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Comparative Genomic and Transcriptomic Analysis Uncovers Metabolic Mechanisms Underlying Drought Tolerance Variation in Maize

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Abstract

Drought stress severely limits maize (*Zea mays* L.) productivity worldwide, yet the molecular mechanisms underlying natural variation in drought tolerance remain poorly understood. We conducted a comprehensive comparative analysis using transcriptome sequencing (RNA-seq) and whole-genome resequencing of two inbred maize lines with contrasting drought tolerance: drought-tolerant line A193 and drought-sensitive line MP23. Under drought stress, A193 exhibited superior photosynthetic performance and an 89% survival rate compared to only 11% for MP23. Transcriptome analysis identified substantial gene expression differences, with 7279 and 5991 differentially expressed genes (DEGs) between the two genotypes under control and drought conditions, respectively. Whole-genome resequencing identified 5,306,884 single-nucleotide polymorphisms and 1,133,400 insertions/deletions between the two lines. Integration of transcriptomic and genomic data revealed 2050 DEGs exhibiting genomic variations (DEVGs). Functional enrichment analysis revealed significant enrichment in starch and sucrose metabolism, benzoxazinoid biosynthesis, and amino acid metabolism pathways. Thirty DEVGs were identified in starch and sucrose metabolism, with 15 genes upregulated in A193, including beta-amylase, sucrose synthases, and starch synthase. Six DEVGs in benzoxazinoid biosynthesis showed stress-protective upregulation in A193. Additionally, 14 DEVGs in amino acid metabolism displayed genotype-specific expression patterns. Our findings demonstrate that superior drought tolerance in A193 is associated with enhanced metabolic reprogramming. Prioritized drought tolerance genes may provide direct targets for functional investigation or allelic mining.

Keywords: maize; drought stress; transcriptome; whole-genome resequencing; starch and sucrose metabolism

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1. Introduction

Maize (*Zea mays* L.), native to Central America, is widely cultivated in tropical and temperate regions of the world [1]. It has become the largest food crop in China, accounting for 23% of total national grain production [2]. However, maize is often affected by abiotic stresses such as drought, salinity, heat, and low temperature during its growth.

These stresses adversely affect plant growth and development. For example, drought stress induces a variety of adverse symptoms that inhibit the growth and development of maize and reduce biomass, leaf area, photosynthetic capacity, and nutrient uptake and utilization, thereby ultimately affecting final yield [3–5]. Drought is one of the major obstacles to increasing maize production. Studies show that maize yield can suffer losses ranging from 30% to 90% under drought stress, depending on its intensity, duration, and the crop growth stage [6]. The drought-resistant alleles *ZmSRO1d*-R, *ZmRtn16*, and *ZmVPP1* contribute to drought resistance in maize and provide an important resource for strengthening drought resistance [7–9]. Despite a diverse array of genetic resources existing in maize [8], the molecular and genetic mechanisms of natural variation in maize drought tolerance are largely unknown. Therefore, it is important to understand the molecular mechanisms of drought tolerance and to explore dominant variations in drought tolerance. Water-saving irrigation is a primary strategy adopted during drought periods, and the breeding of drought-tolerant varieties is a supportive measure that aids in its implementation [10]. Thus, enhancing drought tolerance in maize is a promising strategy for breeders to increase productivity.

Plant responses to drought stress involve complex physiological and molecular adaptations that span across multiple biological scales, from cellular metabolism to whole-organism morphology [11–13]. Physiologically, drought-tolerant plants typically exhibit improved water use efficiency, enhanced osmotic adjustment, and maintained photosynthetic capacity under water deficit conditions [14–16]. These adaptive responses are coordinated by intricate transcriptional regulatory networks involving stress-responsive transcription factors, phytohormone signaling, and metabolic reprogramming [17–19]. Maize employs diverse mechanisms to combat water scarcity, primarily regulated at the molecular level. Abscisic acid (ABA) signaling plays a central role: studies have highlighted genes like *ZmDREB2.7*, *ZmNAC111*, and *ZmPTPN* positively influencing drought tolerance, often by regulating ABA-responsive pathways, enhancing water usage, or coordinating antioxidant production [20–22]. Root architecture, crucial for water uptake, is modulated by genes such as *ZmDRO1*, which influences root growth angle, and *ZmVPP1* and *ZmTIP1*, promoting root development and hair elongation, respectively [9,23,24]. Stomatal closure, a short-term avoidance strategy, is regulated by such genes as *ZmSRO1d*, promoting reactive oxygen species (ROS) accumulation in guard cells to limit water loss [7]. Furthermore, mechanisms involve modulating cuticular wax biosynthesis (e.g., *ZmGL6*, *ZmEREB46*, *ZmFDL1*, *ZmSRL5*) to reduce non-stomatal transpiration and adjustment of flowering time (e.g., *ZmEXPA4*) to escape drought during reproductive stages [25–29]. These advancements, largely propelled by multiomics and genetic dissection, provide key molecular targets for enhancing maize drought resilience.

Recent advances in high-throughput sequencing technologies have facilitated comprehensive transcriptomic profiling of drought-responsive gene expression patterns, uncovering the intricate molecular mechanisms that underlie stress tolerance [30]. Transcriptome analysis has emerged as a powerful approach for dissecting the molecular basis of drought tolerance in crops, providing insights into differentially expressed genes (DEGs), regulatory pathways, and metabolic networks activated under stress conditions [31–34]. In the analysis of plant responses to abiotic stress through RNA sequencing, de novo assembly strategies are instrumental in uncovering species-specific transcripts and novel genes, whereas reference genome-based approaches yield more precise gene expression quantification and alternative splicing analysis. Integration of the two provides comprehensive insights into the molecular mechanisms of stress adaptation [35,36]. However, most studies have focused on single genotypes or limited

comparisons, leaving gaps in our understanding of genotype-specific drought response mechanisms that underlie natural variations in stress tolerance. Furthermore, the integration of transcriptomic data with other omics levels—such as metabolomics—is critical for constructing a holistic view of plant stress responses [37]. For instance, integrated analyses have revealed that drought stress triggers the coordinated upregulation of key biosynthetic pathways (e.g., flavonoids) and their underlying regulatory genes, which are crucial for antioxidant capacity and osmotic adjustment [33]. Recent bioinformatic advances, particularly co-expression network analysis (e.g., WGCNA), leverage these multiomics datasets to identify central hub genes and functional modules controlling complex traits like drought tolerance [38].

Metabolic reprogramming is a central mechanism in plant drought adaptation, with carbohydrate metabolism being particularly important for maintaining cellular homeostasis under water stress [39,40]. Starch and sucrose metabolism pathways are crucial for osmotic adjustment, energy provision, and signaling during drought stress, with differential regulation patterns of these pathways often distinguishing drought-tolerant from drought-sensitive genotypes [41–43]. Similarly, secondary metabolite biosynthesis, including benzoxazinoid production, and amino acid metabolism pathways contribute to stress tolerance by mediating cellular protection and metabolic flexibility [44,45]. Despite significant progress in understanding drought responses in maize, the molecular mechanisms underlying natural variation in drought tolerance remain incompletely characterized. Most studies have focused solely on either transcriptional responses or genetic variations, limiting our ability to identify functionally relevant polymorphisms that contribute to stress tolerance differences. Furthermore, the specific metabolic pathways and regulatory networks that distinguish drought-tolerant from drought-sensitive genotypes require further elucidation for targeted breeding strategies.

To understand the molecular mechanisms governing drought tolerance in maize, we performed a comprehensive analysis of transcriptome and whole-genome resequencing in two inbred maize lines (A193 and MP23) with contrasting drought tolerance. This study aimed to clarify genes with distinct variations at both transcriptomic and genomic levels and uncover key metabolic pathways in response to drought in maize.

2. Materials and Methods

2.1. Plant Materials

Two inbred maize lines with contrasting drought tolerance, the drought-tolerant line S4·4A (A193, from Tang SPT germplasm) and the drought-sensitive line NX110 (MP23, from Yugoslavia germplasm) were used in this study [46]. Maize plants were soil-cultivated in a phytotron (14 h light, 28 °C and 10 h night, 26 °C; 80% humidity) at Yangzhou University. Briefly, maize seeds were soaked in saturated anhydrous $CaSO_4$ and incubated overnight to promote germination. To evaluate drought tolerance, once the radicle (root) reached approximately 1 cm in length, the germinated seeds were sown in white plastic boxes (length × width × depth: 30 cm by 20 cm by 10 cm) filled with 3900 g of mixed substrate (soil–nutrient solution ratio of 5:1). The nutrient solution contained urea (1.087 g/L), potassium fertilizer (0.0932 g/L), and potassium dihydrogen phosphate (3.289 g/L). Six germinated seeds were planted on one side of each white plastic box, and consistent spacing between plants in each row was maintained.

2.2. Drought Treatment

Seedlings of A193 and MP23 were subjected to well-watered (CK) and drought stress (Dr) treatments. Under CK conditions, seedlings were irrigated with 300 mL of water every two days to maintain optimal soil moisture at approximately 20%. Under

Dr conditions, seedlings were irrigated with 300 mL of water on the fifth and seventh days after sowing, and watering was subsequently discontinued. The third fully expanded leaves were collected from the seedlings when the soil moisture content was 10% (13 days after sowing). The collected leaves were immediately snap-frozen in liquid nitrogen and stored at −80 °C for subsequent transcriptome analyses. Each treatment was conducted with three biological replicates. Water was resumed to allow plants to recover, when there was obvious difference in the wilting degree of the leaves between the two lines (21 days after sowing). At this point, the drought-tolerant line A193 maintained relative vigor, exhibiting only mild wilting with leaves largely retaining green coloration and uprightness. In stark contrast, the sensitive line MP23 displayed severe wilting phenotypes, characterized by more than 50% plants exhibiting extensive leaf curling, desiccation, and browning (Supplementary Figure S1). Survival rates were evaluated two days post-rewatering (23 days after sowing).

2.3. Determination of Plant Growth and Environmental Factors

Soil water content was measured every 2 days within 23 days after sowing using a portable handheld FDR soil moisture meter (Hengmei Technology Co., Ltd., Kunshan, China), and seedlings were also photographed to document morphological changes. Fifteen days after sowing, net photosynthesis rate (NPR), stomatal conductance (SC), and transpiration rate (TR) were measured on the third fully expanded leaf using an LI-6800 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). Water use efficiency (WUE) is defined as the ratio between NPR and TR.

2.4. Transcriptome Analysis

For transcriptome analysis, total RNA was extracted from 12 samples, which included both genotypes (A193 and MP23) under CK and Dr treatments, as described above. For library construction, cDNA-sequencing libraries were prepared from 1.5 μg of total RNA per sample using a TruSeq paired-end mRNA-seq kit (Illumia, San Diego, CA, USA). The process included mRNA purification, fragmentation, cDNA synthesis, adapter ligation, and library amplification. Sequencing was performed on an Illumina Nova Seq platform (Shanghai Personal Biotechnology Co., Ltd., Shanghai, China). Raw reads were filtered using Trim-galore (version 0.6.10; Babraham Bioinformatics, Cambridge, UK) to obtain clean reads. HISAT2 (version 2.1.1; Johns Hopkins University, Baltimore, MD, USA) was used to map the clean reads to the maize reference genome (Zm-B73-REFERENCE-NAM-5.0) [47]. FeatureCounts (version 2.0.1; Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) was used to count the read counts mapped to each gene. Gene expression levels were quantified using fragments per kilobase of transcript per million mapped reads (FPKM) [48]. Correlation analysis was performed using Pearson correlations in the R package (version 2.5.6) "psych." To identify DEGs associated with drought response and genotypic differences, we performed pairwise comparisons among the four groups: CK-A193 vs. CK-MP23, CK-A193 vs. Dr-A193, CK-MP23 vs. Dr-MP23, and Dr-A193 vs. Dr-MP23. DEGs were identified using the DESeq2 package (version 1.48.2) in R (version 4.4.0), with selection criteria of $|\log_2(\text{fold change})| > 1$ and q-value < 0.05 [49]. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed with OmicShare tools (https://www.omicshare.com/tools; accessed on 11 March 2025).

2.5. Genome Resequencing

Genomic DNA extracted from young leaves of A193 and MP23 was sequenced on the DNBSEQ-T7 platform (BGI Group, Shenzhen, China), achieving average coverage of ~13.0 and 7.5-fold, respectively. Initial processing of the raw sequencing reads involved

adapter trimming and removal of low-quality bases using Trim Galore (version 0.6.10; Babraham Bioinformatics, UK). The resulting high-quality reads were then mapped to the reference genome Zm-B73-REFERENCE-NAM-5.0 using the Burrows–Wheeler Aligner (BWA, version 0.7.17-r1188; Wellcome Sanger Institute, Hinxton, UK). Alignment files (BAM format) were subsequently sorted and indexed with SAMtools (version 1.6; Wellcome Sanger Institute, UK). Single-nucleotide polymorphism (SNP) detection was performed using a Genome Analysis Toolkit (GATK, version 4.2.6.1; Broad Institute, Cambridge, MA, USA), using HaplotypeCaller to generate genomic variant call format (GVCF) for each sample, followed by using CombineGVCFs and GenotypeGVCFs to produce a unified variant call format (VCF) file. Finally, the identified variants underwent filtering using VariantFiltration (version 4.0.3.0). Annotation of variations was conducted using SnpEff (version 5.1d).

2.6. Quantitative Real-Time PCR (qRT-PCR) Assay

To corroborate the transcriptome profiling data, the expression patterns of four candidate genes were experimentally validated by qRT-PCR with primers specifically designed for each target (Supplementary Table S1). Leaf samples were collected at 13 days after sowing, with three independent biological replicates included for each time point. Total RNA was isolated with an RNAsimple Total RNA Kit (Tiangen, Beijing, China) and treated with gDNA wiper Mix (Vazyme, Nanjing, China) to remove genomic DNA contamination. First-strand cDNA was synthesized using qRT SuperMix II (Vazyme). Amplification was carried out on a CFX96 Real-Time PCR Detection System (Bio-Rad, Munich, Germany). ZmTubulin1 was used as the internal reference gene, and relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

2.7. Statistical Analysis

Statistical analyses were performed using Microsoft Excel (version 16.0; Microsoft Corporation, Redmond, WA, USA). The significance of differences was evaluated using Student's *t*-test. GraphPad Prism (version 8.0.2; GraphPad Software, San Diego, CA, USA) was used for data visualization, and the results are presented as means ± standard error of the mean (SEM) from biological replicates.

3. Results

3.1. Comparison of Drought Tolerance Between the Two Inbred Maize Lines Under Different Water Supply Conditions

To elucidate differential responses to drought stress, we examined morphological changes in the two inbred maize lines with contrasting drought tolerance. The drought-tolerant A193 line consistently exhibited superior growth compared to the sensitive MP23 line, which displayed significant leaf drooping, wilting, and curling observable from 15 days after sowing (Figure 1A). When a marked disparity in leaf wilting severity was observed between the two inbred maize lines at 21 days after sowing (Figure 1A), a rehydration protocol was implemented. After a 2-day recovery (Figure 1A), MP23 showed limited recovery and sustained severe damage compared to A193. Under Dr, A193 achieved an 89% survival rate, markedly surpassing the 11% observed in MP23 ($p = 0.01$, Figure 1B). Collectively, the phenotypic evidence and survival rate data confirm a pronounced difference in drought tolerance between A193 and MP23.

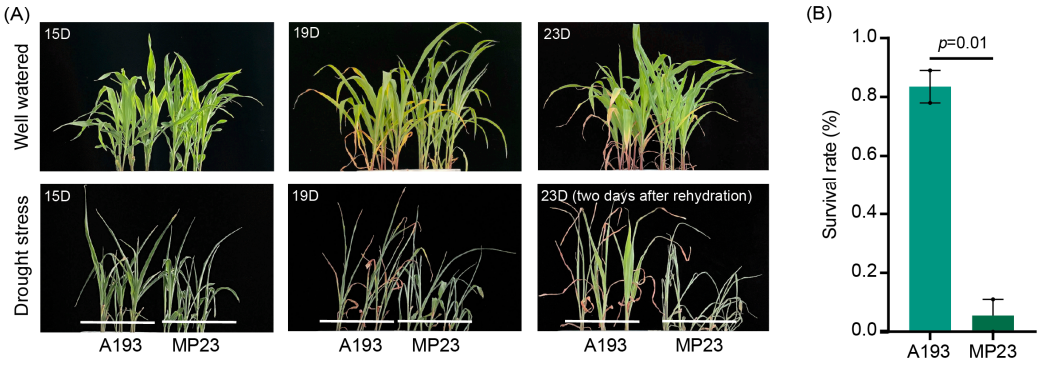

Figure 1. Analysis of drought-resistant phenotypes of A193 and MP23. (**A**) A193 and MP23 under drought stress. A193 and MP23 were placed in the same box to observe and compare them under well-watered (CK) and drought stress (Dr) conditions. Photographed on days 15, 19 and 23 after sowing. (**B**) A193 and MP23 were rehydrated on the 21st day after sowing, The survival rate of A193 and MP23 under Dr was counted two days after rehydration (23 days after sowing). Student's *t*-test was used for statistical analysis.

3.2. Photosynthetic Responses and Water Use Efficiency of Inbred Maize Lines Under Drought Stress

To elucidate the physiological basis underlying the differential drought tolerance between A193 and MP23, we compared their photosynthetic performance under CK and Dr conditions (Figure 2). Under CK conditions, there was no significant difference in NPR between the two inbred maize lines (Figure 2A). However, drought stress led to a significant decline in photosynthetic capacity for both lines. The NPR of A193 decreased by approximately 19%, whereas MP23 exhibited a dramatic 72% reduction (p = 0.0451, Figure 2A). Under CK conditions, the SC of MP23 was significantly higher than in A193 ($p = 2.42 \times 10^{-5}$, Figure 2B). Drought stress caused marked reductions in SC for both lines, with MP23 experiencing an 85% reduction compared to A193's 53% (p = 0.0521, Figure 2B). The TR also differed significantly between two lines under CK conditions, with MP23 exhibiting approximately 39% higher transpiration than A193 ($p = 1.50 \times 10^{-5}$, Figure 2C). Drought stress resulted in substantial reductions in TR for both lines, with MP23 showing a more pronounced relative decrease (p = 0.0502, Figure 2C). Notably, WUE showed contrasting responses between two lines. Under CK conditions, A193 displayed significantly higher WUE than MP23 ($p = 1.94 \times 10^{-5}$, Figure 2D). Drought stress induced an increase in WUE in both lines, with A193 consistently maintaining higher WUE values than MP23 under both CK and Dr conditions ($p = 1.19 \times 10^{-5}$, Figure 2D). Collectively, these results demonstrate that A193 possesses superior photosynthetic performance and water use efficiency under Dr conditions, contributing to its enhanced drought tolerance.

3.3. Transcriptome Analysis of the Two Inbred Maize Lines Under Different Water Supply Conditions

To elucidate the molecular mechanisms underlying the differential drought tolerance between A193 and MP23, we performed RNA-seq analysis on the third fully expanded leaves from both inbred lines under CK and Dr conditions. A total of 11 samples were sequenced (one biological sample for A193 under Dr is missing due to the low quality of RNA), generating 72.98 Gb of clean high-quality data. The Q30 base percentage surpassed 96.72% across all samples, and GC content ranged from 50.84% to 53.44%. A mapping rate of 86.50% to 89.94% was achieved when aligning the clean reads to the B73 reference genome (Zm-B73-REFERENCE-NAM-5.0) (Supplementary Table S2). Quality assessment revealed high reproducibility among biological replicates, with Pearson correlation coefficients

ranging from 0.96 to 0.99 (Figure 3A). Principal component analysis (PCA) based on gene expression profiles (FPKM values) distinctly separated four experimental groups, with PC1 and PC2 explaining 29.7% and 26.1% of the total variance, respectively. The PCA plot in Figure 3B demonstrates the tight clustering of biological replicates within each group and clear distinction between different treatments and genotypes.

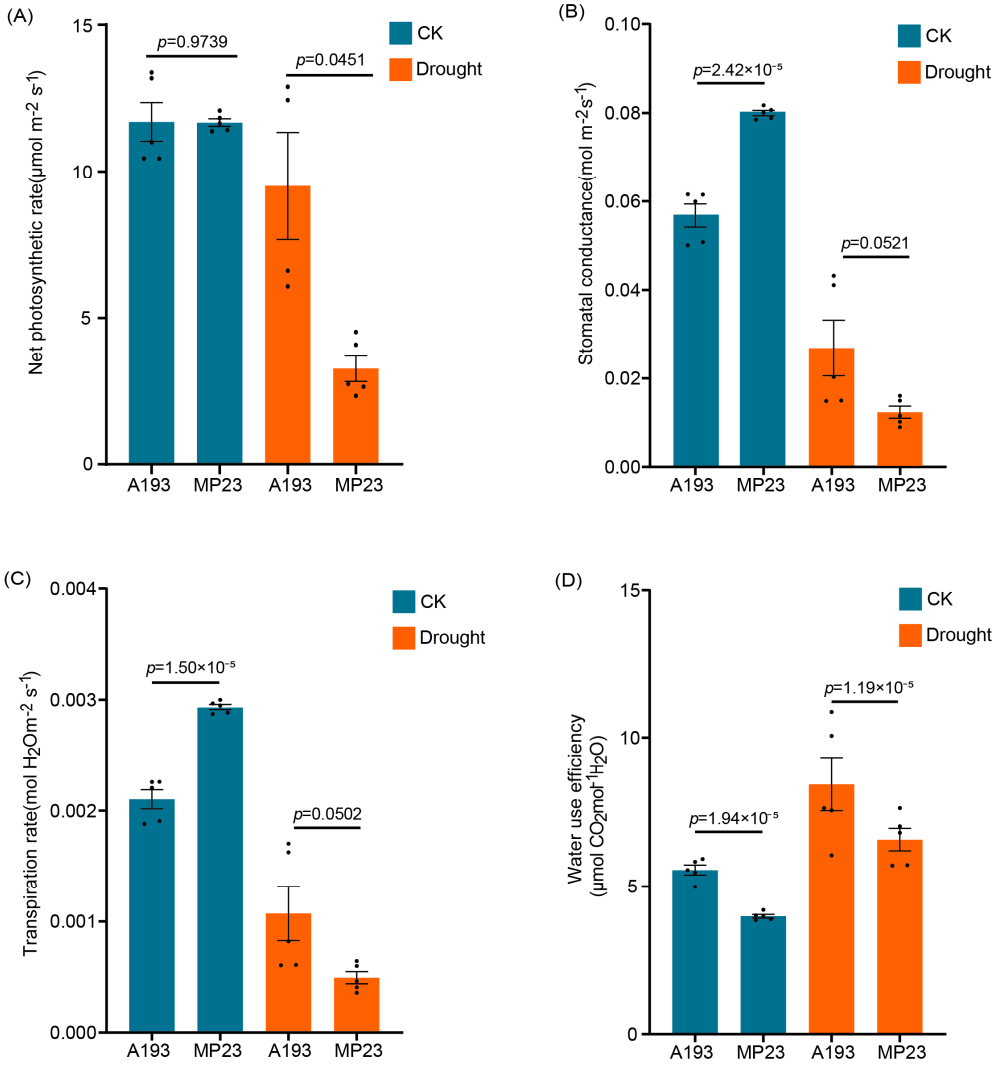

Figure 2. Photosynthetic physiological indexes and water use efficiency of A193 and MP23 under CK and Dr conditions. (**A**) Net photosynthetic rate (NPR), (**B**) stomatal conductance (SC), and (**C**) transpiration rate (TR) were measured 15 days after sowing. (**D**) Water use efficiency (WUE) was calculated from net photosynthetic rate and transpiration rate. Student's *t*-test was used for statistical analysis.

3.4. Differential Expression of Genes Between the Two Inbred Maize Lines Under Different Water Supply Conditions

Under CK conditions (CK-A193 vs. CK-MP23), 3424 upregulated and 3855 downregulated genes were identified, reflecting the inherent transcriptomic differences between genotypes. Under Dr (Dr-A193 vs. Dr-MP23), 2967 and 3024 genes were up- and downregulated, respectively (Figure 3C). In response to drought stress, A193 showed 3161 upregulated and 2471 downregulated genes (CK-A193 vs. Dr-A193), whereas MP23 exhibited 3145 upregulated and 2602 downregulated genes (CK-MP23 vs. Dr-MP23), indicating robust transcriptional reprogramming in both genotypes (Figure 3C). Venn diagram analysis revealed both shared and unique transcriptional responses (Figure 3D). Notably,

897 and 1245 genes were uniquely regulated in A193 and MP23 under Dr conditions, respectively. Additionally, we identified 1834 unique DEGs under CK conditions and 877 unique DEGs under Dr between the genotypes. Importantly, 406 genes were commonly regulated across all four comparisons, constituting a core set of potentially crucial for drought response mechanisms (Supplementary Table S3). These findings suggest that these genes undergo significant transcriptional changes in response to drought, involving both common and genotype-specific regulatory pathways.

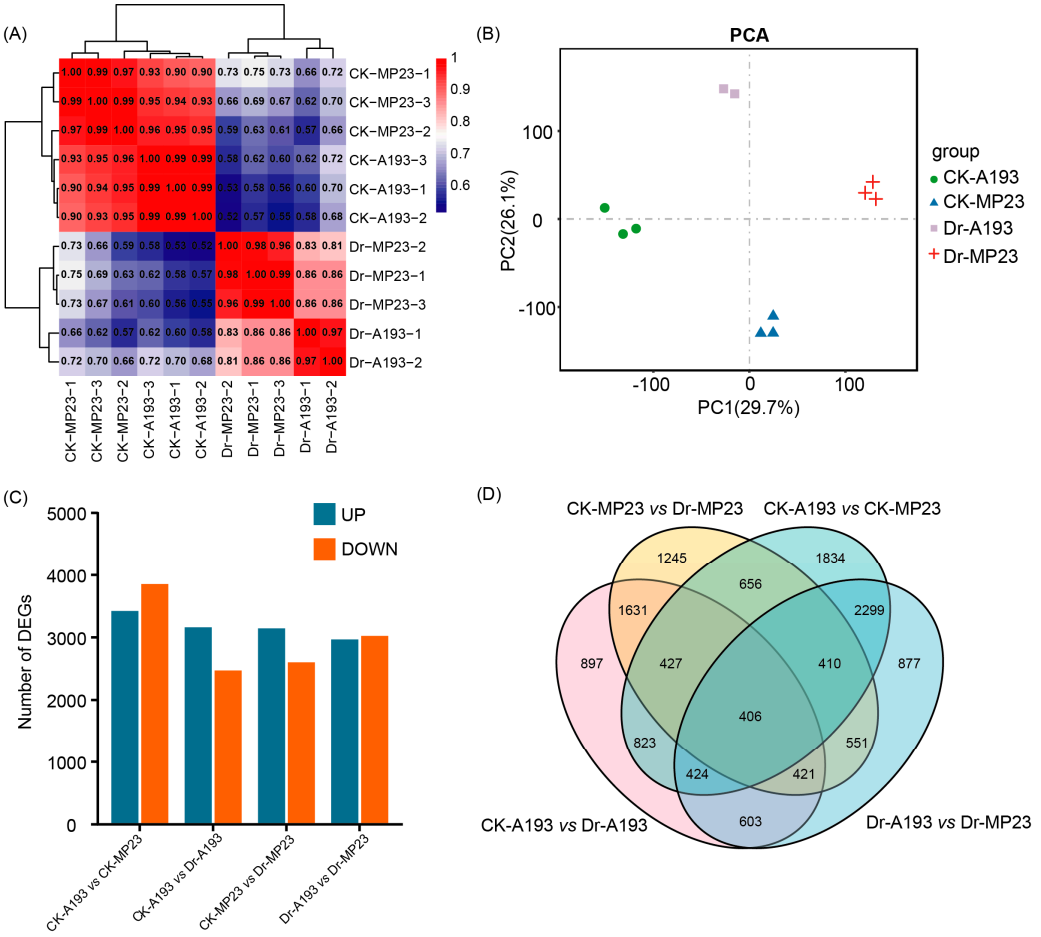

Figure 3. Transcriptome analysis of A193 and MP23. (**A**) Pearson correlation analysis among biological replicates of A193 and MP23 under CK and Dr conditions. The color scale on the right represents the Pearson correlation coefficients (*r*). Values of $r > 0.75$ are indicated by red and $r < 0.75$ by blue. (**B**) Transcriptome principal component analysis (PCA) of A193 and MP23 under CK and Dr conditions. (**C**) Number of differentially expressed genes (DEGs) identified in the four comparisons (upregulated genes highlighted in green box and downregulated genes highlighted in orange box). (**D**) Venn diagram of all DEGs in the four comparison groups. Overlapping portions of the graph represent the number of DEGs in common among the comparison groups.

To elucidate the molecular mechanisms underlying drought responses in A193 and MP23, we conducted comprehensive functional annotation analyses of the identified DEGs, including GO enrichment, KEGG pathway analysis, and transcription factor (TF) identification. GO enrichment analysis revealed significant functional categories affected by drought stress (Figure 4A). Under CK conditions (CK-A193 vs. CK-MP23), only "photosystem I" was significantly enriched, indicating minimal baseline differences in gene function between genotypes. However, drought stress induced dramatic changes in both genotypes (CK-A193 vs. Dr-A193 and CK-MP23 vs. Dr-MP23), with 13 photosynthesis-related GO terms co-enriched, including "photosystem I reaction center" and "photosynthetic electron

transport in photosystem I." The chloroplast showed the highest enrichment significance, underscoring the profound impact of drought stress on chloroplast function. In contrast, under Dr conditions (Dr-A193 vs. Dr-MP23), DEGs were significantly enriched in "hydrolase activity, hydrolyzing O-glycosyl compounds" and "oxidoreductase activity," suggesting differential metabolic adjustments between the genotypes. KEGG pathway enrichment analysis identified key metabolic pathways involved in drought responses (Figure 4B). Ten major pathways were significantly enriched across all four comparisons, including photosynthesis, carbon fixation by Calvin cycle, carbon metabolism, starch and sucrose metabolism, and fatty acid metabolism. The enrichment patterns revealed context-specific pathway regulation. In the drought response comparisons (CK-A193 vs. Dr-A193 and CK-MP23 vs. Dr-MP23), carbon metabolism exhibited the highest enrichment significance, highlighting its central role in drought adaptation. Conversely, starch and sucrose metabolism was most significantly enriched when comparing genotypes under drought stress (Dr-A193 vs. Dr-MP23), indicating that differential carbohydrate metabolism may contribute to their contrasting drought tolerance. Overall, these findings suggest extensive metabolic reprogramming under drought stress, with particular emphasis on photosynthesis, carbon metabolism, and carbohydrate metabolism pathways. A total of 325 genes encoding transcription factors (TFs) from 45 families were differentially expressed between A193 and MP23 under drought stress (Dr-A193 vs. Dr-MP23, Figure 4C). The TF families most represented included ERF, MYB, bHLH, WRKY, and NAC, which are well-known regulators of plant stress responses. Notably, ERF family members showed the highest number of DEGs (32 genes), with predominantly downregulated expression patterns.

3.5. Genomic Variations Between the Two Inbred Maize Lines Through Whole-Genome Resequencing

To investigate the genomic basis of differential drought tolerance between A193 and MP23, we performed whole-genome resequencing of both inbred lines. High-quality sequencing generated 29.4 and 17.2 Gb of clean data, with average coverage of ~13.0- and 7.5-fold for A193 and MP23, respectively (Supplementary Table S4). Mapping the sequences to the B73 reference genome (Zm-B73-REFERENCE-NAM-5.0), we identified extensive genomic variations, including 5,306,884 single-nucleotide polymorphisms (SNPs) and 1,133,400 insertions/deletions (InDels). Variant annotation was performed using SnpEff to assess their functional impact (Supplementary Tables S5 and S6). To identify candidate genes potentially contributing to drought tolerance differences, we integrated resequencing data with transcriptome results. Among the 2815 DEGs responsive to drought stress, 2050 (72.8%) harbored genomic variations, designated DEVGs. Specifically, 1284 DEVGs had variations in the 3" untranslated region (3'UTR), affecting mRNA stability or regulation, while 1426 DEVGs harbored missense mutations that alter protein sequences. Among these, 1944 DEVGs contained SNPs, 1438 DEVGs had InDels, and notably, 1332 DEVGs exhibited both SNP and InDel polymorphisms (Supplementary Table S7). KEGG pathway analysis of the 2050 DEVGs highlighted eight significantly enriched categories (q-value < 0.05, Figure 5A). Biosynthesis of secondary metabolites and metabolic pathways showed the highest enrichment, indicating extensive metabolic reprogramming. Notably, starch and sucrose metabolism was prominently enriched, consistent with transcriptome-only analysis, reinforcing its role in drought response divergence. Additional significantly enriched pathways included benzoxazinoid biosynthesis, galactose metabolism, alanine, aspartate and glutamate metabolism, monoterpenoid biosynthesis, and glycosaminoglycan degradation. These results suggest that genomic variations in A193 and MP23 may confer divergent metabolic capabilities related to drought tolerance. Among the 2050 DEVGs, there were 187 encoded transcription factors representing 37 families (Figure 5B). The most abundant TF families included bZIP (20 genes, predominantly downregulated), ERF (18 genes), bHLH

(14 genes), and G2-like (13 genes). These TF families are known for their crucial roles in stress responses. Other well-represented families included MYB, C2H2, WRKY, and NAC, recognized for their functions in plant stress tolerance.

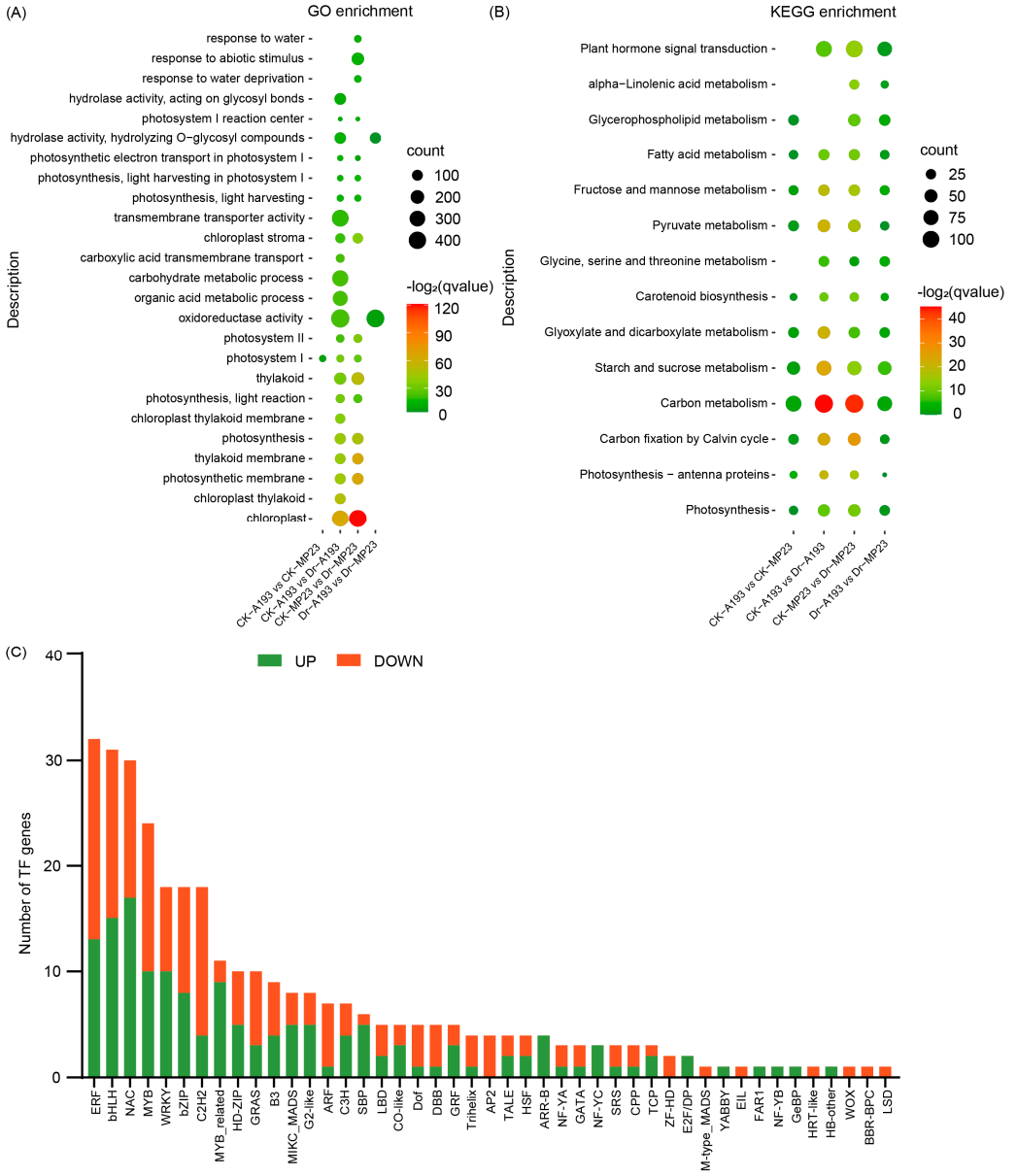

Figure 4. Analysis of DEGs between A193 and MP23. (**A**) Gene Ontology (GO) functional enrichment of DEGs among comparison groups. (**B**) Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment of DEGs among comparison groups. Only q-values < 0.05 are shown. The color of the bubbles represents the significance of enrichment: the redder the color, the higher the significance of enrichment. The size of the bubbles represents the number of DEGs: the larger the bubble, the greater the number of DEGs. (**C**) The number of up- or downregulated genes encoding transcription factors (TFs) with differential expression between A193 and MP23 under Dr conditions.

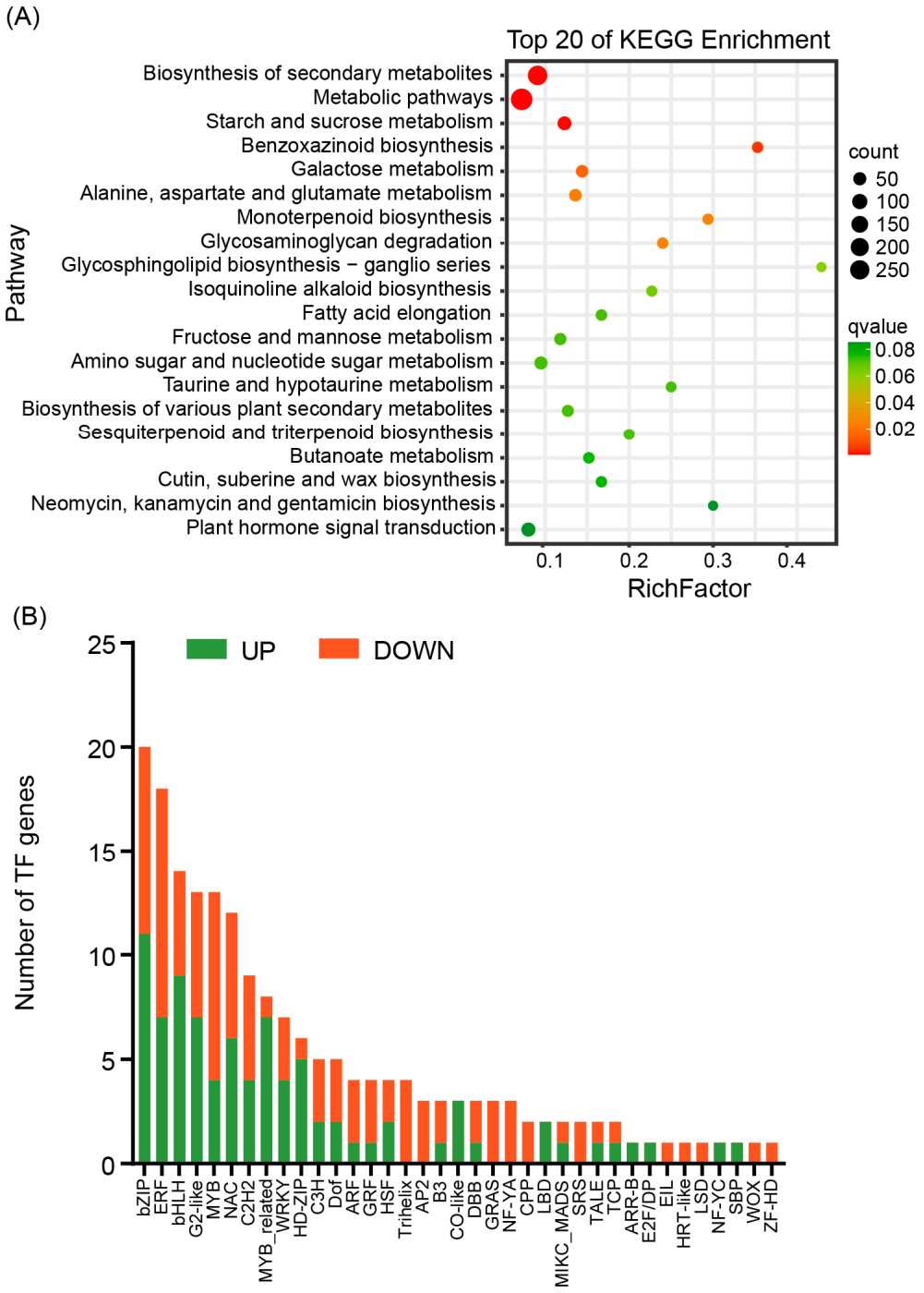

Figure 5. Whole-genome resequencing was performed to investigate genomic variation between A193 and MP23. (**A**) KEGG pathway enrichment analysis of the genes exhibiting both expression differences and genomic variations (DEVGs). The color of the bubbles represents the significance of enrichment: the redder the color, the higher the significance of enrichment. The size of the bubbles represents the number of DEVGs: the larger the bubble, the greater the number of DEVGs. (**B**) The number of upregulated and downregulated DEVGs encoding TF between A193 and MP23.

3.6. Analysis of DEVGs in Starch and Sucrose Metabolism

Given the prominent enrichment of starch and sucrose metabolism, we conducted a detailed analysis of this pathway due to its critical roles in drought tolerance, including osmotic adjustment, stomatal regulation, and maintenance of photosynthetic efficiency [43,50,51]. A total of 30 DEVGs involved in starch and sucrose metabolism were identified between A193 and MP23 under different conditions (Figure 6A). Heatmap analysis revealed distinct expression

patterns between the two genotypes (Figure 6A). Specifically, 15 DEVGs were upregulated in A193 under drought stress, with minimal change or downregulation in MP23. These A193-specific drought-responsive genes encode key enzymes in carbohydrate metabolism: beta-amylase (*Zm00001eb003440*), sucrose synthase 4 isoform X2 (*Zm00001eb016250*), sucrose synthase 2 (*Zm00001eb016290*), alpha-amylase precursor (*Zm00001eb255870*), beta-glucosidases (*Zm00001eb285520* and *Zm00001eb334460*), hexokinase 6 (*Zm00001eb293670*), ramosa 3 isoform X1 (*Zm00001eb327910*), granule-bound starch synthase 1 (*Zm00001eb378140*), and endo-1,4-beta-glucanase (*Zm00001eb395290*). Notably, *Zm00001eb003440* expression increased significantly in A193 under drought compared to MP23 ($p = 0.0058$), and was accompanied by both SNP and InDel variations (Figure 6B). Similarly, *Zm00001eb016250* was robustly upregulated in A193, remaining at basal levels in MP23 under drought ($p = 0.0002$), with one SNP in its genomic sequence (Figure 6C). The sucrose synthase 2 gene (*Zm00001eb016290*) exhibited the most pronounced difference, with expression levels reaching ~120 FPKM in A193 versus ~10 FPKM in MP23 under drought ($p = 3.92 \times 10^{-6}$), with SNP variations detected in different gene regions (Figure 6D). The preferential upregulation of these genes in the drought-tolerant A193 line suggests their potential role in enhancing drought tolerance through improved carbohydrate mobilization and metabolism.

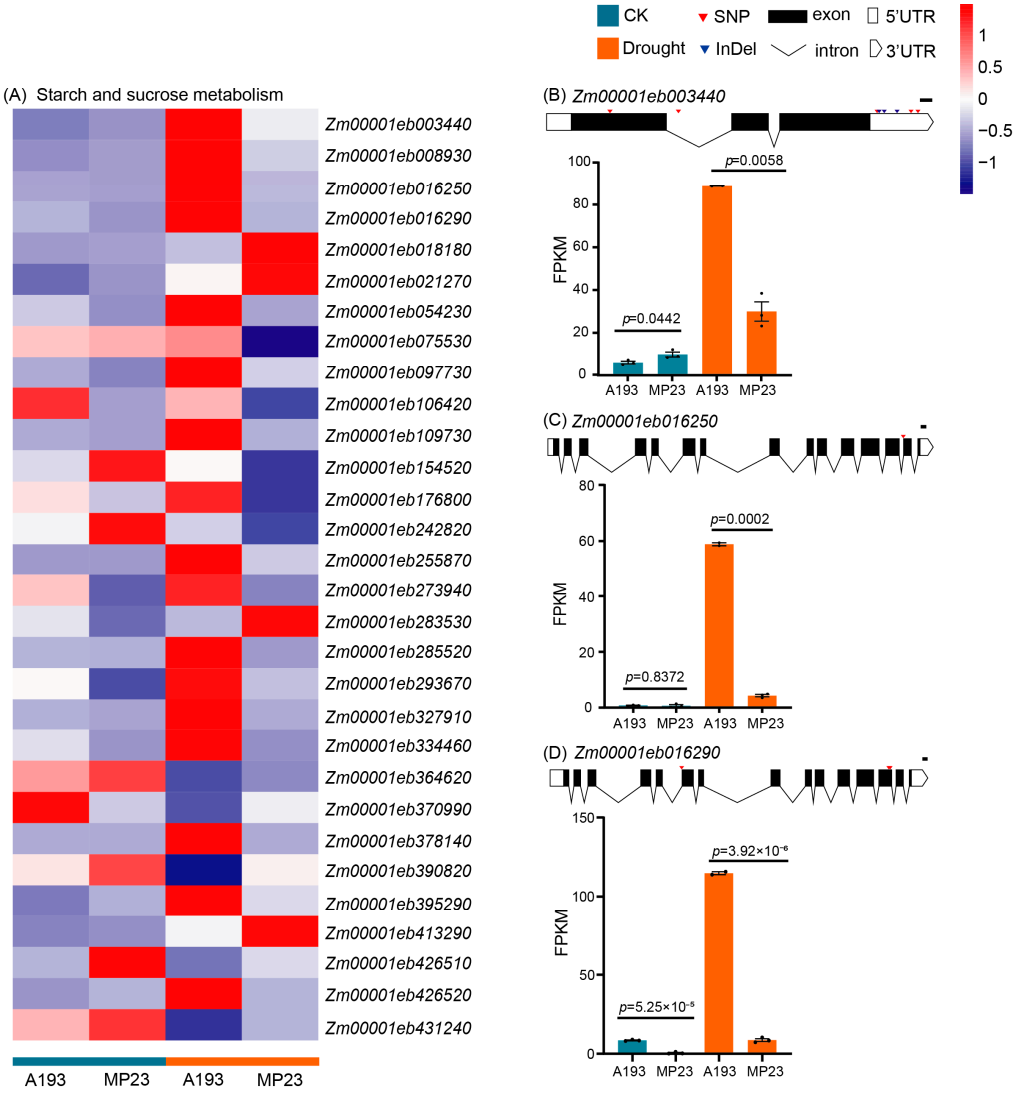

Figure 6. Analysis of DEVGs related to starch and sucrose metabolic pathways. Heatmap shows expression of DEVGs related to (**A**) starch and sucrose metabolism. The genetic structures and expression

levels of (**B**) *Zm0001eb003440*, (**C**) *Zm0001eb016250*, and (**D**) *Zm0001eb016290* are specifically mapped out. Student's *t*-test was used for statistical analysis.

3.7. Analysis of DEVGs in Benzoxazinoid Biosynthesis and Alanine, Aspartate, and Glutamate Metabolism

Maize defense responses to drought stresses are significantly influenced by the benzoxazinoid biosynthesis pathway [52]. In the KEGG enrichment analysis, the benzoxazinoid biosynthesis pathway was found to be significantly enriched. A total of six DEVGs were identified in this pathway (Figure 7A). Among these, one DEVG encoding DIMBOA UDP-glucosyltransferase BX9 (*Zm00001eb033030*), one encoding benzoxazinone synthesis13 (*Zm00001eb116010*), and another encoding cytokinin-N-glucosyltransferase 1 (*Zm00001eb304010*) showed upregulation in A193 and downregulation in MP23 under Dr conditions. Conversely, three DEVGs encoding oxidoreductase (*Zm00001eb165550*, *Zm00001eb165580*, and *Zm00001eb373400*) were downregulated in A193 and maintained low expression in MP23. In summary, the significant upregulation of glycosyltransferase and benzoxazine synthesis genes along with the downregulation of oxidoreductase genes suggests their crucial role in mitigating drought-induced damage.

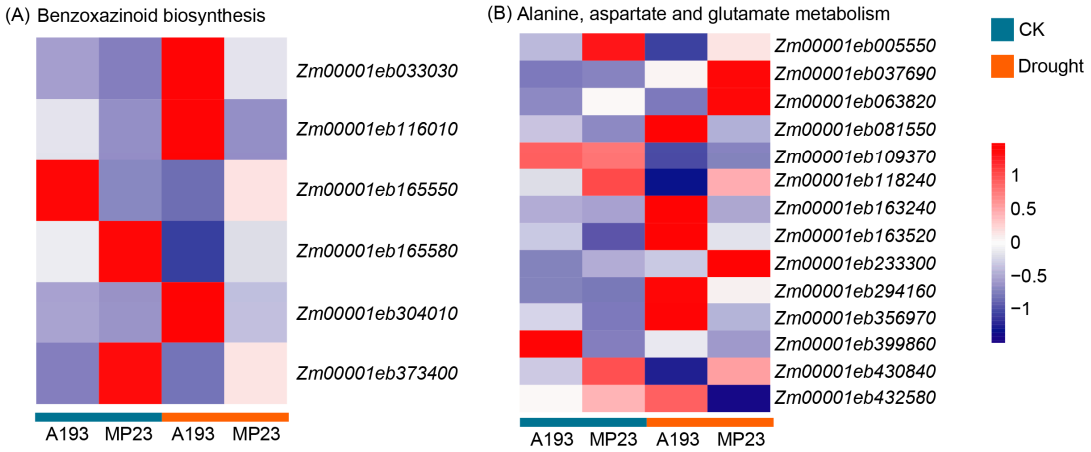

Figure 7. Analysis of DEVGs related to benzoxazinoid biosynthesis and alanine, aspartate, and glutamate metabolism pathways. Heatmap shows expression of DEVGs related to (**A**) benzoxazinoid biosynthesis and (**B**) alanine, aspartate, and glutamate metabolism.

Based on the gene function enrichment analysis, alanine, aspartate, and glutamate metabolism was significantly enriched, and 14 DEVGs were identified in this pathway (Figure 7B). Among these, five DEVGs were upregulated only in A193 under Dr conditions, including genes encoding asparagine synthetase 2 (*Zm00001eb163240*) and carbamoyl-phosphate synthase large chain, chloroplastic (*Zm00001eb356970*), indicating that genetic differences between A193 and MP23 contribute to differential drought response. Notably, three DEVGs exhibited similar expression patterns, being upregulated in MP23 and downregulated in A193: these included *Zm00001eb005550* (encoding alanine-glyoxylate aminotransferase 2 homologue 1, mitochondrial) and *Zm00001eb118240* (encoding alanine aminotransferase 8). Furthermore, three DEVGs were upregulated only in MP23 under Dr conditions, comprising DEVGs encoding pyridoxal phosphate (PLP)-dependent transferase superfamily protein (*Zm00001eb037690*), L-aspartate oxidase, chloroplastic (*Zm00001eb063820*), and succinate semialdehyde dehydrogenase isoform X1 (*Zm00001eb233300*). Additionally, one DEVG encoding glutamine synthetase root isozyme 5 isoform X1 (*Zm00001eb399860*) was upregulated only in A193 under CK conditions, while another encoding glutamine synthetase, chloroplastic isoform X1 (*Zm00001eb432580*) was downregulated only in MP23 under Dr conditions. Thus, the differential expression of

these DEVGs likely underlies the divergence in drought tolerance between the two inbred maize lines. Additionally, qRT-PCR experiments were used to validate the expression pattern of four candidate genes. There was a strong positive correlation ($p < 0.05$) for each gene between the RNA-seq and qRT-PCR. The Pearson correlation coefficients (r) ranged from 0.963 to 0.989 (Supplementary Figure S2). These results indicated the reliability of our transcriptomic profiling data.

4. Discussion

Drought is a common abiotic stress that significantly impairs plant growth and development. Compared with wheat, maize exhibits higher sensitivity to drought, a trait most pronounced during the reproductive stage and consistently observed across both arid and non-arid ecosystems [53]. Drought typically results in limited growth, leaf wilting and yellowing, reduced biomass, and decreased photosynthetic activity in maize. Consequently, both production development and yield increase are drastically reduced under drought stress. This study provides comprehensive insights into the molecular mechanisms underlying differential drought tolerance in maize through an integrative multiomics approach. Our findings reveal that the superior drought tolerance of inbred line A193 compared to MP23 is underpinned by complex physiological adaptations and extensive metabolic reprogramming involving coordinated regulation of carbohydrate metabolism, secondary metabolite biosynthesis, and amino acid metabolism pathways. The damage caused by drought stress in maize involves a complex interplay of physiological responses and gene regulation, with the most noticeable changes being morphological alterations that serve as visible indicators of plant stress tolerance capacity [54]. In our study, the morphological responses to drought stress clearly differentiated the two inbred lines, with MP23 showing pronounced leaf drooping, wilting, and curling as early as 15 days after sowing, while A193 maintained relatively normal leaf architecture and turgor pressure throughout the stress period. These morphological differences reflect underlying cellular and tissue-level adaptations that contribute to drought tolerance [55]. Furthermore, the limited recovery observed in MP23 after rehydration, characterized by sustained morphological damage compared to the rapid recovery of A193, indicates that the drought-sensitive line experienced more severe structural damage to leaf tissues and cellular components during the stress period. This irreversible morphological damage likely reflects underlying differences in cell membrane stability, protein integrity, and metabolic homeostasis maintenance under water deficit conditions [56,57]. The physiological characterization clearly demonstrated the contrasting drought responses between A193 and MP23, with A193 exhibiting superior water use efficiency and photosynthetic performance under stress conditions. The dramatically different survival rates (89% vs. 11%) underscore the substantial genetic variation in drought tolerance within maize germplasm. Notably, A193 maintained higher water use efficiency under both CK and Dr conditions, suggesting constitutive physiological advantages that contribute to stress tolerance. This finding aligns with previous studies indicating that water use efficiency is a key physiological trait distinguishing drought-tolerant from drought-sensitive genotypes [58]. The more severe reduction in stomatal conductance and transpiration rate in MP23 under drought stress likely reflects a more drastic water conservation strategy, which paradoxically may limit photosynthetic capacity and overall plant performance under prolonged stress conditions [59].

The integration of transcriptomics and genomics in our study represents a significant methodological advancement in understanding the complex genetic architecture underlying drought tolerance in maize [60]. While traditional single-omics approaches provide valuable insights into either gene expression patterns or genomic variations independently [61], our multiomics strategy offers a more comprehensive and mechanistic

understanding of how genetic polymorphisms translate into phenotypic differences under stress conditions. The identification of 2050 DEVGs that harbor both transcriptional changes and genomic variations provides a refined set of candidate genes with a higher likelihood of functional relevance to drought tolerance. This approach effectively bridges the gap between genotype and phenotype by demonstrating that 72.8% of drought-responsive genes that differ between the two lines also contain sequence variations, suggesting that genomic polymorphisms contribute substantially to expression divergence under stress conditions [8]. Our KEGG pathway analysis revealed a significant enrichment of terms related to the biosynthesis of secondary metabolites (q-value = 1.95×10^{-13}, count = 182). This finding is consistent with a substantial body of literature demonstrating that the induction of secondary metabolite biosynthesis plays a crucial role in a plant's adaptive response to drought stress by enhancing antioxidant activity and mitigating oxidative damage [62]. Similarly, our results demonstrate that metabolic pathways were also significantly enriched in the KEGG analysis (q-value = 3.57×10^{-9}, count = 269). This observation is highly congruent with the well-documented fact that drought-induced metabolic reprogramming serves as a central mechanism for enhancing drought tolerance, primarily through its governing role in phytohormone signaling networks [63]. The power of this integrative approach is particularly evident in our pathway-level analysis, where genes involved in starch and sucrose metabolism, benzoxazinoid biosynthesis, and amino acid metabolism showed both expression differences and sequence variations between the drought-tolerant and drought-sensitive lines. Starch and sucrose metabolism, which is essential for plant growth and high yields, is strongly influenced by drought stress [64]. In this study, starch and sucrose metabolism pathway was significantly enriched in DEVGs, indicating its involvement in the response to drought stress. The beta-amylase gene *AtBAM1* in Arabidopsis degrades transient starch and provides a carbon skeleton for proline biosynthesis, thereby maintaining cell osmotic balance and enhancing plant response to drought stress [65]. Similarly, a beta-amylase gene, *ZmBAM8*, has also been shown to enhance drought tolerance by promoting starch degradation to increase soluble sugar content and accelerating stomatal closure to reduce water loss in maize [50]. *OsBAM4* and *OsBAM10* were upregulated under drought stress, and Yang et al. proposed that the BAM gene family of grasses may be involved in the response to a variety of abiotic stresses [66]. Here, the beta-amylase gene *Zm00001eb003440* showed markedly stronger drought-induced expression in the tolerant line. The key missense variant (p.Lys458Arg) is hypothesized to enhance its protein function. Two sucrose synthase (*SUS*) genes, *Zm00001eb016250* (encoding sucrose synthase 4 isoform X2) and *Zm00001eb016290* (encoding sucrose synthase 2), were identified as drought-responsive candidates in maize. Consistently with the expression patterns characteristic of SUS subfamilies (SUS I, II, III), both genes exhibited significant upregulation under drought stress, with the potential for drought resistance [67]. Genome resequencing revealed a splice region variant and intron variant SNP (c.2253-5C>A) at the junction of the exon and intron of *Zm00001eb016250*. Three SNPs were detected in *Zm00001eb016290*, including a splice region variant and intron variant (c.690-7C>A) and two missense variants. Beyond carbon metabolism, A193 appears to activate a more comprehensive defense network. The significant enrichment of the benzoxazinoid biosynthesis pathway and the upregulation of key genes like *BX9* in A193 indicate a crucial role in enhancing its abiotic stress tolerance. Benzoxazinoids, a class of plant metabolites, are now understood to play a crucial role in drought tolerance [68]. Furthermore, the differential regulation of genes in the alanine, aspartate, and glutamate metabolism pathway suggests fine-tuning of amino acid metabolism, which is crucial for protein synthesis, nitrogen recycling, and the production of other stress-related compounds [69–71]. This multilayered defense—spanning physiology, primary metabolism, and secondary metabolism—collectively contributes to

the resilience of A193. In addition to the pathways discussed above, other significantly enriched pathways—including galactose metabolism, monoterpenoid biosynthesis, and glycosaminoglycan degradation—may also play important roles in the drought stress response. Collectively, they form a complex metabolic regulatory network that enhances plant adaptation to water stress from multiple perspectives. Galactose metabolism contributes to drought tolerance by facilitating the biosynthesis of raffinose family oligosaccharides (RFOs), which reduce leaf transpiration and protect cellular structures under water deficit conditions [72]. Monoterpenoid biosynthesis contributes to the production of drought tolerance-enhancing compounds that may regulate stress-responsive gene expression [73]. Although the role of glycosaminoglycan degradation per se in plant drought tolerance remains unclear, direct experimental evidence demonstrates that the metabolism and remodeling of cell wall matrix polysaccharides, mediated by specific glycoside-hydrolyzing enzymes, are crucial mechanisms for drought adaptation in plants [74]. While the present study did not extensively explore the molecular underpinnings of these pathways, these enzymes offer valuable insights into genotypic variation in drought tolerance and merit further investigation in future research. This entire network may be orchestrated by a suite of TFs from families like bZIP, ERF, and bHLH, which were themselves identified as DEVGs, indicating that the master regulatory switches of the stress response are genetically different between the two lines.

5. Conclusions

In conclusion, this study elucidates the molecular mechanisms underlying differential drought tolerance in maize through integrative transcriptomic and genomic analysis. The drought-tolerant inbred line A193 demonstrated superior physiological performance, including higher water use efficiency and better photosynthetic maintenance under stress conditions, compared to the drought-sensitive line MP23. The multiomics analysis identified 2050 DEVGs harboring both expression differences and genomic variations between the two lines. Key pathways contributing to drought tolerance divergence include starch and sucrose metabolism, benzoxazinoid biosynthesis, and amino acid metabolism. These findings provide valuable insights into the complex genetic architecture of drought tolerance and identify promising candidate genes for drought resistance improvement programs in maize. Based on our molecular findings, we recommend functional validation of key candidate genes (e.g., *Zm00001eb097730* and *Zm00001eb426520* in starch and sucrose metabolism; *Zm00001eb033030* in benzoxazinoid biosynthesis and *Zm00001eb356970* in amino acid metabolism) using gene editing, overexpression, and complementation assays to establish causal relationships between genetic variants and drought tolerance phenotypes. Future work should focus on such functional validations to further confirm the roles of these candidate genes.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy15092189/s1. Figure S1: Phenotypes of A193 and MP23 under control (CK) and drought stress (Dr) conditions at 21 days after sowing. The drought-tolerant line A193 displays minimal wilting and retains leaf greenness and uprightness, while the sensitive genotype MP23 shows severe wilting, leaf curling, desiccation, and browning. The image was taken at 21 days after sowing. Figure S2: Validation of RNA-seq data by qRT-PCR. A strong positive correlation ($p < 0.05$) was observed between the two methods. Pearson's correlation coefficient (r) for each gene is indicated on the graph. The high correlation values confirm the reliability of the transcriptomic data. *, $p < 0.05$. Table S1: Primers used in this study. Table S2: Transcriptome-sequencing data. Table S3: Information on all differentially expressed genes in the different comparison groups. Table S4: Quality summary of MP23 and A193 whole-genome resequencing. Table S5: Effect numbers of SNP variants by type and region. Table S6: Effect numbers of InDel variants by type and region. Table S7:

Information on drought-responsive genes with genomic variations, showing differential expression between A193 and MP23 under drought stress.

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