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Physiological and transcriptome analysis of changes in endogenous hormone contents and related synthesis and signaling genes during the heat stress in garlic (*Allium sativum* L.)

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Abstract

High-temperature stress severely limits the growth, development, yield, and quality of garlic (*Allium sativum* L.), but the role of hormone signaling in its heat stress response remains unclear. This study examined changes in seven plant hormones and the expression of related genes in garlic leaves ('Xusuan No. 6') under heat stress (38 °C for 0, 2, 4, and 24 h). Growth-promoting hormones, auxin and gibberellic acid, significantly decreased within 2 h of heat stress, while stress-response hormones, including abscisic acid, jasmonic acid, salicylic acid, and ethylene, increased. KEGG pathway analysis revealed significant changes in genes related to hormone biosynthesis and signal transduction, such as *NCED* and *PYR/PYL* in the ABA pathway, *LOX* and *OPR* in JA biosynthesis, *AUX* and *ARF* in IAA signaling, and *ERT* and *ERF* in ethylene signaling. A protein–protein interaction network identified 15 hub genes potentially coordinating hormone regulation under heat stress. These findings provide a basis for functional validation of key hormone-related genes in the garlic heat-stress response and suggest potential genetic targets for the development of heat-tolerant garlic varieties.

Keywords Garlic (*Allium sativum* L.), Hormone, RNA-seq, Gene expression analysis, High-temperature

Introduction

Garlic (*Allium sativum* L.) is an important vegetable and medicinal crop that is widely cultivated throughout the world [1]. Cold weather favors garlic growth, whereas high temperatures during the seedling stage can increase plant respiration and nutrient consumption, hindering subsequent growth and reducing both yield and quality [2]. As global temperatures rise, the adverse effects of high-temperatures on garlic production are becoming an increasingly serious concern.

High-temperature stress has a significant effect on numerous aspects of plant physiology, typically slowing growth, impairing normal development, and reducing crop quality, particularly when combined with drought stress [3]. Plants generate excessive amounts of reactive oxygen species (ROS) under heat stress, leading to various degrees of oxidative damage [4, 5]. Such damage can

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be limited by the plant's antioxidant defense mechanisms, which include enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) that remove superoxide anions and excess H_2O_2 (hydrogen peroxide) to protect normal cellular metabolism [6, 7].

Plants initially respond to high-temperature stress through basal heat tolerance, inducing primary heat stress responses through Ca^{2+} dependent calmodulin and H_2O_2-induced mitogen-activated protein kinases [8]. In addition to the mechanisms of basal heat tolerance, plant hormones also participate in responses to high-temperature stress. Plants often respond to external stresses by adjusting the content and proportion of endogenous hormones, whose downstream signal transduction pathways regulate gene expression and thus alter physiological processes, enabling plant survival [9]. Plant hormones such as abscisic acid (ABA), cytokinin (CTK), gibberellins (GAs), auxin (IAA), and jasmonic acid (JA) participate in the regulation of cold resistance and recovery [10, 11]. Transcription factors (TFs) are key components of hormone signaling pathways, directly binding to *cis*-acting elements in the promoters of downstream target genes [12]. For example, *EIN3/EIL* (Ethylene-insensitive3/EIN3-like) is a key transcription factor in the ethylene signaling pathway [13]. *EIN/EIL* participates in the cold-stress response of *Actinidia deliciosa,* and *AdEIL3*, *AdERF1*, and *AdERF13* significantly enhanced the low-temperature tolerance of transgenic *Arabidopsis thaliana* [14]. Similarly, JA participates in the response of the tea plant (*Camellia silences*) to high-temperature and drought stress [15]. Studies have shown that *MYB* TFs regulate genes related to the metabolic pathways of GA, BR, and JA in rice [16, 17].

Garlic, a member of the *Liliaceae* family, has more than 5000 years of cultivation history; it is a cold-tolerant vegetable whose optimal growth temperature is approximately 10–20℃ [18]. Our previous transcriptome studies on garlic at 0 h, 2 h and 24 h under high temperature stress showed that differential genes were abundant in plant hormone signal transduction pathways. But there have been relatively few studies of its transcriptomic responses to high-temperature stress, especially from the standpoint of phytohormones and associated TFs [19]. In this study, we documented changes in the contents of seven plant hormones in garlic leaves upon exposure to multiple durations of heat stress. We performed transcriptome sequencing of replicated leaf samples to assess the effects of heat stress on global patterns of gene expression and to identify key genes related to hormone synthesis and signaling whose expression changed upon heat exposure. Therefore, this study analyzed the changes in endogenous hormone contents in garlic leaves under heat stress and, in combination with transcriptome data, investigated the expression patterns of genes related to endogenous hormone biosynthesis, metabolism, and signal transduction. These findings provide new insights into the identification of key genes involved in hormone synthesis and signaling pathways, as well as a theoretical basis and technical guidance for enhancing heat tolerance in garlic seedlings through exogenous hormone application.

Materials and methods

Plant materials and growth conditions

Garlic variety 'Xuxuan No. 6' (Surenxuan 202,202) was used as experimental material and stored in the root germplasm resource Center of Xuzhou Institute of Agricultural Sciences in Xuhuai rea. All experiments were performed using garlic plants grown in Xuzhou, Jiangsu province, China (34°58′N, 117°29′E). The bulbs were selected and grown in an artificial climate chamber at 25 °C and 30% relative humidity with a 16 h light/8 h dark photoperiod. For the heat stress, after three weeks, stable, robust plants were then selected chamber at 38 °C to induce heat stress, and each treatment was repeated three times. After exposure to 38 °C for 0 (T0), 2 (T2), 4 (T4), and 24 h (T24), the upper leaves of the garlic plants were removed, frozen in liquid nitrogen, and stored at − 80 °C for later use.

Enzyme activity, hormone content, ROS, and chlorophyll measurements

Leaf samples were collected from three week old seedlings for measurement of auxin (IAA), gibberellic acid (GA_3), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), cytokinin (CTK), and ethylene (ETH) contents using ELISA kits (Meilian Biological Technology, Shanghai, China). Each assay was performed using three replicate leaf samples from each treatment. The activities of POD and SOD and the content of MDA were measured according to the manufacturer's instructions of the relevant assay kits (Beijing Solarbio Technology, Beijing, China). Anthrone colorimetry was used to determine the content of soluble sugar [20]. The accumulation of superoxide anion (O^{2-}) in garlic leaves was analyzed by nitroblue tetrazolium (NBT) staining [21]. The content of chlorophyll in leaves was determined by colorimetry. The same area of garlic leaf tissue was extracted with 95% ethanol/water (v/v), the supernatant was separated after centrifugation, and the absorbance at 665, 649, and 470 nm wavelengths was determined by spectrophotometer [22]. All measurements were performed using three replicate leaf samples from each treatment, and significant differences among treatments were determined using

Duncan's method multiple range test using in SPSS 17.0 software (IBM, Armonk, NY, USA).

RNA extraction, library construction, and RNA-seq

Transcriptome sequencing was performed by Guangzhou Gidio Biotechnology (Guangzhou, China). Total RNA was extracted using a TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Enriched mRNA was broken into short fragments using fragmentation buffer and reverse transcribed into cDNA using random primers. Second-strand cDNA was synthesized using DNA polymerase I, RNase H, dNTP, and buffer. The cDNA fragments were purified using the QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands), then end repaired, A-tailed, and ligated to Illumina sequencing adapters. The ligation products were size selected by agarose gel electrophoresis, amplified by PCR, and sequenced on the Illumina NovaSeq 6000 platform by Guangzhou Kidio Biotechnology (Guangzhou, China) to obtain 150 bp, paired-end reads.

Filtering of clean reads, de novo assembly, gene functional annotation, and enrichment analysis of differentially expressed genes

To get high quality clean reads, reads were further filtered by fastp [23] (version 0.18.0). The parameters were as follows: 1) removing reads containing adapters; 2) removing reads containing more than 10% of unknown nucleotides (N); 3) removing low quality reads containing more than 50% of low quality (Q-value≤20) bases. Transcriptome de novo assembly was performed with short reads assembling program-Trinity [24]. RNAs differential expression analysis was performed by DESeq2 software between two different groups [25]. The genes with the parameter of false discovery rate (FDR) below 0.05 and absolute fold change>2 were considered differentially expressed genes. The "prcomp" package to analysis principal component analysis (PCA). Gene Ontology (GO) is an international standardized gene functional classification system which offers a dynamic-updated controlled vocabulary and a strictly defined concept to comprehensively describe properties of genes and their products in any organism [26]. The calculated *p*-value was gone through FDR Correction, taking FDR ≤ 0.05 as a threshold. GO terms meeting this condition were defined as significantly enriched GO terms in DEGs. KEGG is the major public pathway-related database [27]. The calculated *p*-value was gone through FDR Correction, taking FDR ≤ 0.05 as a threshold. Pathways meeting this condition were defined as significantly enriched pathways in DEGs. Sequence data that support the findings of this study have been deposited in the NCBI (National Center for Biotechnology Information) database accession numbers of RNA-seq data is PRJNA1198391.

Protein–protein interaction

Protein–protein interaction network was identified using String v10, which determined genes as nodes and interaction as lines in a network [28]. The network file was visualized using Cytoscape (v3.7.1) software to present a core and hub gene biological interact [29].

Quantitative real-time polymerase chain reaction

For quantitative real-time polymerase chain reaction (RT–qPCR), total RNA was isolated from garlic leaves using a plant total RNA purification kit, and cDNA was synthesized with the FastQuant RT kit (Tiangen Biotech, Beijing, China). RT–qPCR was performed according to the instructions of the SYBR Premix Ex Taq kit (Takara, Dalian, China). Specific primers were designed using Primer Premier 6.0 software, and all primers are listed in Table S1. The reaction conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, and finally a melting curve analysis (65–95 °C with an increase of 0.5 °C every 5 s). RT–qPCR analysis was performed on the CFX96 Real-Time PCR system (Bio-Rad). Each 20-μL reaction system contained 2 μL of cDNA, 0.4 μL each of forward and reverse primers, 10 μL of SYBR Premix Ex Taq, and 7.2 μL of ddH_2O. Relative gene expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method [30]. The data were analyzed by analysis of variance followed by the least significant difference test performed in SPSS 17.0. The garlic *Actin* gene was used as the internal reference gene [31]. There were three biological replicates for each treatment.

Statistical analyses

Graphs were generated using GraphPad Prism 6.0 software. In bar charts, bars represent the mean values of three biological replicates ± standard deviations.

Results

Physiological changes in garlic leaves during heat stress

Excess ROS production and the resulting oxidative damage, especially to membrane systems, are recognized hallmarks of heat stress. To examine the extent to which our treatments induced heat stress in garlic, we evaluated multiple parameters related to oxidative damage and antioxidant activity (Fig. 1). After 2 h and 4 h of high- temperature stress, there was no significant change in the phenotype of garlic, but after 24 h, garlic appeared wilting, leaf yellowing and other characteristics. As the duration of heat stress increased from 0 to 24 h, POD activity and the content of malondialdehyde (MDA), a lipid peroxidation product, increased in

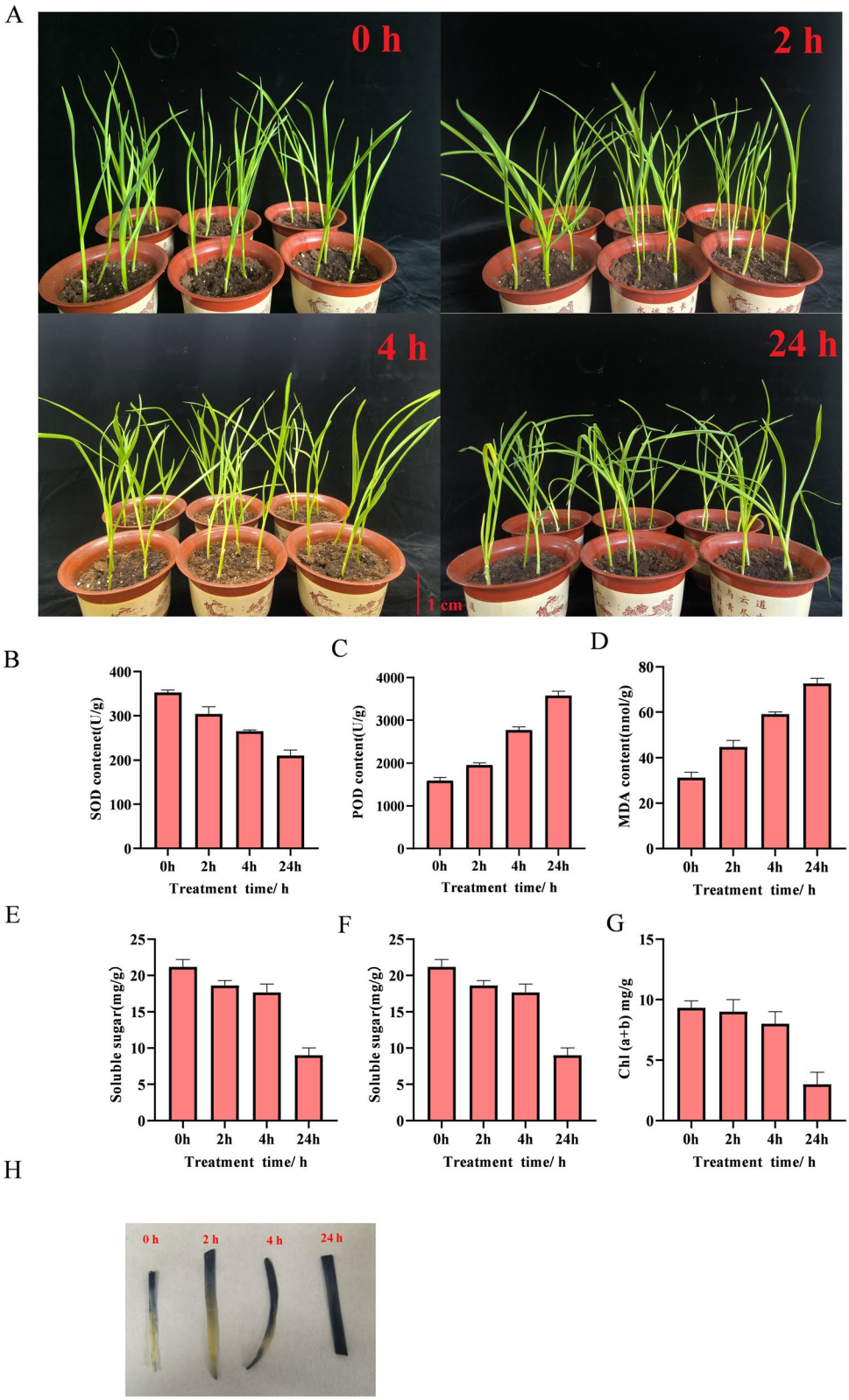

Fig. 1 Physiological changes in garlic seedlings exposed to heat stress. **A** Phenotype of garlic under heat stress for 0 h, 2 h, 4 h and 24 h (**B**) SOD activity. **C** POD activity. **D** MDA content. **E** H2O2 content. **F** Soluble sugar content. **G** Total chlorophyll content. **H** Representative image of NBT-stained tissues

leaves, although SOD activity decreased. H_2O_2 content also increased with increasing stress duration. Soluble sugar content decreased slightly from 0 to 4 h and decreased markedly at 24 h to 67.85% of its 0 h value chlorophyll content also decreased with increasing duration of heat stress, showing a particularly substantial decline at 24 h. NBT staining was used to assess O^{2-} content, and the degree of staining increased markedly from 0 to 24 h. These results are consistent with significant heat stress, ROS production, and oxidative damage in garlic leaves exposed to increasing durations of heat stress at 38 °C.

Effects of high-temperature stress on endogenous hormone content in garlic leaves

The contents of IAA and GA3 decreased significantly in garlic leaves during the early stages of heat stress: IAA and GA3 levels were 60.35% and 49.10% lower, respectively, at 4 h than at 0 h, although the contents of both hormones rose again at 24 h (Fig. 2). By contrast, the

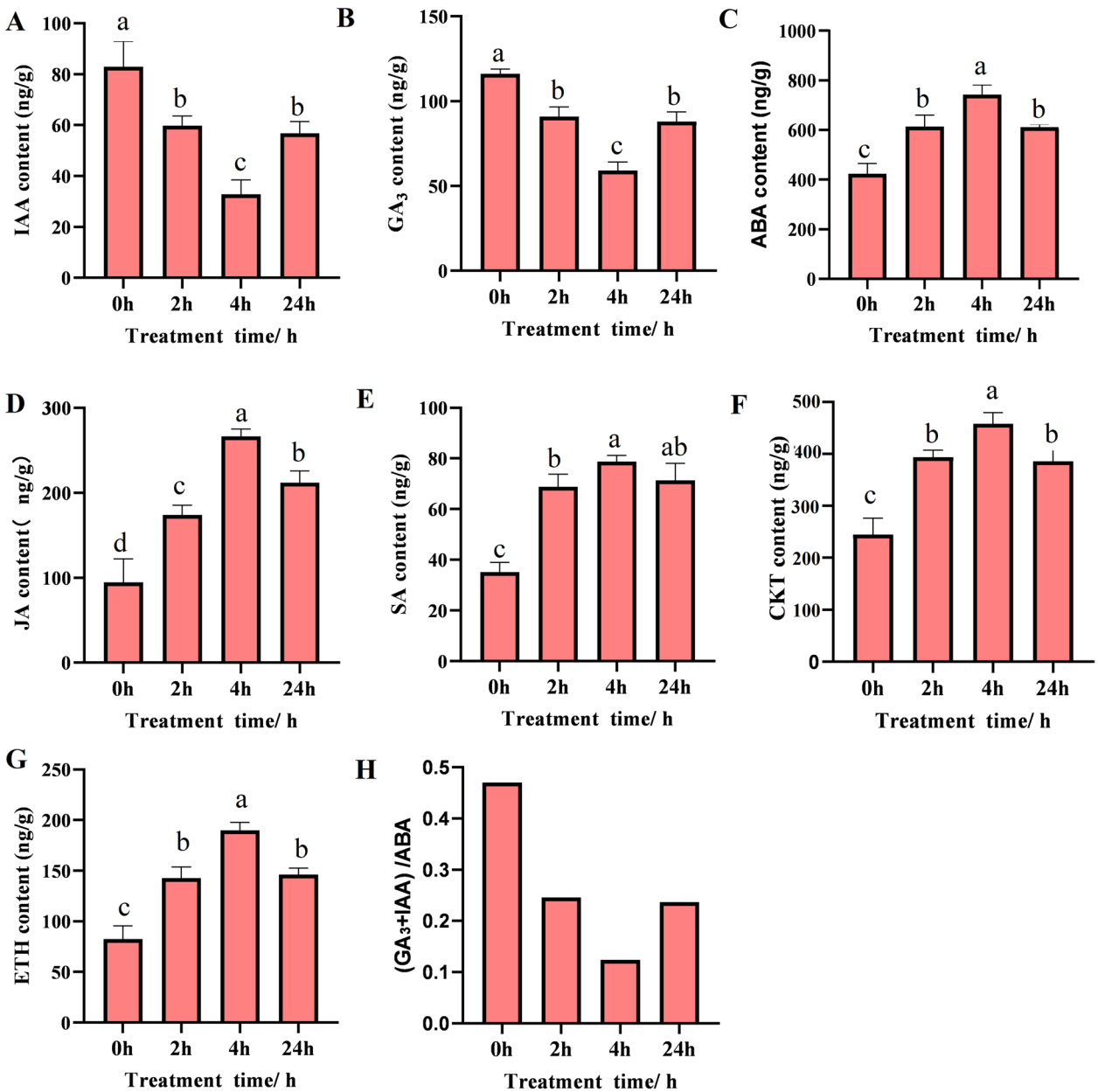

Fig. 2 Contents of (**A**) IAA, **B** GA3, **C** ABA, **D** JA, **E** SA, **F** CTK, and (**G**) ETH and (**H**) the (GA + IAA)/ABA ratio in garlic leaves under exposed to various durations of heat stress

contents of ABA, JA, SA, CTK and ETH all showed a similar trend: decreasing from 0 to 4 h and then increasing slightly at 24 h. (The (GA3 + IAA)/ABA ratio also decreased rapidly from 0 to 4 h of high-temperature stress but increased somewhat after 24 h, suggesting the potential adaptation ability of garlic leaves to high-temperature stress based on hormone level balance.

Transcriptomic sequencing and annotation of unigenes

We next performed transcriptome sequencing of replicated leaf samples from garlic exposed to heat stress for T0, T2, T4, and T 24. After quality filtering of the raw sequencing reads, we obtained more than 750,000,0000 clean reads per sample, with a GC content of approximately 45% and at least 92.58% Q30 bases, confirming the high quality of the sequencing data (Table S2).

Gene expression data from the three biological replicates of each treatment were strongly correlated, with an average Pearson's correlation coefficient of 0.96 ± 0.01 (Fig. 3A). Likewise, a PCA plot showed clear grouping of samples from the same treatment and separation of samples from different treatments (Fig. 3B), although there was some overlap between the 4 h and 24 h samples.

Compared with samples collected at 0 h, there were 18,529, 20,781, and 18,643 DEGs in samples collected at T2, T4, and T24 of heat stress; the largest number of down-regulated genes (15,606) were identified at 4 h, whereas the largest number of up-regulated genes (14,820) were identified at 24 h (Fig. 4A). Complete results for the differential expression analysis are provided in Table S3. A number of genes were differentially expressed in only one of six possible treatment comparisons, and 532 genes were differentially expressed in all treatment comparisons (Fig. 4B).

GO enrichment analysis of DEGs

Enriched GO terms were identified across all DEGs using a threshold of padj ≤ 0.05 (Fig. 5). The top biological process terms were cellular process (GO:0009987), metabolic process (GO:0008152), biological regulation (GO:0065007), and response to stimulus (GO:0050896); the top molecular function terms were binding (GO:0005488), catalytic activity (GO:0003824), and transporter activity (GO:0005215); and the top cellular component terms were cellular anatomical entity (GO:0110065) and protein-containing complex (GO:0032991). Additional enriched GO terms with clear relationships to heat stress included antioxidant activity and protein-folding chaperone. The broad nature of many of the enriched GO terms is consistent with the wide-ranging effects of heat stress on multiple aspects of plant growth and physiology. In the CK-vs-T2 and T2-vs-T4 comparisons, more up-regulated genes were annotated with the enriched GO terms, whereas in the T4-vs-T24 comparison, more down-regulated genes were annotated with the enriched terms, consistent with the large number of down-regulated DEGs at 24 h.

KEGG pathway enrichment of DEGs

We next identified enriched KEGG pathways in the DEGs from the CK-vs-T2, T2-vs-T4, and T4-vs-T24 comparisons (Fig. 6). The top three most significantly enriched KEGG pathways were protein processing in the ER (ko04141; 167 DEGs), plant-pathogen interaction (ko04626; 81 DEGs), and plant hormone signal

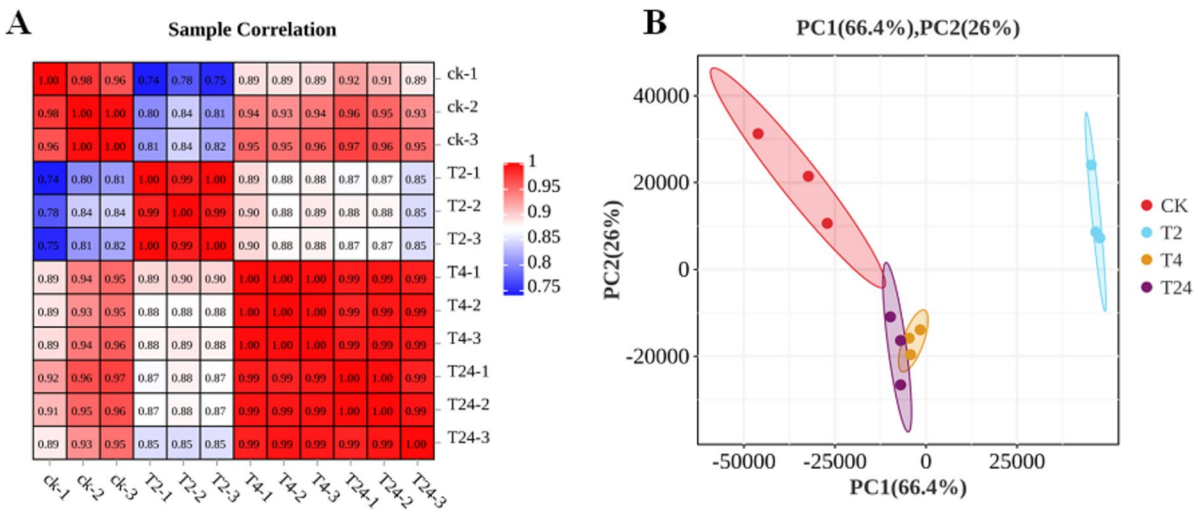

Fig. 3 Global gene expression data from leaves of garlic seedlings exposed to heat stress at T0, T2, T4, and T24. **A** Pearson correlation coefficients for all sample pairs. **B** PCA of gene expression data from the samples in (**A**)

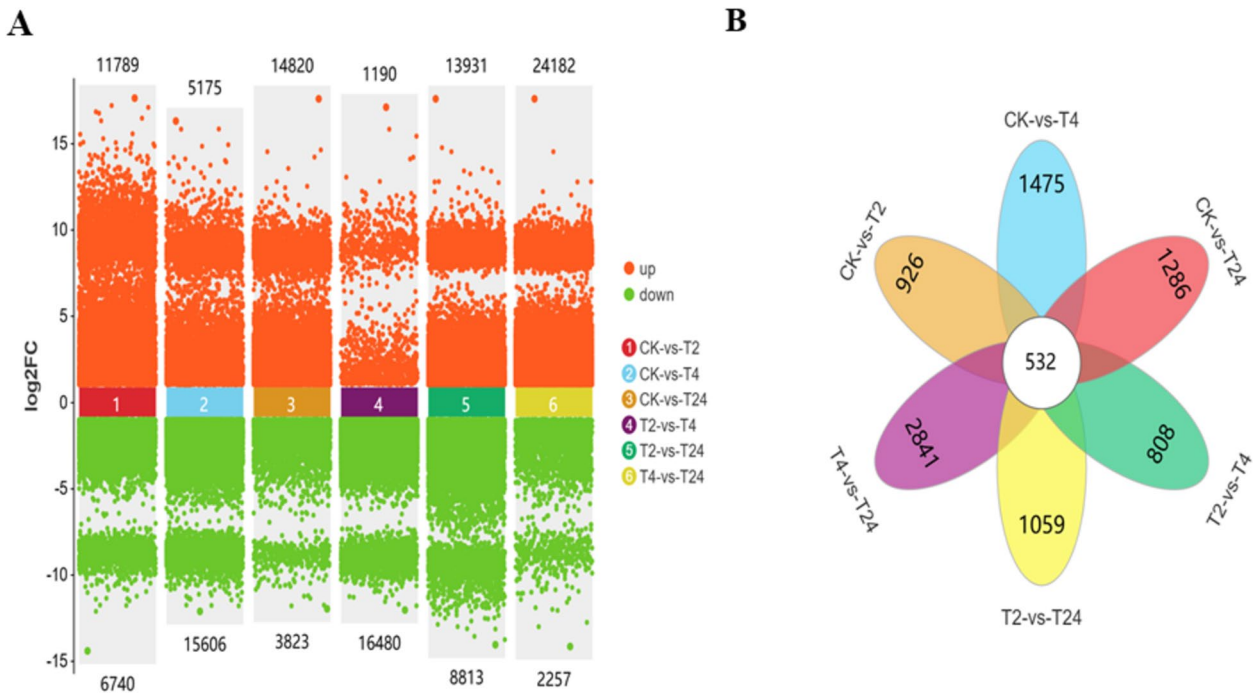

Fig. 4 Differential gene expression in garlic seedlings exposed to heat stress at T0, T2, T4, and T24. **A** Log2 (fold change) values of up and down-regulated genes in six comparisons. **B** Numbers of significantly differentially expressed genes that were unique to individual comparisons or shared among all comparisons

transduction (ko04075; 70 DEGs) for the CK-vs-T2 comparison (Table S4); protein processing in the ER (ko04141; 192 DEGs), ribosome (ko03010; 224 DEGs), and plant-pathogen interaction (ko04626; 123 DEGs) for the T2-vs-T4 comparison (Table S5); and porphyrin metabolism (ko00860; 43 DEGs), photosynthesis (ko00195; 55 DEGs), and one carbon pool by folate (ko00670; 20 DEGs) for the T4-vs-T24 comparison (Table S6). Protein processing in the ER (ko04141) was the most significantly enriched pathway in both the CK-vs-T2 and T2-vs-T4 comparisons, likely reflecting the need to managed misfolded proteins during heat stress. The plant hormone signal transduction pathway (ko04075) was highly enriched in the CK-vs-T2 comparison but was not among the top 20 pathways in the T2-vs-T4 and T4-vs-T24 comparisons, suggesting that changes in hormone-related gene expression occur rapidly after the onset of heat stress.

Clustering of DEGs by temporal expression patterns

We next clustered the DEGs into profiles based on their temporal expression patterns (Fig. 7) and performed KEGG enrichment analysis of DEGs from the resulting profiles (Fig. 8). Genes in profiles one and six first decreased and then increased with increasing duration of high-temperature stress; expression of genes in profile one was lowest at 4 h, whereas that of genes in profile six was lowest at 2 h. Plant hormone signal transduction (ko04075) was among the top 20 enriched KEGG pathways for both of these profiles (Fig. 8A, B). Of the genes in profile one, 4.48% were associated with this pathway, including genes involved in IAA, ET, and JA responses (Table S7). Likewise, 6.45% of the genes in profile six were associated with this pathway, including genes involved in synthesis and response to ABA and ETH (Table S8). Genes in profiles 17 and 15 were rapidly up-regulated at 2 h in response to high-temperatures. Of the genes in profile 17, 5.36% were involved in plant hormone response, including those associated with CK, ABA, and JA responses (Fig. 8C, D) (Table S9). Similarly, 4.26% of the genes in profile 15 were associated with this pathway and included genes involved in synthesis and response to IAA and ABA (Table S10). Genes in profile two were down-regulated throughout the duration of high-temperature treatment (Fig. 8E). Notably, plant hormone signal transduction was the most significantly enriched pathway in profile two, and 20% of its genes were associated with this pathway (Table S11). Genes in profile nine were down-regulated at 24 h (Fig. 8F), and 6.67% of them were associated with plant hormone signal transduction (Table S12); brassinosteroid biosynthesis and zeatin biosynthesis were also significantly enriched in this profile.

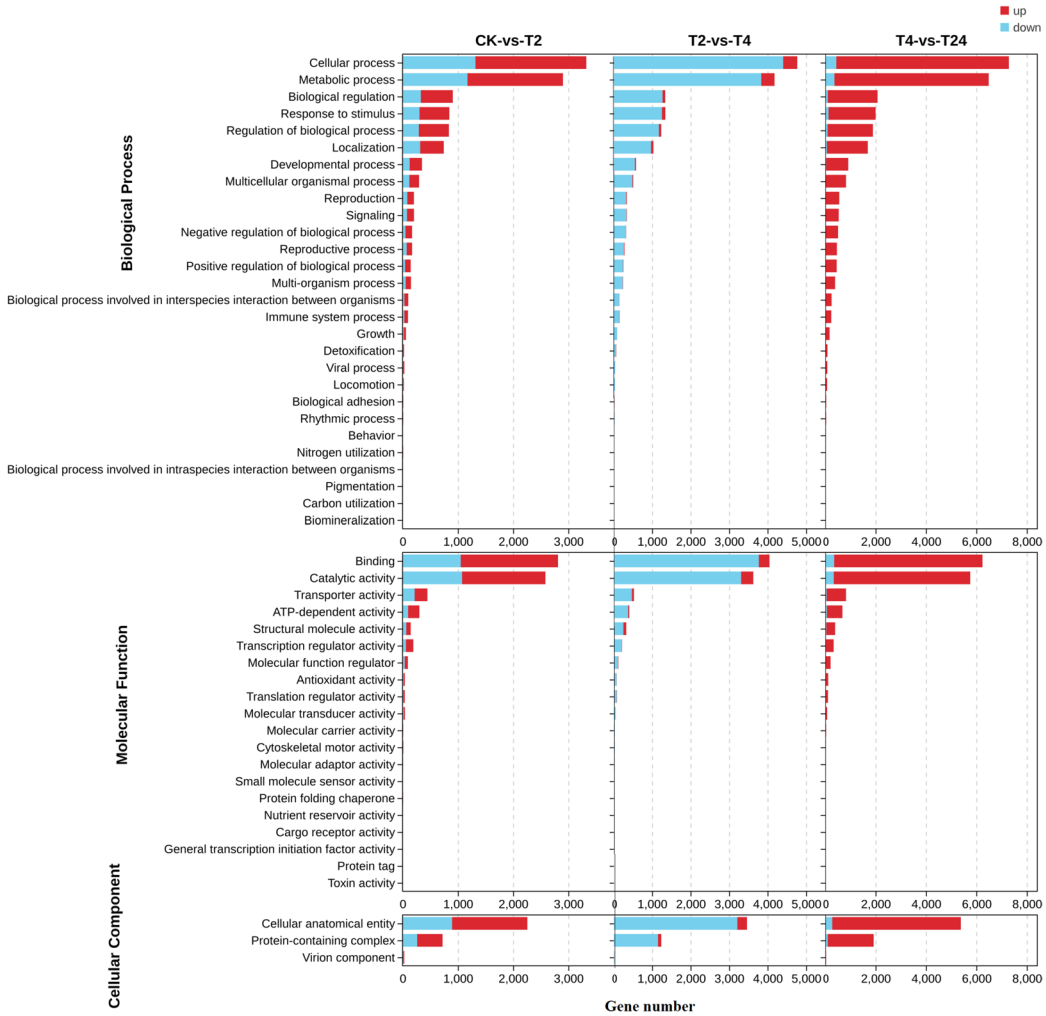

Fig. 5 GO terms significantly enriched in the DEGs. The x axes shows the number of up and down-regulated genes annotated with a given GO term in the CK-vs-T2, T2-vs-T4, and T4-vs-T24 comparisons

Genes related to plant hormone signaling in profile two

DEGs in profile two were down-regulated throughout heat stress and were significantly enriched in 38 KEGG pathways (Fig. 9). Plant hormone signal transduction was the most significantly enriched pathway (ko04075), and we therefore selected the eight DEGs from this pathway for further analysis (Table S13). The signaling pathways in which these genes participate are shown in Figure S1. *Unigene0006526* was annotated as *LG2*, encoding a TGACG-binding (*TGA*) transcription factor whose homologs are involved in the response to JA signaling. *Unigene0040452* (annotated as *GID2*) and *Unigene0056259* (annotated as *PIF4*) encode interacting proteins involved in gibberellin signal transduction. *Unigene0054514* (annotated as *PYL4*) is predicted to encode a PYR/PYL-family ABA receptor involved in the ABA signaling pathway (Fig. 9). *Unigene0048421* (annotated as *RR10*) and *Unigene0079044* (annotated as *XTH24*) are involved in cytokinin and brassinolide signal transduction processes, respectively, and *Unigene0092175* (annotated as *IAA25*) and *Unigene0003541* (annotated as *SAUR*) are involved in IAA signaling.

Differentially expressed transcription factor genes

TFs play a key role in the response to plant hormones, and we therefore identified TF-encoding DEGs in the CK-vs-T2, T2-vs-T4, and T4-vs-T24 comparisons (Fig. 10). A total of 42 TF families were represented among these DEGs, and members of the ERF, MYB, bHLH, NAC, and WRKY families were most abundant (Fig. 10A). Most TF families had more up-regulated than down-regulated members in the CK-vs-T2 and T2-vs-T4 comparisons, whereas most TF families had more down-regulated members in the T4-vs-T24 comparison (Fig. 10B). A particularly large number of bHLH TFs were down-regulated in the latter comparison.

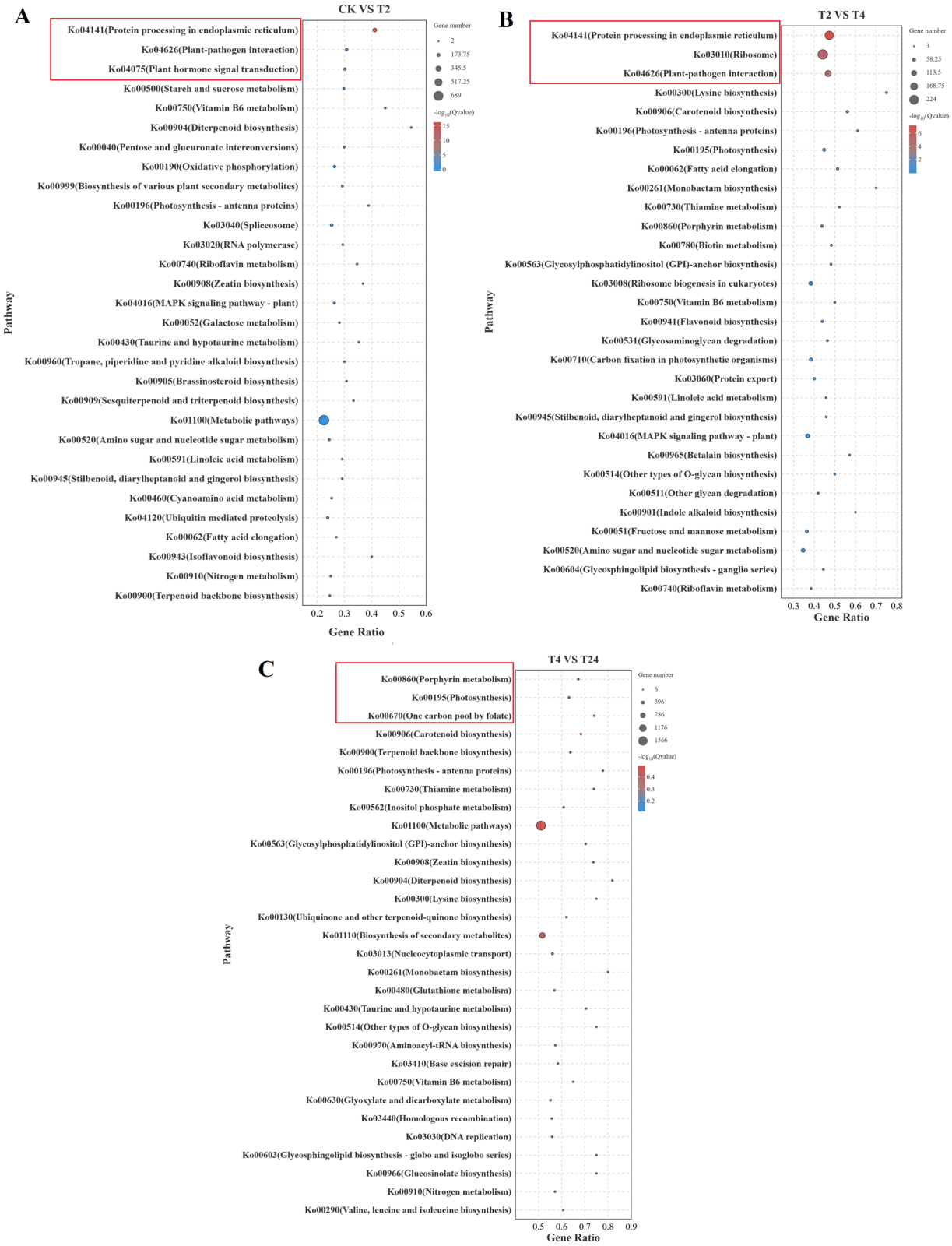

Fig. 6 KEGG pathway enrichment analysis of DEGs from three treatment comparisons. The top 20 enriched pathways for each comparison are shown

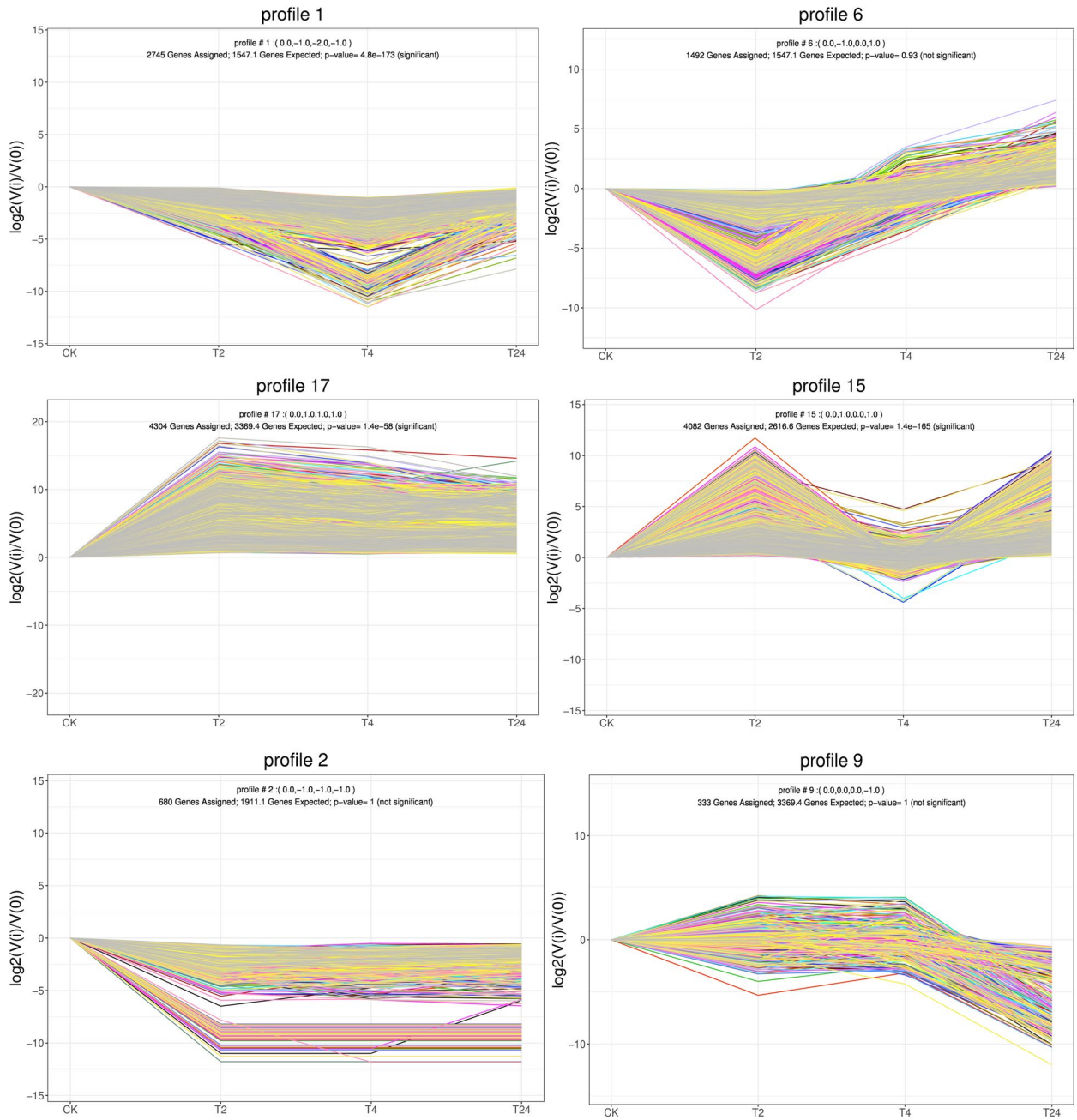

Fig. 7 DEGs were clustered into six profiles based on their temporal expression patterns during heat stress

There were 32 and 21 significantly up-regulated ERF TFs at T2 and T24, respectively (Fig. 10D), and expression of *Unigene0070937* (annotated as the ET-responsive TF *EREBP1*) was significantly up-regulated at all heat-treatment time points. *Unigene0017524* (annotated as the *AP2/ERF* gene *RAP2-6*), and *Unigene0095050* (annotated as the *ERF* gene *CRF4*) were up-regulated at T2 and T4 but down-regulated at T24. Likewise, 21 and 29 MYB TFs were significantly up-regulated at T2 and T24, respectively. Among them, *Unigene0036034* (annotated as *MYB3R1*) was significantly up-regulated at all heat treatment time points, and *Unigene0062921* (annotated as *MYB306*) was down-regulated at all heat treatment time points (Fig. 10E). Meanwhile, in order to better understand the regulatory relationship of transcription factors under high temperature stress, based on *P*-value analysis, five transcription factor genes—*Unigene0070937*, *Unigene0017524*, *Unigene0095050*,

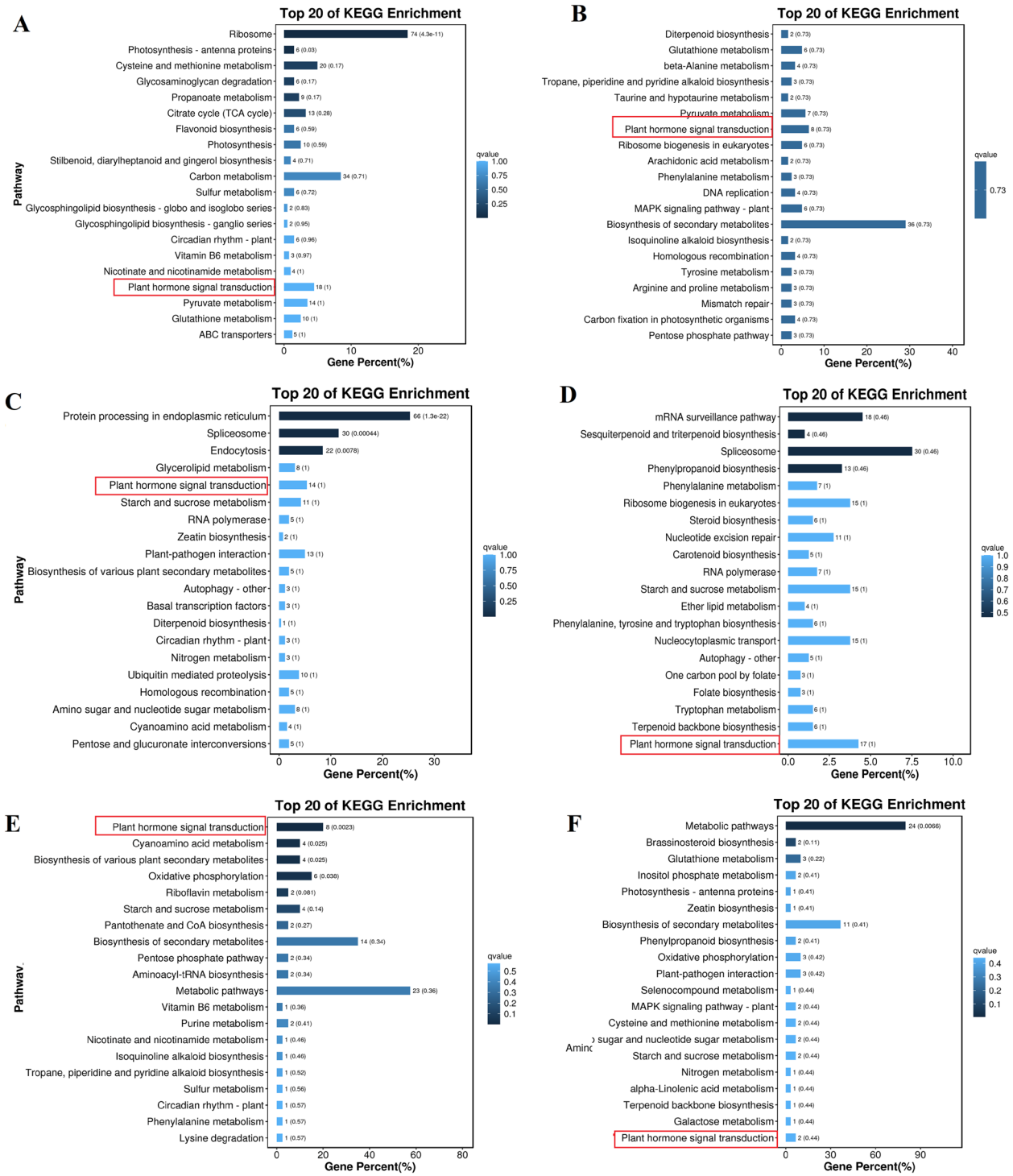

Fig. 8 The top 20 enriched KEGG pathways for each profile are shown. **A**: Profile one (**B**) Profile six (**C**) Profile 17 (**D**) Profile 15 (**E**) Profile two (**F**) Profile nine

Unigene0036034, and *Unigene0062921*—were identified as the most likely regulators of their respective target genes. Among them, *Unigene0070937* potentially targets *Unigene0000490* (*LBD37*), *Unigene0017524* targets *Unigene0025530* (*Bp10*), *Unigene0095050* targets *Unigene0014087* (*AKT2*), *Unigene0036034* targets

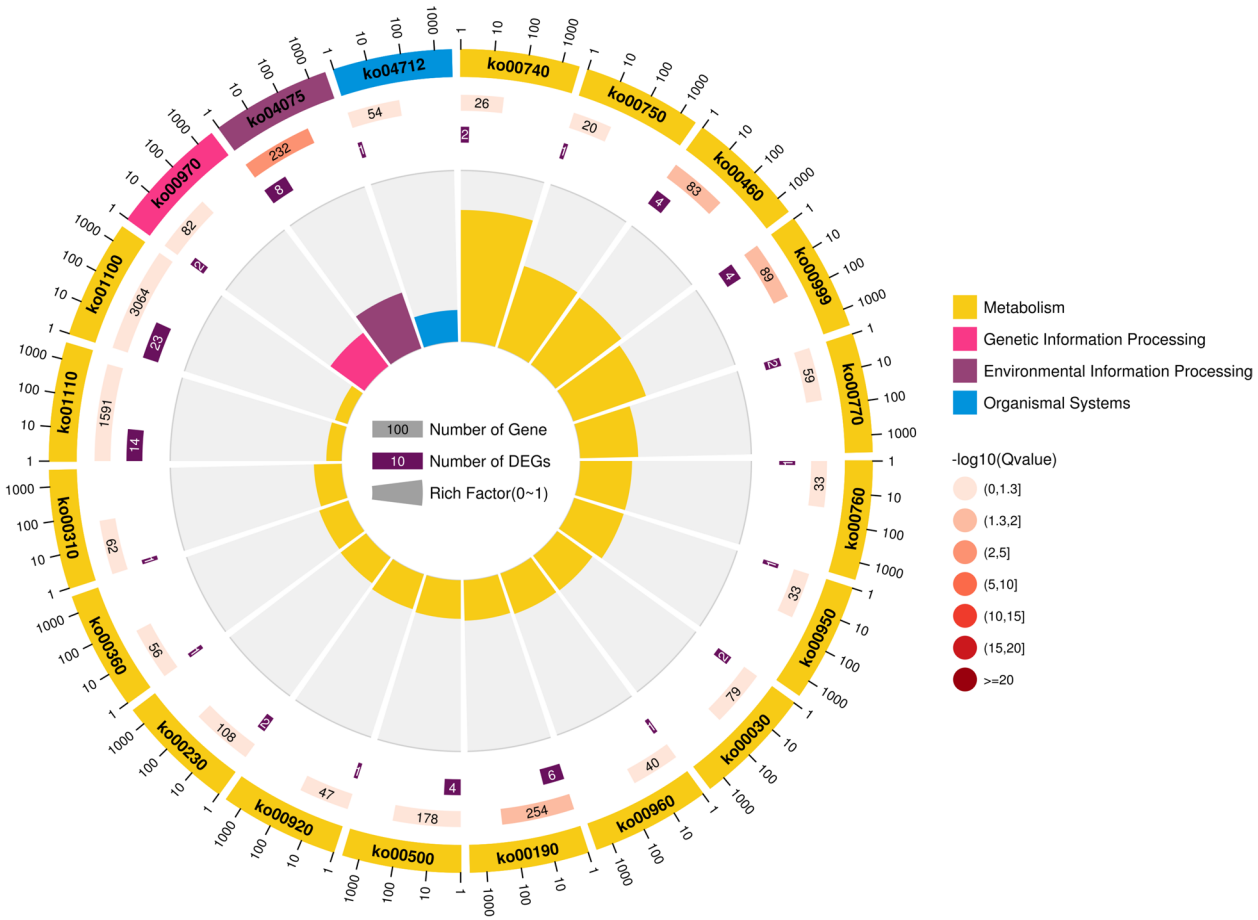

Fig. 9 KEGG pathways enriched in DEGs of profile two. From outside to inside, the tracks represent the KEGG pathways, the total number of genes in each pathway, the number of DEGs in each pathway, and the rich factor associated with the enrichment of each pathway. Colors correspond to four classes of KEGG pathway, as shown in the figure legend

Unigene0000714 (*CYTB5-D*), and *Unigene0062921* is predicted to regulate *Unigene0055696* (*RSZ23*).

DEGs related to hormone synthesis and signaling

We next investigated which specific genes in individual hormone synthesis and signaling pathways were differentially expressed under heat stress. Figures 11 and 12 show the expression profiles of DEGs associated with the synthesis (Fig. 11) and signaling (Fig. 12) of the seven hormones measured in Fig. 2. Multiple genes encoding key enzymes of ABA biosynthesis were up-regulated under high-temperature stress. In particular, genes encoding 9-cis-epoxycarotenoid dioxygenase (*NCED*) and short-chain dehydrogenase/reductase (*SDR*) showed increased expression at 2 and 4 h, broadly consistent with the increased ABA content in heat stressed garlic leaves at these time points (Fig. 11A). Genes encoding the JA biosynthetic enzymes lipoxygenase (*LOX*), allene oxide synthase gene (*AOS*), acyl-coenzyme aoxidase (*ACX*), and multi-functional protein 2 (*MFP2*) were up-regulated under heat stress and may be related to the increased JA content observed under heat stress (Fig. 11C). A number of IAA biosynthesis-related genes encoding anthranilate synthases (*ASA1*), protein S-acyl transferases (*PAT16*), and the flavin-containing monooxygenase *YUCCA8* were differentially expressed in response to heat stress. In particular, the gene encoding *YUCCA8*, which catalyzes the rate-limiting, irreversible formation of IAA, was strongly down-regulated at 2 h, consistent with the marked reduction in auxin content observed after 2 and 4 h of heat stress (Fig. 11D). Genes encoding key enzymes of SA biosynthesis such as isochorismate synthase (*ICS*) and phenylalanine-ammonia lyase (*PAL*) were generally up-regulated, consistent with observed increases in SA content during heat stress (Fig. 11E). GA_3-related DEGs encoding enzymes such as copalyl diphosphate synthase 16 (*CPSf*), kaurene synthetase (*KS1*), and ent-kaurenoic acid oxidase (*KAO*) were broadly down-regulated at 4 h (Fig. 11F),

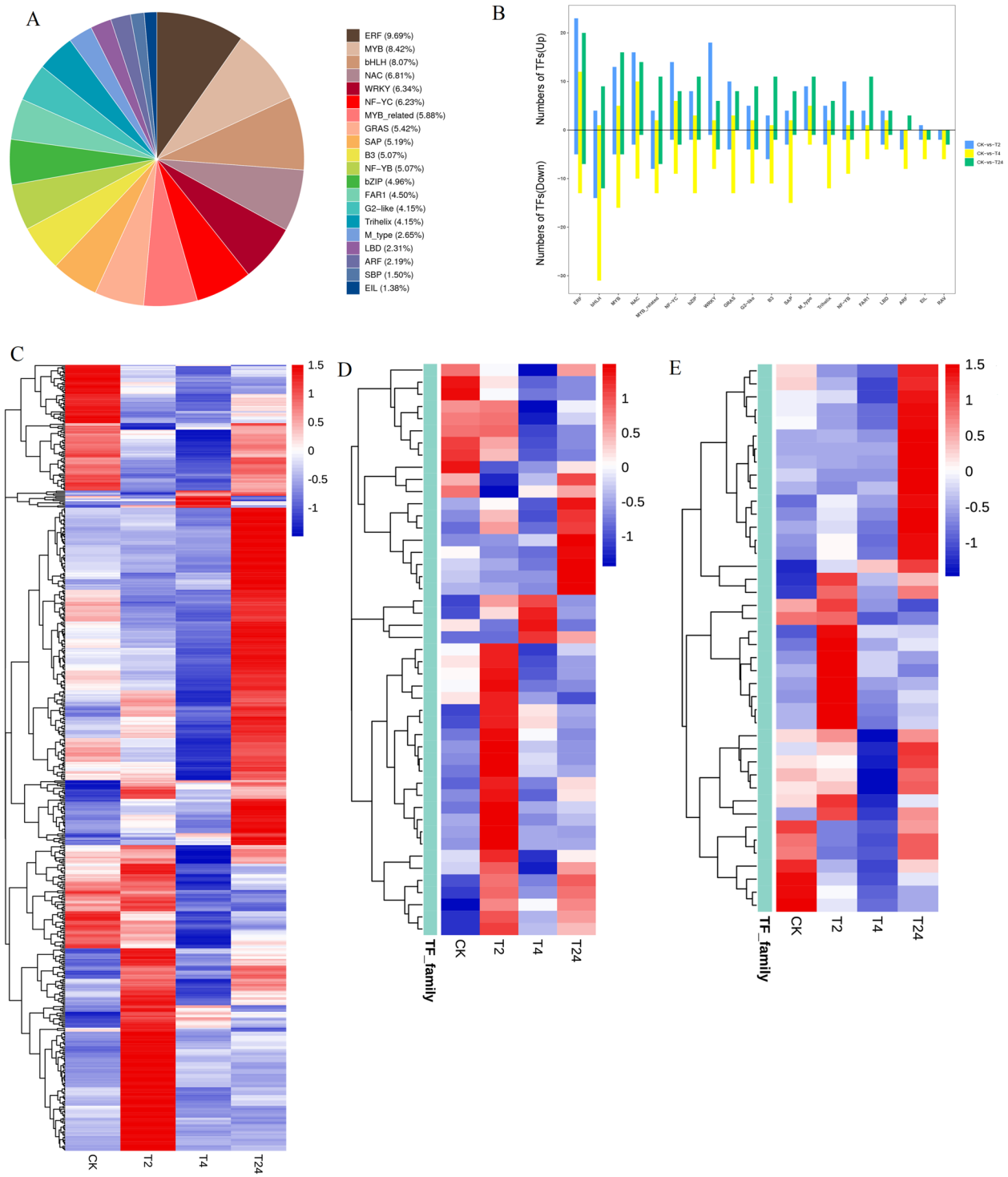

Fig. 10 TFs whose expression was altered under heat stress. **A** Top 20 TF families whose members were differentially expressed in response to heat stress. **B** Numbers of differentially expressed TFs from 20 TF families in three treatment comparisons. **C** Expression heatmap of 594 differentially expressed TFs; columns and rows represent samples and genes, respectively. **D** Expression heatmap of ERF TFs. **E** Expression heatmap of MYB TFs

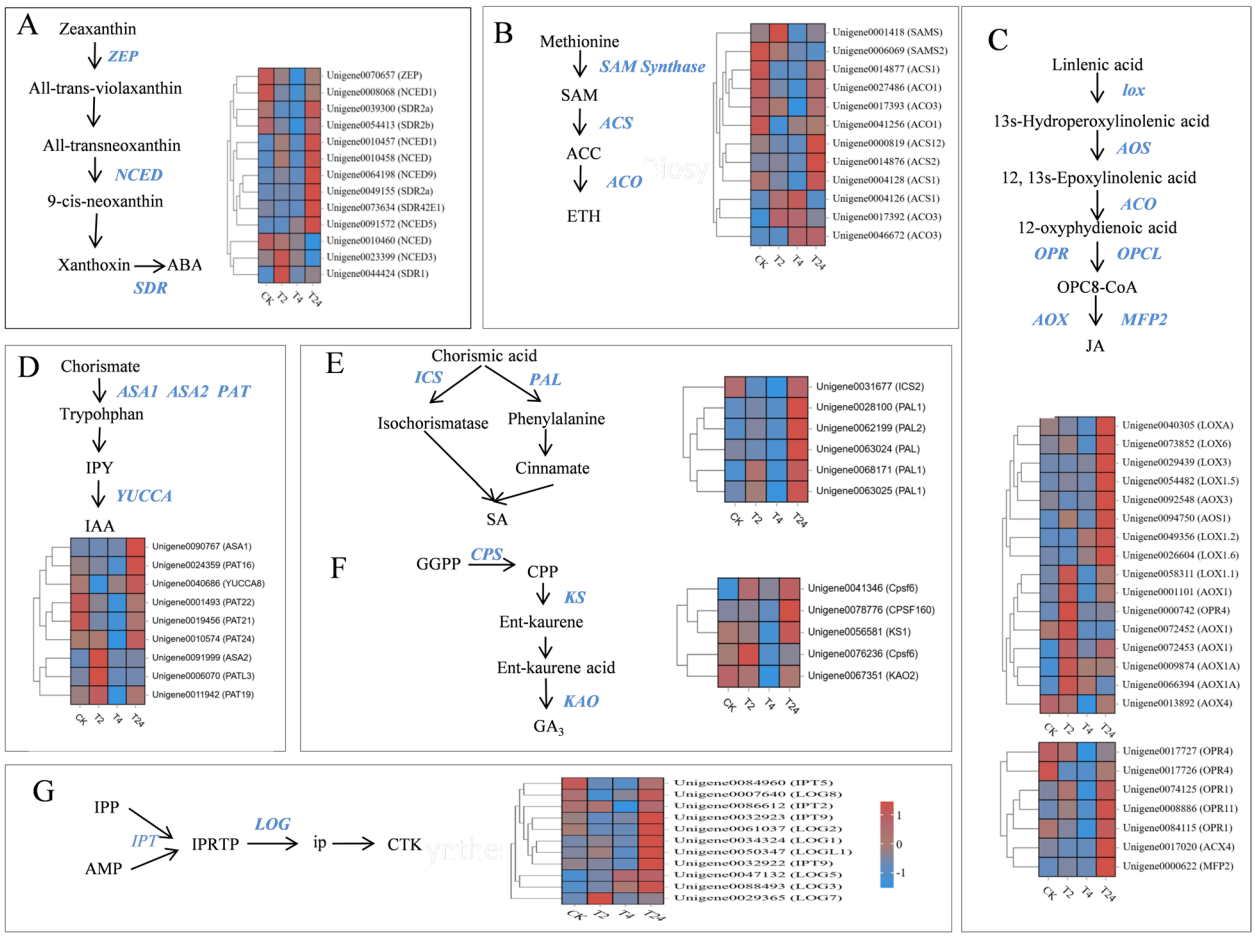

Fig. 11 DEGs associated with the synthesis of seven hormones in garlic under heat stress. **A** ABA, **B** ETH, **C** JA, **D** IAA, **E** SA, **F** GA3 (**G**) CTK

whereas most CK-related DEGs were strongly up-regulated by 24 h, consistent with increasing CK content during heat stress (Fig. 11G).

Genes associated with hormone signaling pathways were also differentially expressed in response to heat stress (Fig. 12). Many IAA signaling and response genes, such as those encoding small auxin up RNA (*SAUR*), Gretchen Hagen 3 (*GH3*), AUX/IAA, and auxin response factor (*ARF)* proteins, were down-regulated in early heat stress, and some were later up-regulated at 24 h (Fig. 12A). *ARR11*, whose encoded protein coordinates crosstalk among CTK signaling pathways, was down-regulated under heat stress, in contrast to the observed increase in leaf CTK content (Fig. 12B). Genes encoding ABA pathway components such as *PYR/PYL* ABA receptors, sucrose non-fermenting 1-related protein kinase 2 (*SnPK2*), and ABA-responsive element binding factors (*ABFs*) were generally up-regulated at late stages of heat stress. ETH-pathway genes, with the exception of *ERT2*, were mostly up-regulated after 2 h of heat stress; these included genes encoding the ethylene receptors *ETR* and *EIN2* and the *EIN3* TF. Expression of the *MYC2* TF in the JA pathway decreased and then increased under heat stress, and most genes encoding *TGA* TFs in the SA pathway were up-regulated after high-temperature stress.

Validation of RNA-seq data

To confirm the transcriptomic results, we performed quantitative real-time PCR (RT–qPCR) on six selected DEGs from hormone biosynthesis and signaling pathways: *Unigene0006069* (*SAMS2*), *Unigene0012691* (*PYL6*), *Unigene0016727* (*PP2C27*), *Unigene0063348* (*PP2C38*), *Unigene0024871* (*EIN2*), and *Unigene0046064* (*ERT2*). The expression patterns of these genes under heat stress were similar in the RNA-seq and RT–qPCR data (Figure S2).

Construction of a PPI network from hormone signaling-related DEGs and identification of hub genes

Phytohormones act synergistically and antagonistically in complex networks to finely regulate plant stress responses [32]. To explore the potential interactions

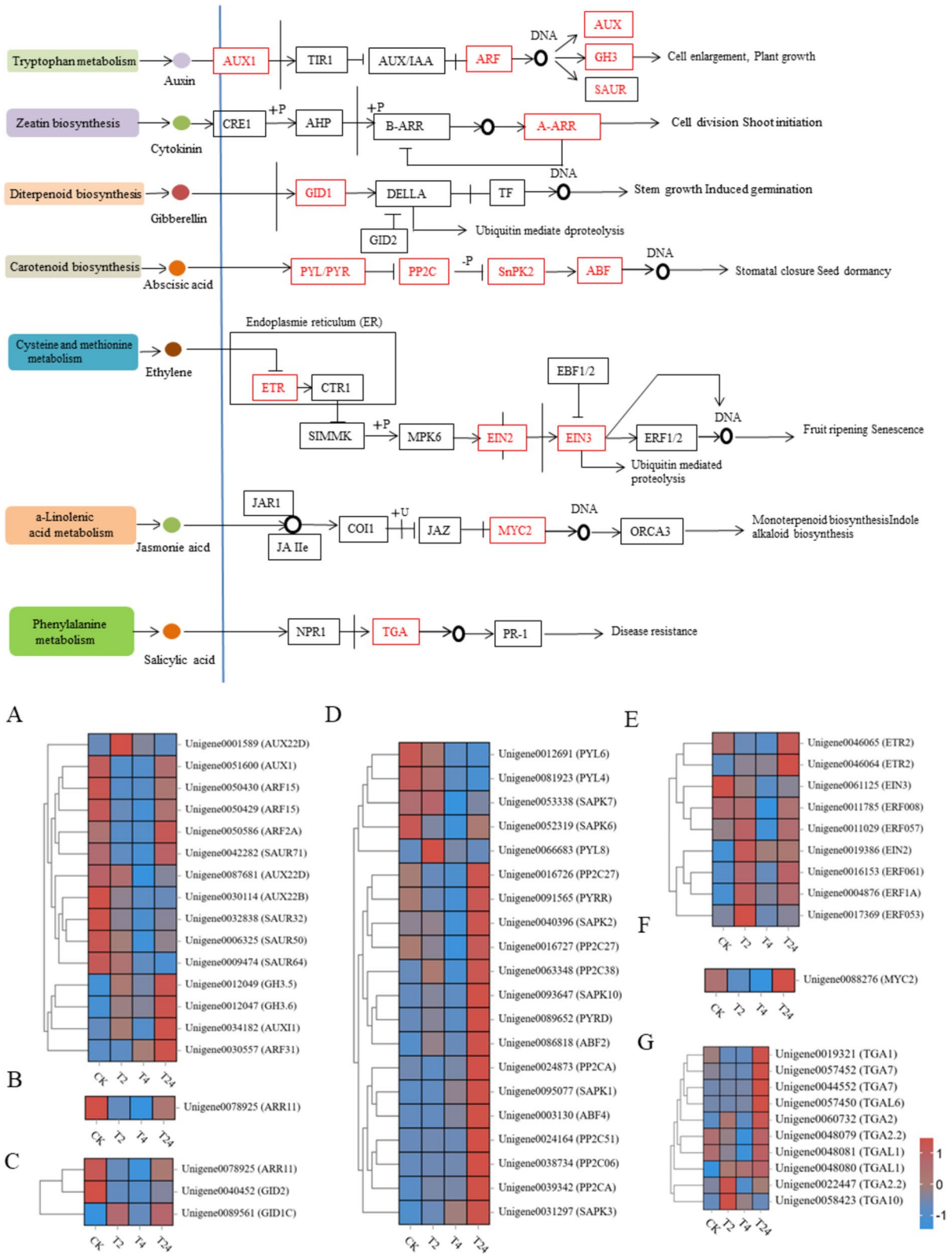

Fig. 12 Analysis of DEGs related to plant hormone signal transduction pathways. The pathways in which their encoded enzymes participate are shown at the top of the figure. **A** IAA (**B**) CTK (**C**) GA3 (**D**) ABA (**E**) ETH (**F**) JA (**G**) SA

among various hormone-related DEGs and identify key regulators of their common network(s), we constructed a protein–protein interaction (PPI) network among the DEGs identified in hormone signaling pathways of the seven measured hormones under high temperature (Fig. 13). We identified 15 hub genes with strong connectivity in the hormone signaling network, including five transcription factor genes: *Unigene053031* (*GSK2*), *Unigene0095581* (*RR10*), *Unigene0003130* (*ABF4*), *Unigene0004598* (*RHT1*), *Unigene0002686* (*PIL13*), *Unigene0017689 (ERS1)*, *Unigene0004597* (*RHT1*), *Unigene0067532* (*RR5*), *Unigene0004597 (RHT1)*, *Unigene0092053 (PHP2)*, *Unigene0015415* (*PHP5*), *Unigene0001832* (*CTR1*), *Unigene0059998* (*RR6*), *Unigene0001195* (*EBF2*), *Unigene0081681* (*AUX22D*), and *Unigene0030114* (*AUX22B*). Three were involved in the IAA signaling pathway, two in the ET signaling pathway, and three in the CK signaling pathway.

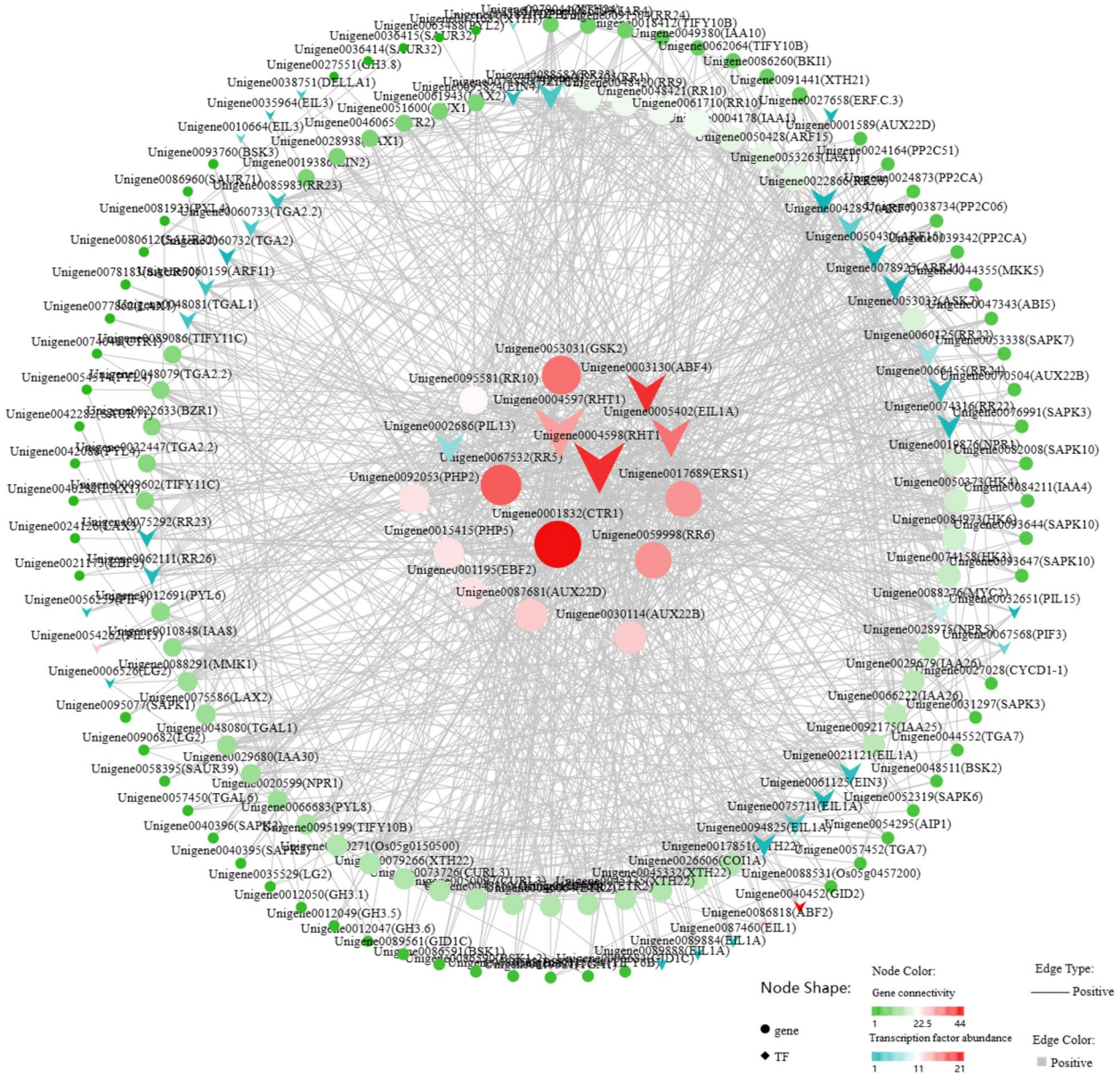

Fig. 13 Predicted PPI network of DEGs related to hormone signaling, with identified hub genes. Each node represents a gene, and each edge represents an interaction between two genes. The size of the node is proportional to its degree of connectivity (i.e., how many edges connect to it). The larger central nodes are therefore likely to be network hubs

Discussion

With the intensification of global warming, high-temperature stress poses increasingly serious threats to plant growth [33]. High-temperature stress damages plant cell membranes, impairing normal physiological functions, and plants respond through a variety of morphological, biochemical, and physiological modifications [34]. MDA is a lipid peroxidation product whose content reflects overall cell damage and antioxidant capacity, and POD and SOD are important antioxidant enzymes [35]. Here, H_2O_2 content, MDA content, and POD activity increased in garlic seedlings under high-temperature stress, confirming that increased ROS production occurred, causing oxidative damage and inducing antioxidant enzyme activity. The observed decrease in SOD activity indicated that POD activity was significantly increased, suggesting that POD may participate in the removal of H_2O_2, but further experiments are needed to verify its dominant role. Soluble sugar and chlorophyll contents decreased with increasing duration of heat stress, although soluble sugar content did not decrease significantly until 24 h, suggesting that short-term high-temperatures had little effect on soluble sugars. Increased respiration and reduced photosynthesis with increasing duration of heat stress could help to explain this drop in soluble sugar content. However, with increasing exposure to high-temperatures, perhaps exacerbated by stomatal closure, leaves may need to consume large amounts of soluble sugar to maintain cell osmotic pressure.

The effects of temperature on plant growth and development are well known, and to some extent, they reflect changes in the levels of endogenous hormones [36, 37], which finely regulate stress responses through a complex network of interacting signaling pathways [38]. IAA and GA_3 are the major hormones that promote plant growth [39]. GA_3 can promote cell expansion and regulate stomatal aperture, reducing transpiration [40]. Contents of IAA and GA_3 were significantly reduced in heat-stressed garlic leaves, suggesting that garlic may resist the effects of high temperature by reducing the synthesis of growth-promoting hormones to slow growth under these conditions. The expression of *ASA*, *PAT*, *CPS* and other genes showed a correlation trend with hormone content, suggesting that they may be involved in regulation, but further functional verification is needed. Interestingly, the expression of *gibberellin-insensitive dwarf1* (*GID1C*) was up-regulated during heat stress, whereas that of *gibberellin-insensitive dwarf2* (*GID2*) was down-regulated. In the GA_3 signaling pathway, *GID1* and *GID2* function in the degradation of DELLA proteins, negative regulators of the GA response [41]. The differential expression of *GID1* and *GID2* suggests that the GA–GID1–DELLA regulatory module plays a key role in the garlic heat-stress response [42].

Plants tend to produce more ABA under heat stress, initiating ABA-dependent signal transduction, and the rapid accumulation of ABA observed here at high temperature suggests that endogenous ABA is also involved in the garlic heat stress response [43]. Genes encoding the ABA-biosynthesis enzyme *NCED* were significantly up-regulated under heat stress, consistent with the observed changes in ABA content, suggesting that *NCED* plays an important role in ABA accumulation under high-temperature stress in garlic. In the ABA signaling pathway, PP2C proteins have a negative regulatory effect, inactivating the downstream SnRK2 protein, which can directly regulate the phosphorylation of *ABF* TFs [44]. Here, the *PP2C* repressor was up-regulated under heat stress, whereas the positive regulators *SnPK2* and *ABF* were down-regulated; nonetheless, *SnPK2A* was significantly up-regulated at 24 h, indicating that this gene was involved in ABA signaling under short-term heat stress.

MYC2 belongs to the bHLH TF family and is a central regulator of the JA signaling pathway, responding to biological and abiotic stresses and regulating secondary metabolite synthesis [45]. The JAZZ–MYC2 module is the best studied JA signal transduction pathway [46]. Here, we found that *Unigene088267* (*MYC2*) was up-regulated under heat stress, suggesting that *MYC2* responds to JA signal and functions in heat stress resistance in garlic. However, the JAZ–MYC2 module depends not only on gene expression levels but also on protein modifications, such as MYC2 phosphorylation, and the mechanisms by which this module influences heat stress responses require further study.

SA also has a critical role in high-temperature responses, promoting the expression of stress-resistance genes and increasing the activity of antioxidant enzymes [47]. Here, we observed significant changes in the expression of *PAL*, which is involved in the early stages of SA biosynthesis, suggesting that *PAL* activity may be responsible, at least in part, for the increased SA content under high-temperature stress. ET is also important for abiotic stress signaling, and *ERF* TFs are integral components of environmental stress signaling cascades, regulating the expression of downstream stress-responsive genes [14, 48]. For example, the two interacting proteins ERF95 and ERF97 participate in an EIN3–ERF95/97–HSFA2 module that regulates basal heat resistance [13]. In our study, numerous *ERF* and *EIN* genes were differentially expressed, suggesting a potential signaling pathway by which increased ET content promotes the expression of stress-related genes to enable the response of garlic to high-temperature stress.

ERF, MYB, bHLH, NAC and WRKY TFs are key regulatory factors in plant responses to abiotic stress, and individual TFs can participate in one or more phytohormone-mediated signaling pathways [49]. In the current study, we identified 594 TFs from 41 families in the leaves of heat stressed garlic, suggesting that they may regulate the gene expression patterns that govern garlic's high-temperature response. The specific roles of these TFs—particularly those identified as network hubs in Fig. 14—warrant additional experimental verification.

In this study, we studied the physiological effects of high-temperature stress on garlic, systematically analyzed the dynamic changes of the contents of seven endogenous hormones in garlic leaves under high-temperature stress, and then analyzed the changes in the expression levels of genes related to the biosynthesis and signal transduction of each hormone based on transcriptome data. The limitations of this study include the following aspects: (1) The heat stress treatments were limited to four time points, and future research should include additional time points to fully understand the dynamic changes; (2) Although several candidate genes related to hormone signaling were identified, functional validation, such as gene knockout or overexpression experiments, has not yet been performed; (3) This study focused on the transcriptional level, without addressing post-translational protein modifications or metabolite-level changes. Therefore, further multi-omics integrative studies will be helpful in comprehensively elucidating the mechanisms underlying garlic's response to high-temperature stress. Moreover, plant hormones do not play a separate role in each life process, and various hormones complement and restrict each other [50]. Therefore, it is of great significance to explore the coordination relationship between endogenous hormones in garlic under high-temperature stress and the molecular mechanism of garlic's endogenous hormone-dependent response to high-temperature stress, which will also be the focus of our future research.

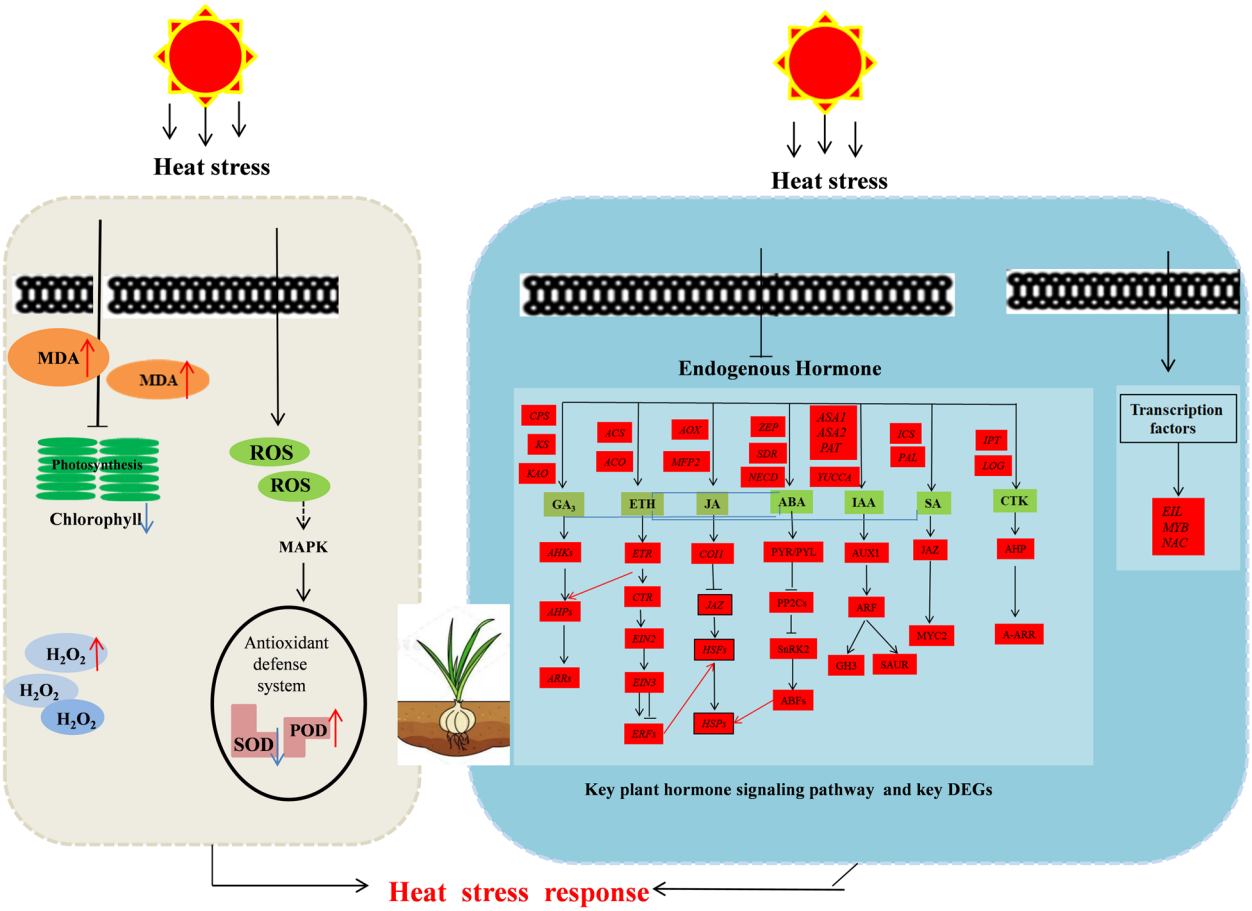

Fig. 14 Key genes that may be related to plant hormone synthesis and transduction in garlic under heat stress. The green box represents the name of each hormone and the red box represents the gene

Conclusion

We analyzed the physiological and transcriptomic responses of garlic seedlings to different durations of high-temperature stress (Fig. 14). H_2O_2 content, POD activity, and MDA content increased under heat stress in garlic leaves, whereas soluble sugar content and SOD activity decreased. POD therefore appeared to be responsible for much of the H_2O_2 scavenging under high-temperature stress. Transcriptomic analysis suggested that plant hormone signaling pathways had an important role in the response of garlic to high-temperature stress, as genes associated with IAA, GA, ET, ABA, JA, SA, and SK synthesis and signal transduction were differentially expressed. MYB, AP2/ERF, and other TF families appeared to participate in heat stress adaptation, regulating the responses to plant hormones such as ET, ABA, and JA. These findings lay a foundation for future functional verification studies and provide a reference for exploring heat-resistant gene resources.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06346-8.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
Not applicable.

Authors' contributions
Qingqing Yang and Feng Yang initiated and designed the research, Qingqing Yang, Ji-De Fan, Canyu Liu, Yongqiang Zhao, and Xinjuan Lu performed the experiments; Jie Ge, Biwei Zhang, Mengqian Li and Yan Yang analyzed the data; Feng Yang contributed reagents/materials/analysis tools; Qingqing Yang wrote the paper; Zhisheng Xu, Ji-De Fan and Feng Yang revised the paper. All authors read and approved the final manuscript.

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Data availability
Sequence data that support the findings of this study have been deposited in the NCBI database accession numbers of RNA-Seq data is PRJNA1198391.

Declarations

Ethics approval and consent to participate
The authors declare that the plant experiments were performed in accordance with national/institutional guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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