Rich Factor and a higher value on the Y-axis indicate a more significant enrichment. The color of the dots corresponds to the Q-value (e.g., red for more significant), and the size of the dots is proportional to the number of DEGs mapped to the respective pathway.

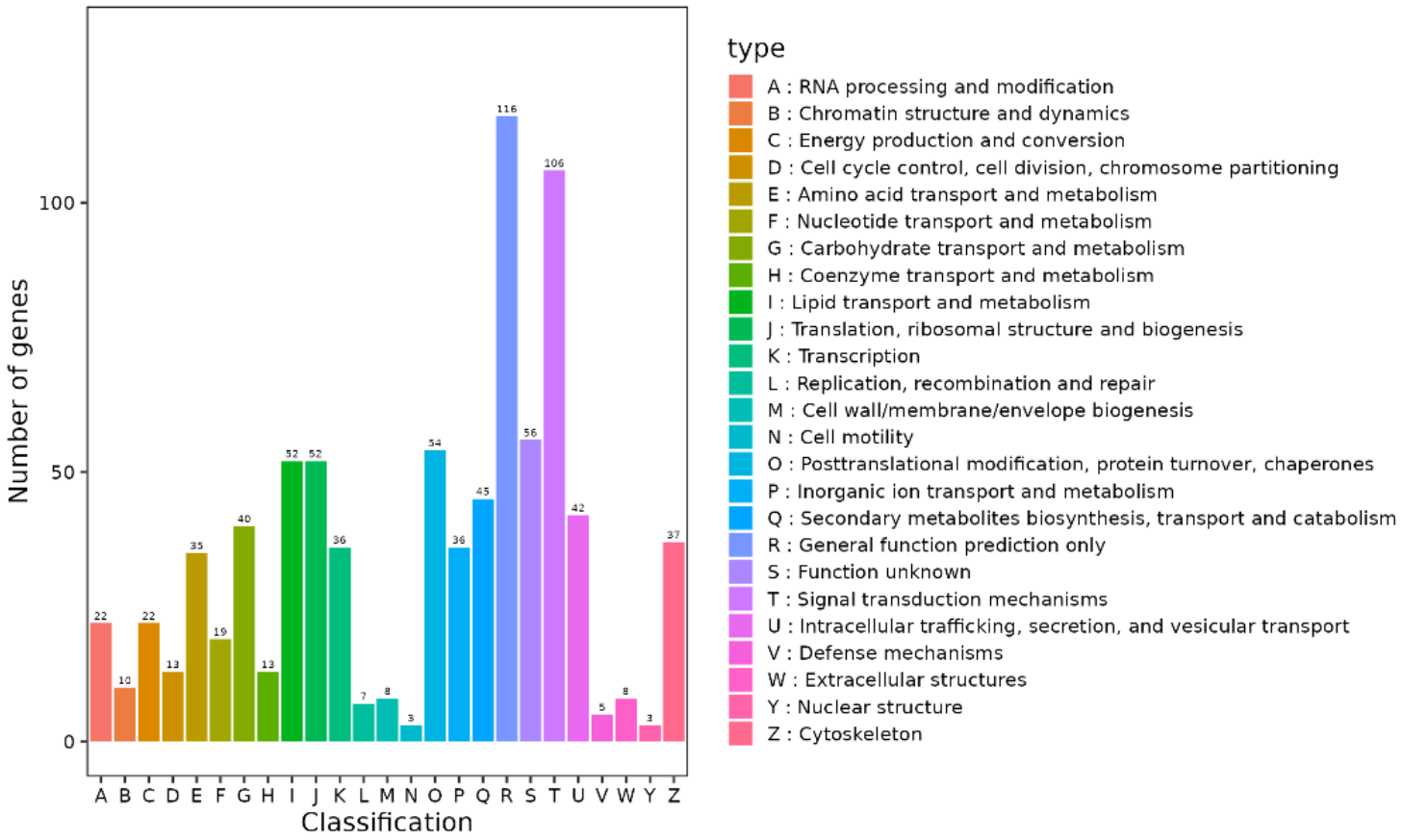


Figure 5

Functional classification of the differentially expressed genes (DEGs) based on the KOG database. The bar chart displays the distribution of DEGs across various KOG functional categories. The Y-axis lists the 25 major KOG categories (denoted by letters A to Z), and the X-axis shows the number of genes assigned to each category.

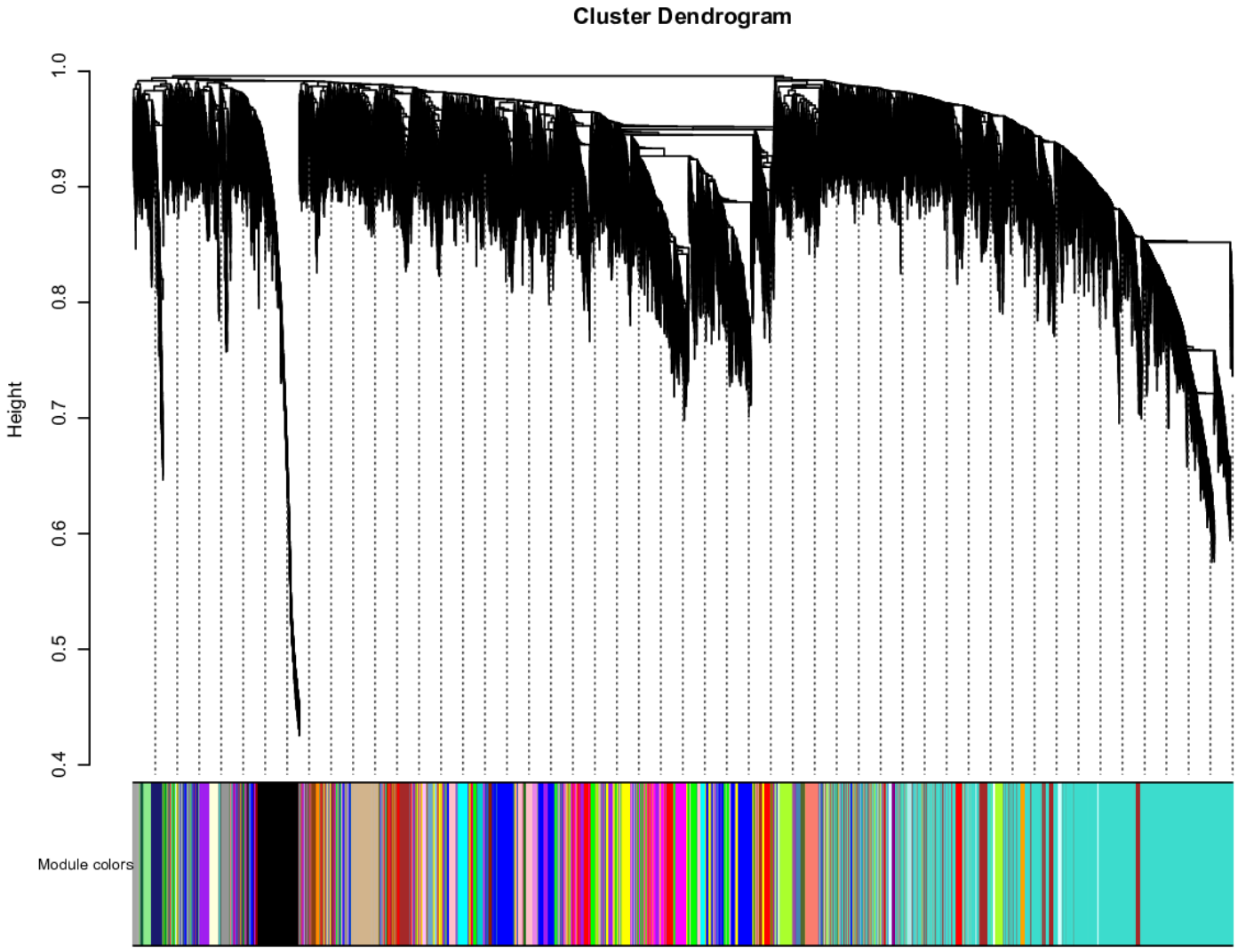


Figure 6

Cluster dendrogram of co-expression modules. The hierarchical clustering tree (dendrogram) of genes is shown. Each leaf in the tree represents one gene. The major branches, identified by different colors below the dendrogram, define distinct co-expression modules. The dynamic tree cut method was used to cut the dendrogram branches and assign genes to modules.