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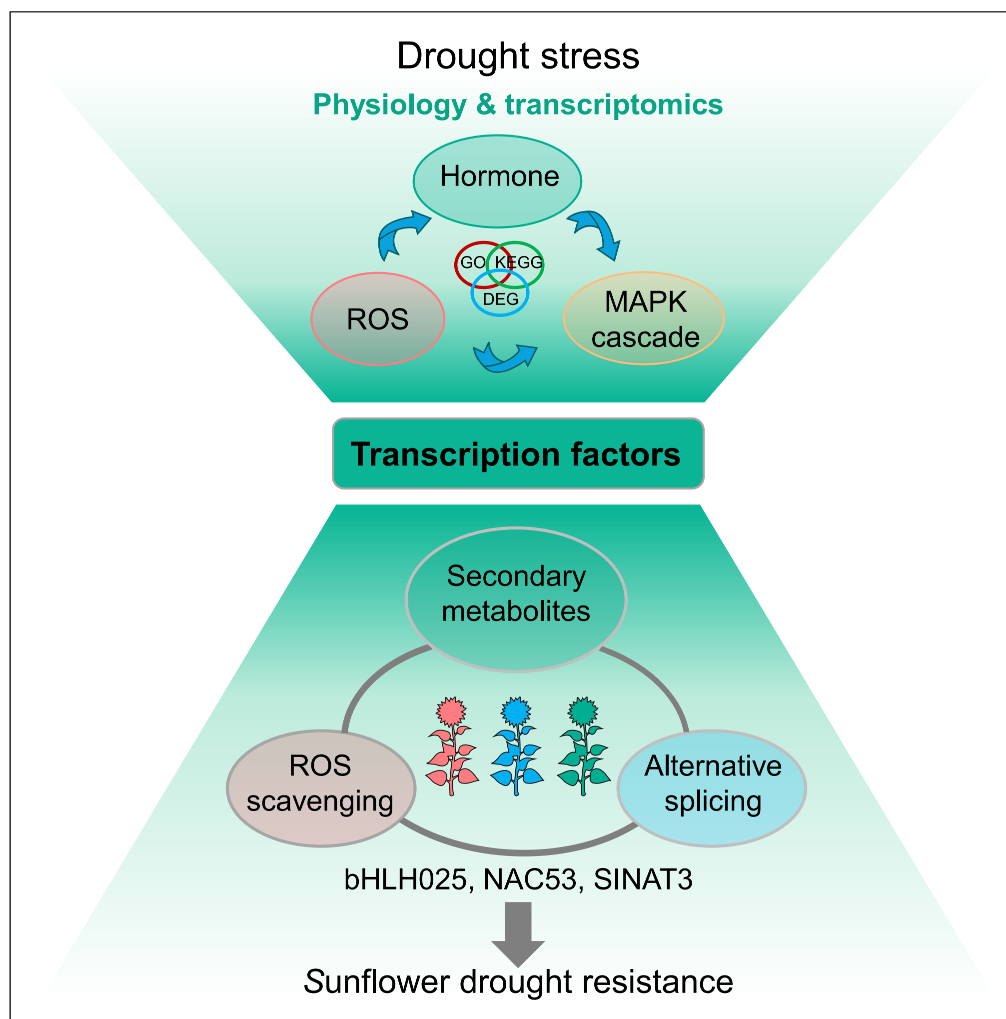

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Highlights

Rehydration can alleviate ROS-damaged sunflowers accumulated by drought stress

DEGs in hormone, MAPK, and secondary metabolite pathways affect drought resistance

bHLH025, *NAC53*, and *SINAT3* may play key roles in sunflower drought resistance

ROS scavenging, MAPK signaling, SMs, and AS aid in sunflower drought resistance

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Physiology and transcriptomics highlight the underlying mechanism of sunflower responses to drought stress and rehydration

Jie Shen,[1] Xi Wang,[1] Huifang Song,[1] Mingyang Wang,[2] Tianzeng Niu,[1] Haiying Lei,[1] Cheng Qin,[1] and Ake Liu[1,3,*]

SUMMARY

Drought can adversely influence the crop growth and production. Accordingly, sunflowers have strong adaptability to drought; hence, we conducted analyses for sunflower seedlings with drought stress and rehydration drought acclimation through physiological measurements and transcriptomics. It showed that drought can cause the accumulation of ROS and enhance the activity of antioxidant enzymes and the content of osmolytes. After rehydration, the contents of ROS and MDA were significantly reduced concomitant with increased antioxidant activity and osmotic adjustment. Totally, 2,589 DEGs were identified among treatments. Functional enrichment analysis showed that DEGs were mainly involved in plant hormone signal transduction, MAPK signaling, and biosynthesis of secondary metabolites. Comparison between differentially spliced genes and DEGs indicated that *bHLH025*, *NAC53*, and *SINAT3* may be pivotal genes involved in sunflower drought resistance. Our results not only highlight the underlying mechanism of drought stress and rehydration in sunflower but also provide a theoretical basis for crop genetic breeding.

INTRODUCTION

Drought is one of the most important abiotic stresses and significantly affects the growth and production of crops, especially in arid and semi-arid regions.[1,2] During the process of long-term adaptation to drought environments, plants have gained drought resistance mechanisms such as drought escape, dehydration avoidance, drought tolerance, and drought recovery.[3] Drought adaptability is the overall performance of crops during drought and rehydration processes, including drought resistance and rapid recovery after rehydration.[4] Due to its severe effects on crops, knowledge of drought response and adaptation is a critical issue that urgently needs to be resolved.

Revealing the physiological and molecular mechanisms of plant responses to drought stress remains a major challenge. Plants rely on a variety of physiological mechanisms to respond to drought stress, including photosynthesis,[5] antioxidant systems,[6] plant hormones,[7] and secondary metabolism,[8] in attempts to alleviate adverse effects on plant growth, development, and yield. Reactive oxygen species (ROS) promote rapid systemic signaling in plants under drought stress.[9] Plants can induce ROS production, which leads to oxidative stress under drought conditions.[10] Plants actively maintain physiological water balance mainly through osmotic regulation[11] and activation of stress response pathways, including plant hormone signaling and antioxidant defense systems for scavenging ROS.[12] Elevated ROS levels not only affect the proteome, metabolic flux, and transcription factors (TFs) but also regulate the level and function of plant hormones.[13] ROS signals coupled with the action of the mitogen-activated protein kinase (MAPK) cascade form a flexible feedback loop that amplifies the effect of hormonal signals. As a multi-functional signaling molecule, MAPK interacts with ROS and hormones to shape acclimation responses to abiotic stress.[9]

In general, multi-omics is considered a useful approach to elucidate the underlying molecular mechanisms of plant responses to drought stress,[14] aiding in identifying candidate genes involved in different abiotic stress tolerances.[15] For example, combined transcriptomics and metabolomics analysis showed that TaSnRK2.10 could enhance the drought resistance of wheat by regulating stomatal aperture and the expression of drought-responsive genes and increase phosphoenolpyruvate supply and promote the degradation of TaERD15 to enhance the drought tolerance of wheat.[16] Several genes related to drought tolerance have been identified using transcriptome technology, such as *NCED*3, *NCED*5, *ABI1*, and *PYL4*, which are involved in abscisic acid (ABA) synthesis and signaling and play essential roles in alleviating drought stress in sunflower (*Helianthus annuus* L.).[17] As a posttranscriptional mechanism, alternative splicing (AS) can further regulate the response to drought in plants.[18,19] For example, a truncated isoform of the zinc-induced facilitator-like 1 transporter can mediate the drought tolerance of *Arabidopsis*.[20] AS occurring in the key circadian rhythm gene CCA1 has been shown to mediate the maize response to drought stress.[21] In a study of sunflower, it was found that different genotypes responded similarly to drought stress, and AS events resulted in their expression differences.[22]

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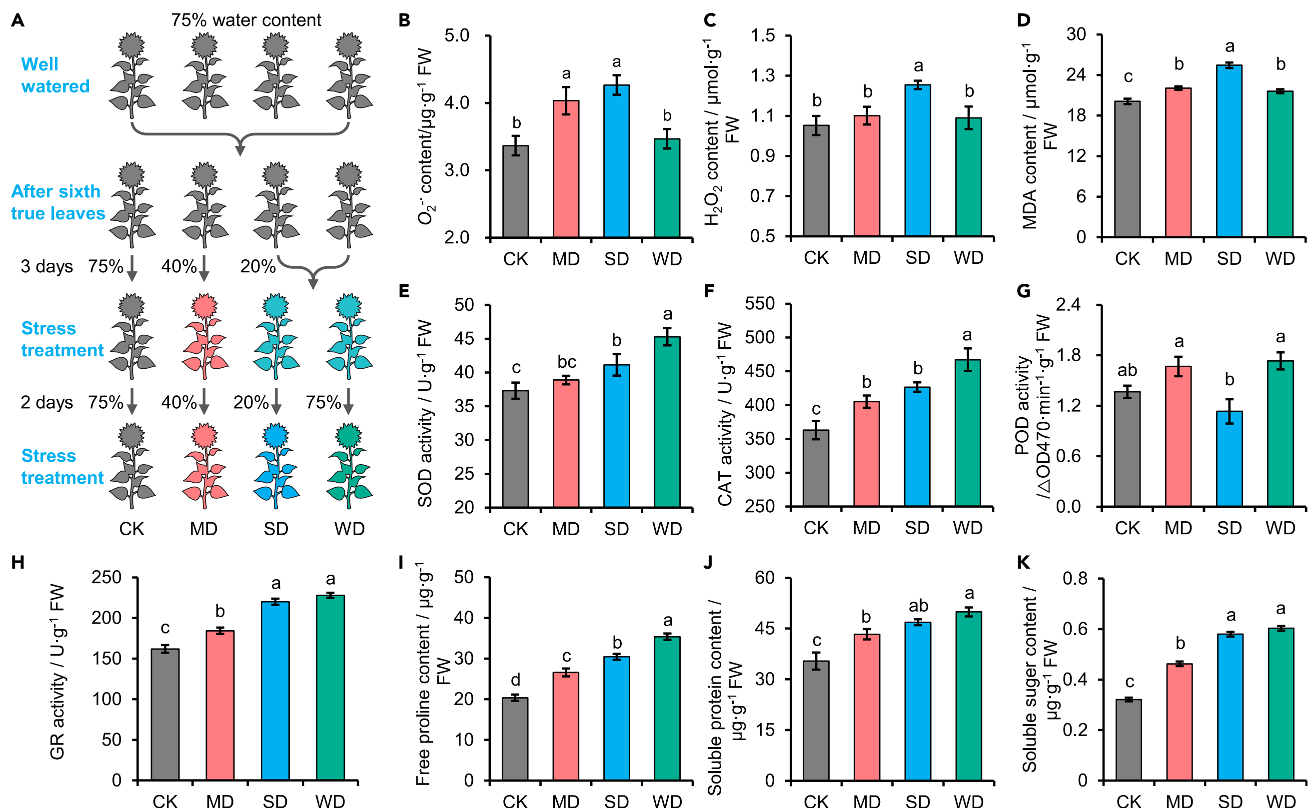

Figure 1. Experimental scheme and analysis of the physiological index

(A) A schematic plot representing the experimental design.

(B and C) Accumulation of reactive oxygen species (ROS), including $O_2^{\cdot-}$ and H_2O_2.

(D) MDA content.

(E–H) Activities of antioxidant enzymes, including SOD, CAT, POD, and GR.

(I–K) Contents of osmotic adjustment substances, including free proline, soluble protein, and soluble sugar. CK, normal water supply; MD, mild drought stress; SD, severe drought stress; WD, rehydrated after severe drought stress. Different lowercase letters indicate significant differences at the p value <0.05 level.

Sunflower is an annual plant belonging to the Asteraceae (Compositae) family, which is the fourth largest source of vegetable oil worldwide and has important economic and ornamental value.[23,24] Sunflower has strong adaptability to various abiotic stresses and can grow under drought, heavy metals, salinity, and other environmental stresses.[23,25–27] It is widely cultivated in Inner Mongolia, Xinjiang, Gansu, and other arid or semiarid regions in China. However, the increasing shortage of water resources has seriously affected the yield and quality of sunflower seeds. Therefore, understanding the underlying molecular mechanism of drought resistance in sunflower would be helpful for improving its drought resistance, with important practical significance for its production.

To date, studies on the drought resistance of sunflower have mainly focused on drought tolerance identification, physiological trait analysis, molecular markers, quantitative trait locus mapping, and expression profiling.[28,29] Seventeen genes involved in the sunflower response to abiotic stimuli have been found, among which nine genes might be associated with responses to water-related stimuli.[23] Accordingly, many sunflower TF families, such as bHLH (basic-helix-loop-helix), WRKY, and bZIP (basic leucine zipper), have experienced multiple gene expansions, which may also play important roles in the strong adaptability of sunflower to various stresses.[30–32] Nevertheless, there are few reports on the drought adaptation and rehydration mechanism of sunflower, and most studies only focus on drought stress. In this study, the potential mechanism of sunflower leaves was analyzed under drought stress and rehydration conditions by integrating physiological and transcriptomic analyses. Our results provide a theoretical basis for elucidating the molecular mechanism underlying drought adaptation in sunflower.

RESULTS

Changes in ROS, antioxidant enzyme activity, and osmotic adjustment substances

Sunflower seedlings under normal water supply (CK), mild drought stress (MD), severe drought stress (SD), and rehydrated after severe drought stress (WD) treatments were obtained as described in the method details (Figure 1A). Based on the observation of phenotype, sunflower seedlings displayed no obvious phenotypic change, which further indicated that sunflower has strong drought resistance. To further study the effects of drought and rehydration on sunflower seedlings, we measured physiological indices, including ROS accumulation,

antioxidant enzyme activity, and osmotic adjustment substances, of sunflower seedlings (Figure 1). ROS accumulated significantly in sunflower seedling leaves with the aggravation of drought stress (Figures 1B–1D). In addition, the contents of $O_2^{\cdot-}$, H_2O_2, and malondialdehyde (MDA) peaked in the SD treatment and were significantly increased by 26.73%, 19.23%, and 26.59% compared to CK, respectively. However, WD significantly reduced the ROS content compared to SD ($O_2^{\cdot-}$, H_2O_2, and MDA contents were significantly reduced by 18.75%, 13.12%, and 15.09%, respectively), which returned to the CK or MD level. With the aggravation of drought stress, the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) increased significantly, but the change in peroxidase (POD) was not significant (Figures 1E–1H). Rehydration treatment can significantly increase the activities of these antioxidant enzymes in sunflower seedling leaves. For instance, compared to SD, SOD and CAT activities increased significantly by 10.09% and 9.50% in WD, respectively.

Osmotic adjustment substances significantly accumulated in sunflower seedling leaves under drought stress (Figures 1I–1K). Indeed, the contents of free proline, soluble protein, and soluble sugar increased significantly by 30.56%, 22.36%, and 43.99% in the MD treatment compared to CK, respectively; with the aggravation of drought stress, their contents continued to accumulate, increasing significantly by 49.53%, 32.44%, and 80.62%, respectively, in the SD treatment compared to CK. Furthermore, rehydration treatment increased the content of osmotic adjustment substances again. For instance, the free proline content accumulated significantly by 16.21% under the WD treatment compared to the SD treatment.

Expression profiles of sunflower leaves in response to drought stress

To further investigate the key genes of sunflower in response to drought and rehydration, we performed transcriptomic analysis using sunflower leaves from four treatments, as described in the method details (CK, MD, SD, and WD). As shown in Table S1, 12 cDNA libraries (three biological replicates per treatment) were constructed. Approximately 515.15 million clean data points were obtained after quality control and filtering. The Q30 value of each library was approximately 94%. The mapping ratio ranged from 91.30% to 92.00%, of which 92.35%–94.14% mapped uniquely, meeting the needs for subsequent transcriptome analysis. Both the number of expressed genes and their expression values were characterized in the samples. Across the 12 tested samples, ~44.24% of genes were barely expressed, ~18.16% of genes were expressed at low levels ($0 < \text{FPKM} \leq 1$), ~33.77% of genes were expressed at moderate levels ($1 < \text{FPKM} \leq 50$), and ~ 3.83% of genes were expressed at high levels (FPKM >50) (Figure S1).

SD affects gene expression more greatly than MD

First, 2,589 differentially expressed genes (DEGs) were identified between any two treatments (MD/CK, SD/CK, WD/CK, SD/MD, WD/MD, and WD/SD) based on DESeq with thresholds $|\log_2 (\text{fold change})| > 1$ and p value <0.05. The number of upregulated DEGs was generally lower than that of downregulated DEGs across all comparisons. For instance, there were 203 DEGs (66 up- and 137 downregulated), 1,297 DEGs (377 up- and 920 downregulated), and 1,053 DEGs (244 up- and 809 downregulated) identified in MD, SD, and WD compared to CK, respectively (Figure 2A, MD/CK, SD/CK, and WD/CK). These genes may be regarded as drought-related DEGs. Moreover, 469 DEGs (230 up- and 239 downregulated) were identified in WD compared to SD (WD/SD). Overall, it seems that the expression profiles of MD and CK were more similar, as were those of SD and WD, which was also confirmed by hierarchical clustering of the expression profiles of all the DEGs (Figure 2B). These results indicate that SD treatment may affect gene expression more than MD treatment.

Then, we compared upregulated DEGs among three comparisons (MD/CK, SD/CK, and WD/CK) and found that only 4 upregulated DEGs were shared in all three comparisons (Figure 2C). For instance, expression of the sunflower *PMEI7* gene was continuously upregulated among CK, MD, SD, and WD. The expression of the *CSLA9* gene was upregulated with the aggravation of drought and downregulated after rehydration, but its expression level was still higher than that in CK. For comparison of downregulated DEGs among these three comparisons, there were 55 downregulated DEGs shared in all (Figure 2C). Among them, protein kinases (PKs), such as *SRK2E*, *FLS2*, and *MAPKKK5*, tended to show downregulated expression with the aggravation of drought and upregulated expression after rehydration. Similar trends were also observed for other genes, such as TFs (*ERF280*, *ERF252*, *ERF220* (also named *DREB1D*), *ERF040*, *WRKY53*, *WRKY27*, and *ZAT8*), calcium-binding proteins (*CML38*, *CML19*, and *CAMBP25*), stress-associated proteins (*SAP5* and *SAP12*), *RDUF1*, and *LOX31*. Meanwhile, we further conducted a comparison of WD/SD with the previous three comparisons (Figure S2). It was found that 4 downregulated DEGs in WD/SD were shared with those upregulated DEGs in SD/CK and WD/CK, and 9 upregulated DEGs were shared with their downregulated DEGs in SD/CK and WD/CK. Among them, there were 1 TF (*WRKY41*), 9 genes, including *YUC10*, *U85C2*, *ALP1*, *PUB21*, *FLS2*, *GRXC9*, *SLD1*, *PUB23*, and *PMI10*, and 3 unannotated genes.

TFs play an important role in plants exposed to various abiotic stresses. In total, 28 TF families and 330 members were identified from all 1,781 DEGs among the three comparisons (MD/CK, SD/CK, and WD/CK). As shown in Figure 2D, the top 10 TF families exhibiting different expression levels between any two different treatments were ERF, WRKY, MYB, NAC, C2H2, bHLH, GRAS, C3H, G2-like, and HSF. The differentially expressed TFs presented the largest number in SD/CK among the three comparisons. There were seven shared TF families, namely, ERF, WRKY, MYB, NAC, C2H2, GRAS, and G2-like families, among all three comparisons (Figure 2E), which may further indicate that these TFs are involved in sunflower resistance to drought stress.

Furthermore, we conducted an analysis of the sunflower ERF family and found 288 members, much greater than those in Arabidopsis (122) and rice (138) (Figure 3A).[33] Many of the members of the ERF gene family were derived from segmental (143 genes) or tandem duplication (59 genes) events (Figure 3B; Table S2). We compared the *ERF* gene expression profiles of these duplicate pairs under different treatments, and the results showed that the expression profiles of most segmental duplicate gene pairs (101/122) were significantly divergent (correlation test, $p > 0.05$), but a larger proportion of tandem duplicate genes (15/63) were still significantly correlated after duplication events (correlation test, $p < 0.05$, Table S3). These results may indicate neofunctionalization after *ERF* gene duplications. Then, we compared differentially expressed

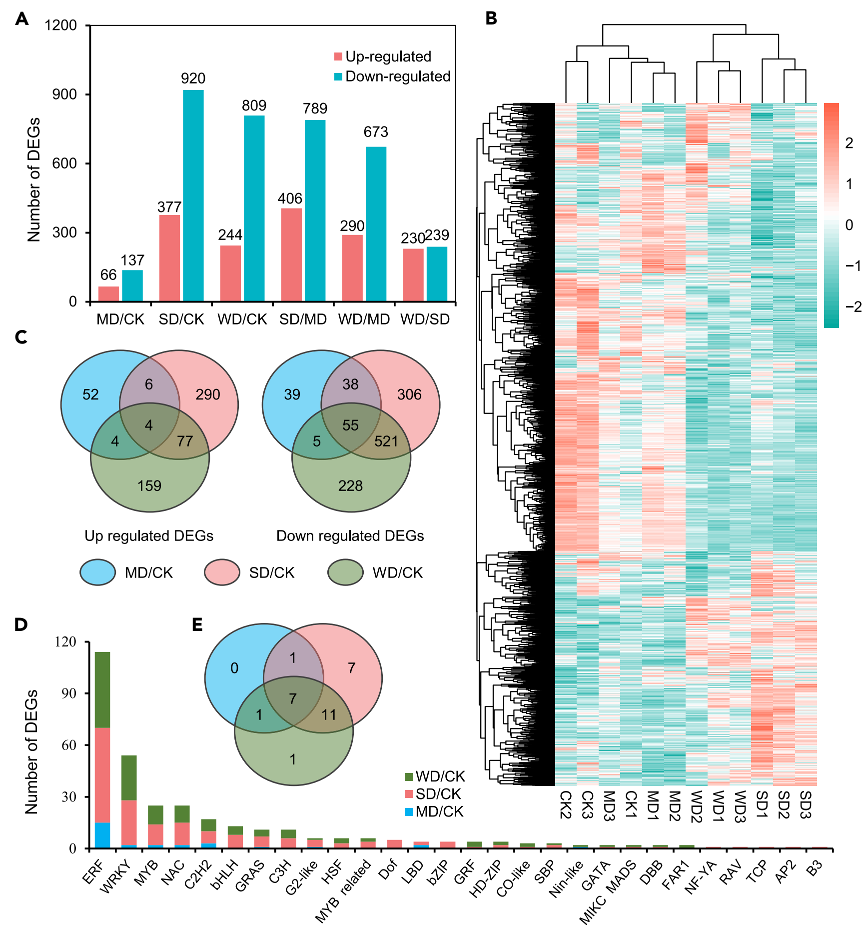

Figure 2. Comparative analysis of DEGs among three comparisons

(A) DEG numbers of six comparisons, including up- and downregulated DEGs.

(B) Expression profiles for all 2,589 DEGs.

(C) Venn comparisons of up- and downregulated DEGs among three comparisons (MD/CK, SD/CK, and WD/CK).

(D) Differentially expressed TFs identified among the three comparisons.

(E) Venn comparison of TF family numbers among the three comparisons.

ERF genes among the three comparisons (15 in MD/CK, 55 in SD/CK, and 44 in WD/CK); there were 62 ERF members in total and 4 members (HaERF252, HaERF220, HaERF040, and HaERF280) shared among them (Figure 3C). We also examined the expression profiles of these 62 ERF members and classified them into five clusters based on expression (Figure 3D). Members of cluster I had high expression in CK and low expression in the other treatments. Cluster V exhibited low expression in CK and high expression in the other treatments, especially SD. The remaining clusters displayed high expression in CK and MD but low expression in SD and WD.

Finally, the reliability of the RNA-sequencing (RNA-seq) results was verified by RT-qPCR analysis among the four treatments in sunflower (Figure 3E). Similar expression trends were observed for the 10 selected genes between RNA-seq data and RT-qPCR, including *LOX31*, *E1314*, *RDUF1*, *ALP1*, *NIP1*, *INO1*, *SAP8*, *FLS2*, *PLA14*, and *GRXC9*, showing a significantly positive correlation ($r = 0.66$, p value $= 1.20 \times 10^{-8}$) for all the 10 genes among the different treatments.

Functional annotation and classification of DEGs

To explore functional differences of DEGs under drought stress and rehydration, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed by hypergeometric tests to identify potential biological functions of these DEGs. First, there were 158, 274, and 296 significantly enriched biological processes (BP) in the three comparisons (MD/CK, SD/CK, and WD/CK), among which 34 BPs were shared, including metabolic process (S-glycoside, glycosinolate, glucosinolate, and isoprenoid), stimulus response to reactions (response to water deprivation, wounding, jasmonic acid (JA), and brassinosteroid (BR)), regulation of transporter activity, and seedling development (Figure 4B; Table S4). Moreover, there were 22 significantly enriched BPs highly related to drought stress in both SD/CK and WD/CK, and 16 of them were shared in these two comparisons (Figure 4B). Additionally, two BPs, namely, regulation of chlorophyll metabolic

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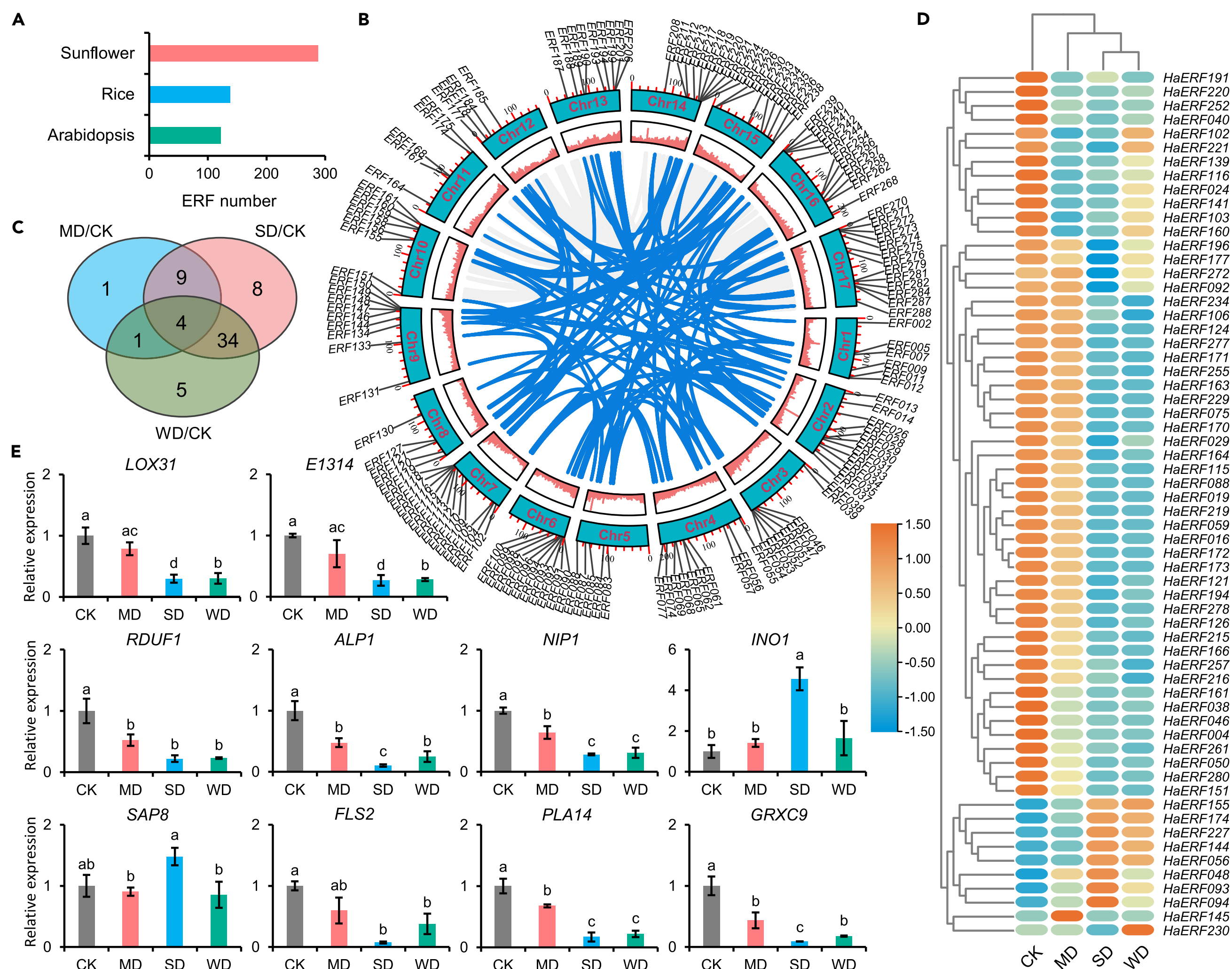

Figure 3. Characteristic analysis of ERF family members and RT-qPCR for RNA-seq validation

(A) ERF numbers in Arabidopsis, rice, and sunflower.

(B) Schematic representation of collinear relationships of sunflower *ERF* genes. Blue and gray lines indicate sunflower ERFs and all genes resulting from segmental duplications. Red lines in chromosome blocks present gene density on each chromosome.

(C) Comparison of differentially expressed ERFs among MD/CK, SD/CK, and WD/CK.

(D) Expression profile of differentially expressed ERFs among the four treatments.

(E) Quantitative analysis of gene expression levels for ten selected DEGs using RT-qPCR. Different lowercase letters indicate significant differences at the *p* value <0.05 level.

process and auxin metabolic process, were significantly enriched only in MD/CK, indicating their potential roles in sunflower responses to moderate drought stress. There were 5 and 4 unique significantly enriched BPs in SD/CK and WD/CK, respectively. The former includes proline metabolic process, water homeostasis, cellular response to water deprivation, response to cytokinin, and calcium ion homeostasis; the latter includes regulation of hormone metabolic process, regulation of lipid biosynthetic process, regulation of hormone biosynthetic process, and BR-mediated signaling pathway. Finally, there were only 106 significantly enriched BPs in the WD/SD comparisons. In particular, the significantly enriched BPs, water transport, response to hydrogen peroxide, cytokinin metabolic process, response to ROS, regulation of chlorophyll metabolic process, auxin metabolic process, calcium ion homeostasis, and response to salicylic acid, were shared with the previous three comparison groups, which may play important roles in the rehydration process (Table S4).

Based on KEGG enrichment analysis, there were 5, 11, and 12 pathways that were significantly enriched in MD/CK, SD/CK, and WD/CK (Figure 4A; Table S5), some of which have been reported to be drought-related pathways. For instance, metabolic pathways and biosynthesis of secondary metabolites (SMs) were shared enriched pathways among the three comparisons. In MD/CK, the other 3 enriched pathways were diterpenoid biosynthesis, glycerophospholipid metabolism, and RNA polymerase (Figure 4C). Among them, RNA polymerase was associated with MD/CK, whereas glycerophospholipid metabolism was shared by MD/CK and WD/CK. In SD/CK and WD/CK, there were 6 other

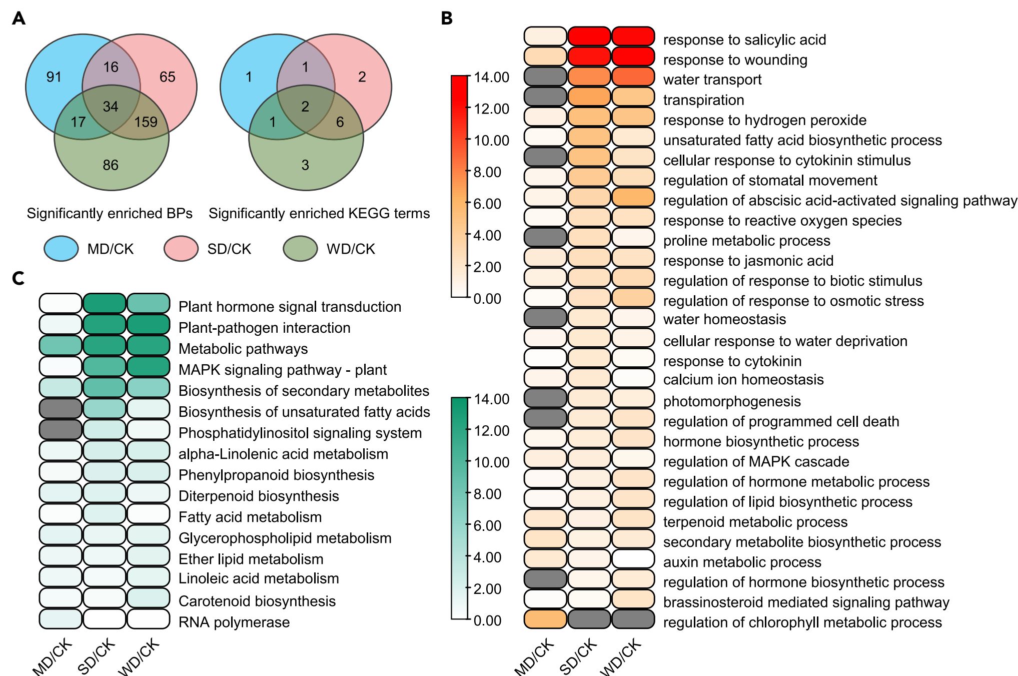

Figure 4. GO term and KEGG pathway enrichment analyses of DEGs identified in three comparisons

(A) Venn diagrams for significantly enriched BPs (biological processes) and KEGG pathways for DEGs among different comparisons.

(B and C) show BP and KEGG pathway enrichment analyses of DEGs in MD/CK, SD/CK, and WD/CK. The color scale in the middle represents the -lg (*p* value).

common pathways that may be involved in the response to severe drought and rehydration, including plant hormone signal transduction, plant-pathogen interaction, MAPK signaling pathway, biosynthesis of unsaturated fatty acids, alpha-linolenic acid (ALA) metabolism, and phenylpropanoid biosynthesis. The phosphatidylinositol signaling system and fatty acid metabolism in SD/CK and ether lipid metabolism, linoleic acid metabolism, and carotenoid biosynthesis in WD/CK were also enriched (Figure 4C). Additionally, there were 8 significantly enriched pathways WD/SD, among which 4 pathways were unique, namely, BR biosynthesis, ascorbate and aldarate metabolism, glutathione metabolism, and lysine biosynthesis (Table S5).

Clustering analysis of functionally related DEGs

The 1,781 DEGs from the three comparison groups (MD/CK, SD/CK, and WD/CK) were subdivided into 8 clusters through K-means clustering analysis based on their expression patterns (Figure 5A). Among these clusters, cluster 3 contained 103 genes displaying a negative response; cluster 4 contained 342 genes that displayed a positive response to soil water deficit. In cluster 3, significantly enriched BPs included localization (glucose import, glucose transmembrane transport, hexose transmembrane transport, monosaccharide transmembrane transport, carbohydrate transmembrane transport, and carbohydrate transport) and response to stimulus (cytokinin-activated signaling pathway, cellular response to cytokinin stimulus, and response to cytokinin) (Table S6). In cluster 4, DEGs significantly enriched in BPs mainly included localization (organic acid transport, carboxylic acid transport, organic anion transport, water transport, and fluid transport) (Table S6). Additionally, there were five and six significantly enriched KEGG pathways identified for clusters 3 and 4, respectively (Figure 5B). For instance, metabolic pathways and plant hormone signal transduction were shared enriched pathways in clusters 3 and 4. Moreover, betalain biosynthesis, biosynthesis of various SMs, and phagosomes were specifically enriched in cluster 3, and biosynthesis of SMs, phenylpropanoid biosynthesis, stilbenoid, diarylheptanoid and gingerol biosynthesis, and tryptophan metabolism were specifically enriched in cluster 4.

We also conducted expression correlation analysis for DEGs from clusters 3 and 4 and regarded any two genes whose Pearson's correlation coefficient (r) value of expression values was greater than 0.9 and p value was less than 0.05 as coexpressed genes. Two gene coexpression networks were constructed for clusters 3 and 4 (Figures 5C and 5D). In cluster 3, the top five connected genes were *TUBB1*, *TUBB2*, *NPF5.2*, *BGH3B*, and *GDL85*, and the top connected TF was *KAN4* (G2-like family) (Figure 5C). In cluster 4, more genes were located in the center of the network, such as *Phox/Bem1p*, *AAP7*, *FB119*, *OFP2*, *UGT85C2*, *NPF5.1*, and *USPAL* (Figure 5D). *ABF2* (bZIP family) and *HAT22* (HD-ZIP family) were the two TF members with the most connections in the network, suggesting their importance in response to drought stress.

Genes related to plant hormone signal transduction, MAPK, and secondary metabolites

In total, 93 DEGs from plant hormone signal transduction were detected based on KEGG pathway enrichment analysis (Figure 6B), especially involving the auxin, cytokinin, ABA, and BR signaling pathways. Eight DEGs displayed a positive response to drought stress and rehydration,

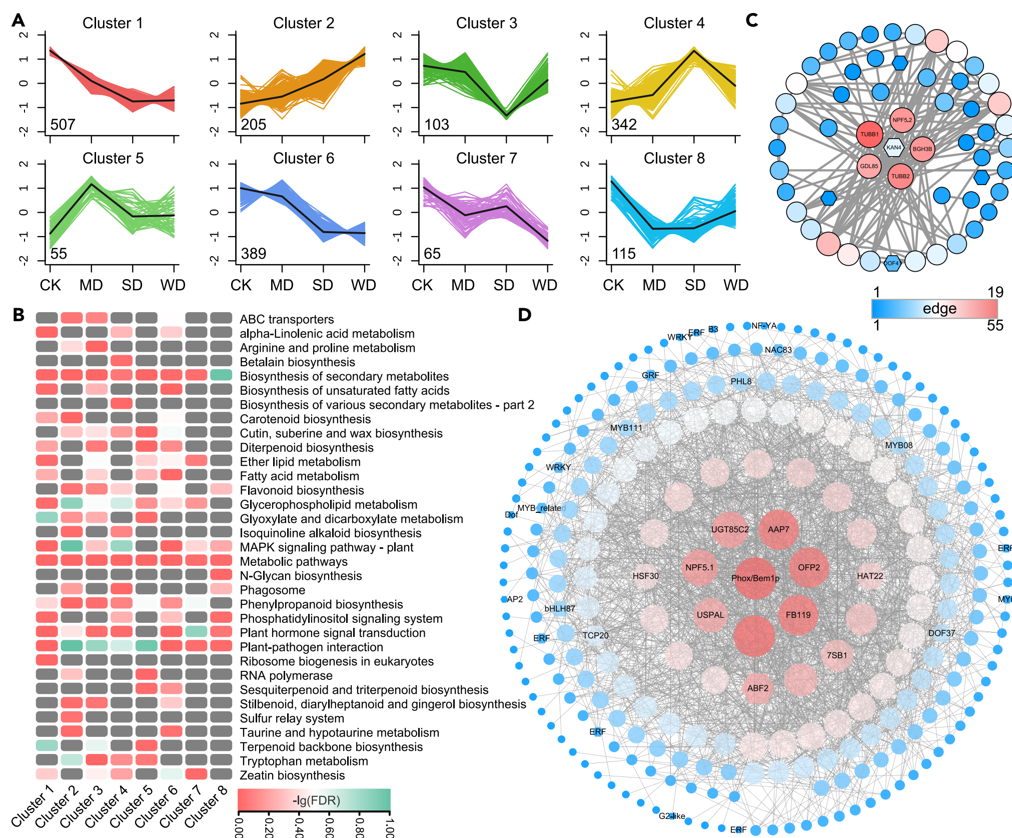

Figure 5. K-means analysis of DEGs identified in three comparisons

(A) Eight clusters reveal specific expression trends. The number in the lower left corner indicates the number of genes in this cluster.
(B) KEGG enrichment analysis for genes from eight clusters.
(C and D) show the coexpression networks for clusters 3 and 4.

including *LECRKS4*, *ABF2*, *IAA1*, *SAUR50*, *PHL8*, *P2C24*, *SAPK2*, and *SAPK3*. Nine DEGs displayed a negative response, including *LECRK1*, *AUX22*, *IAA17*, two *GH3.1s*, *HHO3*, *KAN4*, *ARR17*, and *XTH22*. Ninety-eight DEGs involved in the MAPK signaling pathway were detected, and 7 genes displayed a positive response to drought and rehydration, including *CATA*, *WRKY69*, *RBOHA*, *P2C24*, *P2C06* (*Os01g0583100*), *SAPK2*, and *SAPK3* (Figure 6C). Twenty-eight DEGs involved in ROS-scavenging systems were detected, the majority of which were continuously upregulated or downregulated with drought stress aggravation. However, only five genes (*PER52*, *CSE*, *MED37C*, *CATA*, and *BT1*) showed significant changes after rehydration, which may indicate their potential function in response to drought stress and rehydration (Figure 6A). DEGs acting as transporters or involved in SMs were also characterized, such as ABC transporters and genes from the biosynthesis of unsaturated fatty acids or ALA metabolism. Three ABC transporter genes (*ABCB15*, *ABCG22*, and *ABCG11*) and one gene (*KCR2*) involved in the biosynthesis of unsaturated fatty acids displayed a positive response under drought and rehydration (Figures 6D and 6E). Three genes, *CEQORH*, *4CLL5*, and *DOX1*, displayed a negative response in the ALA metabolism pathway (Figure 6F). All the previously described genes may have certain roles in the response to drought stress and rehydration.

Characterization of AS events and comparative analysis of DEGs and DSGs

AS also plays important roles in responding to environmental stress. Hence, we conducted AS analysis to identify candidate genes that may be involved in drought resistance. In total, 685, 780, and 662 differentially alternative splicing (DAS) events were identified in MD/CK, SD/CK, and WD/CK, which corresponded to 502, 578, and 498 differentially spliced genes (DSGs), respectively (Figure 7A). Then, all these DAS events were further subdivided into 5 patterns, including differentially alternative splicing (DAS) events of alternative 3′ splice site (A3SS), alternative 5′ splice site (A5SS), retained intron (RI), mutually exclusive exons (MXE) and skipped exon (SE). Among these patterns, A3SS and SE accounted for the largest proportion (Figure 7B). To further identify the potential functions for these DSGs, GO and KEGG enrichment analyses were performed, and 19 BPs, such as histone modification and phospholipid biosynthetic and metabolic process, were significantly enriched among all three comparisons (Table S7). In MD/CK and SD/CK, the phosphatidylinositol biosynthetic process, membrane lipid biosynthetic process, regulation of cell shape, and regulation of ABA biosynthetic process were significantly enriched. The GO terms regulation of response to osmotic stress, positive regulation of response to water deprivation, and lipid transport were enriched in SD/CK and WD/CK.

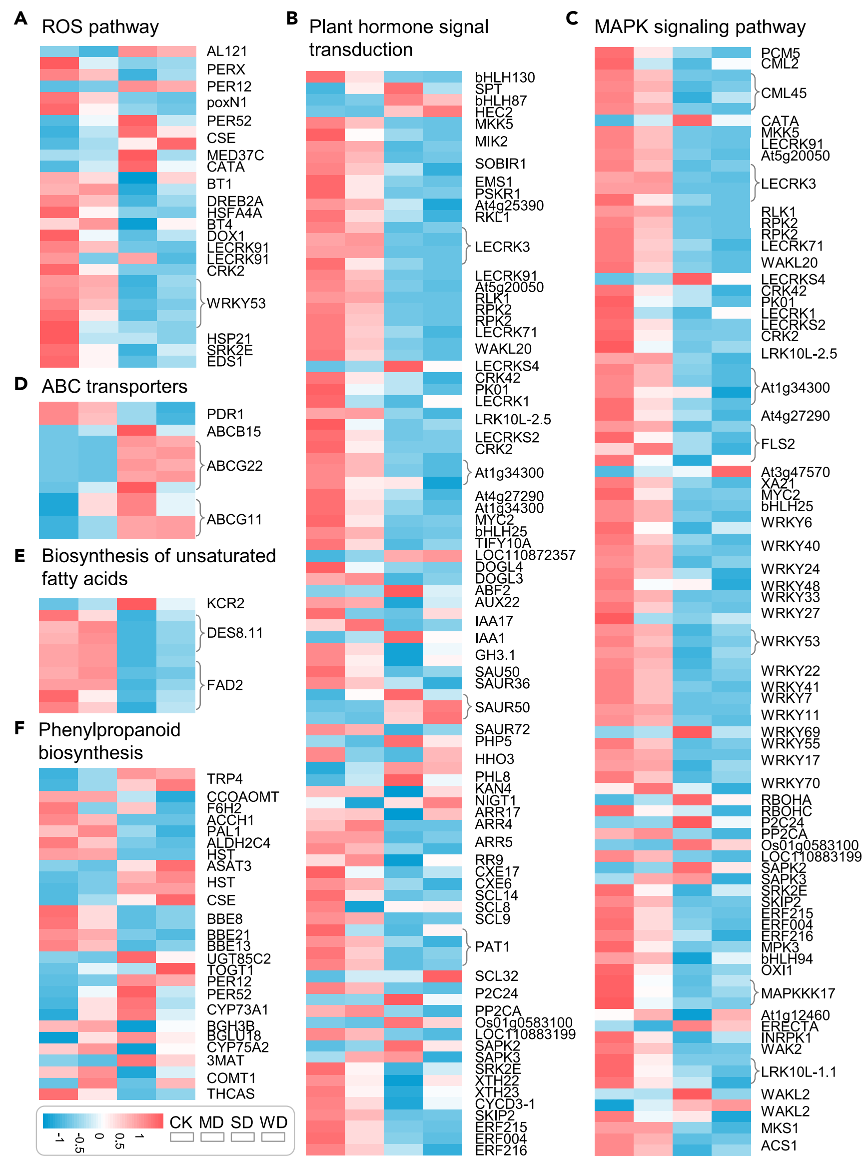

Figure 6. Expression profiles of DEGs

(A) ROS pathway.

(B) Plant hormone signal transduction.

(C) MAPK signaling pathway.

(D) ABC transporters.

(E) Biosynthesis of unsaturated fatty acids.

(F) Phenylpropanoid biosynthesis.

Water homeostasis was specifically enriched in MD/CK, and regulation of protein kinase activity, alternative mRNA splicing via the spliceosome, and response to calcium ions were specifically enriched in SD/CK. Two significantly enriched pathways, the metabolic pathway and biosynthesis of SMs, were shared among the three comparisons (Table S8). However, only one enriched KEGG pathway was shared by MD/CK and SD/CK (lysine degradation). Glycosylphosphatidylinositol-anchor biosynthesis and arginine and proline metabolism were specifically enriched in the MD/CK group. Eight KEGG pathways were specifically enriched in the SD/CK comparison. For instance, cysteine and methionine metabolism, pentose phosphate pathway, sulfur metabolism, and terpenoid backbone biosynthesis. In WD/CK, 3 KEGG pathways were enriched, including 2-oxocarboxylic acid metabolism, starch and sucrose metabolism, and other glycan degradation (Table S8).

To elucidate the relationship between AS and transcriptional regulation, we conducted comparison analysis using the genes that experienced DAS and DEGs in response to drought stress. In total, we found 1, 13, and 6 overlapping genes in the three comparisons (Figure 7C).

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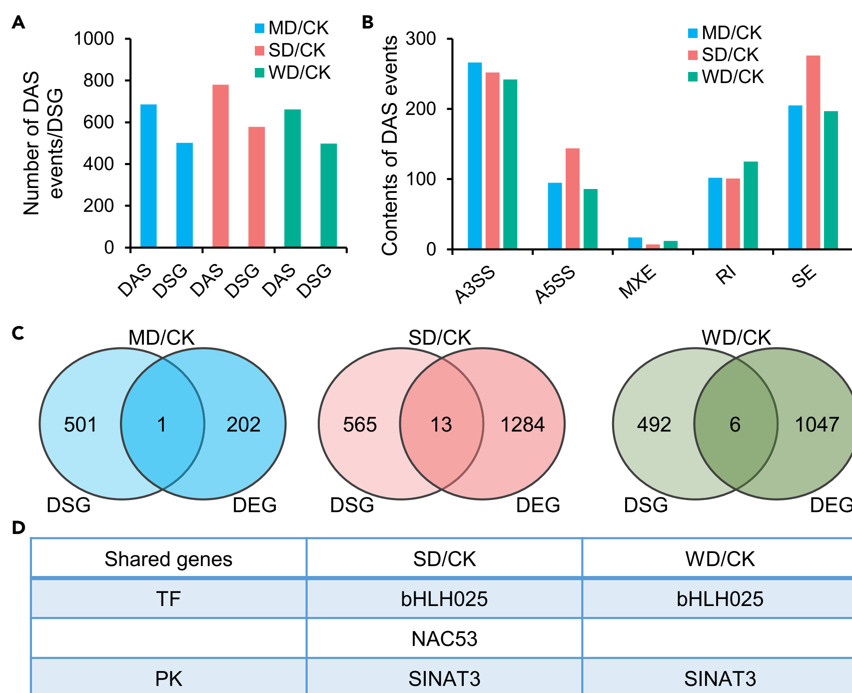

Shared genes	SD/CK	WD/CK
TF	bHLH025	bHLH025
	NAC53	
PK	SINAT3	SINAT3

Figure 7. Effect of drought stress on alternative splicing events in sunflower seedlings

(A) Numbers of differentially alternative splicing (DAS) events and corresponding genes (DSGs).

(B) Distribution of different types of AS events.

(C) Venn diagrams showing overlapping genes between DEGs and DSGs.

(D) TFs and PK genes among DSG-DEG overlapping genes are shown.

Among them, two TFs (*bHLH025* and *NAC53*) and one PK (*SINAT3*) were identified, which may play central roles in the response to severe drought stress (Figure 7D). Interestingly, *bHLH025* and *SINAT3* were also shared between DSGs and DEGs in WD/CK.

DISCUSSION

Drought stress can lead to an insufficient water supply for plants and affect their normal growth.[2] For instance, it can increase the production of $O_2^{\cdot-}$, H_2O_2, ·OH, and other ROS, and the accumulation of ROS can seriously damage plants by increasing lipid peroxidation, protein degradation, and even cell death.[34,35] MDA is a product of cell membrane lipid peroxidation and is an important indicator of the damage to plasma membrane system.[34,35] To prevent such oxidative damage, plants have evolved a complex antioxidant defense system, such as SOD, POD, CAT, and other enzymes.[36,37] In this study, drought stress increased the accumulation of ROS, such as $O_2^{\cdot-}$ and H_2O_2, in sunflower seedlings (Figure 1). Our results showed that rehydration treatment significantly reduced the $O_2^{\cdot-}$, H_2O_2, and MDA contents and increased the activities of SOD, POD, and CAT in sunflower seedlings under drought stress, indicating that rehydration effectively mitigates drought-induced ROS damage by increasing the activity of antioxidant enzymes.

Osmotic adjustment has been considered an important physiological mechanism involved in acclimation to drought stress.[38] The physiological parameters related to osmotic adjustment mainly include free proline, soluble protein, soluble sugar, and osmotic potential,[3,39] the contents of which increase to protect cell structure and function by maintaining cell filling or other physiological mechanisms under drought stress.[40] Similar to the study of Wu et al. (2022) in sunflower[41] and Khan et al. (2019)[6] in rapeseed, the contents of free proline, soluble protein, and soluble sugar were significantly positively related to drought resistance in our study (Figure 1). After rehydration, the contents of osmotic adjustment substances continued to increase, especially free proline. These results indicate that free proline correlated significantly with drought resistance and recovery in sunflower. Based on the previously described physiological results, sunflower seedlings can regulate their antioxidant enzyme activity, ROS content, and osmotic adjustment when responding to drought stress, and rehydration can effectively alleviate this stress damage.

Under drought stress, plants show a variety of regulatory mechanisms coupled with extensive gene expression changes,[42,43] and the number of DEGs reflects the response intensity of the crops. In our study, more DEGs were detected under severe stress than under mild stress and rehydration (Figure 2), indicating that the number of DEGs increased with decreasing water content in potting soil. Plant adaptation to drought stress is closely related to TFs, especially in highly resistant plants, and TFs interact with the promoter-specific elements of resistance genes. Some TFs respond to drought stress by regulating downstream genes.[44] Previous studies have shown that

many TF families play important roles in enhancing drought resistance and improving water use efficiency.[45,46] According to our results, the ERF, WRKY, MYB, NAC, C2H2, GRAS, and G2-like TF families are important in sunflower under drought stress and rehydration. These TFs function as activators or repressors to regulate target genes and form a transcriptional regulatory network in response to abiotic stress response and tolerance.[47] The AP2/ERF family is one of the largest plant-specific TF families,[48] and numerous studies have shown that genes belonging to the AP2/ERF family can improve drought tolerance in *Arabidopsis*[49] and tobacco.[50] Many ERF family members are differentially expressed when exposed to drought stress, and many duplication events in the ERF family may contribute to resistance to drought stress in sunflower seedlings.

As shown in Figure 4, the DEGs were significantly enriched in the signaling pathway of hormones (such as ABA, JA, and salicylic acid), components of the redox system and photosynthetic metabolism under different degrees of drought stress and rehydration, consistent with previous studies in sunflower.[51] Furthermore, the proline metabolic process was only significantly enriched under severe drought stress, and the response by ROS was significantly enriched under severe drought stress and rehydration compared to CK (Figure 4), which further explains the results of our physiological analysis.

Pathway enrichment analyses of both DEGs and DSGs showed that metabolic pathways and biosynthesis of SMs may be involved in the drought stress response (Figure 4; Table S8). In our study, the biosynthesis of unsaturated fatty acids, plant hormone signal transduction, and ALA metabolism pathways were significantly enriched under severe drought stress and rehydration compared to CK (Figure 4). Accordingly, polyunsaturated fatty acids are associated with plant adaptation to abiotic stress.[52] ALA is also a precursor of JA, which can alleviate damage due to drought stress through ROS scavenging, stomatal closure, and other multiple strategies.[53] In addition, numerous DEGs were identified as being involved in plant hormone signal transduction pathways, especially ABA signaling pathways (Figure 6). ABA plays a key role in the response to drought stress[54,55] and can effectively improve water use efficiency and drought resistance.[56] Among the core components of ABA signaling, overexpression of *PYL2*, *PYL8*, *PP2C*, *SnRK2*, and *ABF* enhances drought resistance in plants.[57] In our study, *P2C24*, *SAPK2*, and *SAPK3* and an *ABF* gene were upregulated with increasing drought severity and then downregulated after rehydration, which indicates that they play positive regulatory roles in the ABA signaling pathway and drought resistance. Similar results have been reported in sorghum and ginseng.[58,59] Notably, the ABA pathway plays an important role in the MAPK signaling pathway and ROS-scavenging systems.[60] MAPK is a signaling molecule that interacts with ROS and hormones to form adaptive responses.[9] As depicted in Figure 6, 7 genes (*CATA*, *WRKY69*, *RBOHA*, *P2C24*, *P2C06* (*Os01g0583100*), *SAPK2*, and *SAPK3*) in the MAPK signaling pathway and 5 genes (*PER52*, *CSE*, *MED37C*, *CATA*, and *BT1*) in ROS-scavenging systems showed a positive response to drought stress, which may indicate their potential function in response to drought stress and rehydration. Hence, as important members of the plant stress signal network, plant hormones, MAPK, and ROS crosstalk with each other to alleviate drought stress in sunflower.

It has been reported that AS plays an important role in coping with various environmental stresses by affecting transcript abundance, increasing transcriptome diversity, and enhancing the functional diversity of proteins.[61–63] Accordingly, AS and transcription regulation functions occur relatively independently under abiotic stress.[64,65] The AS of genes in tea leaves was triggered by drought stress and enhanced the transcriptome adaptation in response to stress.[64] In our study, more AS events occurred under severe drought than under mild drought or rehydration (Figure 7A), indicating that the number of AS events increases along with drought severity and decreases after rehydration. A3SS and SE were the most abundant AS patterns of the total AS events under the different water conditions (Figure 7B), consistent with a previous study in soybean.[61] Hence, these changes may play pivotal roles in the response to drought stress. To date, the biological functions of AS in plants have been defined in many genes, especially those involved in the regulation of stress responses, such as TFs.[64] In comparisons between DSGs and DEGs, we found only a few shared TFs, such as two TFs (*bHLH025* and *NAC53*) and one PK (*SINAT3*), indicating that they may function in the response to drought stress through both transcriptional and posttranscriptional regulation.

In conclusion, we propose a model for the underlying mechanism of sunflower seedlings responding to varying levels of drought stress (Figure 8). First, drought stress can cause the accumulation of ROS (such as $O_2^{\cdot -}$ and H_2O_2). As a secondary signal, ROS can interact with hormone signals and the MAPK cascade to activate drought-related TFs (such as *bHLH025* and *NAC53*). Next, the TFs further activate the expression of downstream-related genes, such as ROS scavenging, SMs, and other genes, and extensively promote the drought resistance of sunflower. Moreover, AS may also play important roles in drought resistance. Overall, our results not only deepen insight into the underlying mechanism of drought stress and rehydration in sunflower seedlings but also provide a theoretical basis for genetic breeding and water-efficient irrigation of this crop.

Limitations of the study

Through physiological measurement and transcriptomics for sunflower, we have identified some potential genes positively or negatively responding to drought stress and rehydration and highlighted the underlying regulatory mechanisms. However, the biological function of these genes remained to be verified through genetic modification.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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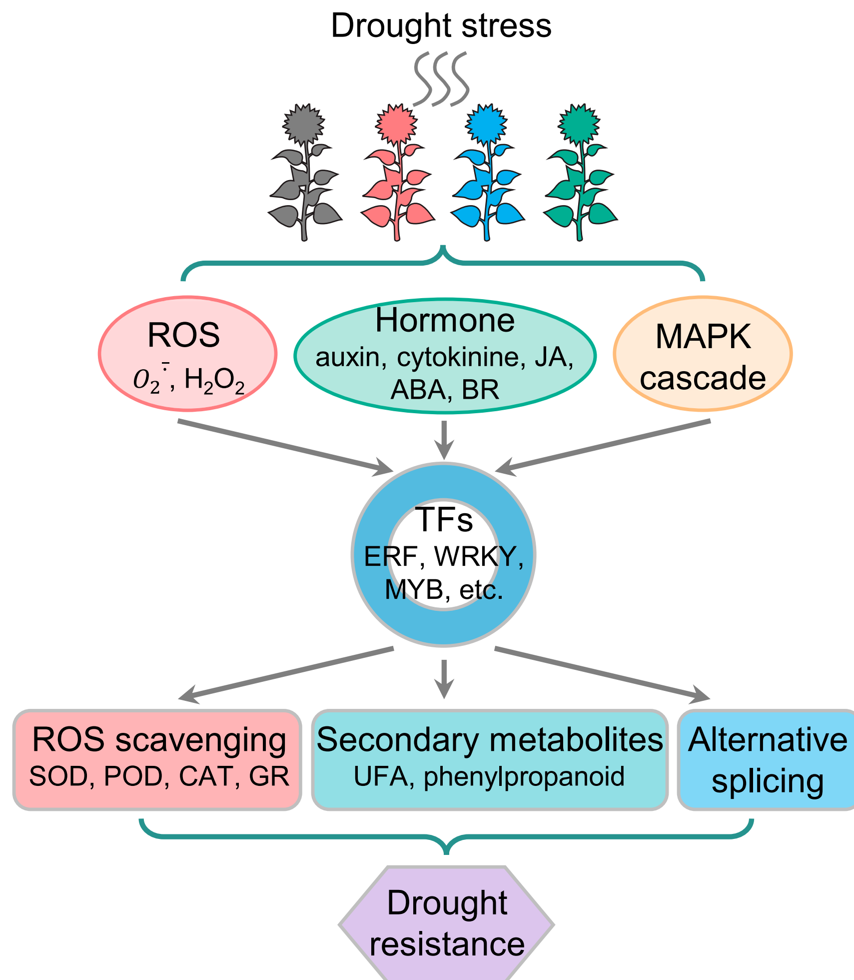

Figure 8. Possible response mechanisms of sunflower seedlings under drought stress

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108112.

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AUTHOR CONTRIBUTIONS

J.S.: Data curation, formal analysis, funding acquisition, investigation, methodology, project administration, roles/writing – original draft, writing – review & editing. X.W.: Data curation, formal analysis, investigation. H.S.: Formal analysis, methodology, validation. M.W.: Formal analysis, investigation. T.N.: Resources. H.L.: Formal analysis. C.Q.: Methodology. A.L.: Conceptualization, formal analysis, funding acquisition, methodology, project administration, resources, software, supervision, validation, visualization, roles/writing – original draft, writing – review & editing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Seeds of sunflower cultivar MH8361	ZHONG KE MAO HUA company, Hebei, China	N/A
Chemicals, peptides, and recombinant proteins		
Ethylenedinitrilotetraacetic acid (EDTA)	Sigma-Aldrich	Cat#E6758
Hydroxylamine Hydrochloride	Sigma-Aldrich	Cat#HX0770
2-Thiobarbituric acid	Sigma-Aldrich	Cat#T5500
Sulfosalicylic acid	Sigma-Aldrich	Cat#390275
Ninhydrin	Sigma-Aldrich	Cat#NX0403
Coomassie Brilliant Blue G-250	Sigma-Aldrich	Cat#1.15444
Guaiacol	Sigma-Aldrich	Cat#G5502
Nitro-blue tetrazolium	Sigma-Aldrich	Cat#N6876
Trichloroacetic acid	Sigma-Aldrich	Cat#T6399
4-aminobenzenesulfonic acid	Sigma-Aldrich	Cat#09180
1-naphthylamine	Sigma-Aldrich	Cat#N9005
Potassium iodide (KI)	Sigma-Aldrich	Cat#P2963
Polyvinylpyrrolidone	Sigma-Aldrich	Cat#81440
Ethanol	Sigma-Aldrich	Cat#E9508
Anthrone	Sigma-Aldrich	Cat#52445
Acetic acid	Sigma-Aldrich	Cat#A6283
Toluene	Sigma-Aldrich	Cat#TX0750
2-Mercaptoethanol	Thermo Fisher	Cat#21985023
Acetone	Thermo Fisher	Cat#T_702A060015
Anhydrous ethanol	Thermo Fisher	Cat#E/0550DF/15
Critical commercial assays		
Total Miniprep Kit	Axygen	Cat#RH175821
RNAprep Pure Plant Plus Kit	Tiangen	Cat#DP452
PrimeScript™ 1st Strand cDNA Synthesis Kit	Takara	Cat#6210B
Deposited data		
Raw data generated in this study	This study	NCBI Sequence Read Archive:PRJNA869183. (the SRA accession NO. for each sample is listed in Table S1).
Oligonucleotides		
Primers used are shown in Table S9	This study	N/A
Software and algorithms		
Cutadapt	N/A	http://cutadapt.readthedocs.io/en/stable/
HISAT2	Kim et al., 2019[66]	http://ccb.jhu.edu/software/hisat2/index.shtml
DESeq	Wang et al., 2010[67]	https://www.bioconductor.org/packages//2.10/bioc/html/DESeq.html
ASprofile	Florea et al., 2013[68]	N/A
rMATS	Shen et al., 2014[69]	N/A
Metware	N/A	https://cloud.metware.cn

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cytoscape v.3.8.2	Otasek et al., 2019[70]	N/A
Primer3 software	Untergasser et al., 2012[71]	http://primer3.ut.ee
SPSS software	IBM	V19.0.0

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Ake Liu (akeliu@126.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Raw data generated in this study have been deposited at NCBI and are publicly available before the date of publication. Accession numbers are listed in the key resources table.

The original code was attached as supplementary file.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The sunflower cultivar MH8361 was used as the material for drought stress experiments. The potting soil (Pindstrup Mosebrug, Ltd., Denmark) used contained more than 98% sphagnum moss and less than 2% impurities. The soil was weighed and quantified before seeds were planted, and 2 seeds were sown in each pot. Then, they were cultured in a growth chamber (25°C, 16 h light/20°C, 8 h dark cycle). Water was replenished regularly every day to ensure normal seedling growth.

METHOD DETAILS

Drought stress treatments

The seedlings were randomly divided into four groups with three replicates each after the sixth true leaves fully expanded. A natural progressive drought was imposed by withholding watering based on daily measurements of pot weight. The drought stress treatments included a normal water supply (CK, 75 $\pm$ 5% water content), mild drought stress (MD, 40 $\pm$ 5% water content) and severe drought stress (SD, 20 $\pm$ 5% water content) treatments applied for five days, and another treatment (WD, water content of 75 $\pm$ 5%) in which the plants were rehydrated with a normal water supply for 2 days after 3 days of severe drought stress (Figure 1A). During this process, the pots were weighed regularly (every day) to maintain the water content in the four different drought treatments. After the experimental treatments, two leaves of the last fully expanded sunflower leaves were collected, immediately frozen in liquid nitrogen, and stored at -80°C until further physiological and RNA sequencing.

Detection of physiological indicators

The production rate of O_2^{-} was detected by the hydroxylamine method.[72] 0.1 g of leaf tissue was macerated in a mortar with 65 mM phosphate buffer (pH 7.8) and ground fully. After centrifuging at 12000 $\times g$ for 15 min, supernatant was mixed with 65 mM phosphate buffer (pH 7.8) and 10 mM hydroxylamine chloride, and incubated at 25°C for 20 min. Then, the reaction mixture was added with 17 mM 4-aminobenzenesulfonic acid and 7 Mm alpha-naphthylamine, and mixed well. After incubating at 30°C for 30 min, absorbance was measured at 530 nm using a spectrophotometer. The content of H_2O_2 was obtained based on the acetone extraction method.[28] 0.5 g of leaf tissue was macerated in a mortar with 0.1% (w/v) trichloroacetic acid solution. After centrifuging at 12000 $\times$ g for 12 min, supernatant was mixed with 0.1 M potassium phosphate buffer (pH 7.0) and KI solution; absorbance was measured at 390 nm using a spectrophotometer. The malondialdehyde (MDA) content was measured by the thiobarbituric acid chromogenic method.[73] Briefly, 0.2 g of penultimate leaves, a small amount of quartz sand, and 0.1% trichloroacetic acid were mixed together and ground in an ice bath. Then, 0.5% thiobarbituric acid was added to the mixture, followed by a boiling-water bath for 15 min, and then centrifuged at 1000 $\times$ g for 15 min after cooling to room temperature. The absorbance values of the supernatant at 532 nm and 600 nm were measured using 0.5% thiobarbituric acid as the control.

Approximately 0.1 g of penultimate leaves, a small amount of quartz sand, and 50 mM phosphate buffer (containing 0.1 mM ethylenediaminetetraacetic acid and 1% polyvinylpyrrolidone) were mixed together and ground in an ice bath. Then, the mixture was centrifuged at 15000 $\times$ g for 15 min at 4°C. The supernatant was used to determine the amounts of SOD, CAT, POD, GR and soluble proteins. The activities of SOD and CAT were detected by the nitroblue tetrazolium photoreduction method and UV spectrophotometry.[74] The activities of POD

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were assessed by the guaiacol colorimetric method.[75] The activity of GR was detected based on the method of Foyer and Halliwell (1976).[76] Soluble proteins were determined using Coomassie Brilliant Blue G-250 colorimetry.

To determine soluble sugar content, 0.1 g of penultimate leaves was accurately weighed in a centrifuge tube with 80% ethanol solution. After water bath for 30 min at 80°C and centrifuging the extract at 3500 × *g* for 10 min, the supernatant was transferred into a new centrifuge tube. Then 80% ethanol solution was added to the precipitation, repeat extraction as above. The supernatant was for the determination soluble sugar content by anthrone-sulfuric acid method.[77] The detection of free proline was performed by the acid ninhydrin method.[77] Briefly, 0.2 g of fresh leaves were placed in a mortar and ground with a small amount of quartz and 3% sulfosalicylic acid on ice. Placed in a boiling water bath for10 min. After cooling, the grinding liquid was centrifuged at 2000 × *g* for 10 min. Supernatant, glacial acetic acid and acid ninhydrin were mixed and then placed in a boiling water bath for 30 min. After cooling to room temperature, toluene was added to the mixture in the dark for extraction. The absorbance of the toluene phase (red) was measured at 520 nm using a spectrophotometer.

RNA isolation and transcriptome sequencing

Total RNA was extracted from the collected samples of all groups using a total Miniprep Kit (Axygen Bioscience, USA) according to the manufacturer's instructions. RNA integrity and purification were determined using an Agilent 2100 BioAnalyzer (Agilent Technologies, USA). High-quality RNA with an RNA integrity number of > 8 and of sufficient quantity was used to construct a sequencing library. After completion of library construction, PCR amplification was performed to enrich the library fragments. Library selection was conducted according to fragment size (300 ~400 bp). The size and total concentration of the library were quality inspected using an Agilent 2100 BioAnalyzer and fluorescence quantification, respectively. After construction of the library, next-generation sequencing technology based on the Illumina HiSeq™ 2000 Sequencing platform was used, and paired-end (PE) sequencing was performed on these libraries.

Transcriptome evaluation and gene expression analysis

Raw RNA-Seq data were collected and deposited in the NCBI (National Center for Biotechnology Information) database under Bioproject PRJNA869183 (the SRA accession NO. for each sample is listed in Table S1). The Cutadapt program (http://cutadapt.readthedocs.io/en/stable/) was used to filter the data, including removing adapters, unknown nucleotides, and low-quality (Q-value ≤ 20) bases. The obtained clean reads were further mapped to the sunflower reference genome[78] (downloaded from the NCBI genome database) using HISAT2 (http://ccb.jhu.edu/software/hisat2/index.shtml).[66] HTSeq statistics were applied to compare the read count value of each gene as the original expression amount of the gene. FPKM (fragments per kilobases per million fragments) was employed to normalize gene expression levels, making them comparable among genes or samples. Differentially expressed genes (DEGs) were analyzed using DESeq (https://www.bioconductor.org/packages//2.10/bioc/html/DESeq.html)[67] with the criteria $|\log_2\text{-fold-change}| > 1.0$ and P value < 0.05.

Alternative splicing (AS) analysis

After read mapping and transcript assembly, AS events were identified and classified using ASprofile.[68] SE, RI, MXE, A5SS, and A3SS events were analyzed by rMATS[69] and screened by FDR < 0.05. Genes involved in any DAS event were considered differentially spliced genes (DSGs).

Functional enrichment analysis of DEGs and DSGs

Functional enrichment analysis was conducted for the identified DEGs and DSGs to predict their potential functions and biological pathways. Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp/kegg) enrichment analyses of all DEGs and DSGs were carried out using an online tool from Metware (https://cloud.metware.cn). The significance for each GO term and KEGG pathway was calculated against the whole-genome background by the hypergeometric test (p value < 0.05). The p value calculation was performed with FDR correction.

TF family analysis and gene coexpression network analysis

TFs and their families were predicted by comparison with the Plant Transcription Factor Database (PlantTFDB). Gene coexpression network analysis was performed by calculating r of expression values ($\log_2$(FPKM+1)) between any two genes under different treatments to reveal the relationship between target genes (File S1). An r value greater than 0.9 and a p value less than 0.05 were selected as the screening thresholds. The networks were visualized using Cytoscape v.3.8.2.[70]

Quantitative RT−qPCR validation

To verify the reliability of the transcriptome sequencing results, we randomly selected 10 DEGs for quantitative real-time PCR (RT−qPCR) analysis. Total RNA of 12 samples of sunflower leaves was extracted using the RNAprep Pure Plant Plus Kit following the manufacturer's instructions (Tiangen Biotech, China). After confirming the integrity of the RNA, reverse transcription was carried out using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio), and RT−qPCR was performed using AceQ® qPCR SYBR Green Master Mix (Vazyme Biotech Co.,

Ltd.). Gene-specific primers were designed using Primer3 software (http://primer3.ut.ee);[71] the primer sequences are shown in Table S9. All reactions were carried out in three replicates, and gene expression levels were calculated by $2^{-\Delta\Delta Ct}$.

QUANTIFICATION AND STATISTICAL ANALYSIS

Bioinformatic analysis was described in the method details section. SPSS (V19.0.0) software was used for statistical analysis. Expression differences were analyzed by one-way ANOVA and LSD's test, and a p value < 0.05 was considered statistically significant.