Analysis of drought and heat stress response genes in rice using co-expression network and differentially expressed gene analyses

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Research Article

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Abstract

Studies on *Oryza sativa* (rice) are crucial for improving agricultural productivity and ensuring global sustenance security, especially considering the increasing drought and heat stress caused by extreme climate change. Currently, the genes and mechanisms underlying drought and heat resistance in rice are not fully understood, and the scope for enhancing the development of new strains remains considerable. To accurately identify the key genes related to drought and heat stress responses in rice, multiple datasets from the Gene Expression Omnibus (GEO) database were integrated in this study. A co-expression network was constructed using a Weighted Correlation Network Analysis (WGCNA) algorithm. We further distinguished the core network and intersected it with differentially expressed genes and multiple expression datasets for screening. Differences in gene expression levels were verified using quantitative real-time polymerase chain reaction (PCR). *OsDjC53, MBF1C, BAG6, HSP23.2*, and *HSP21.9* were found to be associated with the heat stress response, and it is also possible that *UGT83A1* and *OsCPn60a1*, although not directly related, are affected by drought stress. This study offers significant insights into the molecular mechanisms underlying stress responses in rice, which could promote the development of stress-tolerant rice breeds.

Key Message

This study integrated multiple datasets obtained from the GEO database and performed bioinformatics methods to predict and validate five key genes associated with the response to heat stress in plants.

Introduction

Rice, scientifically known as Oryza sativa, is a vital cereal that is extensively grown and serves as a fundamental source of sustenance for approximately 50% of the global population(Ashkani, Rafii et al. 2015, Li, Tian et al. 2018). In recent decades, with the rapid increase in the total population of Earth and the demand for sustenance security, the importance of rice genetics and breeding has become increasingly critical(Huang, Yang et al. 2016). Studies focusing on the generation of advanced rice types aim to improve the yield, quality, and resilience of rice plants to biotic and abiotic stressors, such as pests, diseases, salt, drought, and heat(Vo, Rahman et al. 2021, Raj and Nadarajah 2022). With the deterioration of global climate, the frequency and severity of drought and heat waves are expected to increase in many rice-growing regions(Zandalinas, Fritschi et al. 2021, Saud, Wang et al. 2022). Rice is an aquatic plant predominantly grown in lowland areas that are often subjected to flooding, making the crop more vulnerable to drought and heat stress(Ji, Wang et al. 2012, Jagadish, Murty et al. 2015, Sahebi, Hanafi et al. 2018).

Advances in rice breeding biotechnology and genetic cultivar improvement have played a significant role in increasing the drought resistance of rice while enhancing its ability to adapt to hot environments (Shen, Xie et al. 2022, Wang and Han 2022). Research into the molecular mechanisms underlying drought and heat adaptability in rice can facilitate the creation of novel rice cultivars with improved stress tolerance(Kim, Chung et al. 2020, Liu, Lyu et al. 2020).

Several key genes that confer drought tolerance in rice have been identified. *UGT85E1-* and OsWRKY5-mediated enhancement of abscisic acid response has been shown to improve drought stress tolerance(Liu, Dong et al. 2021, Lim, Kang et al. 2022). *OsNAR2.1* serves a fundamental role in nitrate absorption and translocation; thus, its expression level is positively correlated with drought resistance in rice(Chen, Qi et al. 2019). *OsRINGzf1* regulates aquaporins during drought stress(Chen, Xu et al. 2022). The expression levels of photosynthesis-related genes, such as *CA1*, also change under drought stress(Li, Liu et al. 2020, Auler, Nogueira do Amaral et al. 2021). Overexpression of *Arabidopsis UBC32* improves drought tolerance in rice(Chen, Liu et al. 2021). These genes are involved in various processes such as hormone signaling pathways, osmotic regulation, and photosynthesis.

OsRab7-mediated modulation of osmolytes, antioxidants, and genes that respond to abiotic stress can lead to improved grain yield and enhanced ability to withstand heat in transgenic rice(El-Esawi and Alayafı 2019). *OsTT1* plays a protective

role against heat stress by eliminating denatured proteins that are cytotoxic and preserving thermal response processes in cells(Li, Chao et al. 2015), and *OsNTL3* and *OsbZIP74* have a similar mechanism(Liu, Lyu et al. 2020). *HES1* maintains the stability of the photosynthetic system under high-temperature stress(Xia, Liu et al. 2022). These genes are associated with heat shock proteins, antioxidant enzymes, protein synthesis, and photosynthesis.

In summary, research on drought and heat durability in rice is critical for ensuring global food security, adapting to extreme climate change, and improving agricultural productivity(Tyczewska, Woźniak et al. 2018). Previous studies have provided valuable insights into the physiological and molecular aspects of stress responses in rice(Lakshmanan, Cheung et al. 2016). However, one significant gap and limitation in the current literature is the incomplete identification and understanding of the key genes and regulatory networks involved in drought and heat stress responses in rice. Although many stress-responsive genes have been identified, they represent only a small fraction of the vast number of genes in rice. Existing studies are unable to compare the significance of these genes in stress responses. This limits our ability to develop targeted strategies for enhancing stress tolerance in rice varieties.

To comprehensively analyze the molecular mechanisms underlying drought and heat responses in rice, a set of RNA-seq data from the Gene Expression Omnibus (GEO) database was selected, which contained different gradients of drought and heat treatments, and compared with multiple datasets that were subjected to either drought or heat stress. The integration of diverse datasets and the utilization of advanced analytical techniques allow us to overcome the limitations of individual studies and provide a more holistic view of the molecular mechanisms underlying stress responses in rice. The present study enhances our understanding of the molecular mechanisms underlying drought and heat stress adaptation in rice and can be useful in discovering new and more important genes that could serve as candidates for genetic breeding purposes.

Materials and Methods

4.1. Data collection

Multiple gene expression profiling datasets, including high throughput sequencing (Illumina HiSeq 2000/ Illumina HiSeq 4000/ Illumina NovaSeq 6000) and array datasets (Affymetrix Rice Genome Array Platforms), were sought and retrieved from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). These included GSE221542, GSE168650(Kan, Mu et al. 2022), GSE136746(Ps, Sv et al. 2017), GSE41648(Sharma, Borah et al. 2021), GSE14275(Hu, Hu et al. 2009), GSE159816(Zu, Lu et al. 2021), GSE93917(Wang, Li et al. 2020), GSE83378(Wei, Chen et al. 2017), and GSE121303(Chung, Jung et al. 2016) (Table 1). Gene symbols for these GEO datasets were annotated using the National Center for Biotechnology Information (NCBI), Rice Annotation Project database (RAP-db) (https://rapdb.dna.affrc.go.jp/), and the Rice Genome Annotation Project (RGAP) (http://rice.uga.edu/index.shtml). The data were processed using R (version 4.2.3) and RStudio (version 2023.03.0) software. GSE221542 contains 15 samples, including three water levels and two heat levels, each with three replicates.

Table 1
Raw data information from GEO.

Name	Dataset	Cultivar	Tissue	Samples
GSE221542	GSE221542	Nipponbare	whole shoot	all
GSE168650X	GSE168650	NIL-TT2HJX	developing aerial tissues	heat vs control
GSE168650-32	GSE168650	NIL-TT2HPS32	developing aerial tissues	heat vs control
GSE136746-N22	GSE136746	Nagina22	panicle	heat vs control
GSE41648-Ann	GSE41648	Annapurna	seedling	heat vs control
GSE14275	GSE14275	ZhongHua 11	seedling	heat vs control
GSE159816-WT	GSE159816	wild type	leaf	drought vs control
GSE159816-idr11	GSE159816	idr1-1	leaf	drought vs control
GSE93917-nadk1	GSE93917	osnadk1	leaf	drought vs control
GSE93917-WT	GSE93917	wild type	leaf	drought vs control
GSE83378-MILT	GSE83378	MILT1444	panicle	drought vs control

4.2. WGCNA of drought/heat response genes

Using the WGCNA(Langfelder and Horvath 2008) package in R (version 4.2.3), a WGCNA co-expression network was constructed using the following steps. First, the average expression of each gene under different levels of drought or heat stress was calculated, and genes that did not exhibit any changes in expression were filtered out. Second, normalization of gene expression levels to a range of 0–1 was followed by the calculation of Pearson's correlation coefficients, which is used to measure the similarity of co-expression between genes. Third, to ensure a scale-free network distribution, an appropriate beta value was selected for the adjacency matrix weights to construct a Topological Overlap Matrix (TOM) for module clustering and segmentation. Finally, to select modules related to drought or heat responses, the relationship between each network module and the sample phenotype was analyzed.

GO terms were used to enrich selected genes (Tian, Liu et al. 2017). The analysis results were presented using the R package "clusterProfiler" for visualization(Yu, Wang et al. 2012). KEGG enrichment(Kanehisa and Goto 2000) analysis was also performed using the R package "clusterProfiler" (Yu, Wang et al. 2012). Using the CytoHubba(Chin, Chen et al. 2014) plugin of Cytoscape (3.9.1), based on the shortest paths, every gene of the key module was scored using the MCC method, and the top 20 hub genes were selected.

4.3. DEG analysis with DESeq2 and GO enrichment in R

DEG analysis was performed using the R package DESeq2(Love, Huber et al. 2014). Raw count data from the RNA-seq experiments were imported into R, and genes with low expression were filtered using the "filterByExpr" function. Next, the "DESeqDataSetFromMatrix" function was used to create a DESeq2 object, which was then used to estimate size factors and dispersions using the "estimateSizeFactors" and "estimateDispersions" functions, respectively. A false discovery rate (FDR) cutoff of 0.05 was applied to identify genes that were significantly differentially expressed, based on an absolute log2 fold change ≥ 1 and an adjusted p-value ≤ 0.05. All data analyses were performed using R software (version 4.2.3).

GO enrichment analysis was also performed to analyze DEGs using a previously described approach(Yu, Wang et al. 2012, Tian, Liu et al. 2017).

4.4. Intersection of hub and DEGs for candidate key genes

The top 20 hub genes from the filtered key modules were compared with the DEGs obtained from the filtering process. Based on their intersection and the candidate key genes along with their log2 fold change values were obtained. The Rice Gene Index (RGI) (https://riceome.hzau.edu.cn/) was used to determine the gene ID corresponding to the rice gene(Yu, Chen et al. 2023).

We searched for datasets on drought or heat treatments in the GEO database (Table 1). Count data were processed using the same method as above but not filtered for log2 fold change ≥ 1 and p-value ≤ 0.05. For array data, online GEO2R analysis was performed, and a matrix table containing the log2 fold change, p-value, and adjusted p-value data was downloaded.

Using the "pheatmap" package in R (version 4.2.3), the log2 fold change calculated from the different array or count data treatments were clustered and plotted. Key genes with high and stable expression levels were selected for further experiments.

4.5. Plant Materials

Oryza Sativa, Jiahe 102 (State Key Laboratory of Hybrid Rice, Wuhan, China) was used as a model rice variety subjected to appropriate environmental conditions, drought stress, and heat stimulation and for RNA extraction for quantitative real-time PCR (qRT-PCR).

The seed coat was removed, sterilized, and soaked in a dark room at 37°C for 24 h. The seeds were allowed to germinate at 25°C in the dark for 48 h and then transferred to a culture bottle for cultivation in the Murashige and Skoog (MS) medium at 25°C for 14 days. The control group was directly sampled. For the drought treatment group, on the evening of the 13th day, the samples were cultured for 12 h in the MS medium containing 0.3 M mannitol. For the heat treatment group, on the 14th day, rice seedlings cultured in normal MS medium were exposed to a temperature of 37°C for 1 h and then sampled.

4.6. RNA extraction

Whole shoot tissues (100 mg) of rice from different treatment groups were weighed and placed in a grinding tube containing steel beads. The grinding tubes were immersed in liquid nitrogen for 10–20 min. Finally, the samples were freeze-ground at − 20°C for 120 s and returned to liquid nitrogen for storage. RNA extraction was performed using *the FastPure Universal Plant Total RNA Isolation Kit* (Vazyme, Nanjing, China), and the extracted total RNA was stored at − 80°C.

4.7. Quantitative real-time PCR

cDNA was synthesized using *the Revert Aid First Strand cDNA Synthesis Kit* (Thermo Scientific, Watham, MA, USA). qRT-PCR analysis was performed using a LightCycler 96 (Roche, Basel, Switzerland). *eEF1* was used as the reference gene. Gene sequences were searched using Phytozome (https://phytozome-next.jgi.doe.gov/), and qRT-PCR primer sequences were designed using the primer blast tool of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers used in this study are listed in Table 2.

Table 2
Primer information for qRT-PCR.

Gene name	MSU-ID	Group	5' primer	3' primer
eEF1	LOC_Os03g08010	Reference	GATGATCTGCTGCTGCAACAAG	GGGAATCTTGTCAGGGTTGTAG
BAG6	LOC_Os02g15930	Green	GTTGAAAGTAGTGTGTCAGCT	AAGGATACTGATGAGTCCCC
HSP23.2	LOC_Os04g36750	Green	GGTGGAGGTGGAGGACAA	CCAGAACCTGCCGTAGGA
OsDjC53	LOC_Os06g09560	Green	GATTTCCTCGGCGAGATGG	ACGAACAGCTGCTGCAA
MBF1C	LOC_Os06g39240	Green	AGGTTGAGCGGCAACATC	CGCATCGCCTGGTTCAC
HSP21.9	LOC_Os11g13980	Green	CGTACGGCTACGGCTACAT	TCCTTCCAGTCGCACCTC
UGT83A1	LOC_Os03g55030	Darkmagenta	GGCGTCCTCAACGAGAAG	CAGACGAGGTCGAAGATGATG
OsCPn60a1	LOC_Os12g17910	Darkmagenta	CAAGGCTGTCCTTCAGGATATT	TGTCCCAAGTTGCTCTTCAG

Relative expression level of target genes was calculated based on the 2−ΔΔCt method for normalization(Livak and Schmittgen 2001). The normalized qRT-PCR data was analyzed using a t-test to determine statistically significant differences in gene expression between the control and experimental groups.(Wilson and Worcester 1942) Statistical significance was set at $p < 0.05$.

Results

2.1. Construction of co–expression network

The workflow followed in this study is demonstrated in Fig. 1.

Weighted Correlation Network Analysis (WGCNA) was utilized in this study to analyze the GSE221542 dataset, with a scale-free topology model fitting degree of 0.8 and a soft threshold of 30 selected for network construction (Fig. 2A-B). A hierarchical clustering process was used to create a tree-like structure representing genes. Subsequently, gene modules were determined using the dynamic cutting method, followed by calculation of the eigenvector value of each module. Similar modules were then merged to identify distinct modules, which were assigned different colors for better visualization (Fig. 2C).

2.2. Co–expression network module analysis

Six modules, namely black (1550 genes), green (1646 genes), dark orange (3658 genes), dark magenta (535 genes), royal blue (9432 genes), and gray (234 genes), were obtained. The modules showed either positive or negative correlation with drought or heat stress, and the genes within these modules were either upregulated or downregulated, suggesting that the genes respond differently under different stress conditions. The green module with heat and the dark magenta module with drought had the highest positive correlation coefficients (0.98 and 0.71, respectively) (Fig. 3A). According to the scatter plots, the genes in the green module were highly correlated with heat stimulation, whereas the genes in the dark magenta module showed a weak association with drought stress (Fig. 3B-E). The other modules showed low correlation with heat or drought stress (Figure S1).

Cytoscape software was used to process the dark magenta and green modules separately and visualize the co-expression network obtained from WGCNA. Genes in the network were scored using the Maximal Clique Centrality (MCC) method, and the top 20 hub genes with the highest correlations with other genes were selected (Fig. 3F-G). These genes were located at the most central positions in the co-expression network and may play a central regulatory role in drought and heat stress.

In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that genes in the green module are involved in processes such as protein synthesis in the endoplasmic reticulum and RNA splicing, whereas genes in the dark magenta module are involved in essential processes such as carbon metabolism, synthesis of amino acids and coenzyme factors, and glycerolipid metabolism. In addition, both gene modules are involved in carbon fixation in photosynthetic organisms (Fig. 4A). The results of the Gene Ontology (GO) enrichment analysis showed that genes in the green module were related to biological processes such as cellular response to stimuli, phosphorylation, and signaling, whereas genes in the dark magenta module were involved in as phosphorus metabolism and phosphate-containing compound metabolic processes. Although genes of both modules are expressed in the cytoplasm and vesicles; genes of the green module are expressed in membrane-bound organelles only. In green module, the expressed proteins exhibited transferase activity and nucleic acid binding, whereas those in the dark magenta module exhibited catalytic activity and metal ion binding (Fig. 4B).

Construction of the gene co-expression network narrowed the range of candidate genes, and the 20 hub genes obtained by screening made the follow-up study more convenient.

2.3. Differentially expressed gene (DEG) analysis

Differential gene analysis was performed on two modules of the GSE221542 dataset: severe drought stress and control and prolonged heat stress and control (Fig. 5A). The count data files were downloaded directly from the GEO website. Because the count data can only represent the read count of each gene, normalization is necessary. The expression level of the experimental group was subtracted from that of the control group, and log2 fold-change values were calculated to compare gene expression levels. The preset threshold was used to select DEGs; 484 DEGs had increased expression levels, and 1559 DEGs had decreased expression levels in the drought stress group, whereas 1876 DEGs were upregulated, and 3158 DEGs were downregulated in the heat stress group.

GO analysis indicated that under drought or heat stress, macromolecule metabolism was the most altered biological process, and hormonal responses and other response activities were also altered. The proteins expressed by the two groups of DEGs were mainly binding proteins and mostly located in membrane-bound organelles and vesicles. However, the DEGs in the drought stress group were mostly related to ATP function, whereas those in the heat stress group were involved in nucleic acid and DNA-related functions. (Fig. 5B).

The GO enrichment analysis results for the green module and heat stress-induced DEGs shared many similarities. In terms of biological processes, both involved cellular response activities. Regarding molecular functions and cell components both involve a large proportion of proteins with nucleic acid binding that commonly act on membrane-bound organelles or vesicles. Therefore, the prediction of heat stress-related genes in the green module was expected to be more accurate (Fig. 4B and 5B).

Analyzing DEGs in the dataset is an important basis for the subsequent screening of key genes.

2.4. Further screening of hub genes

The top 20 hub genes in the dark magenta and green modules intersected with the DEGs under drought and heat stress, respectively, resulting in the selection of two candidate key genes associated with drought stress and 20 genes associated with heat stress (Fig. 5C).

Green module: The RNA-seq dataset used was GSE168650(Kan, Mu et al. 2022), which contained RNA-seq data for two different strains of rice subjected to heat treatment and their corresponding controls. The data type was the RAW count. Differential genes were analyzed using the same method without setting a threshold filter to identify key genes and their relative expression levels. GEO2R was used to analyze the expression levels of key candidate genes in multiple array datasets including GSE136746(Ps, Sv et al. 2017), GSE41648(Sharma, Borah et al. 2021) and GSE14275(Hu, Hu et al. 2009).

Cluster analysis was performed separately according to the original data types. Genes with high expression levels and consistent expression levels among different samples were selected from the heatmaps of both RNA-seq and array data (Fig. 6A-B, Figure S1A). Five key genes with the best overall performance were selected: *OsDjC53, MBF1C, BAG6, HSP23.2*, and *HSP21.9*.

Darkmagenta Module: dataset GSE159816(Zu, Lu et al. 2021) was downloaded, which contained two lines of rice subjected to drought treatment and their corresponding controls. We also analyzed the expression levels of key candidate genes in the GSE93917(Wang, Li et al. 2020) and GSE83378(Wei, Chen et al. 2017) array datasets. The results showed that the expression levels of *UGT83A1* and *OsCPn60a1* did not show the same trend in multiple datasets; however, they were classified as key genes for further confirmation (Fig. 6C-D, Figure S2B).

2.5. Verification of key genes

Quantitative real-time PCR (qRT-PCR) was used to verify changes in the expression levels of key genes in rice subjected to drought or heat stress conditions. The results showed that *OsDjC53, MBF1C, BAG6, HSP23.2*, and *HSP21.9* were significantly overexpressed in rice under heat stress conditions (Fig. 7A), whereas the expression levels of *UGT83A1* and *OsCPn60a1* significantly decreased in rice under drought stress conditions (Fig. 7B). In summary, the five candidate genes in the green module may be the key genes associated with heat stress response in rice.

Discussion

A co-expression network was constructed using the WGCNA algorithm, which allowed us to identify the top 20 genes and form a core network. The core network was intersected with DEGs identified from the same dataset to obtain candidate key genes associated with drought and heat stress responses. Furthermore, by analyzing multiple datasets, two key genes that respond to drought stress and five key genes that respond to heat stress were identified among the candidate key genes. The final qRT-PCR results excluded all key drought-related genes and identified *OsDjC53, MBF1C, BAG6, HSP23.2*, and *HSP21.9* as genes associated with heat stress response.

UDP-glycosyltransferases (UGTs) are a class of enzymes that add sugars covalently to a wide range of secondary metabolites(Bowles, Isayenkova et al. 2005). *UGT83A1* is a key gene for yield and drought resistance in rice, and *UGT83A1*-overexpressing lines exhibit strong resistance to drought stress(Dong, Sun et al. 2020). In addition, the expression level of *UGT85E1* first increases and then decreases under drought stress; the *UGT83A1*-overexpressing line can obviously improve the drought tolerance of rice but is more prone to withering(Liu, Dong et al. 2021). A dataset (GSE121303(Chung, Jung et al. 2016)) was subjected to drought stress for 1,2,3 days, respectively. The expression level of this gene fluctuated with drought duration (Figure S3). In conclusion, the overexpression of *UGT83A1* can improve drought resistance in rice; however, *UGT83A1* expression levels do not necessarily increase or decrease when rice is under drought stress. This suggests that *UGT83A1* may be involved in the drought stress response through a complex mechanism influenced by other factors.

OsCPn60a1 may bind to the RuBisCO small and large subunits and is implicated in the assembly of the enzyme oligomer(Aigner, Wilson et al. 2017). Thus, we suggest that changes in *OsCPn60a1* expression levels may indicate changes in photosynthesis but may not necessarily be directly associated with the drought stress response.

The response of rice to heat stress is closely linked to heat shock proteins (HSPs). There is a high degree of homology between HSP21.9 and HSP23.2 proteins (Figure S4). Furthermore, protein motif prediction revealed multiple shared motifs among OsDjC53, MBF1C, BAG6, HSP23.2, and HSP21.9 (Figure S5), indicating that these five proteins potentially interact or cooperate with each other.

HSPs are crucial for plant growth and abiotic stress tolerance(Mansfield and Key 1987, Sarkar, Kim et al. 2009). *OsDjC53* is predicted to belong to the *DnaJ/HSP40* family (RGI). *HSP21.9* and *HSP23.2* belong to the HSP20 family (RGI). HSPs were found to control programmed cell death (PCD) of suspension cells in response to high temperatures and play an important

role in the response to hyperosmotic and heat shock stress by preventing the aggregation of stress-denatured proteins and by disaggregating proteins(Wang, Zhang et al. 2019). *MBF1C* is a multi-protein bridging factor. In *Arabidopsis*, *MBF1C* improves the tolerance to heat and osmotic stress by partially activating or disrupting the ethylene response signal transduction pathway(Suzuki, Rizhsky et al. 2005). *BAGs* (*Bcl-2* associated athanogene) are considered to be adaptor that can form complexes with signaling molecules and molecular chaperones(Kabbage and Dickman 2008). *BAG6* plays a critical role in plant heat tolerance by regulating the accumulation of HSPs and maintaining protein homeostasis under heat stress conditions in *Arabidopsis*(Echevarría-Zomeño, Fernández-Calvino et al. 2016). In other species, these genes are also highly correlated with drought resistance. Thus, increased expression of these genes may improve the ability of rice to resist heat stimulation.

This study has few limitations. The small number of control, heat stress, and drought stress samples in the WGCNA may have resulted in potential statistical errors during the construction of the co-expression network. Furthermore, the limited data available in the GEO validation cohorts underscore the need for more publicly available transcriptome sequencing data. Additional experiments are required to elucidate the mechanisms underlying the response of rice to drought and heat stress. In addition, although our analytical method successfully predicted the heat stress response genes in rice, it did not perform as well in predicting drought stress response genes, possibly because of the limited data used by the WGCNA. Therefore, larger datasets are required for more accurate analyses and predictions.

Overall, our findings provide valuable insights into the molecular mechanisms underlying the response of rice to drought and heat stress and may have important implications for the development of stress-tolerant rice varieties through genetic engineering approaches.

Conclusions

Our approach successfully identified key candidate genes associated with heat stress response in rice. More importantly, our study represents an innovative integration of multiple RNA-seq and array datasets from the GEO database to analyze the key genes associated with drought and heat stress responses in rice. The degree of fit between each module and the corresponding trait (Fig. 3B and E) determined the effectiveness of the obtained key gene (Fig. 7). In addition, the majority of this study did not require gene annotation; therefore, this study may be of greater value to research on unsequenced species with large amounts of RNA-seq data.

Declarations

Supplementary Materials: *Figure S1*: "Gene significance and module membership fit scatter plots. Each gene is indicated by a hollow dot. The x-axis represents the correlation between the module eigengene and the gene expression profile in the different color groups. The y-axis shows the correlation between the gene and different degrees of heat or drought stress." *Figure S2*: "Expression levels of key genes in the array dataset. Expression levels of key genes in the array dataset. Gene names are plotted on the x-axis, and gene expression levels are shown on the y-axis. **(A)** The top, middle, and bottom figures show the three datasets related to heat stress. **(B)** Drought stress." *Figure S3*: "The expression level of *UGT83A1* changed in rice subjected to 1, 2, or 3 days of drought stress. The x-axis represents the duration of drought stress treatment, and the y-axis represents the relative expression levels of genes." *Figure S4*: "Evolutionary tree analysis of 36 rice HSP20 proteins. Closer branch distances indicate higher degrees of homology among proteins." *Figure S5*: "Protein motif prediction. Each block indicates the position and strength of a motif site. The height is calculated to be proportional to the negative logarithm of the p-value of the site, truncated at the height for a p-value of 1e-10."

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Figures

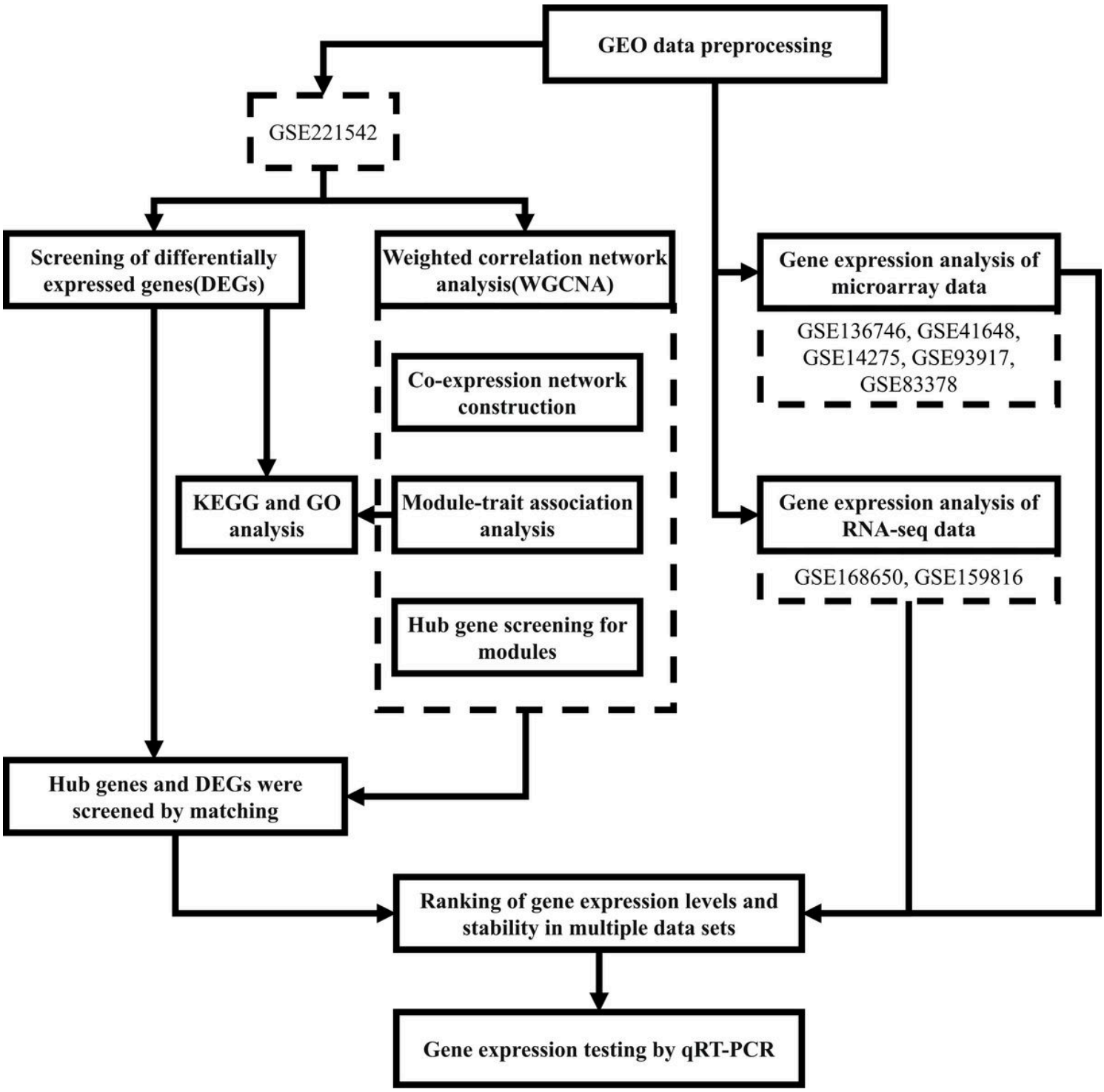


Figure 1

Workflow of the present study.

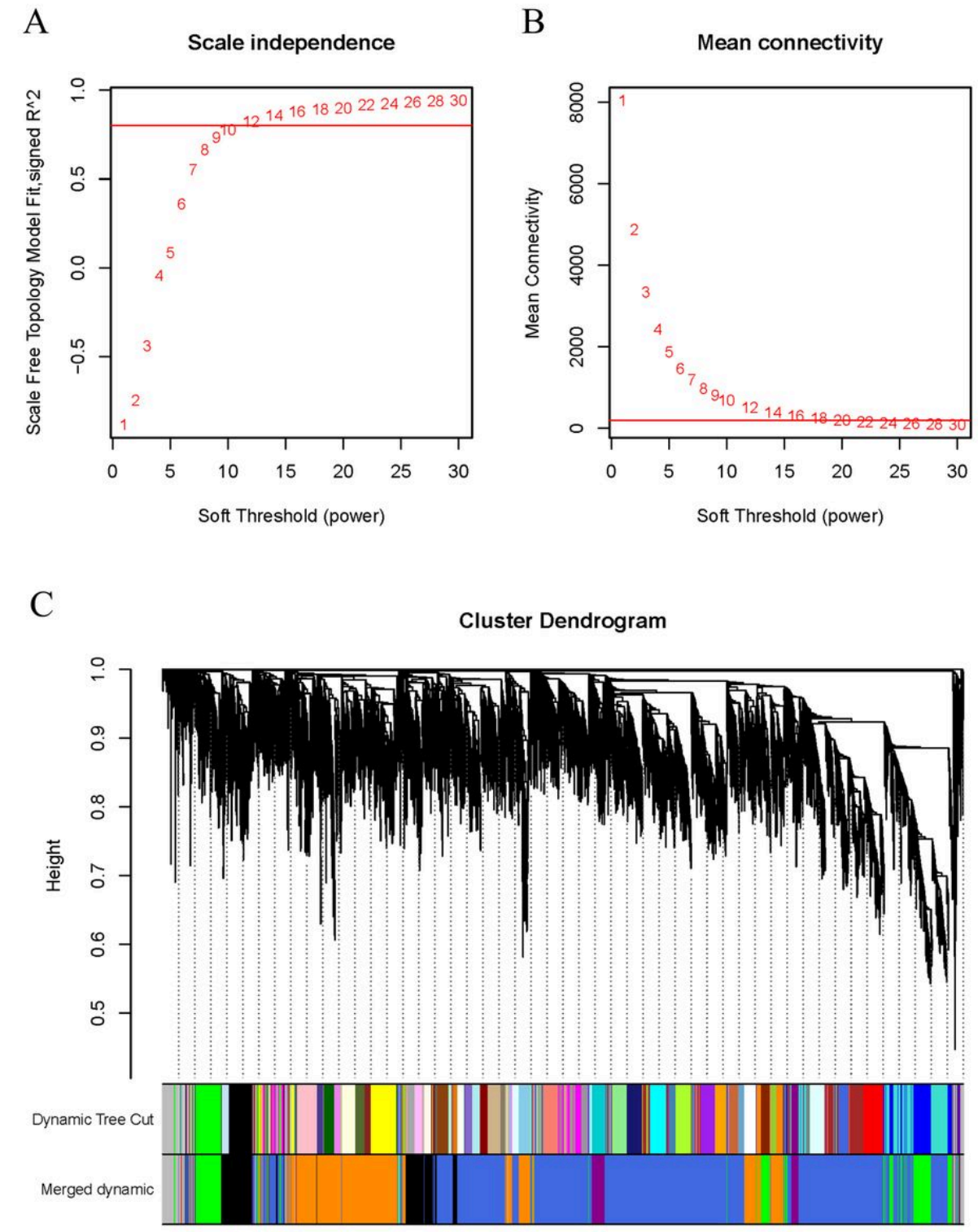
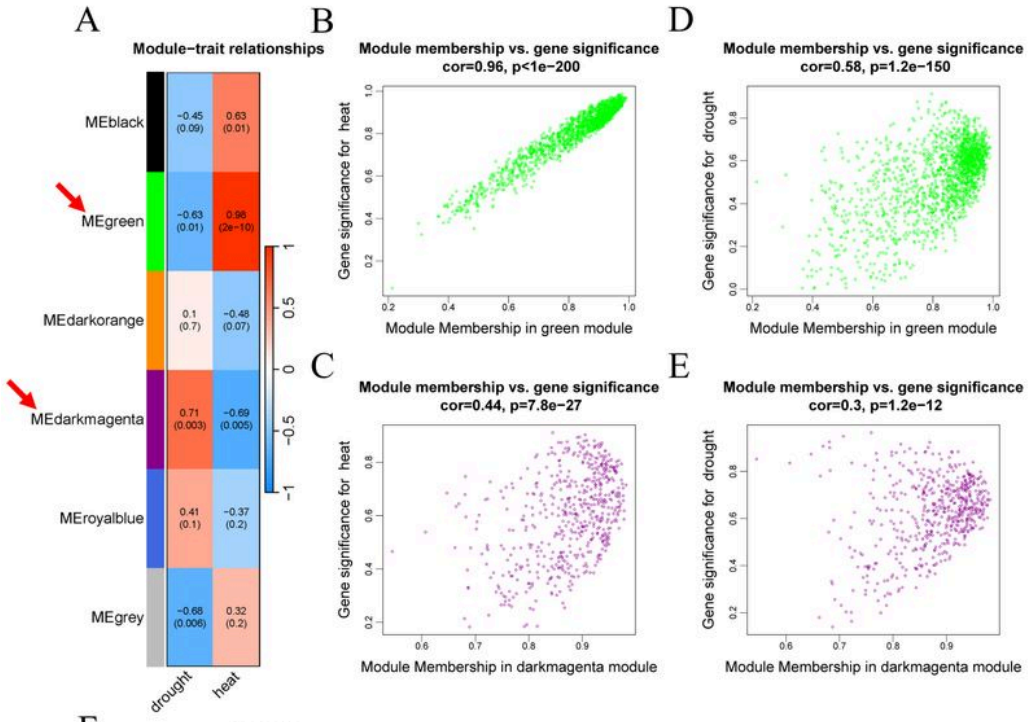
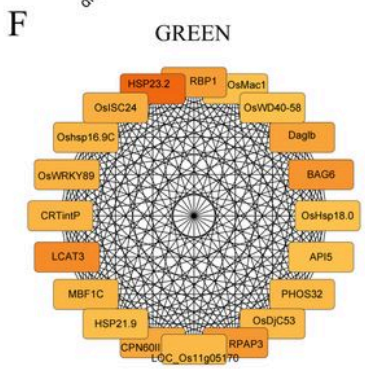


Figure 2

(A, B): Network topology for different soft–thresholding powers. The x-axis represents the weight parameter β. The y-axis in panel **(A)**represents the square of the correlation coefficient between log(k) and log(p(k)) in the corresponding network. The y-axis in panel **(B)**represents the mean of all gene adjacency functions in the corresponding gene module. The approximate scale free topology can be attained at the soft thresholding power of 30 in the genotypes. **(C)**: Gene modules identified by Weighted Correlation Network Analysis (WGCNA). Gene dendrogram obtained by clustering the dissimilarity based on

consensus topological overlap with the corresponding module colors indicated by the color column. Each colored column represents a module, which contains a group of highly connected genes.

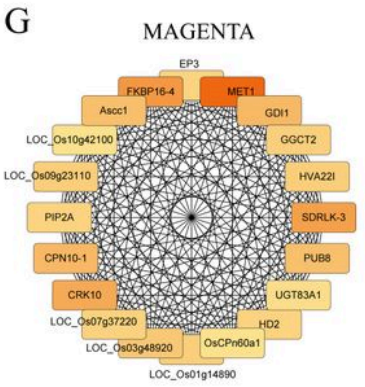




Green

Top 20 in network Turquoise module ranked by MCC method

Rank	ID	Name	Score	Rank	ID	Name	Score
1	LOC_Os04g36750	HSP23.2	2.37E+06	11	LOC_Os06g39240	MBF1C	1.16E+06
2	LOC_Os09g33820	LCAT3	1.82E+06	12	LOC_Os01g04360	Oshsp16.9C	1.15E+06
3	LOC_Os02g15930	BAG6	1.66E+06	13	LOC_Os11g05170	LOC_Os11g05170	1.12E+06
4	LOC_Os05g03910	RPAP3	1.61E+06	14	LOC_Os02g53320	PHOS32	1.12E+06
5	LOC_Os06g03690	RBP1	1.54E+06	15	LOC_Os06g09560	OsDjC53	1.10E+06
6	LOC_Os01g67420	Daglb	1.45E+06	16	LOC_Os11g13980	HSP21.9	1.04E+06
7	LOC_Os10g32550	CPN60II	1.36E+06	17	LOC_Os02g20930	API5	9.92E+05
8	LOC_Os03g02260	OsISC24	1.26E+06	18	LOC_Os06g51100	OsMac1	9.90E+05
9	LOC_Os08g17400	OsWRKY89	1.23E+06	19	LOC_Os02g56880	OsWD40-58	9.83E+05
10	LOC_Os07g31540	CRTintP	1.17E+06	20	LOC_Os01g08860	OsHsp18.0	9.78E+05



Darkmagenta

Top 20 in network Turquoise module ranked by MCC method

Rank	ID	Name	Score	Rank	ID	Name	Score
1	LOC_Os07g07540	MET1	1.59E+02	11	LOC_Os02g02920	HVA22I	8.40E+01
2	LOC_Os07g09040	FKBP16-4	1.23E+02	12	LOC_Os09g23110	LOC_Os09g23110	8.40E+01
3	LOC_Os01g57560	SDRLK-3	1.17E+02	13	LOC_Os04g37580	GGCT2	8.30E+01
4	LOC_Os07g43570	CRK10	1.10E+02	14	LOC_Os07g37220	LOC_Os07g37220	8.10E+01
5	LOC_Os10g41710	CPN10-1	1.00E+02	15	LOC_Os02g15950	EP3	8.00E+01
6	LOC_Os06g21340	GDI1	9.90E+01	16	LOC_Os07g26690	PIP2A	8.00E+01
7	LOC_Os01g13100	Ascc1	9.40E+01	17	LOC_Os07g49460	HD2	8.00E+01
8	LOC_Os02g28720	PUB8	9.40E+01	18	LOC_Os03g55030	UGT83A1	7.30E+01
9	LOC_Os03g48920	LOC_Os03g48920	8.80E+01	19	LOC_Os10g42100	LOC_Os10g42100	7.00E+01
10	LOC_Os01g14890	LOC_Os01g14890	8.40E+01	20	LOC_Os12g17910	OsCPn60a1	7.00E+01

Figure 3

(A): The correlation coefficient and correlation significance between the module and different stress conditions. Each row in the table corresponds to a consensus module, and each column corresponds to drought or heat stress. The red arrows indicate the two modules with the highest correlation. **(B-E)**: Gene significance and module membership fit scatter plot. Each gene is represented by a hollow dot. In **(B, D)** graphs, the x-axis represents the correlation between the module

eigengene and the gene expression profile in the green module. The **(C, E)** graphs correspond to the dark magenta module. In **(D, E)** graphs, the y-axis represents the correlation between the gene and different degrees of drought stress. The **(B, C)** graphs correspond to heat stress. **(F-G)**: Top 20 hub genes obtained from the interaction network analysis. Identification of hub genes using the Maximal Clique Centrality (MCC) method. Genes with the top 20 MCC values were colored orange to yellow. Orange refers to a relatively large MCC value, whereas yellow refers to relatively smaller MCC values. The F network corresponds to the green module, and the G network corresponds to the dark magenta module.

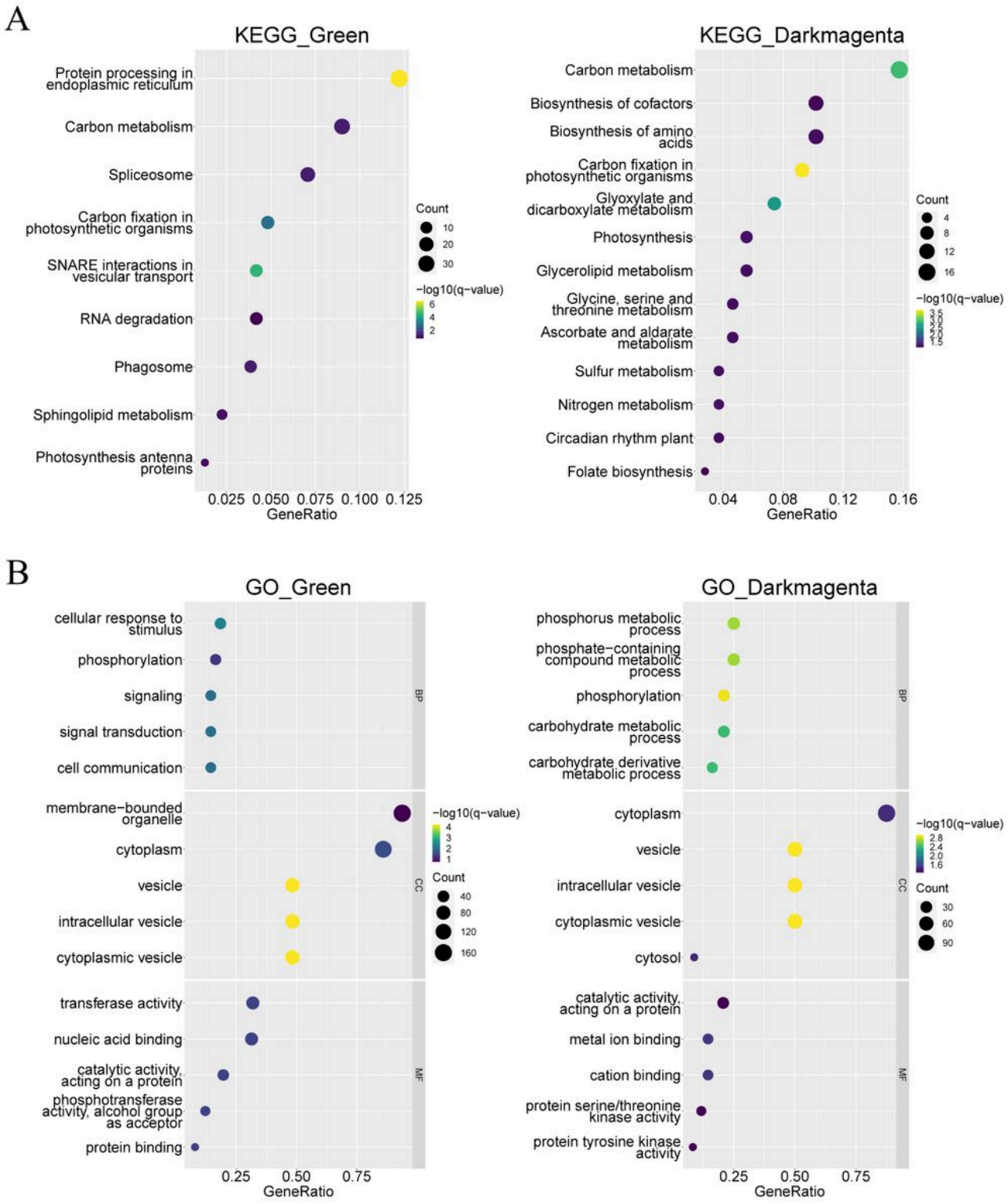


Figure 4

The y-axis shows the biological function of a gene in a cell. The x-axis represents the ratio of the number of genes enriched from the target pathway to the total genes contained in the gene list. The size of bubble area indicates the number of enriched genes. Bubble color indicates enrichment significance. The green module is shown on the left, and the dark magenta module is shown on the right. **(A)**: Bubble map of the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. **(B)**: Bubble map of the gene Ontology (GO) enrichment analysis.

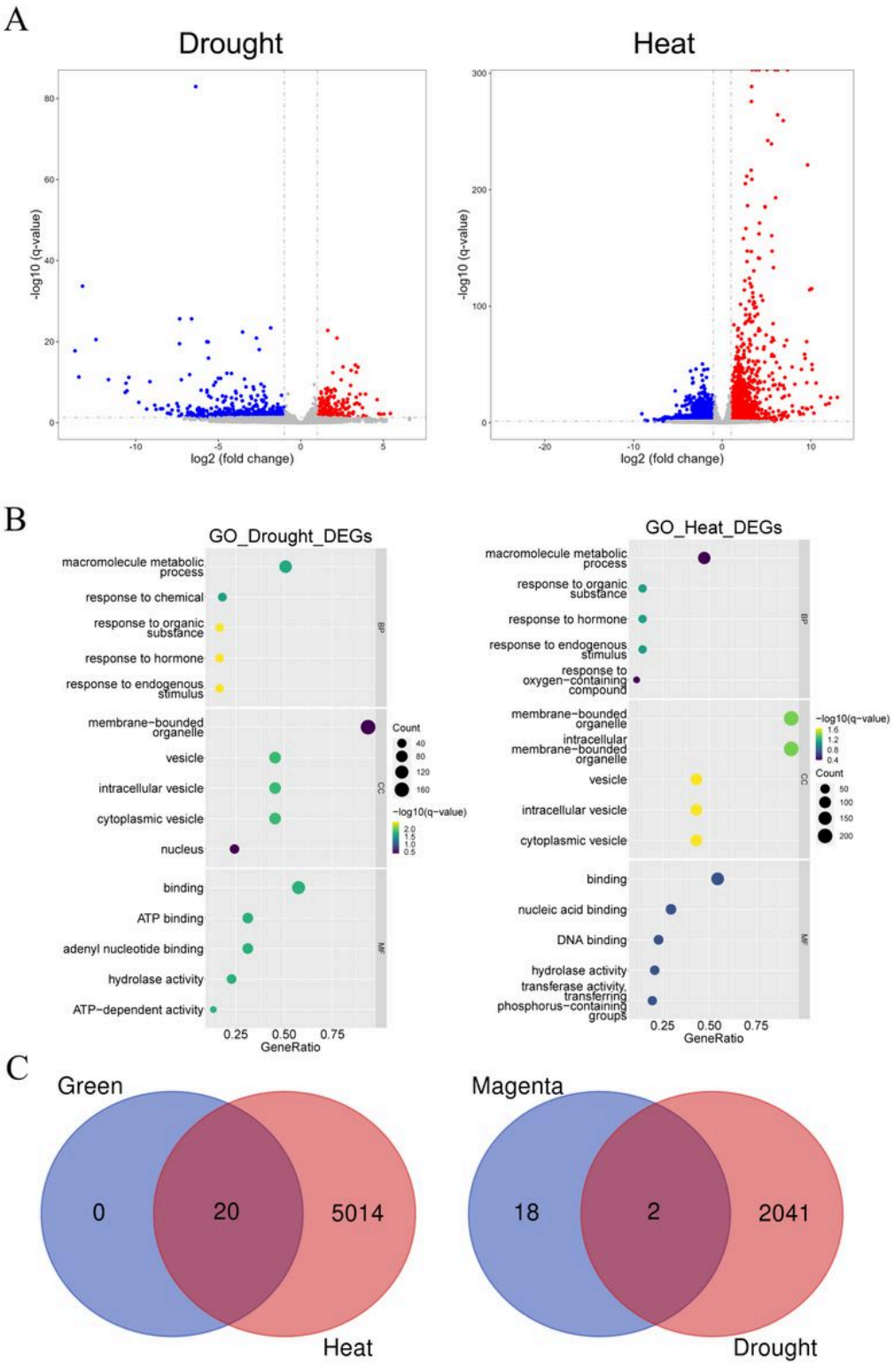


Figure 5

The drought group is shown on the left, and the heat group is shown on the right. **(A)**: Volcano plot of differentially expressed genes (DEGs). Log2 fold change = 1 and p-value = 0.05 were used as truncation criteria. The x-axis represents log2 fold change, and the y-axis represents −log10 q-value. Each dot represents a gene. Red dots represent significantly upregulated genes. Blue dots represent significantly downregulated genes. Gray dots represent genes with no significant differences. **(B)**: Gene Ontology (GO) enrichment analysis bar chart. The y-axis shows the biological function of a gene in a cell. The x-axis represents the ratio of the number of genes enriched from the target pathway to the total genes contained in the gene list. The size of bubble area indicates the number of enriched genes. Bubble color indicates enrichment significance. The left panel shows the GO analysis of drought stress and control DEGs, and the right panel shows the GO analysis of heat stress and control DEGs. **(C)**: Venn diagram of intersection of top 20 hub genes and DEGs. Green module and heat stress DEGs are shown on the left. Dark magenta module and drought stress DEGs are shown on the right.

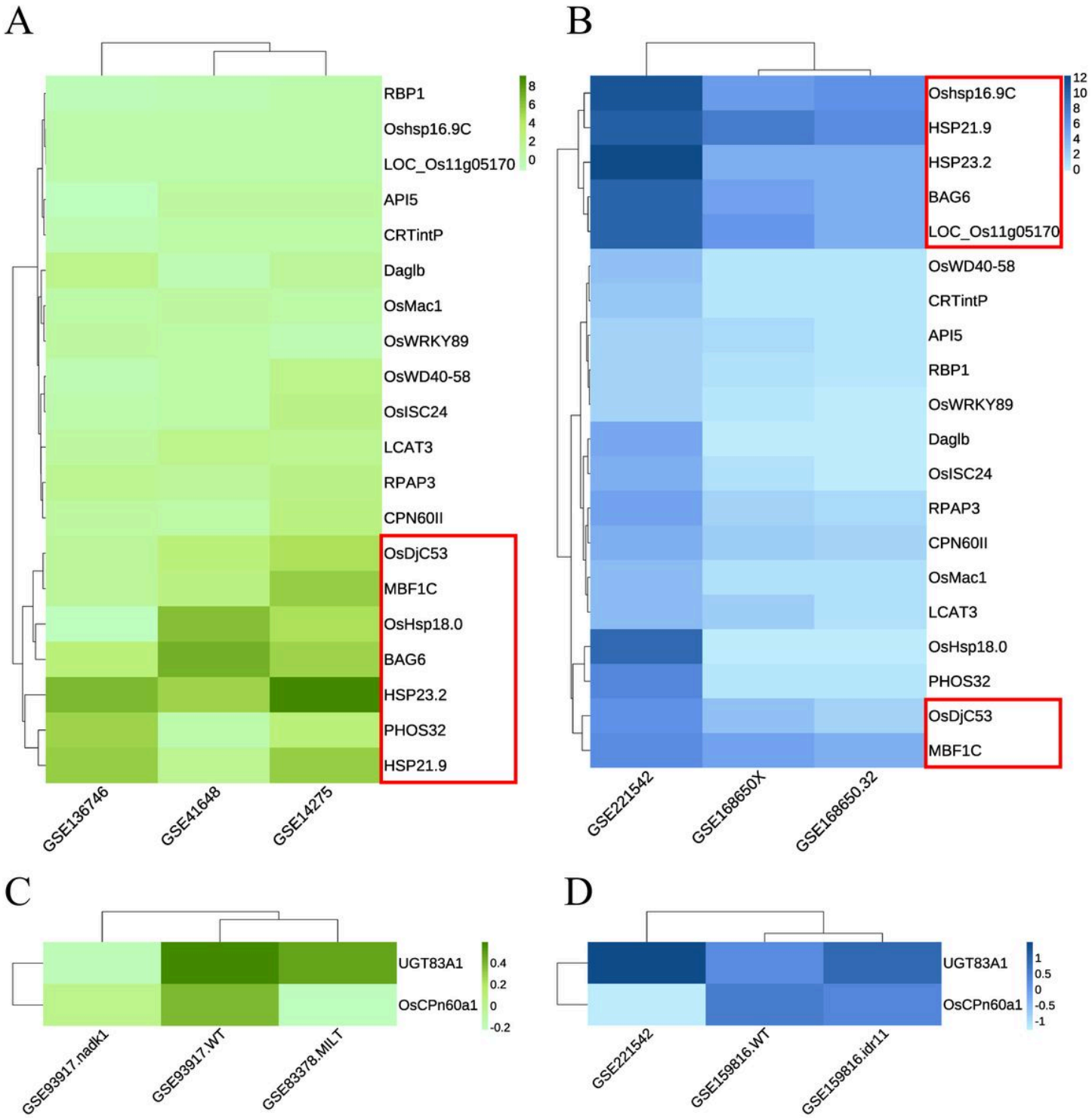


Figure 6

Heat maps indicate the expression of candidate key genes in response to heat or drought stress in array sequencing data or RNA-seq data. The abscissa represents the data set, and the ordinate represents each candidate key gene. The level of gene expression is indicated by the shade of color. Darker colors indicate a higher expression level. **(A, C)** are array sequencing data. **(B, D)** are RNA-seq data. **(A, B)** are heat stress response group. **(C, D)** are drought stress response group. The red boxes indicate genes with high expression levels across multiple datasets.

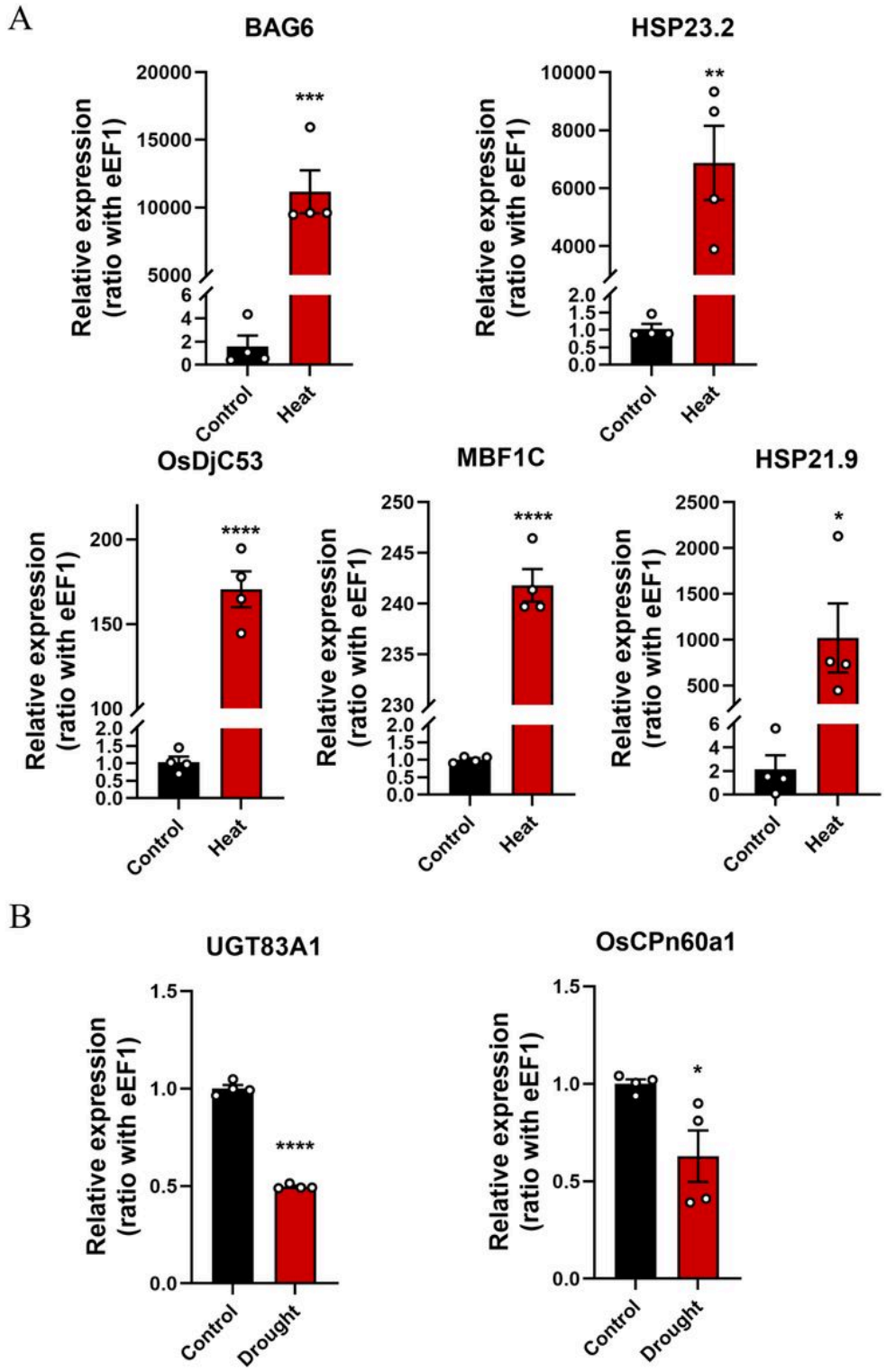


Figure 7

The expression levels of key genes in rice under drought and heat stress conditions were detected by quantitative real-time PCR (qRT-PCR) and compared with those in the control group. *p< 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. **(A)**: Heat stress-related genes, including *BAG6, HSP23.2, OsDjC53, MBF1C,* and *HSP21.9*. **(B)**: Drought stress-related genes, including *UGT83A1* and *OsCPn60a1*.

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