

Article

Transcriptome Analysis of Germinated Maize Embryos Reveals Common Gene Responses to Multiple Abiotic Stresses

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Abstract

Maize seeds undergo a rapid germination phase, spanning from dry seeds to radicle protrusion, which is highly susceptible to various abiotic stresses. However, the specific distinctions between responses to different abiotic stresses and the genes commonly activated across multiple stresses remain largely unreported. Here, we performed a transcriptome analysis using germinating embryos subjected to low-temperature stress (LTS), high-temperature stress (HTS), drought stress (DS), and salinity stress (SS) at two key germination stages: the imbibition saturation stage and radical protrusion stage. By comparing these samples to dry embryos, we first identified germination-related genes active at both stages. Subsequently, we compared stressed samples to non-stressed controls under standard germination conditions to exclude genes influenced solely by developmental progression. This ultimately identified 1226, 2418, 1298, and 850 DEGs stress-responsive differentially expressed genes (srDEGs) at the imbibition saturation stage for LTS, HTS, DS and SS, respectively, alongside 1995, 1437, 1741 and 1555 srDEGs at the radicle protrusion stage. Through a cross-stress comparison, we identified 214 to 1563 single-stress-responsive, 35 to 414 dual-stress-responsive, and 33 to 243 triple-stress-responsive srDEGs. Notably, we detected 44 and 235 common stress-responsive (co-srDEGs) across all four stresses at the imbibition saturation and radicle protrusion stage, respectively. These co-srDEGs were primarily associated with reactive oxygen species (ROS) metabolism, hormone signaling, and transcriptional regulation. Among the co-srDEGs, we identified 20 transcription factors (TFs) representing 11 families, which may serve as critical candidate genes for regulating multi-stress tolerance. The expression of four TFs was further verified by qPCR analysis. These findings not only highlight the differences and similarities in the regulatory networks underlying LTS, HTS, DS and SS during germination but also provide essential candidate genes for elucidating the mechanisms of seed germination in response to multiple abiotic stress.

Keywords: maize; germination; abiotic stress; transcriptome sequencing; differently expressed genes



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1. Introduction

Maize is a major food crop for agricultural production worldwide, and its yield largely depends on seed vigor, which is characterized by rapid germination and successful seedling

establishment [1]. Seed germination is a brief yet critical process comprising three phases; it begins with rapid water imbibition by the dry seed (Phase I), transitions into a period of activated metabolism after imbibition saturation (Phase II), and concludes with renewed water uptake and radicle emergence (Phase III) [2]. Germination *sensu stricto* is considered complete once the radicle penetrates the seed coat [2]. It is well established that abiotic stresses such as low temperature, high temperature, drought, and salinity have significant negative effects on seed germination [2]. Exploring their underlying mechanism will facilitate the breeding of stress-tolerant maize varieties.

Upon exposure to abiotic stress during germination, seeds produce elevated levels of reactive oxygen species (ROS), while their ROS-scavenging capacity is weakened [3], which results in excessive ROS accumulation and oxidative stress, causing electrolyte leakage and cell membrane damage [4]. Abiotic stress also alters the level of antioxidants, such as catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD), further impairing ROS scavenging and reducing germination ability [5,6]. Plant hormones, including abscisic acid (ABA), gibberellin (GA), auxin, jasmonic acid (JA), cytokinin and ethylene, mediate signaling pathways essential for growth and stress survival [7]. Among them, ABA is the most critical stress-related hormone during germination; it activates ABA-responsive genes and enhances seed tolerance to abiotic stress [3,8,9]. For instance, *ZmABA2*, a gene involved in ABA biosynthesis, functions to promote radical growth under osmotic stress during germination [8]. Similarly, the stress-responsive NAC transcription factor genes, *ZmSNAC1* and *ZmNAC55*, which are ABA-responsive genes, function to improve tolerance to drought stress during germination [9,10]. These studies underscore the crucial roles of ROS and hormones in regulating seed germination under environmental stresses.

Cloning stress-responsive genes from various plants has advanced our understanding of the molecular mechanisms underlying different abiotic stresses [11–29]. Under low-temperature stress (LTS), key functional proteins such as the C-repeat-binding factors OsCBF3, ATP-binding cassette transporter ZmMRPA6, and dehydrin ZmDHN15 have been shown to alleviate the inhibitory effect of low temperature on seed germination [11–14]. Under high-temperature stress (HTS), seeds employ strategies such as the heat shock transcription factor (HSF) gene *TaHsfA2h* in wheat and the *ZmHsf06* and *ZmHSF21* in maize to promote the expression of heat shock proteins for enhancing thermotolerance [15–17]. Under drought stress (DS), germinating seeds display upregulated genes including *AtHSA6b*, the mitogen-activated protein kinase (MPK) gene *ZmMPK5*, the abscisic acid stress ripening (ASR) gene *OsASR5*, the *CIRCADIAN CLOCK-ASSOCIATED* gene *OsCCA1*, the WRKY transcription factor gene *TaWRKY53*, and the DEAD-box RNA helicase gene *TaDEAD-box57-3B* to modulate ABA biosynthesis, signaling and ROS homeostasis in response to stress [8,18–22]. Under salinity stress (SS), tolerance regulators such as the NAC transcription factor genes *ANAC047* and *RhNAC31*, the suppressor of the *ABAR-overexpressor 1* (*AtSOAR1*), the ethylene response factor gene *AtERF96*, the salt tolerance-related protein (STRP) gene *AtSTRP*, the small heat shock protein (SHSP) gene *OsSHSP1* and *OsSHSP2*, and the RNA helicase gene *OsRH58* are activated to enhance expressions of various stress-related genes [23–29]. However, research on these genes has predominantly focused on single stress response, and the identification of common genes responsive to multiple abiotic stresses remains limited [30–32].

Advances in genomics, transcriptomics, and proteomics have gradually deepened our understanding of the molecular mechanisms underlying multi-stress responses [33]. For example, a large-scale meta-analysis integrating expression data from 11 stresses identified 561 differentially expressed genes (DEGs) responsive to multiple stresses [34]. Integrated transcriptomic and proteomic analyses have also been conducted to investigate the effects of multiple stresses on maize and rice seedlings, along with the genes and

proteins affected [35]. Furthermore, certain proteins associated with cell membrane or transcription factors (TFs) have recently been shown to function in cross-tolerance to several abiotic stresses during the seedling stage [36–39]. In rice, the NAC transcription factor ONAC023 is induced by drought and heat stresses during seedling and reproductive stages, targeting genes involved in water transport, ROS homeostasis, and alternative splicing [36]. The integral membrane tetraspanin (TET) protein OsTET5 enhances seedling tolerance to salinity and drought stresses by maintaining an elevated potassium-to-sodium ratio and redox homeostasis [37]. In wheat, overexpression of the ERF transcription factor TaERF3 improves seedlings' tolerance to salinity and drought through interaction with a GCC-box in the promoters of downstream stress-related genes, activating their expressions [38]. In maize, an MYB transcription factor ZmMYBR24 has been reported to regulate seed germination under saline, alkali, and low-temperature conditions [39]. Despite these advances, the effects and interrelationship of low temperature, high temperature, drought, and salinity stress on maize seed germination, as well as the common genes responsive to these abiotic stresses, remain largely unknown.

In this study, we surveyed the physiological phenotypes of seed germination and seedling growth under four abiotic stresses: low temperature, high temperature, drought, and salinity. Using transcriptomics, we identified stress-responsive DEGs (srDEGs) at two key germination stages: imbibition saturation and radicle protrusion. Furthermore, we focused on common stress-responsive DEGs (co-srDEGs) across all four stresses at both stages and analyzed their expression patterns under different stress conditions. These findings provide insights into potential stress-tolerance genes and the regulatory mechanisms governing seed germination under multiple abiotic stresses.

2. Materials and Methods

2.1. Plant Materials and Stress Treatments

Seeds of the maize inbred line B73 were harvested at physiological maturity in Zhuozhou, Hebei Province, China. Prior to germination assays, seeds were sterilized with 1% NaClO solution for 10 min and then rinsed thoroughly with deionized water. For each assay, 50 seeds were sown in sterile sand within a plastic box, covered with an additional 1 cm layer of sand, and incubated in a growth chamber under a 16 h/8 h light/dark photoperiod. All germination experiments included three independent biological replicates.

For the temperature-treated germination assay, the standard germination temperature for maize, as defined by the International Seed Testing Association (ISTA), is 25 °C [40]. To determine appropriate stress temperatures, we conducted a germination assay across a range of 9 temperatures from 10 °C to 40 °C (spanning ± 15 °C from the standard) at an interval of 3.5 °C or 4.0 °C.

To evaluate the effect of drought stress, seeds were germinated in sand with water contents of 2%, 3%, 4%, 7%, 12%, 17%, and 22% at 25 °C.

To evaluate the effect of salinity stress, seeds were germinated at 25 °C and watered with NaCl solution at concentrations of 0, 50, 150, 200, 250, and 350 mM.

2.2. Evaluation of Seed Physiological Indicators

A seed was considered germinated when the coleoptile emerged from sand surface. The germination percentage was calculated according to the ISTA standard as the ratio of germinated seeds to the total seeds tested [40]. Germination was recorded until the percentage plateaued. If germination was completed within 7 days, the fresh weight (FW) and dry weight (DW) of the shoot, root and seed residue were measured on the seventh day. If germination exceeded 7 days, weights were measured once the germination count

had stabilized. Graphpad Prism 8 software was used to plot data, and SPSS 13.0 was used for statistical analysis via ANOVA and Duncan's multiple comparison tests.

To distinguish germination stage, the relative water content (RWC) during germination was measured. At each time point, ten seedlings were sampled for FW measurement then dried to a constant weight to obtain the DW. RWC was calculated as follows: $100\% \times (FW - DW)/FW$.

2.3. Transcriptome Sequencing and Gene Expression Analysis

Embryos were sampled at the imbibition saturation and radicle protrusion stages under four stress conditions. Sampling times were as follows: 18 and 68 h after imbibition (HAI) for low temperature; 12 and 31 HAI for high temperature; 22 and 70 HAI for drought; and 12 and 55 HAI for salinity stress. For each condition, two biological replicates were collected, with each replicate comprising 30 pooled embryos ground in liquid nitrogen. Total RNA was extracted using the Polysaccharide Polyphenol Plant Total RNA Extraction Kit (Tiangen, Beijing, China).

A strand-specific RNA-Seq library was constructed and sequenced on an Illumina platform (Annoroad, Beijing, China) to generate 150 bp paired-end reads. High-quality reads were aligned to the maize B73 AGPv4 reference genome using HISAT2 2.1.0 with the parameter “-rna-strandness RF” [41]. Gene expression levels were quantified with HTseq-count (version 2.0.3) using uniquely mapped reads and “--stranded reverse” parameters [42]. Then, raw read counts were converted to fragments per kilo-base of transcript per million reads of base pairs mapped (FPKM). Transcriptome data for dry embryos (non-germination control) and embryos germinated under standard conditions at 14 and 40 HAI (non-stress conditions) were obtained from our previous work [43].

2.4. Hierarchical Clustering and Functional Annotation Enrichment Analysis

Hierarchical clustering and principle component analysis (PCA) were performed in R (v4.4.2) using normalized gene expression values ($\log_2(\text{FPKM} + 1)$) with the prcomp function and the pvclust package (v2.2-0) [44]. Differential expression analysis was conducted with the DESeq2 package (v3.20) using raw read counts, applying the threshold of false discovery rate (FDR) < 0.05 and $\log_2(|\text{fold change}|) \geq 1$ to identify DEGs [45]. Gene Ontology (GO) enrichment analysis of stress-related DEGs was performed using the agriGO online tool (<http://systemsbiology.cau.edu.cn/agriGOv2/>, accessed on 14 September 2024) with Fisher's test. The terms with an FDR < 0.05 were considered significantly enriched.

2.5. Quantitative RT-PCR Validation

Total RNA was extracted from embryos at the imbibition saturation stage (14, 18, 12, 12 and 22 HAI for standard, low-temperature, high-temperature, saline, and drought conditions, respectively) and at the radicle protrusion stage (40, 68, 31, 55 and 70 HAI for the same respective conditions). cDNA was synthesized using oligo-dT primers and the HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). qPCR was performed under the following conditions: pre-denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. Four genes with high expression levels under all four stresses were selected for validation. Gene expression was calculated using quantitative methods ($2^{-\Delta\Delta C_t}$) with three biological replicates. The maize *ACTIN* gene was used as an internal control. All quantitative primers are listed in Table S1.

3. Results

3.1. The Effect of Temperature, Salinity, and Moisture on Maize Seed Germination

To assess the effect of temperature on B73 seed germination, we tested nine temperatures ranging from 10 °C to 40 °C at an interval of 3.5 °C or 4.0 °C (Figure 1A–D). Seven germination traits were evaluated: germination percentage (GP), seedling dry weight (SLDW) and fresh weight (SLFW), shoot dry weight (STDW) and fresh weight (STFW), and root dry weight (RDW) and fresh weight (RFW). All traits initially increased and then decreased as temperature rose (Figure 1A–C). The highest GP was observed at 25 °C, which was therefore set as the standard germination temperature (Figure 1A). Germination was strongly inhibited at 17.5 °C and 36 °C, with GP reduced to 70~80%, and SLFW and SLDW significantly lowered (Figure 1A–D). Consequently, 17.5 °C and 36 °C were selected as the LTS and HTS conditions, respectively.

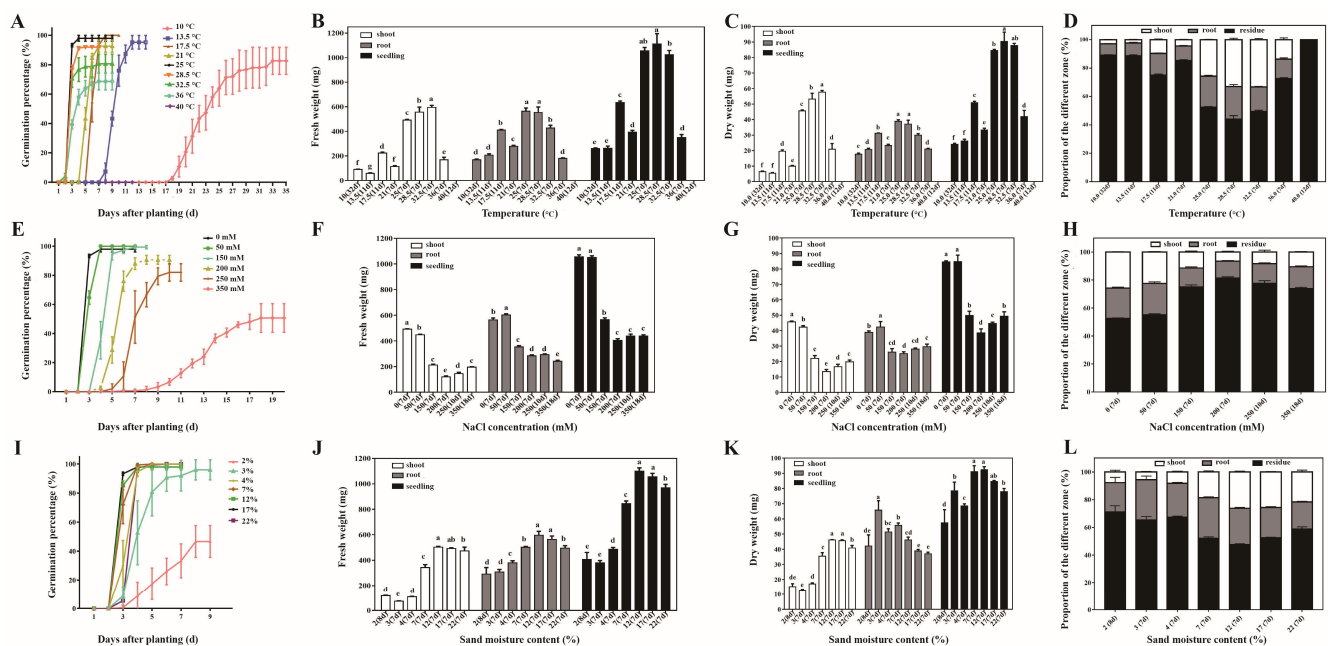


Figure 1. Physiological traits of B73 seeds germinated at different temperatures, salinity concentrations, and sand moisture contents. Measurements include germination percentage (A,E,I); fresh weight of shoot, root and seedling (B,F,J); dry weight of shoot, root and seedling (C,G,K); and the proportional biomass allocation to shoot, root and residual seed in the seedling (D,H,L). Different lowercase letters within a given trait indicate significant difference according to Duncan's test ($p < 0.05$).

To examine the effect of SS, seeds were germinated under six NaCl concentrations from 0 mM to 350 mM at 50 mM intervals (Figure 1E–H). The GP remained at 97% at a NaCl concentration below 200 mM but declined significantly as the concentration increased from 200 mM to 350 mM (Figure 1E). All seedling traits, including SLDW, SLFW, STDW, STFW, RDW and RFD, decreased with rising NaCl concentration, leading to a reduction in the ratio of SLDW to residual seed dry weight (Figure 1E–H). Notably, with 250 mM treatment, GP, all measured seedling traits, and seed dry matter conversion efficiency were significantly reduced; this concentration was therefore selected as the representative SS condition (Figure 1E–H). Interestingly, at 50 mM NaCl, GP and seedling traits were slightly higher than those in the untreated control (Figure 1E–G), suggesting that a low concentration of NaCl might have a mild stimulatory effect on seed germination.

To evaluate the effect of DS, germination was tested at seven sand moisture levels from 2% to 22% (Figure 1I–L). SLFW approximately doubled as moisture increased from 4% to

7%, peaked between 12% to 17% moisture, and slightly decreased thereafter (Figure 1J,K). The highest proportion of seedling mass relative to total germinated seed mass (seedling plus residual endosperm) was also observed at 12–17% moisture, indicating optimal seed-to-seedling conversion efficiency under these conditions (Figure 1J,L). Concerning GP, it significantly reduced only at 2% moisture compared to the 12–17% range (Figure 1I–L); therefore, 2% moisture was selected as the representative DS condition.

3.2. Transcriptome of Germinated Embryos in Response to Temperature, Salinity, and Drought Stresses

To investigate the impact of high temperature, low temperature, salinity and drought stresses on B73 seed germination, we measured seed water content from 0 HAI to 40 HAI (Figure 2A). We focused on two key stages: the imbibition saturation stage, defined by the inflection point of the water absorption curve (28% water content), and the radicle protrusion stage, when radicles had emerged in more than 50% of germinated seeds (Figure 2A,B). Under standard germination conditions (25 °C, 16% sand moisture), these stages occurred at 14 and 40 HAI, respectively. Under stress, the times shifted to 18 HAI and 68 HAI for low temperature, 12 HAI and 31 HAI for high temperature, 12 HAI and 55 HAI for salinity, and 22 HAI and 70 HAI for drought conditions, respectively (Figure 2A,B). Embryos were collected at these time points, resulting in a total of 16 samples (4 stress treatments $\times$ 2 stages $\times$ 2 biological replicates) for transcriptome sequencing (Figure 2B; Table S2).

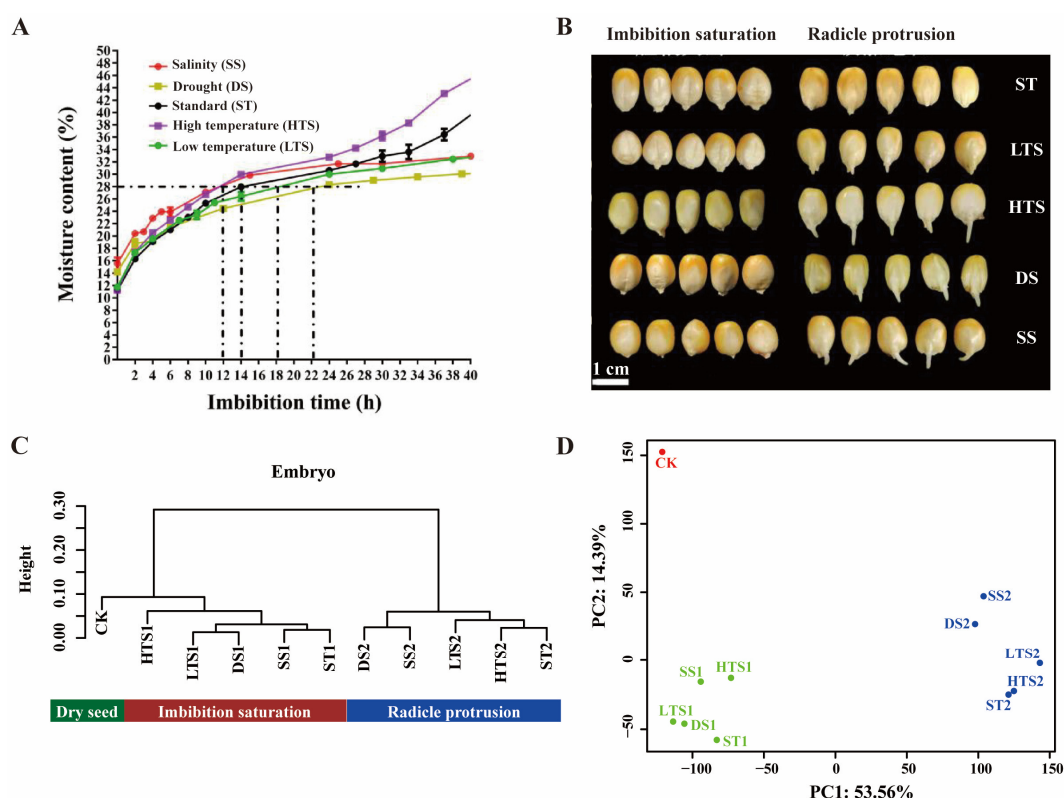


Figure 2. Embryo transcriptome analysis under four stresses at two key stages of germination. (A) Change in water content during maize seed germination. The dotted line represents the time point of imbibition saturation. (B) Phenotype of germinating embryos under various treatments. (C,D) Hierarchical clustering (C) and principal component analysis (PCA) (D) of germinated embryos samples under standard treatment (ST; non-stress control), low-temperature stress (LTS), high-temperature stress (HTS), drought stress (DS) and salinity stress (SS) at the imbibition saturation stage (stage 1) and radicle protrusion stage (stage 2). Dry embryos (CK) serve as non-germinated controls.

The uniquely mapped reads were then used to estimate the normalized gene expression values as indicated by FPKM. A gene was considered expressed if its FPKM ≥ 1 in at least one sample [43]. The high correlations between biological replicates ($R^2 = 0.99$) confirmed the data's reliability (Figure S1), and the average FPKM value from two replicates served as the gene expression level.

Dry embryos and germinated embryos from the standard germination at 14 and 40 HAI served as non-germination and non-stress germination controls, respectively; their transcriptome data were generated from our previous study [43]. In total, 23,990 genes were found to be expressed in at least one sample, representing ~80% of all annotated genes (Table S3). Hierarchical clustering and principal component analysis (PCA) of these expressed genes revealed that samples primarily clustered by germination stage rather than by stress treatment. Imbibition saturation stage samples showed a closer resemblance to the dry seed sample (CK) than to the radical protrusion samples (Figure 2C,D). Within each stage, samples from all five treatments (four stresses plus standard germination) showed similar clustering relationships to each other.

3.3. Differentially Expressed Genes Under High-Temperature, Low-Temperature, Salinity and Drought Stresses Compared to Standard Germination

To identify genes responsive to abiotic stresses during germination, we first defined germination-associated DEGs (gmDEG) by comparing germinated embryos to dry embryos (Figure S2, Table S4). For standard germination, this yielded 6722 (3074 up, 3648 down) at the imbibition saturation stage and 12,481 gmDEGs (6949 up, 5532 downregulated) at the radicle protrusion stage [39] (Figure S3). A similar analysis under stress conditions identified 5609 (2690 up, 2919 down) and 12,928 gmDEGs (7280 up, 5648 downregulated) under LTS (Figure S3); 6624 (3421 up, 3203 down) and 12,529 gmDEGs (6955 up, 5574 down) under HTS; 5742 (2671 up, 3071 down) and 11,472 gmDEGs (6321 up, 5151 down) under DS; and 5133 gmDEGs (2582 up, 2551 down) and 11,226 gmDEGs (6381 up, 4845 down) under SS at the imbibition saturation stage and radicle protrusion stage, respectively. In total, 18,702 unique gmDEGs were identified across all comparisons (Table S5).

To identify stress-responsive genes, we then compared gmDEGs from each stress condition to those from the standard germination control (Figure 3). This comparison identified 1226, 2418, 1298 and 850 DEGs at the imbibition saturation stage, and 1995, 1437, 1741 and 1555 DEGs at the radicle protrusion stage for LTS, HTS, DS and SS, respectively (Figure 3). These DEGs were considered as stress-responsive gmDEGs (srDEG) specific to each germination stage.

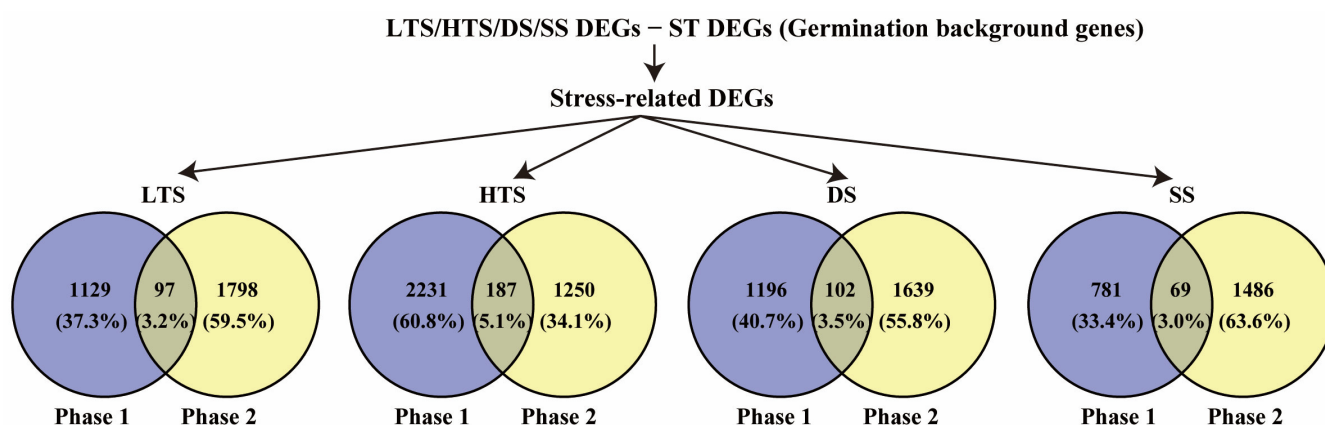


Figure 3. Venn diagram exhibiting stress-responsive differential expression genes (srDEGs) under low-temperature stress (LTS), high-temperature stress (HTS), drought stress (DS) and salinity stress (SS) at the imbibition saturation stage (stage 1) and radicle protrusion stage (stage 2).

To further gain insights into the functional pathways associating with the srDEGs, we performed Gene Ontology (GO) enrichment analysis. At the imbibition saturation stage, srDEGs from LTS were significantly enriched in terms related to nucleosome assembly (GO:0006334, $p = 0.027$), single-organism processes (GO:0044699, $p = 0.027$), and response to abiotic stimulus (GO:0009628, $p = 0.027$) (Figure 4A). In contrast, HTS srDEGs were associated with protein refolding (GO:0042026, $p = 1.1 \times 10^{-5}$), cellular detoxification processes (GO:1990748, $p = 3.0 \times 10^{-4}$), and cellular oxidant detoxification (GO:0098869, $p = 6.1 \times 10^{-4}$) (Figure 4A). For DS, enriched GO terms included response to inorganic substance (GO:0010035, $p = 0.016$), response to oxygen-containing compounds (GO:1901700, $p = 0.029$), and response to water deprivation (GO:0009414, $p = 0.04$) (Figure 4A). The SS srDEGs were mainly enriched in oxidation reduction (GO:0055114, $p = 6 \times 10^{-21}$), response to hormone stimulus (GO:0009725, $p = 1.0 \times 10^{-14}$), and cellular response to stress (GO:0033554, $p = 6.2 \times 10^{-11}$) (Figure 4A).

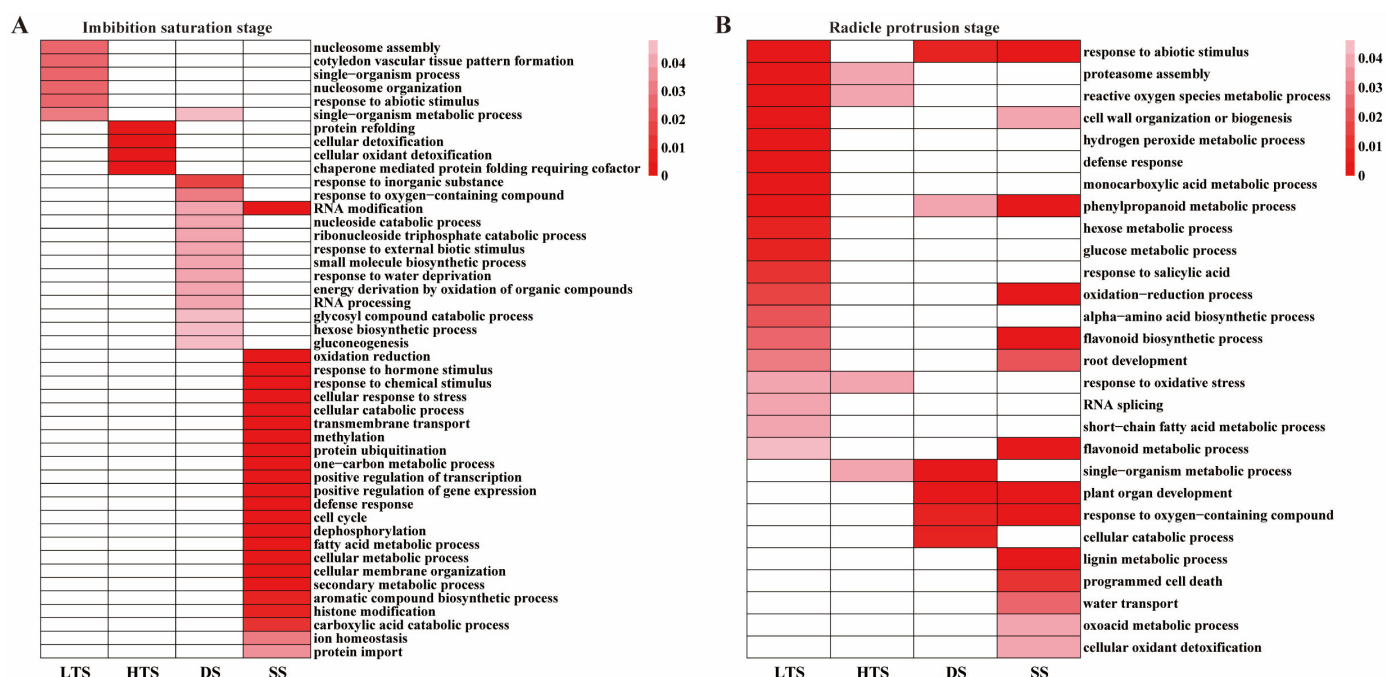


Figure 4. GO enrichment analysis of stress-responsive DEGs under low-temperature stress (LTS), high-temperature stress (HTS), drought stress (DS) and salinity stress (SS) at the imbibition saturation stage (A) and radicle protrusion stage (B). The color gradient indicates the level of statistical significance.

At the radicle protrusion stage, LTS srDEGs were significantly enriched for response to abiotic stimulus (GO:0009628, $p = 1.3 \times 10^{-7}$), proteasome assembly (GO:0043248, $p = 2.0 \times 10^{-5}$), and ROS metabolic processes (GO:0072593, $p = 3.9 \times 10^{-4}$) (Figure 4B). HTS srDEGs were also linked to ROS-related processes, including ROS metabolic processes (GO:0072593, $p = 0.04$) and response to oxidative stress (GO:0006979, $p = 0.04$). Meanwhile, DS srDEGs were significantly enriched in pathways relating to single-organism metabolic processes (GO:0044710, $p = 2.5 \times 10^{-3}$), plant organ development (GO:0099402, $p = 9.3 \times 10^{-4}$), and response to oxygen-containing compounds (GO:1901700, $p = 8.5 \times 10^{-3}$) (Figure 4B). SS srDEGs were mainly enriched in phenylpropanoid metabolic processes (GO:0009698, $p = 8.6 \times 10^{-8}$), plant organ development (GO:0099402, $p = 1.80 \times 10^{-4}$), and lignin metabolic processes (GO:0009808, $p = 6.8 \times 10^{-5}$) (Figure 4B). Notably, seeds activated a more extensive defensive response at the radicle emergence stage than at the imbibition stage, with greater enrichment of pathways re-

lated to programmed cell death, cell wall organization or biogenesis processes, and ROS metabolism (Figure 4B).

3.4. Identification of Common srDEGs in Response to Multiple Abiotic Stresses

To identify common genes responsive to multiple abiotic stresses, we compared the srDEGs across all four stresses. At the imbibition saturation stage, we identified 44 srDEGs responsive to all four stresses; these were classified as common stress-responsive DEGs (co-srDEGs) for further analysis (Figure 5A,C; Table S6). Concurrently, we identified 201 srDEGs responding to three stresses, with the largest subset (76 srDEGs) responding simultaneously to low-temperature, high-temperature and drought stresses, and the smallest number (33) responding simultaneously to low-temperature, drought, and salinity stresses (Figure 5A,C). A total of 966 srDEGs responded to two stresses (Figure 5A,C). Among them, the largest overlap (414 srDEGs) was between low-temperature and drought stresses, followed by 319 srDEGs common to high-temperature and salinity stresses (Figure 5A,C). In contrast, only 35 to 79 srDEGs were shared between other two stress conditions (low temperature and salinity, low temperature and high temperature, drought and high temperature, and drought and salinity) (Figure 5A,C). These results suggest that at the imbibition stage, the physiological mechanisms of response to low temperature and drought were more similar, while those of high temperature and high salinity were more similar. Finally, we identified 1563, 389, 214 and 460 srDEGs that were uniquely responsive to high-temperature, low-temperature, salinity and drought stresses, respectively (Figure 5A,C). The higher number of unique srDEGs for high temperature indicates that maize seeds' response to this stress is distinct from their response to the others.

At the radicle protrusion stage, we identified 235 co-srDEGs that responded to all four stresses (Figure 5B,D; Table S7). Notably, only one co-srDEG from this stage, *ZmJRL8* (Zm00001d035561) encoding a phytoagglutinin, overlapped with the co-srDEGs identified from the imbibition saturation stage (Figure S4). We also identified 482 srDEGs responding to three stresses, of which 243 responded to LTS, DS, and SS (Figure 5B,D). This suggests that the response mechanisms among these three stresses are relatively similar compared to those involving HTS. Furthermore, we identified 857 srDEGs responding to two stresses (Figure 5B,D) and 2073 srDEGs specific to a single stress, with 730, 485, 498 and 360 srDEGs unique to LTS, HTS, DS, and SS, respectively (Figure 5B,D). The higher number of unique srDEGs for LTS indicates that at the radicle protrusion stage, the response to this stress diverges from the response to the others.

To further characterize the co-srDEGs identified under the four stress conditions, we generated a heatmap using normalized expression values to visualize their expression patterns (Figure 6A). We observed that the 44 co-srDEGs at the imbibition saturation stage and the 235 co-srDEGs at the radicle protrusion stage tended to be significantly induced under one or two specific stresses, while showing only slight induction or even a decrease in expression under the remaining stress conditions (Figure 6A). To gain deeper insights into the functional pathways associated with these genes, we performed GO analysis to find that the 235 co-srDEGs at the radicle protrusion stage were mainly enriched in pathways related to various defense responses, oxidation–reduction process, secondary metabolic processes, cellular detoxification, and binding processes (Figure 6B). On the other hand, no pathway was statistically enriched according to the formal GO analysis for the 44 co-srDEGs of the imbibition saturation stage. However, through manual gene annotation, we found that these co-srDEGs were primarily involved in phytohormone response, detoxification and antioxidant defense, and cell wall modification (Table S6).

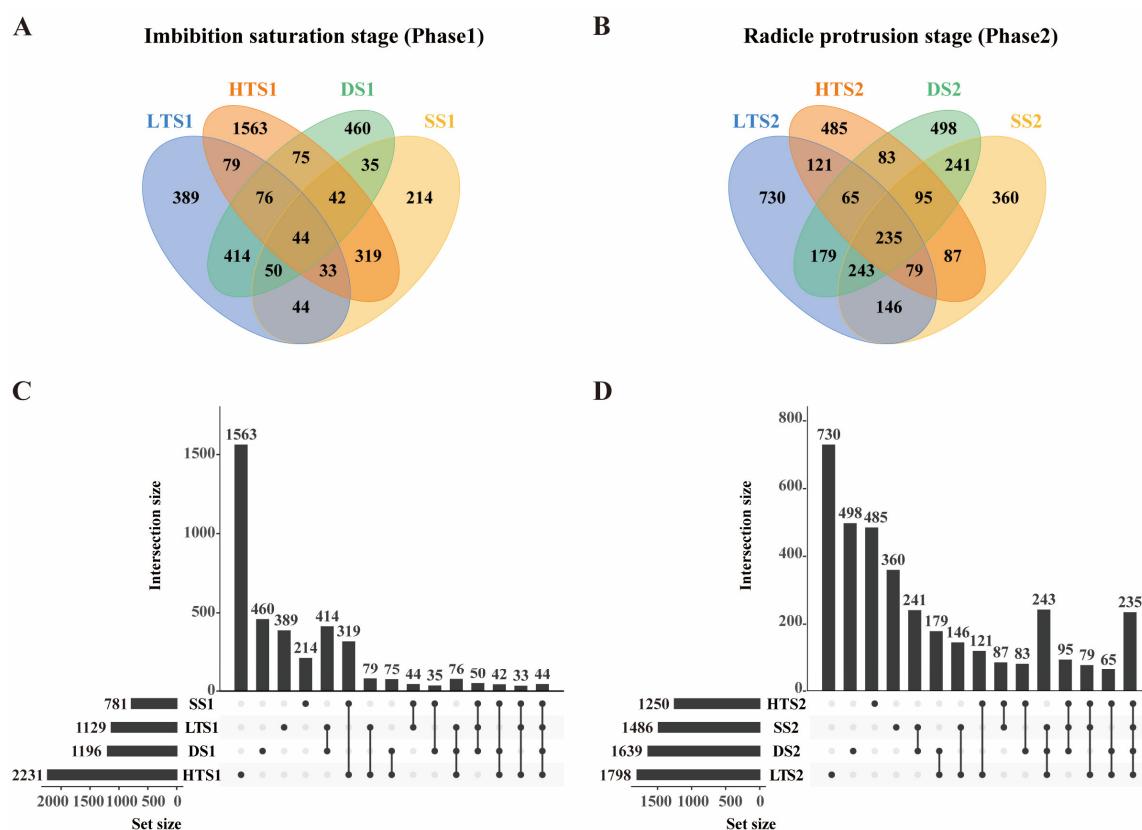


Figure 5. Venn diagram (A,B) and upset plots (C,D) show the number of stress-related DEGs in low-temperature stress (LTS), high-temperature stress (HTS), drought stress (DS), and salinity stress (SS) at the imbibition saturation (stage 1) and radicle protrusion stage (stage 2).

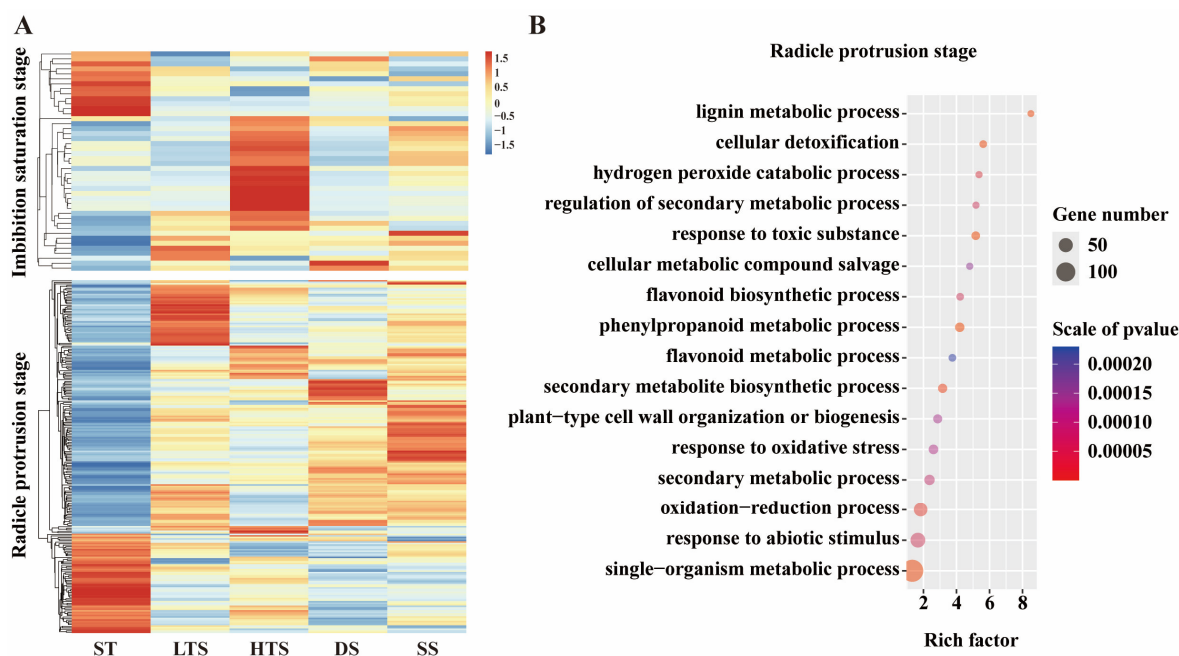


Figure 6. Common stress-responsive DEGs (co-srDEGs) across four abiotic stresses. (A) Expression profiles of the co-srDEGs in response to low-temperature stress (LTS), high-temperature stress (HTS), drought stress (DS), and salinity stress (SS) at the imbibition saturation stage and radicle protrusion stage. In the heatmap, red represents upregulation, while blue represents downregulation. (B) GO enrichment analysis of the co-srDEGs at the radicle protrusion stage. The different colors of the bubbles represent varying degrees of statistical significance.

3.5. Common Stress-Responsive Transcription Factors During Germination

TFs are regarded as central regulators for controlling downstream gene expression in response to stresses [46]. Among the 44 co-srDEGs at the imbibition saturation stage and the 235 co-srDEGs at the radicle protrusion stage, we identified 20 TFs belonging to 11 TF families. We designated these common stress-responsive TFs (co-srTF) (Figure 7A,B). Notably, members of the bHLH family were the most abundant, with six genes representing the highest proportion of the identified TFS (Figure 7A). To validate the reliability of transcriptome data, we randomly selected four co-srTFs for qRT-PCR analysis (Figure 7C). The results showed that the expression pattern of these genes was highly consistent with the their corresponding FPKM values from the transcriptome data across all conditions of standard germination: LTS, HTS, DS and SS (Figure 7C).

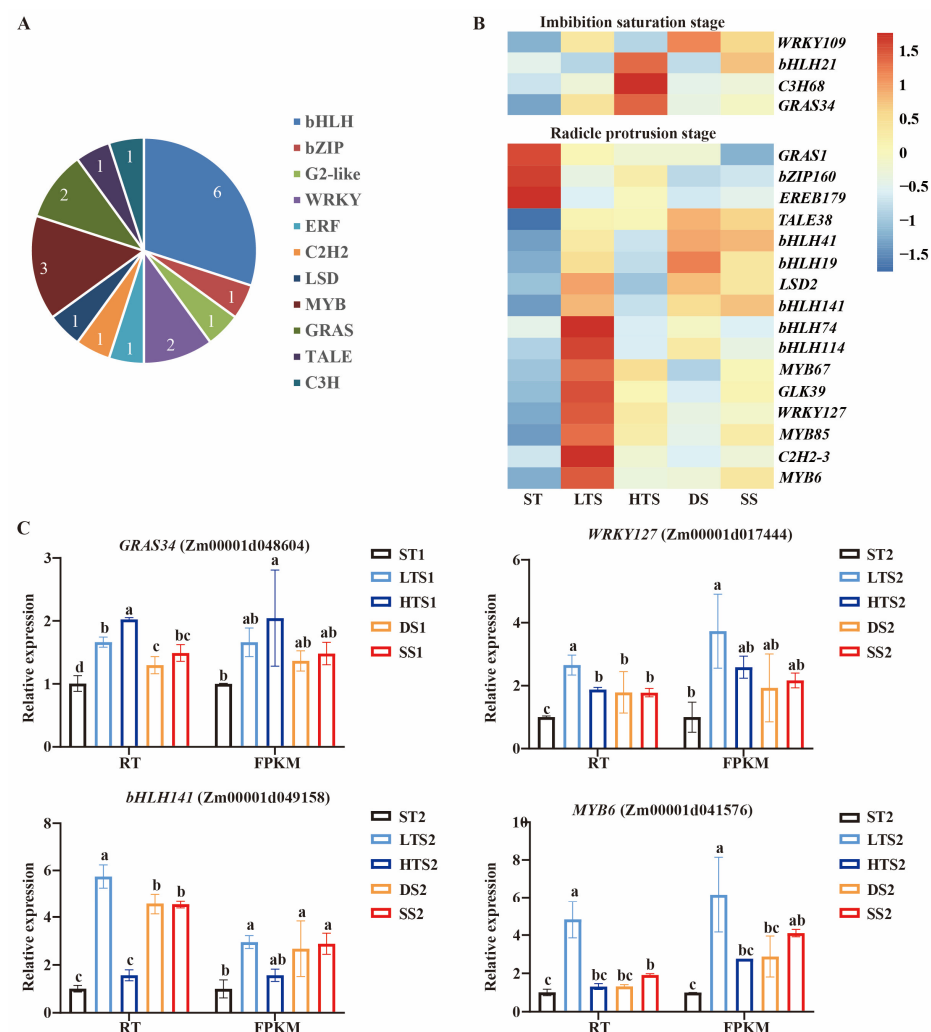


Figure 7. Common stress-responsive transcription factors (co-srTFs) across four abiotic stresses. (A) Gene family classification of the identified co-srTFs. (B) Expression profiles of the co-srTF genes in response to low-temperature stress (LTS), high-temperature stress (HTS), drought stress (DS), and salinity stress (SS). Standard germination (ST) served as the non-stress control. Data are from the transcriptome sequencing, where red represents upregulation and blue represents downregulation. (C) Relative expressions levels of four co-srTF genes in response to the four stresses, which were confirmed by RT-qPCR analysis (RT) to validate the RNA sequencing data. Log₂-transformed FPKM values and $-\Delta\Delta C_t$ values were normalized against the ST control. Data are presented as means $\pm$ SDs ($n = 3$ biological replicates). Different lowercase letters on the bars indicate statistically significant differences ($p < 0.05$ according to Duncan's multiple range test using SPSS Statistics 26.0).

4. Discussion

4.1. ROS Signaling Plays a Vital Role in Multiple Stress Tolerance During Germination

ROS are essential signaling molecules that initiate seed germination by maintaining ROS homeostasis and modulating dormancy status [47–49]. Additionally, ROS production is a central signaling hub in response to abiotic stresses, regulating oxidative signaling to influence metabolic and defense pathways [50–52]. However, understanding of ROS function during germination under multiple abiotic stresses remains limited. In this study, we identified 44 and 235 co-srDEGs responsive to all four stresses at the imbibition saturation and radicle protrusion stage, respectively. GO analysis revealed that the co-srDEGs at the radical protrusion stage were enriched in ROS-producing and ROS-scavenging pathways (Figure 6). At the imbibition saturation stage, functional annotation associated two co-srDEGs with detoxification and antioxidant defense. These findings indicate that ROS pathways play essential roles during seed germination under multiple stresses, particularly at the radical protrusion stage.

When seeds take up water in adverse environments, ROS are produced in the mitochondria of embryo cells during the early stages and in the plasma membrane during the late stages [49]. Mitochondria are not only the energy-supplying organelles for germination; they also act as intracellular systems that generate mitochondrial ROS (mit-ROS) for sensing environmental signals [49]. In germinating seeds, ROS originate from the mitochondrial electron transfer chain (mtETC) and respiratory burst oxidase homologues (RBOH) [49,53,54]. While the mtETC pathway is known to be involved in early germination, reports on mtETC genes regulating stressed germination are scarce [49,54]. In the model plant *Arabidopsis*, *RbohD* expression is induced by salinity to inhibit germination via ROS accumulation [54]. In this study, we found that at the radicle protrusion stage, dozens of co-srDEGs involved in ROS production responded to all four stresses (LTS, HTS, DS, and SS). These included ubiquinone oxidoreductase core subunit (NDUF) genes *NDUFS1*, *NDUFA9*, *NDUFA1* (encoding components of mitochondrial Complex I) and *RBOH* (encoding nicotinamide adenine dinucleotide phosphate (NADPH) oxidase) (Figure 6, Table S7). These genes showed significantly elevated expressions under stresses at the radical protrusion stage (Figure 6), indicating that they may specifically influence ROS levels during germination under multiple abiotic stresses.

Environmental stress markedly elevates ROS levels in plants [50]. To maintain ROS within physiological range, plants have evolved enzymatic and non-enzymatic mechanisms to efficiently scavenge excess ROS, thereby maintaining ROS homeostasis and alleviating ROS damage [51,52]. The enzymatic mechanism primarily relies on the functions of a series of antioxidant enzymes such as SOD, CAT, POD, ascorbate peroxidase (APX), and glutathione peroxidase (GPX) [55]. SOD catalyzes the conversion of superoxide to H_2O_2 and O_2 , while POD or CAT subsequently converts H_2O_2 into H_2O [55]. Previous studies have shown that overexpression of genes related to ROS scavenging significantly improves stress tolerance [54,56]. We identified 9 POD encoding genes with significantly elevated expressions among the 244 co-srDEGs at the radicle protrusion stage (Table S7), indicating that POD-mediated ROS scavenging may play a central role in maintaining ROS homeostasis during stressed germination.

Upon sensing stress, plant cells trigger ROS production, which activates secondary signaling pathways involving Ca^{2+} and phytohormones [47]. ABA and GA are the primary hormones regulating germination, often inhibiting or promoting the processes by modulating ROS accumulation [57,58]. Some studies have shown that ROS signaling interacts with these hormone pathways during germination [54,57,59]. For example, the *Abscisic Acid-Insensitive 4-RbohD/Vitamin C Defective 2* module inhibits germination under salinity stress in *Arabidopsis* [54], while *OsRbohB*-mediated ROS production interacts with ABA

signaling to increase seed weight and germination in rice under drought [59]. Although we did not identify ABA signaling genes among the 244 co-srDEGs, their involvement cannot be ruled out. Recent studies have shown that indole-3-acetic acid (IAA) and JA signaling genes can enhance the expression of ABA synthesis and signaling genes to promote ROS-scavenging and increase salinity and drought stress tolerance during germination [60]. Interestingly, we identified 4 IAA and JA signaling genes among the co-srDEGs (Figure 6), which may provide insights into the crosstalk between ROS and hormone signaling under stressed germination.

4.2. Potential Transcription Factors Regulating Multiple Stress Tolerance in Germinating Maize Seeds

Intracellular signaling pathways (e.g., Ca^{2+} , ROS, and hormones) transmit extrinsic stress signals from the cell wall and cell membranes to TFs in the nucleus [61]. In crops, six major TF families—WRKY, MYB, DREB, bZIP, ABF and NAC—are primarily involved in stress tolerance by binding to the promoters of stress-responsive genes [62]. In this work, we found that the co-srTF included members of the WRKY (two DEGs), MYB (three DEGs) and bZIP (one DEG) families, which were simultaneously regulated by four stresses at either the imbibition stage or radical protrusion stage (Figure 7) and may have as candidate genes governing seed germination under multiple abiotic stresses.

Recent studies highlight the roles of the WRKY family in multi-stress tolerance, such as the *AtWRKY46* regulating lateral root development under salinity stress in Arabidopsis [63] and the *TaWRKY76* regulating root morphology under drought and salinity stresses in wheat [64]. Among the four co-srTFs identified at the imbibition saturation stage, we found *WRKY109*, which has been reported to associate with stress response by reducing nickel toxicity in maize [65], showing higher expressions under drought and salinity stresses than under other stresses during germination (Figure 7). These expression patterns suggested that this TF responds to drought and salinity stresses. Furthermore, STRING database analysis suggests *WRKY109* may interact with a GAI-RGA-and-SCR (GRAS) transcription factor identified in our co-srTF set at the imbibition saturation stage (Figure S5); exploring this relationship will be a priority for future studies.

At the radicle protrusion stage, *MYB6* and *MYB85* displayed more transcript amounts under LTS, indicating a potential function in responding to cold stress (Figure 7). While protein predictions for *MYB6* and *MYB48* yielded an unknown protein, the Arabidopsis homolog of *MYB6*, *AtMYB59*, has been reported to regulate root elongation and stress responses by negatively regulating of calcium signaling [66]. Since calcium signaling is a vital factor in multi-stress response [67], this provides clues for further investigation on the functions of these two MYBs.

Notably, we found that bHLH TF family was the largest co-srTF family during germination, with one gene at the imbibition saturation stage and five at the radicle protrusion stage (Figure 7). Specifically, *bHLH41* and *bHLH19* were highly activated under drought and salinity stresses, while *bHLH141* was more responsive to cold and salinity stresses (Figure 7B). As bHLH transcription factors often form homodimers or heterodimers to function together [68], verifying their interaction relationships and downstream targets for these six co-srTFs is a worthwhile future endeavor. We also analyzed the binding elements in the promoters of hormone- and ROS-related co-srTFs and found that most of them contained binding elements for WRKY, MYB, and bHLH transcription factors (Figure S6), which provides a foundation for further studies on their regulatory relationships.

In summary, we propose a regulatory model (Figure 8) in which various abiotic stresses trigger ROS production in germinated embryo cells. This likely activates ROS signaling that interacts with the IAA and JA hormone pathways, subsequently modulating TF-mediated transcriptional networks and downstream metabolism to enhance stress tolerance during

seed germination. These findings expand our understanding of distinct stress responses and provide potential co-srTF and their underlying regulatory mechanisms for maize seed germination under multiple abiotic stresses.

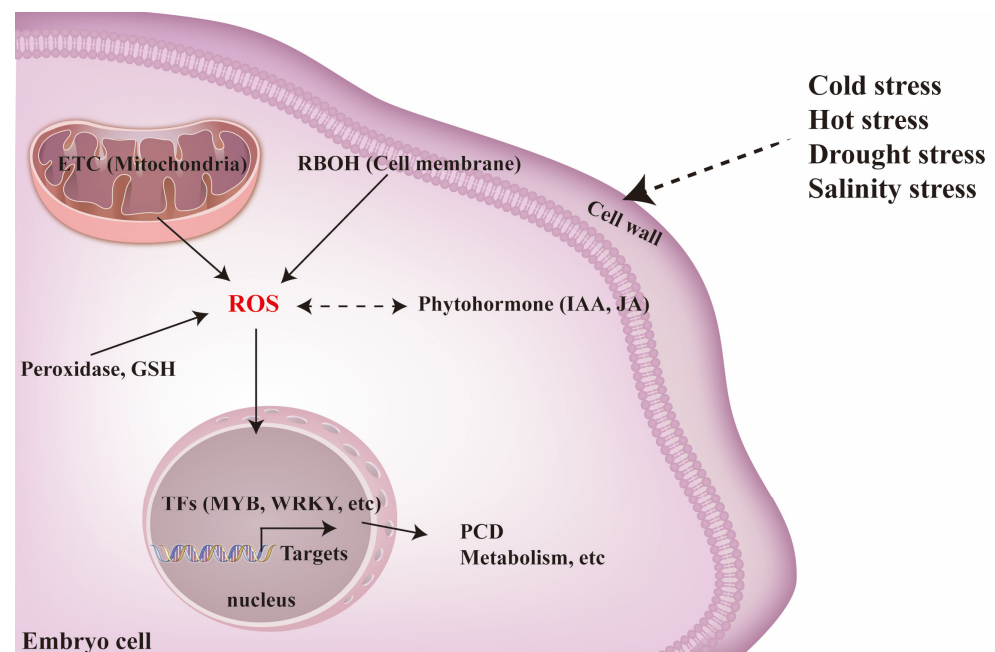


Figure 8. A proposed model for ROS signaling mediated by stress-responsive transcription factor (TF) networks during seed germination (drawn using Figdraw 2.0 and Adobe Illustrator 2023 software). Under abiotic stresses, ROS signaling interacts with the IAA and JA pathways to activate downstream TFs regulation, metabolic pathways, and other physiological events, thereby enhancing the stress tolerance of germinating seeds. The arrows indicate the possible regulatory relationships.

5. Conclusions

We screened 44 and 235 co-srDEGs across four abiotic stresses (LTS, HTS, DS, and SS) at two germination stages: the imbibition saturation stage and radicle protrusion stage. These co-srDEGs are likely involved in activating ROS and hormone signal pathways, which in turn modulate TF-mediated regulatory networks and downstream metabolic processes, thereby enhancing multiple stress tolerance during seed germination. These findings not only provide essential candidate genes but also deepen our understanding of the regulatory mechanisms commonly activated across multiple stresses during maize seed germination.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy16010040/s1>, Figure S1: Correlation between biological replicates; Figure S2: Volcano plot representing the differentially expressed genes (DEGs) from embryos collected for the imbibition saturation stage and the radicle protrusion stage under standard treatment (ST), low-temperature stress (LTS), high-temperature stress (HTS), drought stress (DS), and salt stress (SS), respectively; Figure S3: Venn diagram representing the differentially expressed genes (DEGs) from embryos collected under standard treatment (ST), low temperature stress (LTS), high temperature stress (HTS), drought stress (DS) and salt stress (SS) at imbibition saturation (BA) and radicle protrusion (CA) stages; Figure S4: Stage common (imbibition saturation and radicle protrusion stage) DEGs responding to low-temperature stress (LTS), high-temperature stress (HTS), drought stress (DS) and salt stress (SS); Figure S5. Predicting the interacting proteins of WRKY109 through the STRING database; Figure S6. Predicting the binding elements in the promoters of hormone- and ROS-related co-srTFs for WRKY, MYB, and bHLH transcription factors using the FIMO database; Table S1. Primers used in this study; Table S2: Summary of transcriptome sequencing;

Table S3: Genes expressed at two stages of germination in maize embryos under four stresses; Table S4: Germinating DEGs (gmDEG) found by comparing germinated embryos with dry embryos under four treatments; Table S5: Expression of DEGs at two stages of germination in maize embryos under four stresses; Table S6: Expression of common stress-related DEGs under four stresses at the imbibition saturation stage; Table S7: Expression of common stress-related DEGs under four stresses at the radicle protrusion stage.

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Data Availability Statement: The data that support the findings of this study are available at <https://ngdc.cnca.ac.cn/> with accession number subCRA048719 (uploaded on 9 September 2025 and released on 22 December 2025).

Conflicts of Interest: The authors declare no conflict of interest.

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