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Transcriptome analysis reveals differential gene expression in tomato under high-temperature stress

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Abstract: Tomato is a major global crop, extensively cultivated in China. However, the molecular mechanisms underlying its responses to high-temperature stress remain poorly understood. This study investigates these mechanisms by examining a heat-resistant tomato variety, Hm 2-2 (R), and a heat-sensitive variety, BY 1-2 (S), under high temperature (40 °C). Total RNA was extracted from samples taken at 0 and 24 h post-treatment, followed by RNA-sequencing (RNA-seq). Differentially expressed genes (DEGs) were screened based on the criteria of $|\log_2$ fold change$| \geq 2$ and false discovery rate ≤ 0.05. Gene ontology (GO) function annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway enrichment analysis were performed to explore the biological significance of these DEGs. The results revealed 6 038 upregulated and 2 866 downregulated DEGs in the R-0 (Hm 2-2 plants treated at 40 °C for 0 h) vs. R-24 (Hm 2-2 plants treated at 40 °C for 24 h) group and 5 297 upregulated and 3 920 downregulated DEGs in the S-0 (BY 1-2 plants treated at 40 °C for 0 h) vs. S-24 (BY 1-2 plants treated at 40 °C for 24 h) group, respectively. GO enrichment analysis indicated that the majority of DEGs were associated with biological processes, followed by cellular components and molecular functions. KEGG pathway analysis identified 130, 131, 89, and 115 regulatory (or altered) pathways in the R-0 vs. R-24, S-0 vs. S-24, S-0 vs. R-0, and S-24 vs. R-24 group comparisons, respectively. Notably, pathways related to protein processing in the endoplasmic reticulum and plant hormone signal transduction were significantly enriched, suggesting their critical roles in the tomato's response to heat stress. Moreover, 156 transcription factors (TFs) implicated in heat stress response were identified, spanning various TF families such as MYB, AP2-EREBP, b-ZIP, bHLH, NAC, and WRKY. Quantitative RT-PCR analysis of 14 randomly selected DEGs validated the RNA-seq results confirming the reliability of the data. In summary, this study provides valuable insights into the molecular mechanisms of tomato's responses to high-temperature stress, laying a crucial foundation for future research in this area.

Keywords: gene expression profile; heat response; quantitative RT-PCR; RNA-seq; transcription factors

Tomato (*Solanum lycopersicum* L.) is a globally significant crop, widely cultivated in China. The fruit is highly valued for its rich nutritional content, distinctive flavour, and high levels of vitamins, carbohydrates, minerals, and organic acids, making it a staple in the diet of Chinese residents (Todaka et al. 2024). In 2021, China produced 66.09 million tons of tomatoes, with the country leading the world in tomato cultivation area, reflecting the rapid development of the tomato industry (Niu et al. 2022). Tomato growth and development are optimal at temperatures between 15 °C and 30 °C, with deviations

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from this range negatively impacting its growth and development (Su et al. 2021a). As global temperatures continue to rise, high temperatures pose a significant threat to crop productivity (Djanaguiraman et al. 2018). Research has shown that elevated temperatures can affect tomato seed vitality, even completely inhibiting germination in extreme cases (Qian et al. 2002). High temperatures not only impede normal plant growth but also suppress photosynthesis, ultimately reducing tomato yield and quality (Zandalinas et al. 2018). The accelerating pace of global warming has made high summer temperatures a major limiting factor in tomato cultivation, as plants struggle to adapt to these changing environmental conditions (Zhou et al. 2010).

High temperatures not only cause evident external damage to plants but also lead to the degradation of chloroplast structures, disruption of the photosynthetic system, and accumulation of intracellular reactive oxygen species. These conditions damage the cell membrane system and disrupt metabolic functions and physiological processes, negatively affecting plant growth, yield, and in severe cases, leading to plant death (Higashi et al. 2015; Li et al. 2018; Zhao et al. 2020; Huang et al. 2022). Plants exposed to high temperatures rapidly sense and transmit heat signals, inducing the expression of heat-responsive genes, including transcription factors (TFs), protein kinases, and heat shock proteins (HSPs), to regulate cellular and metabolic homeostasis, thereby enhancing their resistance to heat stress (Mittler et al. 2012; Kan et al. 2023; Seth & Sebastian 2024; Kong et al. 2025). Current research on tomato heat stress has primarily focused on the effects of high temperatures on growth and development, fruit quality, physiological and biochemical indices, and the screening of corresponding heat-resistance identification indices and heat-resistance gene localization (Camejo et al. 2005; Lv 2021; Vijayakumar et al. 2021; Mesa et al. 2022; Jiang 2023; Graci & Barone 2024; Madasamy Raja et al. 2025). However, there is limited research on the molecular mechanisms underlying tomato's responses to high temperatures (Ding et al. 2022; Meng et al. 2022; Wang et al. 2023; Wen et al. 2024; Li et al. 2025b). Transcriptome sequencing technology involves reverse transcribing mRNA into cDNA, allowing for the rapid and comprehensive acquisition of sequence information and the quantification of nearly all cDNA in a specific sample. This technology is instrumental in studying the differential expression of functional elements within the genome, as well as in classifying transcriptional profiles and analysing responses to various stress conditions (Hawkins & Hon 2010; Annadurai et al. 2012). RNA-sequencing (RNA-seq) has emerged as a crucial tool for studying gene transcription and expression due to its speed, accuracy, and capacity to generate large datasets (Wei et al. 2020; Jiang et al. 2024; Bu et al. 2025). It has been extensively used to investigate heat stress resistance in various plant species, including *Arabidopsis thaliana* (Rao et al. 2023), *Oryza sativa* (Qiu et al. 2023), *Zea mays* (Wu et al. 2020), *Triticum aestivum* (Paul et al. 2022), *Solanum melongena* (Zhang et al. 2020), *Solanum tuberosum* (Jian et al. 2023), *Gossypium hirsutum* (Liang et al. 2021; Zhang et al. 2022). However, there are relatively few studies utilising RNA-seq technology to explore heat resistance in tomatoes. For example, Wen et al. (2019) combined traditional quantitative trait locus (QTL) mapping with QTL-seq analysis and RNA-seq, identifying four candidate genes (*SlCathB2*, *SlGST*, *SlUBC5*, and *SlARG1*) associated with heat resistance in major QTLs. Bizouerne et al. (2020) used RNA-seq to study the effects of light and temperature on tomato seed vitality during harvest, generating gene ontology (GO) and Mapman annotations on ITAG 4.0 for data analysis. These annotations serve as valuable resources for future data mining. Almeida et al. (2021) examined the changes in wild-type tomato fruits under transient heat stress at the transcriptome, cellular, and metabolite levels, identifying several heat stress response genes linked to metabolite changes, including genes involved in fruit maturation regulation. Despite these advances, the molecular mechanisms by which heat-resistant genes regulate tomato heat resistance remain unclear. Therefore, it is essential to screen for heat-resistant genes in tomatoes using transcriptome analysis to analysis to deepen our understanding of this process.

In this study, two tomato inbred lines, Hm 2-2 and BY 1-2, with distinct differences in heat resistance, were used as experimental materials. A total of 12 samples from these lines, before and after heat stress treatment, were analysed using RNA-seq. Differentially expressed genes (DEGs) were screened, followed by functional annotation analysis to identify key TFs involved in the tomato's response to heat stress. This study provides a theoretical foundation for future research on the molecular mechanisms of tomato heat resistance.

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MATERIAL AND METHODS

Plant materials and high-temperature treatment. In this study, two tomato inbred lines, Hm 2-2 (R) and BY 1-2 (S), with evident differences in heat resistance, were used as experimental materials (Chen et al. 2022). In March 2024, tomato seeds were sown in a culture chamber within the light culture room of the Key Laboratory at Yichun University. The day and night temperatures in the chamber were maintained at 28–30 °C and 15–17 °C, respectively, with 50% humidity, a 12/12-h dark cycle, and a light intensity of 30 000 lx. When the seedlings reached the 5–6 leaf stage, robust plants with consistent growth were selected and subjected to high-temperature treatment in an artificial climate incubator set at 40 °C. Leaves from the two inbred lines were collected at 0 h (R-0 and S-0) and 24 h (R-24 and S-24) after treatment. Three biological replicates were set, yielding a total of 12 samples, which were immediately flash-frozen in liquid nitrogen and stored at –80 °C for subsequent RNA-sequencing.

RNA extraction, quality evaluation, cDNA library construction, and RNA-sequencing. Total RNA was extracted from the leaf tissue of six samples from each of the four lines (R-0, R-24, S-0, and S-24) using an RNA extraction kit (R6827, Omega Bio-Tek, USA) following the manufacturer's protocol. Each experiment was conducted in three biological replicates. RNA integrity, the presence of DNA, and protein contamination were assessed by 1% agarose gel electrophoresis. The concentration and purity of the extracted RNA were measured using a Nanodrop microspectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, USA), and RNA integrity (RIN value) was determined using an Agilent 2100 bioanalyzer (Agilent Technologies, USA). Eukaryotic mRNA containing poly(A) tail was enriched using oligo(dT) magnetic beads, followed by fragmentation of the mRNA using a buffer solution. The first strand of cDNA was synthesised from the fragmented mRNA using random oligonucleotides as primers in the M-MuLV reverse transcriptase system. The RNA strand was then degraded by RNaseH, and the second cDNA strand was synthesised in the DNA polymerase I system using dNTPs as raw material. The resulting double-stranded cDNA was purified and end-repaired, and an A-tail was added before ligation with sequencing adapters. Approximately 200 bp cDNA fragments were screened using AMPure XP beads for PCR amplification, and the PCR products were purified again using AMPure XP beads. The final library was sequenced on the Illumina HiSeq2500 platform (Gene Denovo Biotechnology Co., Ltd., Guangzhou, China).

Transcriptome assembly and annotation. To ensure high data quality, the raw sequencing data were filtered before analysis to reduce the interference caused by invalid data. The raw reads were quality-controlled using fastp (Chen et al. 2018), and low-quality reads (more than 50% of bases with a quality value $Q \leq 20$) were filtered out to obtain clean reads. These clean reads were then aligned to the ribosomal RNA database using the short reads comparison tool Bowtie2 (Langmead & Salzberg 2012), with no mismatches allowed, to remove reads matching ribosomal sequences. The remaining unmapped reads were used for transcriptome analysis. Clean reads were mapped to the reference tomato (*S. lycopersicum*) genome version ITAG4.0 (https:// solgenomics.net/ftp/tomato_geno me/annotation/ITAG4.0_release/) using HISAT2 (Kim et al. 2015) software. Gene expression levels in the samples were calculated as Fragments Per Kilobase of transcript Per Million Fragments mapped (FPKM) values using Stringtie (Pertea et al. 2015) software based on the HISAT2 alignment results. The FPKM value represents the expression level of each gene in the sample.

Sample relationship analysis. Principal component analysis (PCA) was conducted using R (http://www.r-project.org/) software based on the gene expression data to study the relationships between samples through dimensionality reduction. Additionally, Pearson correlation coefficients were calculated between each pair of samples using the expression data, and these correlations were visually represented in heat maps. This analysis helped assess the repeatability between samples and identify any potential outliers.

Expression profile and enrichment analysis of DEGs. Differential expression analysis was conducted using DESeq2 (Love et al. 2014) software, with input data consisting of read count data obtained from gene expression level analysis. The analysis process involved three main steps: (1) normalisation of read counts; (2) calculation of hypothesis testing probability (*P*-value) based on the model; and (3) multiple hypothesis testing to obtain the false discovery rate (FDR). Genes with a FDR < 0.05 and $|\log_2 \text{foldchange (FC)}| \geq 2$ were considered DEGs.

To determine the primary biological functions of the DEGs, all DEGs were mapped to the GO database (http://www.geneontology.org/) and assigned GO terms. The number of DEGs associated with each

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GO term was calculated to generate a list of DEGs with specific GO functions. Hypergeometric tests were then applied to identify GO terms significantly enriched among the DEGs compared to the background. Since different DEGs coordinate their biological functions *in vivo*, pathway-based analysis was conducted to further explore the biological roles of these differential genes. Pathway enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway as the unit, applying hypergeometric tests to identify pathways significantly enriched in DEGs compared to the overall background. This analysis helps pinpoint the most critical biochemical metabolic pathways and signal transduction pathways involved in the activity of the differential genes.

Verification of RNA-seq data by quantitative real-time PCR (qRT-PCR). To validate the reliability of the RNA-seq data, quantitative real-time PCR (qRT-PCR) analysis was conducted on a randomly selected set of 14 genes (*HSP22.7* (*Solyc01g102960.3*), *GAPA* (*Solyc02g020940.3*), *PMEI11* (*Solyc03g083770.1*), *ABP19A* (*Solyc03g123410.1*), *CAB6A* (*Solyc05g056050.3*), *PSAG* (*Solyc07g066150.1*), *LE25* (*Solyc10g078770.2*), *JA2L* (*Solyc07g063410.3*), *WRKY24* (*Solyc06g066370.4*), *bZIP53* (*Solyc01g100460.3*), *ERF110* (*Solyc04g071770.3*), *MYB15* (*Solyc03g005570.3*), *HSF30* (*Solyc08g062960.4*), and *BT4* (*Solyc02g092460.3*)). Specific primers for these genes were designed using Primer 5 software (Premier, Canada) and synthesised by Sangon Biotech (Shanghai, China). cDNA was synthesised from 1 μg of total RNA using the StarScript III All-in-one RT Mix with gDNA Remover reagent Kit (GenStar, Beijing, China). qRT-PCR was then performed using the StepOne Real-Time PCR Instrument (Applied Biosystems, Thermo Fisher, USA) and corresponding software, with the 2× RealStar Fast SYBR qPCR Mix (with High ROX) (GenStar, Beijing, China) as the reaction mix. Each sample was analysed in triplicate. The tomato *Actin* (Solyc03g078400) gene was used as the internal control (Yan et al. 2020). The relative expression levels of the genes across three biological replicates were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). The sequences of the 14 pairs of primers used for qRT-PCR are listed in Table S1 in Electronic Supplementary Material (ESM).

RESULTS

Experimental design and phenotypic characterization. Plants exhibited different phenotypic symptoms after treated at 40 °C high temperature for 24 h. As shown in Figure 1, leaves of the Hm 2-2 and BY 1-2 plants treated at 40 °C for 0 h showed no obvious symptoms. However, one or two leaves of the Hm 2-2 plants treated at 40 °C for 24 h showed wilting symptoms, but almost all the leaves of the BY 1-2 plants treated at 40 °C for 24 h showed wilting symptoms. These results indicate that tomato Hm 2-2 and BY 1-2 plants may respond differently to high-temperature stress.

Figure 1. Phenotypic symptoms of Hm 2-2 and BY 1-2 tomato seedlings before and after treated at 40 °C high temperature
R-0 – Hm 2-2 plants treated at 40 °C for 0 h; R-24 – Hm 2-2 plants treated at 40 °C for 24 h; S-0 – BY 1-2 plants treated at 40 °C for 0 h; S-24 – BY 1-2 plants treated at 40 °C for 24 h

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RNA extraction and quality assessment. The quality analysis of total RNA extracted from tomato samples, both before and after high-temperature treatment, demonstrated that the RNA was intact, with no signs of degradation or protein contamination (Figure S1 in ESM), as confirmed by agarose gel electrophoresis. The concentrations, measured using a Nanodrop spectrophotometer, were all above 100 ng/μL (Table S2 in ESM), with OD260/OD280 and OD260/OD230 ratios indicating high RNA purity. Additionally, the RNA content for each sample exceeded 1 μg, meeting the requirements for constructing twelve libraries and suitable for subsequent experiments.

RNA quality control data statistics. Twelve cDNA libraries were constructed and sequenced using the Illumina HiSeq 2500 platform. An overview of the RNA-seq data is presented in Table 1. The R-0, R-24, S-0, and S-24 groups yielded approximately 40 040 068; 39 138 831; 39 092 054, and 38 349 559 raw reads, respectively, which corresponded to 39 892 723; 39 020 033; 38 963 658; and 38 233 107 clean reads. The average GC content across these groups was 43.00, 42.29, 42.91%, and 42.35%, respectively. The Q20 (98.40, 98.63, 98.32%, and 98.42% for R-0, R-24, S-0, and S-24 groups, respectively) and Q30 (95.08, 95.79, 94.86%, and 95.10% for R-0, R-24, S-0, and S-24 groups, respectively) values were high, indicating the transcriptome data quality was excellent. The rRNA content was less than 3% across all samples, minimising interference in the subsequent bioinformatics statistics (Table S3 in ESM). Clean reads were mapped to the tomato genome using HISAT2 software, with mapping rates of approximately 93.01, 92.25, 93.11%, and 93.80% for R-0, R-24, S-0, and S-24 groups, respectively. The proportion of unmapped reads was low, being 3.39, 3.75, 3.09%, and 3.52%, respectively, and the mapping results were consistent across all libraries (Table S4 in ESM).

Sample relationship analysis. PCA was conducted on the 12 samples, as shown in Figure 2A. The analysis revealed significant differences between the two varieties before and after the treatment, while samples subjected to the same treatment clustered closely together, indicating good repeatability within each group. Pearson correlation coefficients were calculated between each pair of samples (Figure 2B), further confirming the high repeatability of the biological replicates. The global distribution of gene expression levels, evaluated using FPKM values, was similar across all samples (Figure 2C), demonstrating consistent expression trends and robust repeatability.

Table 1. Overview of the sequence assembly after Illumina sequencing

Sample	Raw reads	Clean reads	Q20	Q30	GC content
			(%)		
R-0-1	41 449 070	41 283 548	98.33	94.91	43.01
R-0-2	39 912 050	39 764 382	98.38	95.03	43.07
R-0-3	38 759 084	38 630 240	98.49	95.31	42.91
$R\text{-}0_{means}$	40 040 068	39 892 723	98.40	95.08	43.00
R-24-1	36 041 090	35 917 834	98.68	95.93	42.27
R-24-2	39 739 624	39 622 846	98.55	95.53	42.33
R-24-3	41 635 780	41 519 418	98.67	95.90	42.28
$R\text{-}24_{means}$	39 138 831	39 020 033	98.63	95.79	42.29
S-0-1	39 291 702	39 156 818	98.31	94.86	42.89
S-0-2	36 590 354	36 486 080	98.26	94.65	42.93
S-0-3	41 394 106	41 248 076	98.39	95.08	42.90
$S\text{-}0_{means}$	39 092 054	38 963 658	98.32	94.86	42.91
S-24-1	38 942 172	38 818 190	98.31	94.82	42.35
S-24-2	38 756 944	38 629 388	98.21	94.55	42.49
S-24-3	37 349 560	37 251 742	98.75	95.93	42.21
$S\text{-}24_{means}$	38 349 559	38 233 107	98.42	95.10	42.35

Q20 – the percentage of bases with a quality value greater than or equal to 20; Q30 – the percentage of bases with a quality value greater than or equal to 30; GC – the proportion of guanine (G) and cytosine (C) bases among all the bases in the genome

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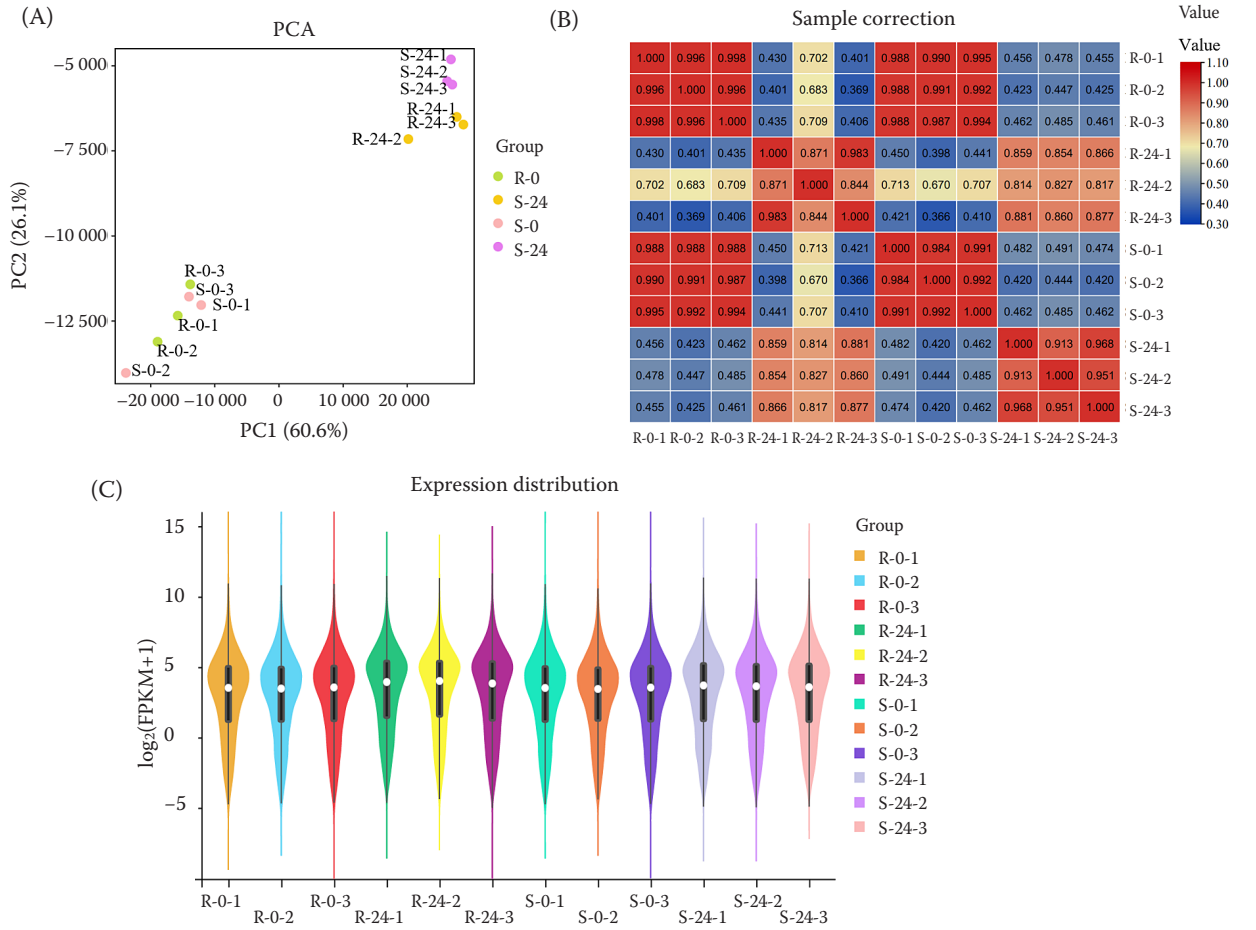

Figure 2. Sample relationship analysis: principal component analysis (PCA) analysis of twelve expression samples (A), sample correlation heat map (B), violin illustration of gene expression in different groups (C)
R-0 – Hm 2-2 plants treated at 40 °C for 0 h; R-24 – Hm 2-2 plants treated at 40 °C for 24 h; S-0 – BY 1-2 plants treated at 40 °C for 0 h; S-24 – BY 1-2 plants treated at 40 °C for 24 h

Analysis of DEGs. Differential expression analysis was performed using the DESeq2 package with criteria set at FDR < 0.05 and $|\log_2 FC| \geq 2$ DEGs. A total of 8 904 DEGs (6 038 and 2 866 significantly upregulated and downregulated genes, respectively) were identified in the R-0 vs. R-24 comparison, 9 217 DEGs (5 297 and 3 920 significantly upregulated and downregulated genes, respectively) in the S-0 vs. S-24 comparison, 775 DEGs (397 and 378 significantly upregulated and downregulated genes, respectively) in the S-0 vs. R-0 comparison, and 2 257 DEGs (972 and 1 285 significantly upregulated and downregulated genes, respectively) in the S-24 vs. R-24 comparison (Figure 3A). A Venn diagram revealed that 56 genes were differentially expressed across all four groups (Figure 3B). Additionally, 5 519, 74, 403, 79, 589, and 207 DEGs were identified in the following comparisons: R-0 vs. R-24 and S-0 vs. S-24; R-0 vs. R-24 and S-0 vs. R-0; R-0 vs. R-24 and S-24 vs. R-24; S-0 vs. S-24 and S-0 vs. R-0; S-0 vs. S-24 and S-24 vs. R-24; S-0 vs. R-0 and S-24 vs. R-24, respectively. The volcano plots displayed a clear distribution pattern of upregulated and downregulated genes in all four sets of comparisons, with a significantly higher number of DEGs in the R-0 vs. R-24 and S-0 vs. S-24 comparisons than in the S-0 vs. R-0 and S-24 vs. R-24 comparisons (Figure 3C). However, despite these differences, the overall distribution patterns remained similar across the four groups. Hierarchical clustering analysis further highlighted the overall expression patterns of DEGs (Figure 3D), demonstrating that more genes were upregulated in the experimental groups than those in the control groups. This upregulation of genes suggests that heat stress induced the expression of resistance genes. These findings provide a clear depiction of the

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overall gene expression patterns across the different comparisons.

Verification of RNA-seq data by qRT-PCR. To verify the reliability of gene expression data obtained through RNA-seq analysis, 14 DEGs were randomly selected for qRT-PCR analysis, with three biological replicates for each reactions. As depicted in Figure 4, several genes including *HSP22.7*, *LE25*, *JA2L*, *WRKY24*, *bZIP53*, *ERF110*, *MYB15*, *HSF30*, and *BT4* were upregulated after high-temperature treatment. Conversely, genes such as *GAPA*, *PMEI11*, *ABP19A*, *CAB6A*, and *PSAG* exhibited downregulated following high-temperature treatment. The qRT-PCR results were consistent with the RNA-seq data, and the correlation coefficients between the qRT-PCR and RNA-seq data were both greater than 0.9, confirming the reliability of the gene expression patterns observed under high-temperature stress treatment.

GO analysis of DEGs. We annotated the function of GO for all DEGs across comparison groups using the GO database. Accordingly, all DEGs were classified into three categories: biological processes (24), molecular functions (12), and cellular components (17). The distribution patterns of the number and type of enriched GO terms were similar across the four comparison groups (Figure 5). In terms of biological processes, metabolic processes, cellular processes, and single-organism processes dominated

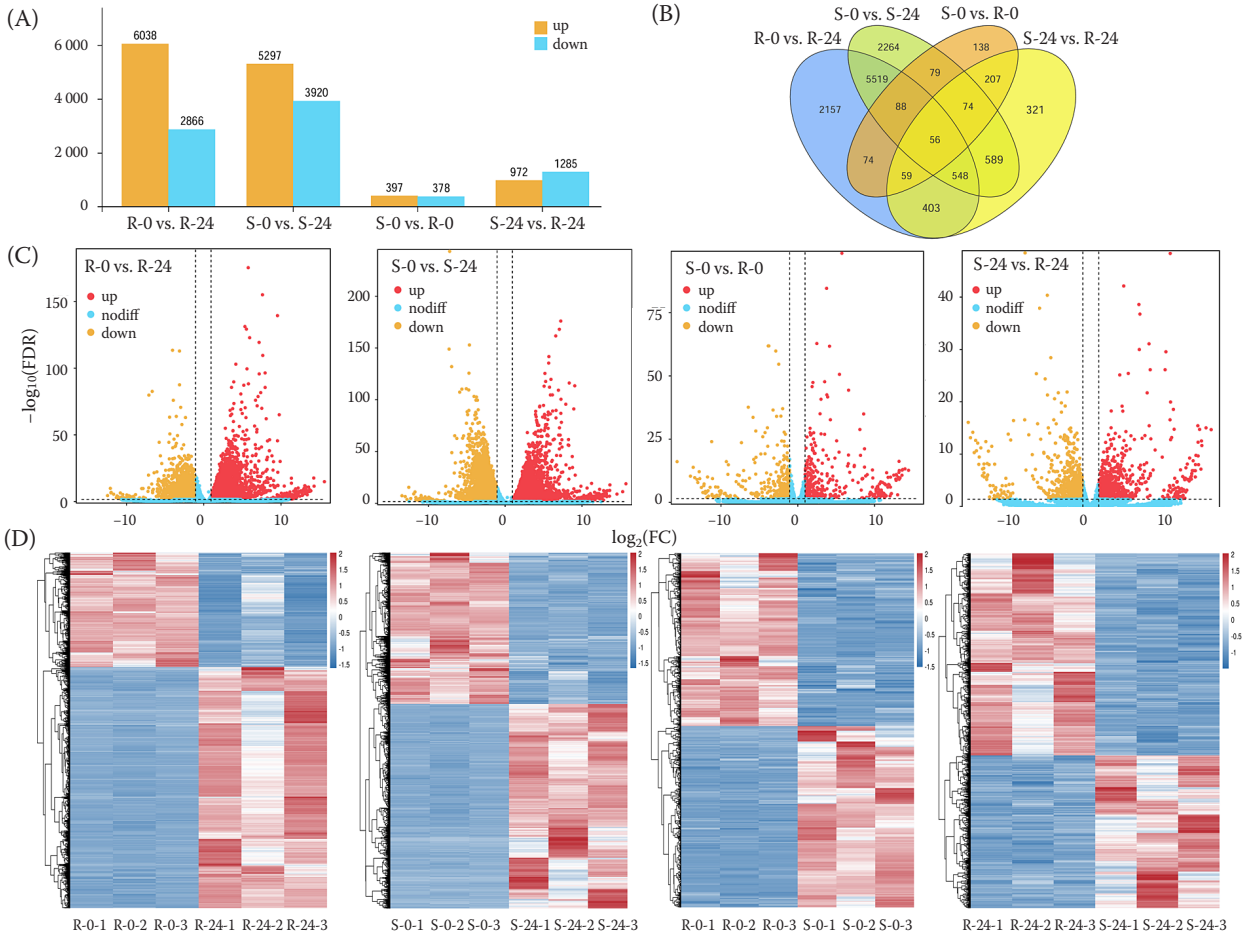

Figure 3. Differential expression analysis between treatments: comparison of the number of up- and down-regulated genes in different groups (A; yellow and blue points represent up- and down-regulated genes, respectively), Venn diagram of DEGs (B), volcano plots of DEGs between treatments (C; red and blue points represent up- and down-regulated genes, respectively; blue points represent no significant), clustering heat map of DEGs between treatments (D; red colour represents highly expressed genes; blue colour represents low-expressed genes. From red to blue: $\log_{10}$ (FPKM+1) is from large to small. R-0 – Hm 2-2 plants treated at 40 °C for 0 h; R-24 – Hm 2-2 plants treated at 40 °C for 24 h; S-0 – BY 1-2 plants treated at 40 °C for 0 h; S-24 – BY 1-2 plants treated at 40 °C for 24 h

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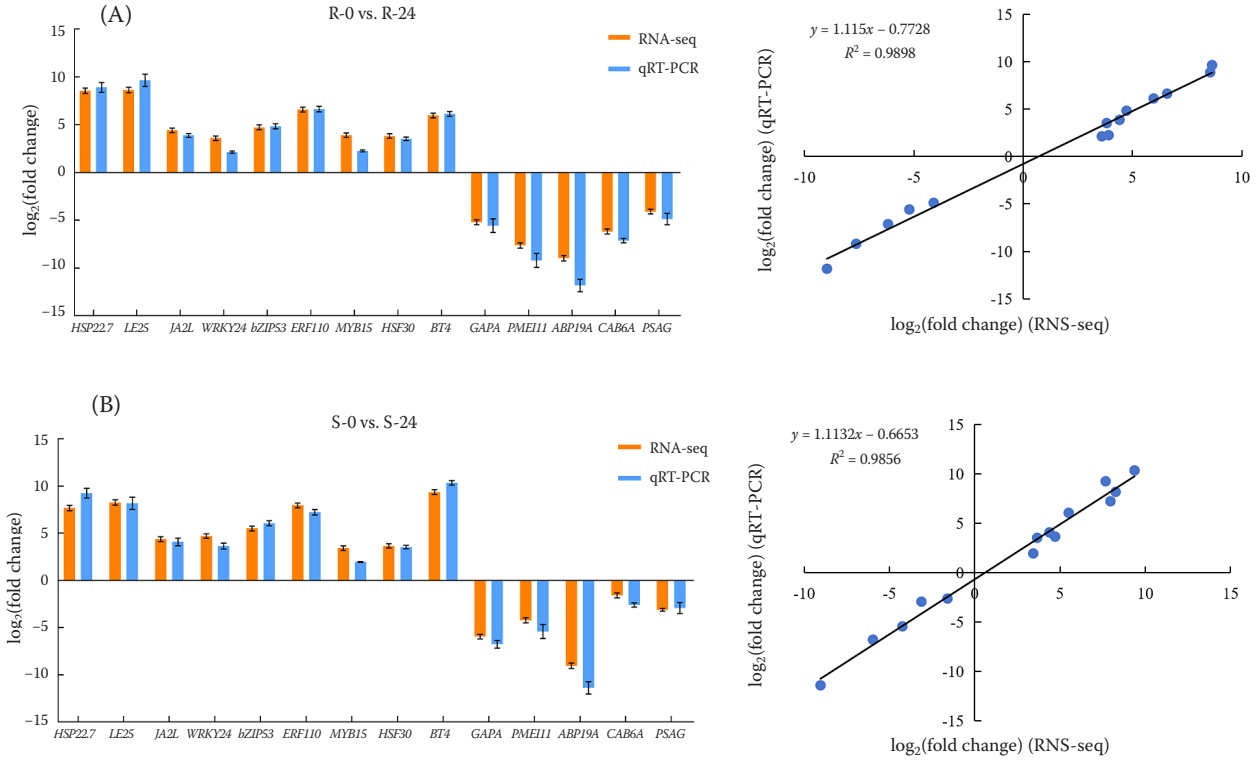

Figure 4. Quantitative real-time PCR validation: comparison of the R-0 vs. R-24 (A), comparison of the S-0 vs. S-24 (B) Red and blue points represent up- and down-regulated genes, respectively; blue points represent no significant; the yellow column represents RNA-seq data, and the blue column represents qRT-PCR data; the experiments were performed in triplicate; R-0 – Hm 2-2 plants treated at 40 °C for 0 h; R-24 – Hm 2-2 plants treated at 40 °C for 24 h; S-0 – BY 1-2 plants treated at 40 °C for 0 h; S-24 – BY 1-2 plants treated at 40 °C for 24 h; the data are presented as the mean ± SE (n = 3)

the GO categories across the comparison groups. For molecular functions, catalytic activity, binding, and nucletic acid binding transcription factor activity were the most prominent. In the cellular components category, cell, cell part, and organelle were the major categories.

In GO functional enrichment analysis of the R-0 vs. R-24 comparison, several GO terms were significantly enriched and contained a substantial number of DEGs. These terms included plastid thylakoid (GO:0031976), NADP metabolic process (GO:0006739), pyridine-containing compound metabolic process (GO:0072524), rRNA metabolic process (GO:0016072), pyridine nucleotide metabolic process (GO:0019362), nicotinamide nucleotide metabolic process (GO:0046496), and oxidoreduction coenzyme metabolic process (GO:0006733) (Figure 6A). Similarly, in the S-0 vs. S-24 comparison, such GO terms were NADP metabolic process (GO:0006739), nicotinamide nucleotide metabolic process (GO:0046496), pyridine nucleotide metabolic process (GO:0019362), pyridine-containing compound metabolic process (GO:0072524), oxidoreduction coenzyme metabolic process (GO:0006733), cofactor metabolic process (GO:0051186), coenzyme metabolic process (GO:0006732), and plastid thylakoid (GO:0031976) (Figure 6B). For the S-0 vs. R-0 comparison, the enriched GO terms with a significant number of DEGs were associated with catalytic activity (GO:0003824), oxidoreductase activity (GO:0016491), inflammatory response to antigenic stimulus (GO:0002437), acute inflammatory response to antigenic stimulus (GO:0002438), hypersensitivity (GO:0002524), and acute inflammatory response (GO:0002526) (Figure 6C). Lastly, in the S-24 vs. R-24 comparison, the GO terms significantly enriched and containing a substantial number of DEGs included oxidoreductase activity (GO:0016491), mitochondrial membrane part (GO:0044455), mitochondrion (GO:0005739), mitochondrial part (GO:0044429), mitochondrial membrane (GO:0031966), mitochondrial envelope (GO:0005740), and respiratory electron transport chain (GO:0022904) (Figure 6D).

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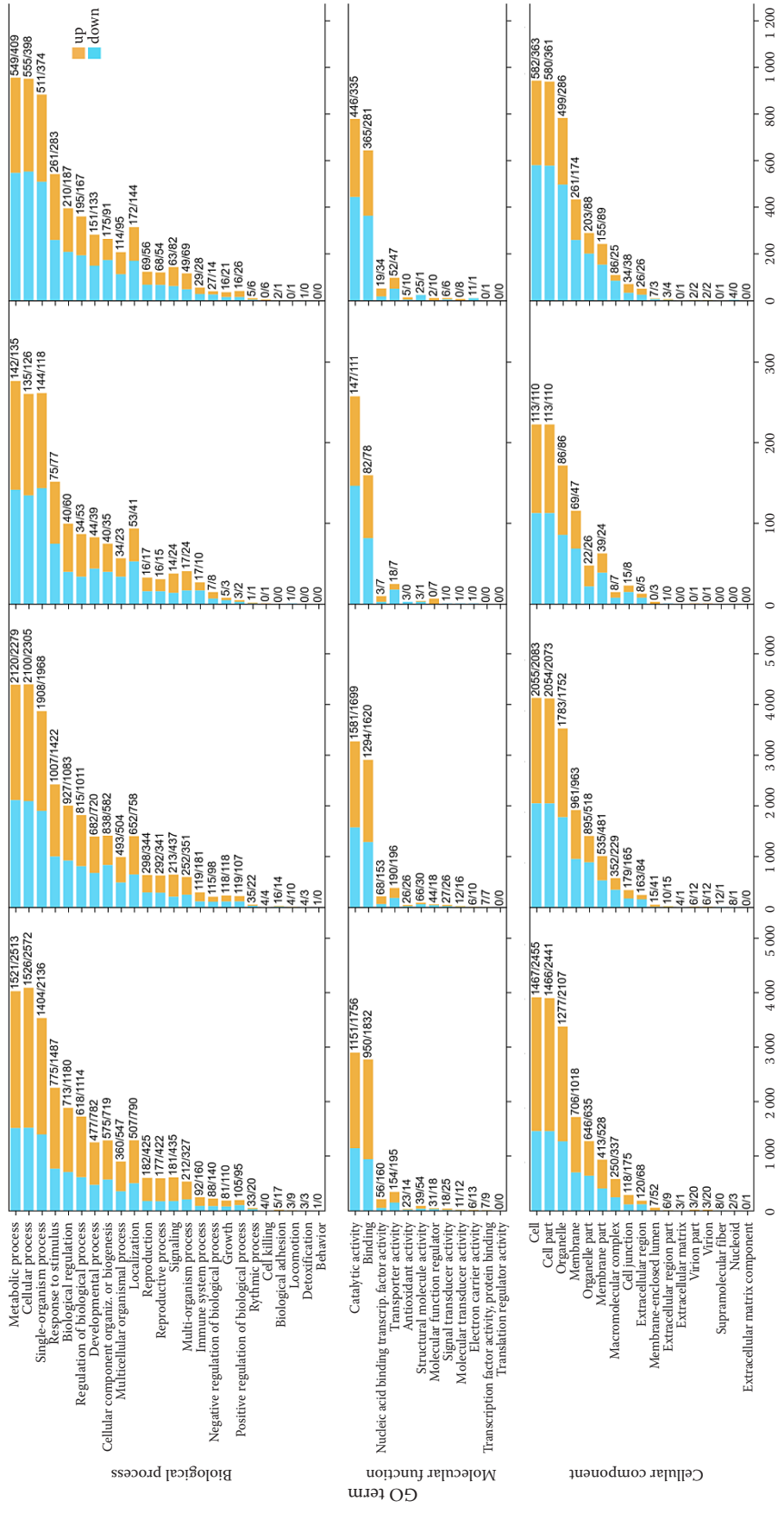

Figure 5. Gene ontology (GO) function annotation analysis in four comparisons (R-0 vs. R-24, S-0 vs. S-24, S-0 vs. R-0, and S-24 vs. R-24)
The yellow column represents up-regulated differentially expressed genes (DEGs), and the blue column represents down-regulated DEGs; R-0 – Hm 2-2 plants treated at 40 °C for 0 h; R-24 – Hm 2-2 plants treated at 40 °C for 24 h; S-0 – BY 1-2 plants treated at 40 °C for 0 h; S-24 – BY 1-2 plants treated at 40 °C for 24 h

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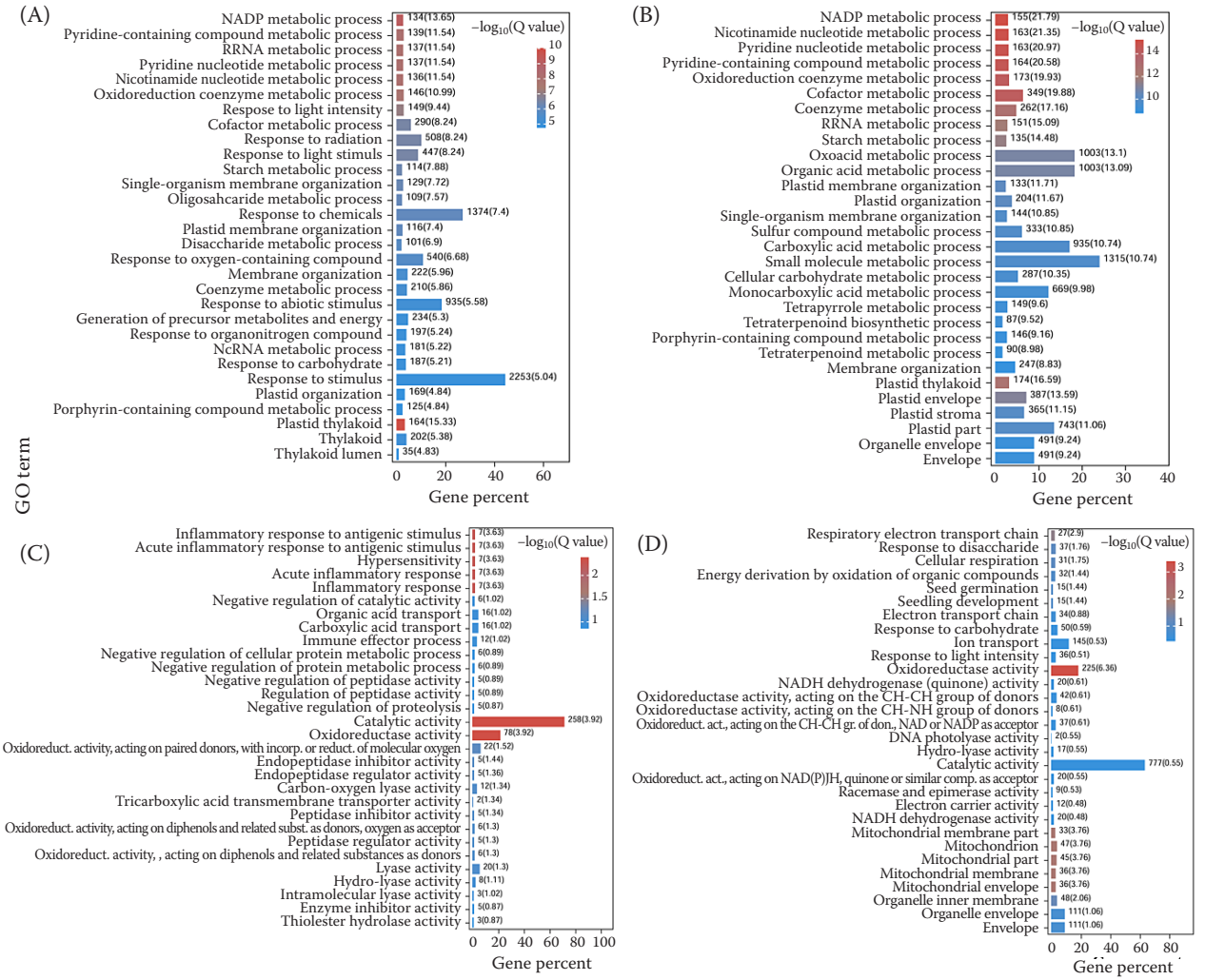

Figure 6. Gene ontology (GO) functional enrichment analysis: the R-0 vs. R-24 comparison (A), the S-0 vs. S-24 comparison (B), the S-0 vs. R-0 comparison (C), the S-24 vs. R-24 comparison (D)

R-0 – Hm 2-2 plants treated at 40 °C for 0 h; R-24 – Hm 2-2 plants treated at 40 °C for 24 h; S-0 – BY 1-2 plants treated at 40 °C for 0 h; S-24 – BY 1-2 plants treated at 40 °C for 24 h

KEGG pathway analysis of DEGs. To further understand the biological and signal transduction pathways involved in tomato's response to heat stress, we conducted a KEGG pathway enrichment analysis for DEGs across the comparison groups and compared them to the entire transcriptomic context (Figure 7). In the R-0 vs. R-24 comparison, we identified 130 pathways, with the most DEGs being enriched in pathways such as "carbon metabolism" (113), "protein processing in endoplasmic reticulum" (96), "biosynthesis of amino acids" (83), "spliceosome" (77), and "starch and sucrose metabolism" (62) pathways (Figure 8A). Among these, 12 pathways had Q values < 0.05, indicating significant enrichment (Table S5 in ESM). For the S-0 vs. S-24 comparison, 131 pathways were identified, with the majority of DEGs being enriched in "metabolic pathway" (937), followed by "biosynthesis of secondary metabolites" (537), "carbon metabolism" (126), "protein processing in endoplasmic reticulum" (93), and "biosynthesis of amino acids" (89) pathways (Figure 8B). Moreover, 12 of 131 pathways had Q values < 0.05 (Table S6 in ESM). In the S-0 vs. R-0 comparison, we identified 89 pathways, with significant enrichment in "biosynthesis of secondary metabolites" (42), "beta-alanine metabolism" (11), "valine, leucine and isoleucine degradation" (11), "mismatch repair" (11), and "homologous recombination" (11) (Figure 8C). However, only seven pathways had Q values < 0.05 (Table S7 in ESM). In the S-24 vs. R-24 comparison,

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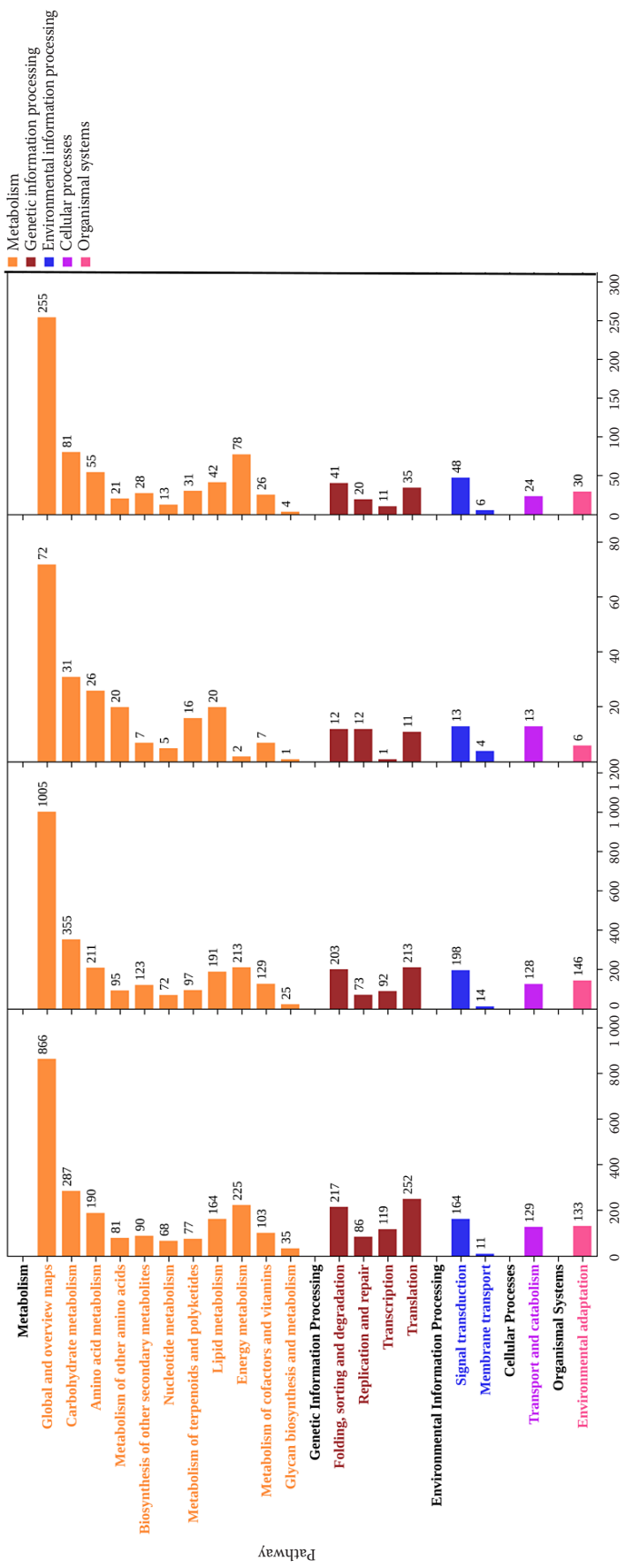

Figure 7. Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotation analysis in four comparisons (R-0 vs. R-24, S-0 vs. S-24, S-0 vs. R-0, and S-24 vs. R-24)
R-0 – Hm 2-2 plants treated at 40 °C for 0 h; R-24 – Hm 2-2 plants treated at 40 °C for 24 h; S-0 – BY 1-2 plants treated at 40 °C for 0 h; S-24 – BY 1-2 plants treated at 40 °C for 24 h

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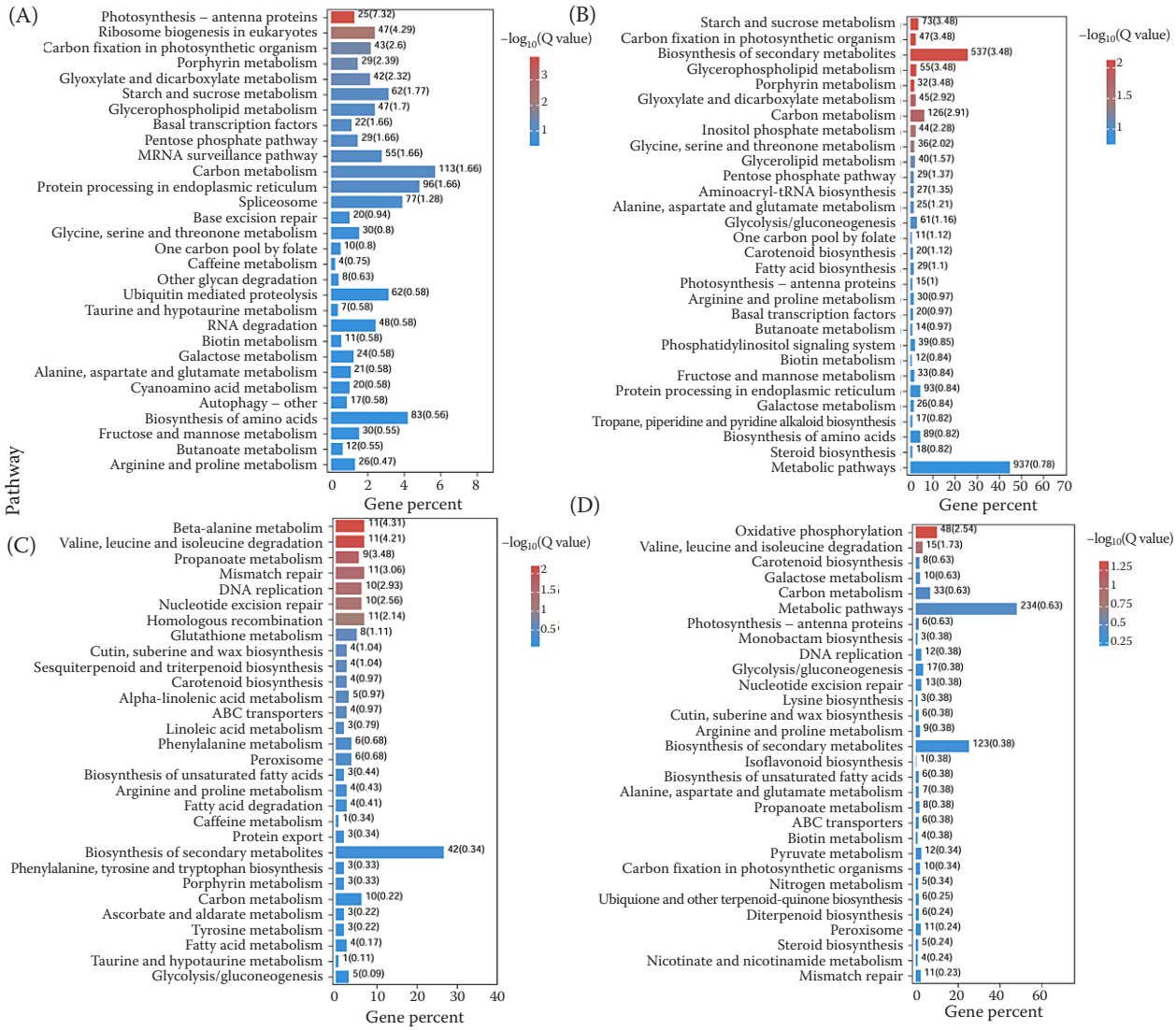

Figure 8. Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis: the top thirty KEGG pathways containing the largest number of differentially expressed genes (DEGs) in R-0 vs. R-24 comparison (A), the top thirty KEGG pathways containing the largest number of DEGs in S-0 vs. S-24 comparison (B), the top thirty KEGG pathways containing the largest number of DEGs in S-0 vs. R-0 comparison (C), the top thirty KEGG pathways containing the largest number of DEGs in S-24 vs. R-24 comparison (D)

The first 30 pathways with the smallest Q value were mapped; the ordinate coordinate was the pathway, and the abscess coordinate was the percentage of the number of these pathways in the number of all differential genes; the darker the colour, the smaller the Q value, and the values on the column were the number and Q value of the pathway; R-0 – Hm 2-2 plants treated at 40 °C for 0 h; R-24 – Hm 2-2 plants treated at 40 °C for 24 h; S-0 – BY 1-2 plants treated at 40 °C for 0 h; S-24 – BY 1-2 plants treated at 40 °C for 24 h

115 pathways were identified, with DEGs being most enriched in "metabolic pathway" (234), "biosynthesis of secondary metabolites" (123), "oxidative phosphorylation" (48), "carbon metabolism" (33), and "glycolysis/gluconeogenesis" (17) (Figure 8D). However, only two pathways had Q values < 0.05 (Table S8). The *Q* values from the KEGG enrichment analysis indicate that although the level of enrichment in some pathways may not be the highest compared to that in others, the "metabolic pathway" consistently shows significant enrichment of the most DEGs (Table S5–S8 in ESM).

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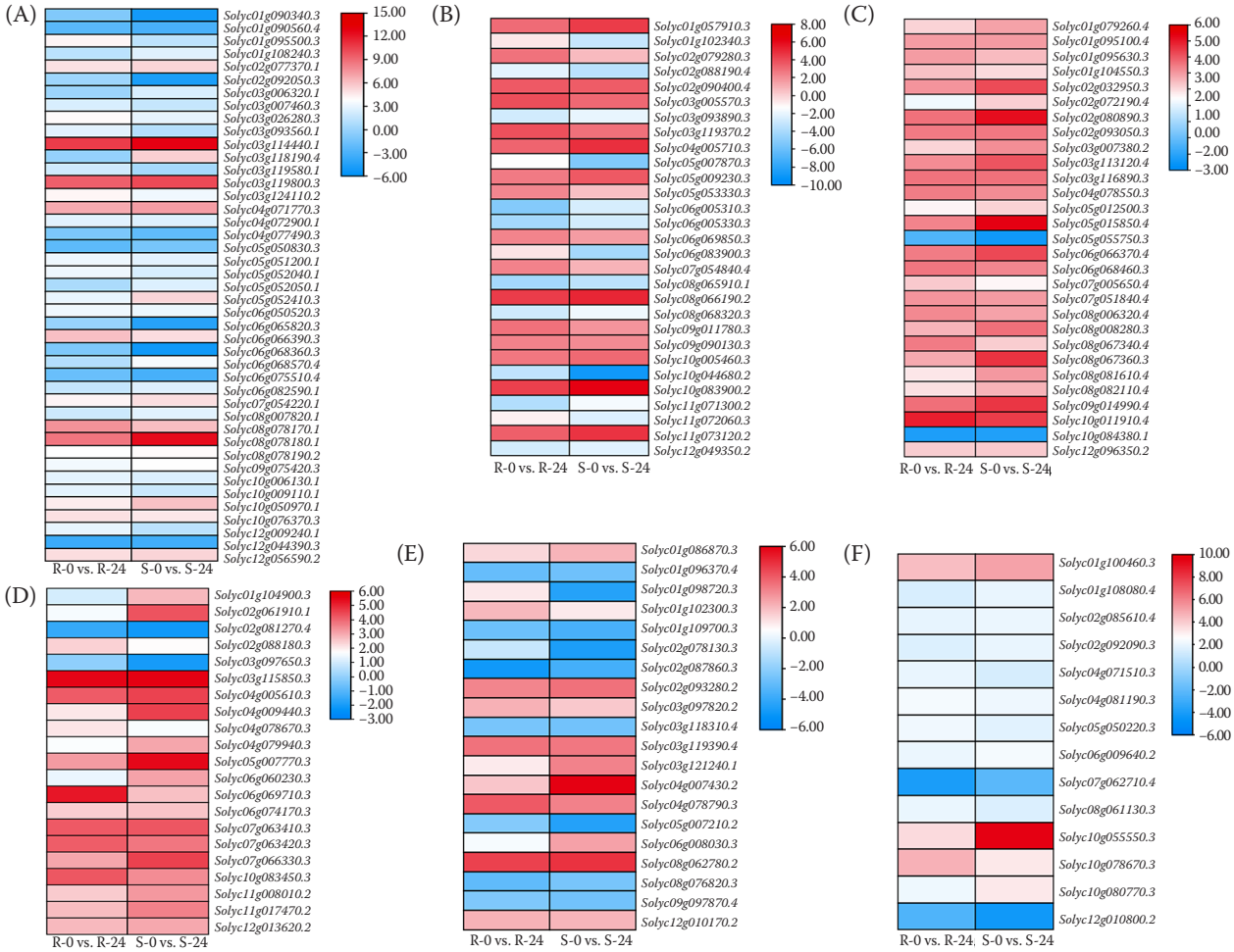

Figure 9. Stratified clustering of differentially expressed genes (DEGs) expression profiles of 6 different transcription factor gene families identified in tomato: AP2-EREBP transcription factor gene family (A), MYB transcription factor gene family (B), WRKY transcription factor gene family (C), NAC transcription factor gene family (D), bHLH transcription factor gene family (E), b-ZIP transcription factor gene family (F)

The genes with a FDR < 0.05 and $|\log_2\text{foldchange(FC)}| \geq 2$ were considered DEGs; R-0 – Hm 2-2 plants treated at 40 °C for 0 h; R-24 – Hm 2-2 plants treated at 40 °C for 24 h; S-0 – BY 1-2 plants treated at 40 °C for 0 h; S-24 – BY 1-2 plants treated at 40 °C for 24 h

Protein processing in the endoplasmic reticulum pathway. During protein processing in the endoplasmic reticulum pathway, 86 and 81 DEGs were induced and upregulated by high-temperature stress in the R-0 vs. R-24 and S-0 vs. S-24 comparisons, respectively. Most of these DEGs, including *HSP90*, *HSP70*, *HSP40*, and *sHSF*, were associated with heat stress response (Figure S2 in ESM). However, the expression patterns of different genes varied between the two comparisons, with genes such as *Sec13/31*, *PDIs*, and *EDEM* being upregulated in the S-0 vs. S-24 comparison but did not show any significant change in the R-0 vs. R-24 comparison. Conversely, *Sec62/63*, *ERManI*, *IRE1*, *NEF*, *HSP70*, and *RMA1* were upregulated in the R-0 vs. R-24 comparison, with no significant changes in the S-0 vs. S-24 comparison. These findings provide valuable insights into the mechanisms underlying tomato's resistance to high-temperature stress.

Plant hormone signal transduction pathway. In the plant hormone signalling pathway, 71 genes were upregulated and 25 genes were downregulated in the R-0 vs. R-24 comparison, while 78 genes were upregulated and 33 genes were downregulated

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in the S-0 vs. S-24 comparison. Most DEGs, such as auxin-responsive *AUX1*; cytokine-responsive *CRE1* and *B-ARR*; gibberellin-responsive *GID1* and *TF*; abscisic acid-responsive *PYR/PYL* and *SnRK2*; ethylene-responsive *ETR*, *CTR*, *EBF1/2*, *EIN3*, and *SIMKK*; brassinosteroid-responsive *TCH4*; salicylic acid-responsive *NPR1*, were upregulated in both R-0 vs. R-24 and S-0 vs. S-24 comparisons (Figure S3 in ESM). However, there were notable differences in the expression of certain DEGs between the two comparisons. For example, salicylic acid-responsive *PR-1* was upregulated in the S-0 vs. S-24 comparison but downregulated in the R-0 vs. R-24 comparison. The expression of jasmonic acid-responsive *JAR1* was downregulated in the R-0 vs. R-24 comparison, whereas jasmonic acid-responsive *JAZ* was upregulated and *CYCD3* was downregulated in the S-0 vs. S-24 comparison, with *JAR1* showing no significant change.

Gene expression analysis of transcription factors. TFs are DNA-binding proteins that interact with the cis-acting elements of genes to either activate or inhibit gene transcription. They play a crucial role in regulating various biological pathways. In this study, more than 1 000 TFs were identified, with the majority belonging to different TF families such as MYB, AP2-EREBP, b-ZIP, bHLH, NAC, and WRKY. Among these, the AP2-EREBP subfamily (part of the ERF family), with 43 DEGs, stood out as the largest TF family responding to heat shock in the leaves of tomato seedlings under high-temperature stress (Figure 9A, Table S9 in ESM). Additionally, we identified 29, 29, 21, 20, and 14 DEGs belonging to the MYB, WRKY, NAC, bHLH and bZIP families, respectively (Figure 9B–F, Table S10–S14 in ESM). These TF genes exhibited various expression patterns in the leaves of tomato seedlings, indicating their potential involvement in the heat shock response and crucial role in improving the resistance of tomato to heat stress.

DISCUSSION

With the increasing threat of global warming, agriculture worldwide faces significant challenges due to high temperatures, which severely inhibit crop growth and development, leading to decreased yield and quality (Wang et al. 2020). Therefore, breeding heat-resistant varieties has become a crucial goal in current crop breeding programs. Understanding the molecular mechanisms of heat stress responses and identifying heat-resistant genes can provide the foundation for breeding new heat-resistant varieties. Tomatoes, one of the most important vegetable and fruit crops globally, are particularly sensitive to high-temperature stress, which can drastically reduce both yield and quality. Consequently, studying the molecular mechanisms underlying the heat shock response is essential for breeding tomatoes with improved heat resistance. The advent of next-generation sequencing technology over the past decade has revolutionised our understanding of the mechanisms involved in heat shock responses and the development of heat-resistant plant varieties. In this study, we constructed 12 cDNA libraries from total RNA isolated from tomato leaves and sequenced them using the Illumina HiSeq TM 2500 platform. We identified 8 960 and 9 217 DEGs in the R-0 vs. R-24 and S-0 vs. S-24 comparisons, respectively, indicating that these heat shock-related genes are induced by high temperatures. All DEGs were classified into three GO categories—molecular function, biological process, and cellular component. The most enriched GO terms were related to metabolic processes. The GO functional classification analysis revealed that DEGs across the four groups were associated with various functions, involving many biological processes such as stress resistance and changes in cell membrane integrity, providing further insights into the molecular mechanisms of tomato's responses to heat shock.

Plants have evolved complex self-defence mechanisms to enhance their adaptability to extreme environments when exposed to high temperatures. HSPs are critical proteins synthesised in response to high temperatures and are involved in various environmental stress responses, regulating numerous developmental processes (Hua et al. 2023; Zhu et al. 2023). Based on their apparent molecular weight, plant HSPs are categorised into five main families: HSP40, HSP70, HSP90, HSP100, and small HSPs (sHSPs). These proteins contribute to protein folding in the endoplasmic reticulum, stabilisation of damaged polypeptide chains, protein complex polymerisation, regulation of intracellular protein homeostasis, and chloroplast movement (Ren et al. 2024). Heat shock transcription factors (HSFs) and HSPs play a pivotal role in plant heat shock response. KEGG analysis revealed that DEGs were significantly enriched in the protein processing pathway in the endoplasmic reticulum under heat shock conditions. Genes such as *HSP70*, *HSP90*, *HSP40*, and

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sHSF responded to high temperatures, with their expression levels being upregulated when tomatoes were exposed to high temperatures (Figure S2 in ESM). Previous studies have demonstrated that the overexpression of *HSP70* enhances the tolerance of transgenic plants to high temperatures (Nakashima et al. 2009). For instance, Zhao et al. (2019) cloned *PlHSP70* from herbaceous peony and found that its overexpression improved the heat tolerance of transgenic *Arabidopsis thaliana*, offering a theoretical basis for future genetic manipulation aimed at increasing heat tolerance in *Arabidopsis*. Similarly, Zhang et al. (2023) concluded that tobacco *NtHSP70-8b* plays a significant role in the heat stress response, with its overexpression contributing to improving crop tolerance to heat stress. Jeon et al. (2025) found that overexpression of *Panax ginseng PgHSFA2* increases the expression of HSPs in tobacco and enhances the resistance of transgenic plants to high-temperature stress. These findings align with our results, suggesting that high-temperature treatment disrupts the normal physiological functions of the endoplasmic reticulum, thereby affecting protein synthesis, modification, and proper folding.

High temperature, as a form of abiotic stress, profoundly impact plant growth and survival. Adapting to and tolerating such stress involves complex sensing, signalling, and response mechanisms. Plant responses to abnormal or extreme temperature fluctuations are primarily regulated by plant hormones, which are signalling compounds that regulate key aspects of growth, development, and environmental stress responses. Hormones such as abscisic acid (ABA), auxin, brassinolide (BR), cytokinin (CK), ethylene (ET), gibberellic acid (GA), jasmonic acid (JA), and salicylic acid (SA) are well-known regulators of plant adaptation to environmental conditions (Devireddy et al. 2021; Waadt et al. 2022). ABA, in particular, plays a crucial role in adaptation to high-temperature stress through its core signalling pathway, involving pyrabactin resistance (*PYR*)/pyrabactin resistance-like (*PYL*)/ABA receptor regulatory components (RCAR), protein phosphatase 2C (*PP2C*), and sucrose non-fermentation (*SNF1*)-associated protein kinase 2 (*SnRK2*) (Zhang et al. 2019). Li et al. (2022) reported that SlSnRK2.3 interacts with SlSUI1 to modulate high-temperature tolerance in tomatoes by regulating stomatal movement via ABA. Similarly, Wu et al. (2023) found that the expression of *LcPYLs* from *Liriodendron chinense* was upregulated in response to heat stress. In this study, the expression of *SnRK2* and *PYR/PYC* were downregulated in the R-0 vs. R-24 and S-0 vs. S-24 comparisons (Figure S3 in ESM). Ethylene response factors (ERFs) are also involved in the heat stress response of many plants. Wang et al. (2022) identified *LlERF110* as an important TF in the ethylene signalling pathway from the high-temperature transcriptome of lilies, where its virus-induced gene silencing reduced the basal heat-resistant phenotype and significantly decreased the expression of HSF-HSP pathway-related genes *LlHsfA2*, *LlHsfA3A*, and *LlHsfA5*. Luo et al. (2025) reported that TFs *ERF74/77/108/125* enhance the thermotolerance in rice by regulating common and distinct heat-responsive gene expression. In this study, the expression of *ERF1/2* was upregulated in the R-0 vs. R-24 comparison group (Figure S3 in ESM). JA is another critical plant hormone known not only for its roles in growth and development, and resistance to pathogens and insect grazing (Song et al. 2022; Wan & Xin 2022; Huang et al. 2023), but also for its involvement in coping with various abiotic stresses (Liu et al. 2017; De Domenico et al. 2019; Ali & Baek 2020; Seleiman et al. 2021), including heat stress (Xu et al. 2016; Tian et al. 2020; Su et al. 2021b; Khan et al. 2023). Our findings support this, as the expression of JA signalling gene *JAR1* was upregulated following high-temperature treatment in the R-0 vs. R-24 comparison. Additionally, the genes *JAZ* and *CYCD3* showed differential expression in the S-0 vs. S-24 comparison, with the expression of *JAZ* being upregulated and *CYCD3* downregulated (Figure S3 in ESM). SA has also been extensively studied over the past decade for its role in plant resistance to abiotic stress (Alotaibi et al. 2023; Das et al. 2023; Talaat & Hanafy 2023) and is recognised as an important endogenous signalling molecule in plants, playing a key role in alleviating heat stress (Balfagón et al. 2022; Rasheed et al. 2022; Li et al. 2023; Li et al. 2025a). In this study, the expression of SA signalling gene *NPR1* was upregulated in both R-0 vs. R-24 and S-0 vs. S-24 groups. Moreover, *PR-1* exhibited variable expression, with its expression being downregulated in the R-0 vs. R-24 comparison and upregulated in the S-0 vs. S-24 comparison (Figure S3 in ESM). These findings suggest that plant hormones are crucial components of the tomato's response to high-temperature stress.

To protect themselves from abiotic and biological stresses, plants have evolved complex physiological and biochemical responses to adapt to adverse environmental conditions. A crucial component

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of these adaptive responses is the role of plant TFs and their corresponding cis-regulatory sequences (Seki et al. 2003). TFs, such as NAC, MYB, AP2/ERF, WRKY, and bZIP, are instrumental in mediating plant adaptation to various stresses (Erpen et al. 2018; Baillo et al. 2019; Xu et al. 2022). Xi et al. (2022) isolated the NAC TF gene *ZmNAC074* from maize and demonstrated that it plays a key role in plant heat tolerance. This gene presents itself as a promising candidate for regulating heat tolerance and could be utilised in the genetic improvement of maize and other crops. Similarly, Liao et al. (2017) reported that the *MYB30* TF regulates oxidative and heat stress responses through ANNEXIN-mediated cytosolic calcium signalling in *Arabidopsis*, indicating the broader relevance of MYB TFs in stress response mechanisms. Kong et al. (2025) reported that *PgMYB96* enhanced *Physalis grisea* tolerance to high-temperature stress by activating trithorax-like factor *WD REPEAT CONTAINING5b*. Moreover, Wu et al. (2022) identified *LlWRKY22* from *Lilium longiflorum* as a potential new regulator of heat stress response, which actively participates in establishing heat tolerance by activating itself and the heat-related *LlDREB2B* gene. Eom et al. (2023) further concluded that *BrbZIP-S* from *Brassica rapa* could regulate plant tolerance to dark stress and heat stress. In this study, we identified 156 TFs that may be associated with the tomato's response to high-temperature stress, belonging to different TF families, including MYB, AP2-EREBP, b-ZIP, bHLH, NAC, and WRKY (Figure 9). These findings underscore the critical role of TFs in the tomato's heat stress response and suggest that these TFs represent key candidate regulatory genes that could be targeted for enhancing heat tolerance, as well as for broader genetic improvements in tomato. Finally, we compared samples at 0 h and 24 h after high-temperature treatment; this design may conflate treatment effects with natural developmental changes over time. And in future studies, we will consider the mock-treated 24 h controls to better isolate heat-specific responses.

CONCLUSION

In this study, RNA-seq analysis of total RNA isolated from tomato leaves led to the construction of 12 cDNA libraries, allowing us to identify the changes in gene expression following high-temperature treatment. We investigated the expression levels and types of DEGs and performed GO and KEGG enrichment analyses. The KEGG pathway enrichment analysis of DEGs revealed that the protein processing pathway in the endoplasmic reticulum plays a significant role in the tomato's response to heat stress, primarily through the induction of HSPs such as *HSP40*, *HSP70*, *HSP90*, *HSP100*, and *sHSPs*. Additionally, the plant hormone signalling pathway was found to be crucial in the tomato's response to high temperature. We also identified 156 TFs potentially involved in the tomato's heat stress response, belonging to different TF families, including MYB, AP2-EREBP, b-ZIP, bHLH, NAC, and WRKY. The reliability of the RNA-seq data was further validated by qRT-PCR. Overall, the response to high temperature in the two tomato inbred lines studied is complex and distinct. The findings of this research provide a valuable foundation for the identification and functional analysis of DEGs. Moreover, these results offer critical insights and important clues for the further exploration and identification of candidate genes for heat-resistant breeding in tomatoes.

Data availability. The datasets generated during the current study are available in the National Center for Biotechnology Information (NCBI) repository, in the Sequence Read Archive (SRA) under the PRJNA1150370 Bioproject accession (https://dataview.ncbi.nlm.nih.gov/object/PRJNA1150370?sort_by=status&archive=sra).

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