agronomy

MDPI

Article

Genome-Wide Identification of the *AP2/ERF* Family in Alfalfa and Its Association with Antioxidant Regulation Under Drought Stress

Xia Wang, Yongzhong Luo *, Jianwei Qi, Yuyu Kong, Liya Zhang, Xiaotong Zhou and Shengli Lei

College of Forestry, Gansu Agricultural University, Lanzhou 730070, China; xiaoqi_325_qi@163.com (J.Q.); 1073325020284@st.gsau.edu.cn (Y.K.); 1073324120516@st.gsau.edu.cn (L.Z.); zhouxt@st.gsau.edu.cn (X.Z.); 1073324120535@st.gsau.edu.cn (S.L.)

* Correspondence: luoyzhong@gsau.edu.cn

Abstract

In this study, Alfalfa (*Medicago sativa* L.) was used as the experimental material to systematically identify members of the *AP2/ERF* transcription factor family in its genome and to elucidate the response patterns and potential regulatory networks of this family under drought stress. A total of 756 *AP2/ERF* genes were identified and classified into four subfamilies: AP2, ERF, DREB, and RAV, among which the DREB and ERF subfamilies exhibited significant expansion. Evolutionary analysis indicated that whole-genome duplication (WGD)/segmental duplication was the primary driving force underlying the expansion of this family. Under graded drought treatments, multiple *AP2/ERF* genes were significantly upregulated, with *MsERF210* showing continuously increasing expression as drought intensity escalated. WGCNA revealed that *MsERF210* was highly positively correlated with antioxidant-related modules, including "Glutathione metabolism," "Peroxisome," and "Ascorbate and aldarate metabolism." Further analysis showed that several antioxidant genes, such as *SOD*, *GST*, *ALDH*, and *IDH*, were significantly upregulated under drought stress, and their promoter regions were predicted to be bound by *MsERF210*. Physiological indicator measurements demonstrated that drought stress led to increased accumulation of H_2O_2 and MDA, along with elevated contents of AsA, DHA, GSH, and GSSG, while SOD activity initially increased and then decreased. qRT-PCR validated the expression trends of key genes, confirming the reliability of the transcriptome data. In summary, this study reveals the evolutionary characteristics of the *AP2/ERF* family in alfalfa and identifies *MsERF210* as a key regulator that may enhance alfalfa's adaptability to drought stress by modulating the antioxidant defense system and redox homeostasis. These findings provide important candidate gene resources and a theoretical basis for breeding new drought-resistant alfalfa varieties.

Keywords: AP2/ERF; drought; *Medicago sativa*; antioxidant defense

Check for updates

Academic Editor: Junfei Gu

Received: 5 July 2026
Revised: 12 August 2026
Accepted: 20 August 2026
Published: 2 September 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

1. Introduction

Drought stress is one of the most severe abiotic stresses that limit crop growth, development, and yield formation, posing a serious threat to agricultural production and ecosystem stability [1]. Because plants are sessile and unable to escape drought, they have evolved a complex and elaborate regulatory network for drought response over long-term evolution, encompassing stress signal perception, hormone signal transduction, transcriptional regulation, osmotic adjustment, and antioxidant defense [2,3]. Among these,

transcription factors (TFs), as key regulators of gene expression, can recognize and bind to specific cis-acting elements in the promoter regions of downstream target genes, thereby regulating the expression of a large set of stress-responsive genes and playing a central role in plant adaptation to drought environments [4]. To date, the TF families most closely associated with plant drought responses include bZIP, AP2/ERF, NAC, MYB, and WRKY. These TFs enhance plant tolerance to drought stress by modulating ABA signaling, ethylene signaling, osmolyte biosynthesis, ROS scavenging, and the expression of cytoprotective proteins [5–9].

The AP2/ERF (APETALA2/ethylene-responsive factor) superfamily is a class of TFs that are both plant-specific and ubiquitously present in plants. Members typically contain one or two conserved AP2 DNA-binding domains of approximately 60 amino acids [10]. Based on domain composition and sequence features, the AP2/ERF superfamily is generally divided into the AP2, DREB, ERF, RAV, and Soloists (a few unclassified factors) subfamilies [10,11]. The AP2/ERF family usually exists as a multi-copy gene family in plants; for example, *Arabidopsis thaliana* contains 148 AP2/ERF superfamily members [11], rice (*Oryza sativa*) contains 164 [12], and *Populus trichocarpa* contains 210 [13]. Extensive studies have demonstrated that ERF transcription factors participate in the regulation of plant drought resistance through multiple pathways. For instance, Quan et al. found that overexpression of TSRF1, an ERF subfamily member, improved the drought tolerance of transgenic rice. ERF1 activates the expression of SDR, a key enzyme in ABA biosynthesis; promotes the accumulation of ABA, proline, and soluble sugars; and induces the expression of genes related to MYB, MYC, proline synthesis, and photosynthesis, suggesting that ERF may regulate drought response through an ABA-dependent pathway [14]. Maruyama et al. overexpressed DREB1A in *A. thaliana* and observed alterations in the activities of starch-degrading enzymes, sucrose-metabolizing enzymes, and sugar alcohol synthases, leading to changes in the contents of monosaccharides, disaccharides, and trisaccharides, thereby enhancing plant drought resistance [15]. Moreover, overexpression of ERF improves plant tolerance to drought and salt stress by boosting antioxidant capacity [16]. Collectively, these findings indicate that ERF transcription factors participate in plant drought stress responses by modulating multiple processes, including hormone signaling, osmolyte synthesis, and antioxidant defense.

Alfalfa (*Medicago sativa* L.) is a perennial leguminous forage widely cultivated around the world. Owing to its high biomass yield, excellent nutritional quality, good palatability, and strong nitrogen-fixing ability, it is known as the "king of forages" [17]. With the rapid development of animal husbandry in China, the domestic demand for high-quality alfalfa is growing rapidly; therefore, elucidating the mechanisms by which alfalfa withstands abiotic stress and mining key functional genes have become pressing research priorities [18–21]. To date, several studies have performed preliminary identification of the AP2/ERF family in *Medicago* species. For example, Shu et al. identified 123 AP2/ERF members in the genome of the diploid model species *M. truncatula*, covering four subfamilies: ERF, DREB, AP2 and RAV [22]; Qiu et al. identified 110 members in the genome of the tetraploid alfalfa cultivar "Xinjiang Daye" and classified them into three subfamilies: DREB, ERF, and AP2 [23]. Notably, Xinjiang Daye alfalfa is an autotetraploid; so, the gene family size in a tetraploid genome would be expected to be substantially larger than that in the diploid *M. truncatula*. However, the numbers reported in these studies are remarkably similar, and the criteria for subfamily classification and the nomenclature system are also inconsistent. This discrepancy may arise from differences in genome assembly version, annotation pipeline, domain screening threshold, or redundancy removal strategy, suggesting that current identification of the alfalfa AP2/ERF family still lacks a standardized pipeline and systematic comparison. In addition, Chen et al. found that alfalfa ERF8 and ERF11

improved the tolerance of transgenic tobacco and rice to salt stress [24]; Jin et al. identified 17 *AP2/ERF* genes whose expression responded to salt, drought, and cold stresses, implying that the AP2/ERF family may participate in alfalfa's adaptation to diverse environmental stresses [25]. These studies demonstrate that *AP2/ERF* transcription factors play important roles in the stress responses of alfalfa. Nevertheless, systematic genome-wide identification of the alfalfa *AP2/ERF* gene family, its expression response patterns under drought stress, and its potential downstream regulatory networks remain poorly characterized. In particular, whether AP2/ERF participates in ROS scavenging and redox homeostasis maintenance by regulating antioxidant-related gene modules is still unclear. To address this gap, the present study used Xinjiang Daye alfalfa as the experimental material to systematically identify members of its *AP2/ERF* gene family. By integrating transcriptome data under graded drought treatments, we screened for ERF candidate genes with pronounced drought responsiveness, identified co-expression modules and key functional pathways significantly associated with them, and finally examined the AP2/ERF-mediated antioxidant defense mechanism in conjunction with physiological and biochemical indices including H_2O_2, MDA, SOD, AsA, DHA, GSH, and GSSG. This study will contribute to understanding the molecular basis of alfalfa ERF transcription factors in drought stress responses and provide a theoretical foundation and candidate gene resources for molecular breeding of drought-resistant alfalfa and the creation of superior germplasm.

2. Materials and Methods

2.1. Plant Materials

In this study, Xinjiang Daye alfalfa was used as the experimental material for drought simulation experiments. The experimental conditions were as follows: Four soil moisture treatment gradients were designed: adequate water supply (80–85% of field capacity, CK), mild water stress (60–65% of field capacity, D1), moderate water stress (40–45% of field capacity, D2), and severe water stress (20–25% of field capacity, D3). Six pots were set up per treatment as replicates. On the 14th day of drought stress, robust leaves from each treatment were collected for physiological indicator measurement and qRT-PCR quantification.

2.2. Bioinformatic Analysis of Gene Families

2.2.1. Data Sources and Transcriptome Expression Quantification

The alfalfa genome and its annotation files were downloaded from the FigShare database (https://doi.org/10.6084/m9.figshare.12327602, (accessed on 25 February 2026)). Genome data for *Arabidopsis thaliana* and *M. truncatula* were obtained from the TAIR database (https://www.arabidopsis.org/, (accessed on 25 February 2026)) and Ensembl Plants (https://plants.ensembl.org/, (accessed on 25 February 2026)), respectively. The transcriptome data used in this study were derived from previous experiments conducted by our research group [26], with three biological replicates per treatment and experimental conditions consistent with the drought treatments described above. Expression quantification was performed as follows: Clean reads were aligned to the Xinjiang Daye alfalfa reference genome using Hisat2 (v2.2.1) [27]. Gene alignment counts were calculated using featureCounts (v2.0.3) [28], and FPKM values for each gene were computed based on read coverage.

2.2.2. Identification of the *MsAP2/ERF* Gene Family

AP2/ERF gene sequences of *A. thaliana* were downloaded from the TAIR database (https://www.arabidopsis.org/, (accessed on 16 March 2026)). Blastp 2.17.0 was used to search against the alfalfa genome with an E-value threshold of 1×10^{-5}. Additionally, the Pfam model file (PF00847) for the ERF family was obtained from the Pfam database

(https://pfam.xfam.org/, (accessed on 16 March 2026)). Hmmsearch v3.3.2 was performed against the Xinjiang Daye alfalfa genome with an E-value threshold of 1e-5 to screen for sequences containing the AP2 conserved domain. The retrieved sequences were used to construct an alfalfa-specific Pfam model, followed by another round of Hmmsearch. Finally, the results from Blastp and Hmmsearch were merged, redundant sequences were removed, and domain annotation was performed using NCBI-CDD and InterPro online tools (https://www.ebi.ac.uk/interpro/, (accessed on 17 March 2026)). Sequences containing a complete AP2 domain were defined as ERF candidate members. Furthermore, the PlantTFDB database (https://planttfdb.gao-lab.org/, (accessed on 24 March 2026)) was used to verify whether the candidate genes belonged to the ERF family, and SMART (https://smart.embl.de/, (accessed on 24 March 2026)) was employed to validate the integrity of the AP2 domain. In addition, ERF family members of *M. truncatula* were also identified using the same methodology.

2.2.3. Phylogenetic Tree Analysis

Phylogenetic trees of the *AP2/ERF* gene families from Xinjiang Daye alfalfa, *M. truncatula*, and *A. thaliana* were constructed using IQ-TREE 2 based on the maximum likelihood method [29]. CDS sequences of all *AP2/ERF* gene members were aligned using MAFFT, and the multiple sequence alignment results were trimmed using trimAl [30]. Model selection in IQ-TREE 2 was automatically performed using the ModelFinder Plus (MFP) algorithm to select the optimal nucleotide substitution model. The maximum likelihood method was used to search for the optimal tree topology, and branch reliability was assessed using ultrafast bootstrap (UFBoot) with 2000 replicates and the SH-like approximate likelihood ratio test (SH-aLRT). Phylogenetic trees were visualized using ChiPlot [31]. Physicochemical properties of MsAP2/ERF proteins, including relative molecular mass, theoretical isoelectric point, instability index, and hydrophilicity index, were analyzed using Expasy ProtParam (http://web.expasy.org/protparam/, (accessed on 2 April 2026)) with default parameters.

2.2.4. Gene Structure and Synteny Analysis

Based on the genome annotation file, Perl scripts were used to split the alfalfa genome into four haplotype genomes (hap1-hap4), each containing eight chromosomes (chr1–chr8). Synteny analysis was performed using TBtools 2.390 [32], including synteny among the four haplotypes, synteny between *M. truncatula* and alfalfa hap1, and intra-hap1 synteny. Additionally, MCScanX [33] was used to analyze gene duplication types of MsERFs, including WGD/segmental, dispersed, tandem, and proximal duplication. For the identification of quadruple-allelic *ERF* genes, a graph-based approach was employed by integrating gene family annotation and synteny analysis. Using MCScanX software based on BLASTP results (E-value $< 1 \times 10^{-10)}$ and gene order conservation, syntenic relationships among sub-haplotype genomes were constructed. Subsequently, synteny files were parsed to extract gene pairs located within conserved syntenic blocks, and a gene interaction network was constructed: genes served as nodes, and edges were established if two genes exhibited a syntenic relationship. A breadth-first search (BFS) algorithm was used to identify connected components in the network. When a connected component contained exactly four genes, each originating from a different haplotype, it was designated as a "true 4-allele ERF tetrad." This criterion ensured that the identified genes represented completely retained single-copy homologous genes after polyploidization. The analysis pipeline was implemented using Python 3.10 scripts, utilizing defaultdict and deque for graph traversal.

2.2.5. Annotation of ERF Downstream Target Genes

Plant transcription factor motif PFMs (non-redundant) files were downloaded from the JASPAR 2026 database (https://jaspar.elixir.no/downloads/, (accessed on 11 April 2026)). TBtools 2.390 was used to extract 2000 bp upstream promoter sequences of all genome-wide protein-coding genes. FIMO v5.5.4 [34] was used to scan transcription factor binding sites in the promoter sequences of genome-wide protein-coding genes, with a screening threshold of q-value < 0.05. KofamScan v1.3.0 [35] was used for KEGG Orthology (KO) functional annotation of alfalfa protein sequences, and KEGG pathway enrichment analysis was performed using the ggplot2 v3.3.0 package in R v3.5.1. The binding conformations between ERF and downstream target gene binding sites were predicted using AlphaFold3 [36], and an ipTM score greater than 0.6 was considered indicative of a stable complex. All structural models were visualized using PyMOL 2.5.0 [37].

2.2.6. Differential Gene Expression and WGCNA Analysis

DEGs were identified using DESeq2 v1.22.1 [38], with thresholds of $|\log_2$ fold change$|$ > 1 and false discovery rate (FDR) < 0.05. Weighted gene co-expression network analysis (WGCNA) was performed using the WGCNA v1.71 package in R v4.2.2 with default parameters [39].

2.3. qRT-PCR Quantification and Physiological Indicator Measurement

2.3.1. qRT-PCR Quantification

Total RNA was extracted from leaves using the CTAB method and reverse transcribed into cDNA using a reverse transcription kit (SweScript All-in-One RT Super-Mix, G3337, Wuhan servicebio technology Co., Ltd., Wuhan, China). The qRT-PCR reaction system was 15 µL, and the reaction program consisted of initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 30 s. Primer sequences for target genes were designed using NCBI Primer-BLAST 2.17.0. The alfalfa *Actin* gene was used as an internal reference, and three biological replicates with three technical replicates each were performed. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. qRT-PCR primer information is provided in Table S4.

2.3.2. Physiological Indicator Measurement

MDA content was determined using the thiobarbituric acid (TBA) method [40]. Fresh leaf samples (0.2 g) were ground with quartz sand and 20% trichloroacetic acid (TCA), adjusted to a final volume of 5 mL, and centrifuged at 3000 rpm for 15 min. The supernatant was mixed with an equal volume of 0.5% TBA, heated in a boiling water bath for 10 min, cooled on ice, and absorbance was measured at 450, 532, and 600 nm. Absorbance at 532 nm represented the characteristic absorption peak, while readings at 450 and 600 nm were used to correct for interference from soluble sugars and turbidity, respectively.

AsA and DHA contents were determined following the method of Wang et al. [41]. Leaf samples (0.2 g) were ground in 50 g/L TCA on ice, centrifuged at 4000 rpm for 10 min at 4 °C, and the supernatant was collected for measurement. AsA was measured at 534 nm after a chromogenic reaction. For DHA, samples were first reduced with DTT to convert DHA to AsA, and total ascorbic acid content was determined using the AsA method; DHA content was calculated as the difference between total ascorbic acid and AsA content.

GSH and GSSG contents were determined using the DTNB colorimetric method [42]. Leaf samples (0.2 g) were ground in 5 mmol/L EDTA-TCA extraction solution, adjusted to a final volume of 6 mL, and centrifuged at 4000 rpm for 10 min at 4 °C. Two sets of supernatant were prepared: one with added glutathione reductase (GR) and one without. After adjusting pH to 6.5–7.0 with NaOH, potassium phosphate buffer and DTNB were added

for color development. Total glutathione and GSH contents were measured separately, and GSSG content was calculated as the difference between the two.

SOD activity was determined using the nitroblue tetrazolium (NBT) photoreduction method [43]. Leaf samples (0.2 g) were ground in phosphate buffer (pH 7.8) on ice, centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatant was collected as the enzyme extract. The reaction mixture contained phosphate buffer, EDTA, methionine, NBT, riboflavin, and enzyme extract. After light exposure for 20 min, absorbance was measured at 560 nm. One unit of SOD activity (U) was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction.

H_2O_2 content was determined using the titanium sulfate colorimetric method [44]. Leaf samples (0.2 g) were ground in pre-cooled acetone on ice, centrifuged at 5000 rpm for 10 min at 4 °C, and the supernatant was reacted with titanium sulfate and concentrated ammonia to form a precipitate. The precipitate was washed with cold acetone, dissolved in sulfuric acid, and absorbance was measured at 415 nm. H_2O_2 content was calculated based on a standard curve.

Superoxide anion (O_2^-) content was determined using the hydroxylamine hydrochloride colorimetric method [45]. Leaf samples (0.20 g) were ground in potassium phosphate buffer, centrifuged, and the supernatant was collected. The supernatant was reacted with hydroxylamine hydrochloride, sulfanilamide, and α-naphthylamine to form a pink compound, which was extracted with chloroform. Absorbance was measured at 530 nm, and O_2^- content was calculated based on a standard curve.

3. Results

3.1. Phylogenetic Analysis

A total of 756 *AP2/ERF* genes were identified in the alfalfa genome. Detailed information regarding renaming, subfamily classification, and other features is provided in Table S1. Phylogenetic analysis (Figure 1A) revealed that the *MsAP2/ERF* family members clustered into five major clades. Based on their conserved domains and clustering patterns, these were classified into five subfamilies: AP2, ERF A, ERF B, ERF C, and RAV, comprising 92, 205, 212, 240, and 7 members, respectively. To further distinguish the *ERF* and *DREB* subfamilies, we constructed a phylogenetic tree using MsERFs and *MtERFs*. The results (Figure 1B) showed that *MsERFs* and *MtERFs* grouped into two major branches, corresponding to the ERF subfamily and the DREB subfamily. Among all *MsAP2/ERF* members, 267 were assigned to the *DREB* subfamily and 390 to the *ERF* subfamily.

3.2. Structural Features and Physicochemical Property Analysis

Gene structure analysis (Figure 2A) revealed marked differences among subfamilies. All members of the RAV subfamily exhibited a single-exon, intron less structure. Most members of the ERF and DREB subfamilies also displayed a single-exon, intron less architecture, whereas a few possessed a two-exon, one-intron structure. Isolated cases presented other structural types; for example, some ERF members contained 19 exons and 18 introns, and certain DREB members harbored 16 exons and 15 introns. Compared with other subfamilies, the gene structures of AP2 subfamily members were relatively less conserved, predominantly featuring nine-exon/eight-intron, ten-exon/nine-intron, eight-exon/seven-intron, and seven-exon/six-intron configurations. These observations suggest that the ERF family has acquired considerable structural and functional diversity during evolution.

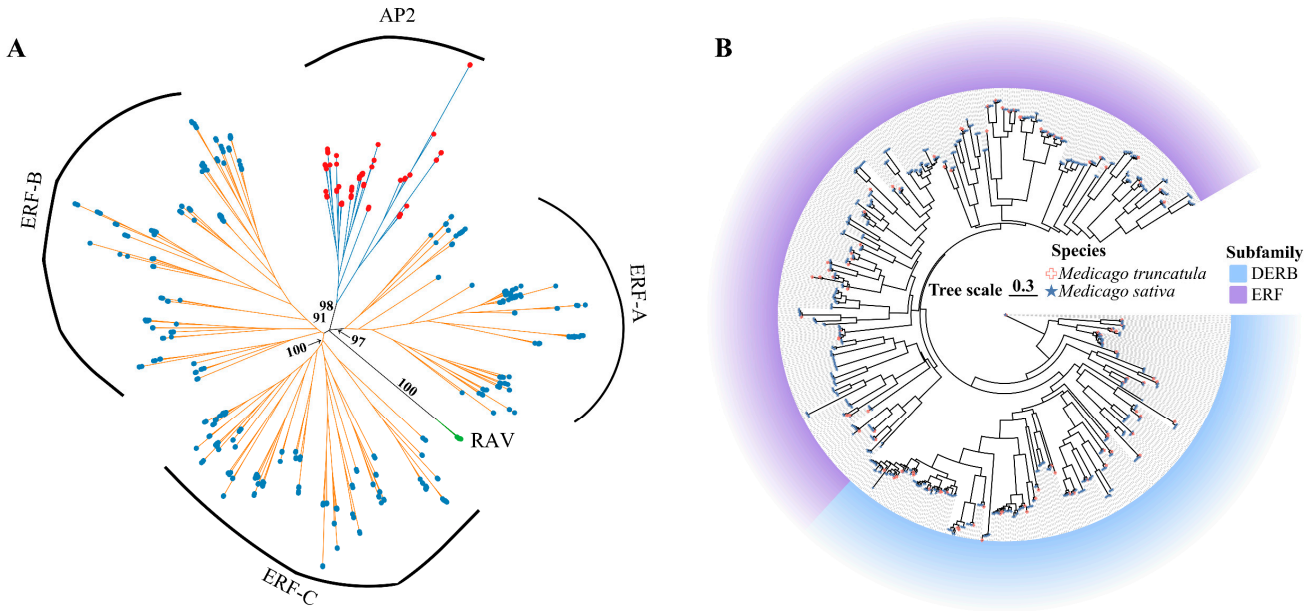


Figure 1. Phylogenetic relationships of *MsAP2/ERFs*. (**A**) Phylogenetic tree of *MsAP2/ERFs*; numbers at nodes indicate bootstrap support values. (**B**) Phylogenetic tree of *MsERFs* and *MtERFs*.

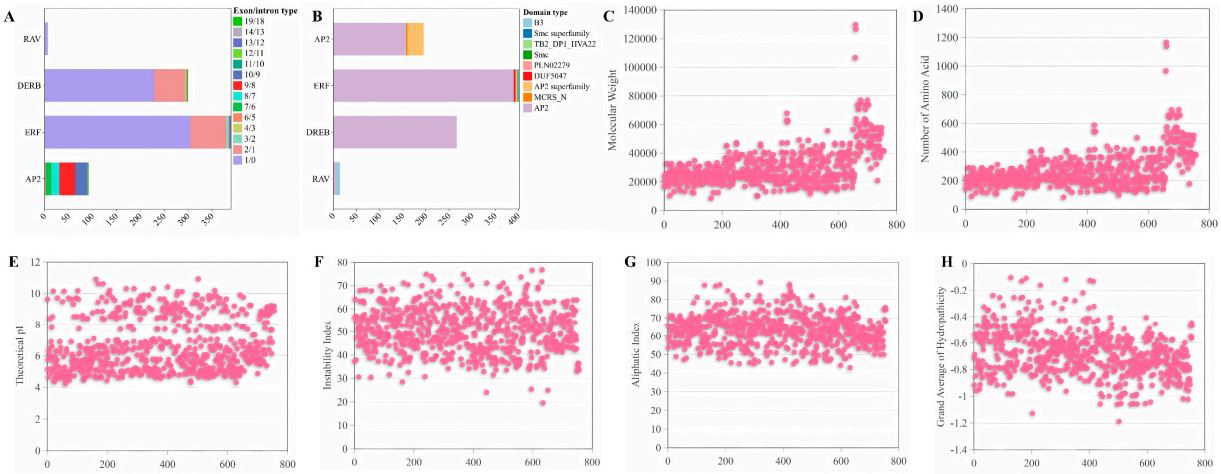


Figure 2. Gene structure, domain organization, and physicochemical properties of MsAP2/ERF proteins. (**A**) Gene structure types of members from the four *MsAP2/ERF* subfamilies; 1/0 denotes a structure with one exon and no intron. (**B**) Domain types of members from the four *MsAP2/ERF* subfamilies. (**C**) Scatter plot of molecular weights of MsAP2/ERF proteins. (**D**) Scatter plot of amino acid residue numbers of MsAP2/ERF proteins. (**E**) Scatter plot of isoelectric points (pI) of MsAP2/ERF proteins. (**F**) Scatter plot of instability indices of MsAP2/ERF proteins. (**G**) Scatter plot of aliphatic indices of MsAP2/ERF proteins. (**H**) Scatter plot of grand average of hydropathicity (GRAVY) values of MsAP2/ERF proteins.

Domain annotation of members from the four subfamilies (Figure 2B) showed that all MsAP2/ERF proteins contained an AP2 domain, consistent with the canonical features of the AP2/ERF transcription factor superfamily. All RAV subfamily members possessed one B3 domain and one AP2 domain, whereas all DREB members contained only a single AP2 domain. Notably, some ERF subfamily members carried additional domains (e.g., SAM) besides the AP2 domain, implying that certain members may have undergone boundary shifts, sequence divergence, or gain/loss of accessory structural features, thereby leading to further functional differentiation.

Physicochemical property analysis of ERF proteins (Figure 2C–H) indicated that the majority of MsAP2/ERF proteins contained between 100 and 400 amino acids; the

largest member comprised 1,166 amino acids and the smallest consisted of 75 amino acids (Figure 2D). Molecular weight exhibited a similar distribution pattern (Figure 2C), with most proteins ranging from 10,000 to 40,000 Da. The isoelectric points (pI) of MsAP2/ERFs largely fell between 4 and 10 (Figure 2E). The instability index showed a broadly scattered distribution (Figure 2F); most MsAP2/ERF members had an instability index between 40 and 60, indicating relatively high instability and thus potential in vitro instability. The aliphatic index was generally concentrated between 40 and 80 (Figure 2G), reflecting a stable overall composition of hydrophobic amino acids across the family. The grand average of hydropathicity (GRAVY) values were mostly below zero (Figure 2H), indicating that most ERF proteins were overall hydrophilic.

3.3. Syntenic Relationships and Duplication Events of the AP2/ERF Gene Family

To reveal the conservation and syntenic features of the *MsAP2/ERF* gene family across the four haplotype genomes, we analyzed the syntenic relationships of *MsAP2/ERF* genes among hap1–hap4. The results (Figure 3A) showed 82 groups of quadruple-allelic *MsAP2/ERF* genes across the four chromosome sets (Table S2), along with extensive homologous gene connections, indicating that the MsAP2/ERF family maintains generally high homology and conservation across different haplotypes. To further explore the evolutionary conservation and lineage-specific differences of the MsAP2/ERF family, we performed a syntenic comparison between *M. sativa* and *M. truncatula*. The results (Figure 3B) revealed 229 syntenic gene pairs between the chromosomes of the two species. The *AP2/ERF* genes exhibited strong homologous correspondence between the two species, suggesting that this family is generally conserved during the evolution of *Medicago* species. Notably, some syntenic links displayed a "many-to-one" correspondence pattern, implying that additional duplication retention events may have occurred in *Medicago* AP2/ERF genes. The syntenic analysis within hap1 (Figure 3C) showed that *MsAP2/ERF* genes were distributed across all eight chromosomes, with 42 collinear gene pairs, indicating that whole-genome duplication and segmental duplication events played important roles in the formation of the *AP2/ERF* gene family. To clarify the primary mechanisms driving the expansion of the *MsAP2/ERF* gene family, we classified and quantified different duplication types (Figure 3D). The results showed that WGD/segmental duplication contributed the most to the expansion of the *MsAP2/ERF* gene family, accounting for the highest proportion at 33%. In addition, dispersed duplication and tandem duplication also accounted for substantial proportions, at 31% and 28%, respectively, while proximal duplication accounted for a relatively low proportion of 8%.

3.4. Regulatory Network and Functional Enrichment Analysis of MsAP2/ERF

To investigate the genome-wide regulatory potential of the *MsAP2/ERF* gene family, we predicted transcription factor binding sites (TFBS) in the promoter regions of all annotated protein-coding genes in the alfalfa genome. The results (Figure 4A) showed that the promoter regions of the 164,632 predicted genes in the alfalfa genome harbor binding sites for 16 classes of transcription factors, including DOF, bZIP, and ERF/DREB. KEGG pathway enrichment analysis of the potential target genes of the AP2/ERF family (Figure 4B) revealed that AP2/ERF target genes were significantly enriched in "Metabolic pathways," "Ribosome," "Phosphate metabolism," and various lipid metabolism pathways, such as glycerophospholipid metabolism and ether lipid metabolism. In addition, DNA repair-related pathways, including mismatch repair and base excision repair, also exhibited high enrichment significance.

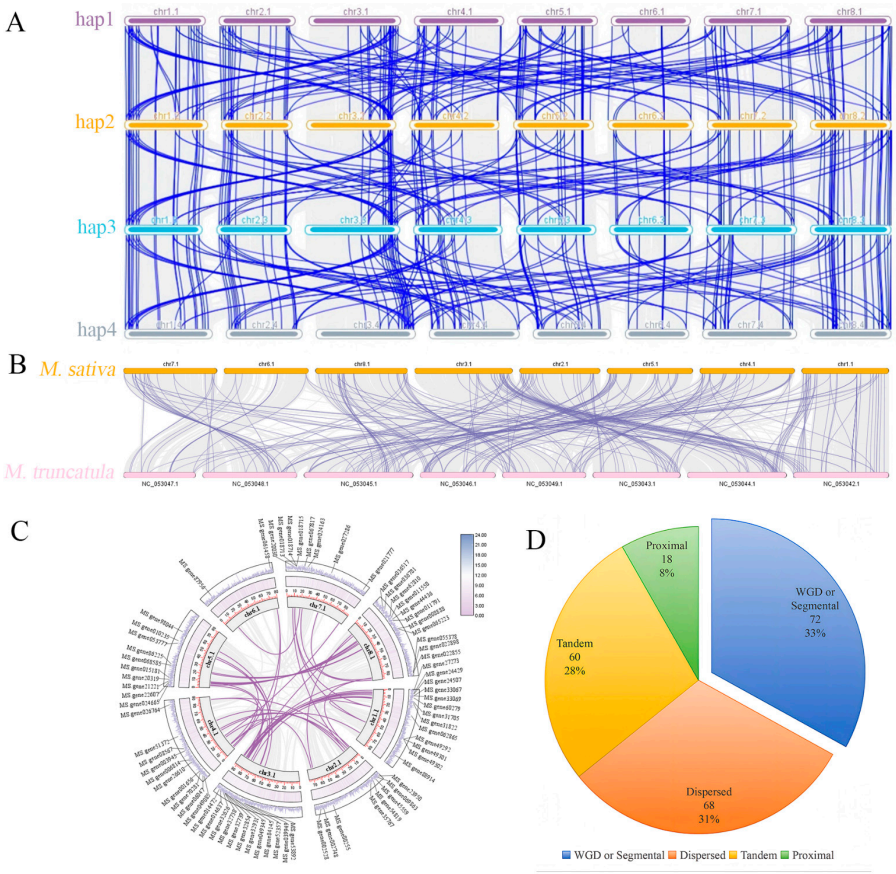


Figure 3. Synteny analysis of *MsAP2/ERFs*. (**A**) Syntenic relationships of *MsAP2/ERFs* across the four haplotype genomes, with blue connecting lines indicating synteny among MsAP2/ERFs across different haplotype genomes (**B**) Syntenic relationships between *MsAP2/ERFs* on hap1 chromosomes of *M. sativa* and *MtAP2/ERFs*, with blue connecting lines indicating synteny between MsAP2/ERFs and MtAP2/ERFs. (**C**) Syntenic relationships among *MsAP2/ERFs* on hap1 chromosomes of *M. sativa*, with purple connecting lines indicating synteny among *MsAP2/ERFs*. (**D**) Duplication types of MsAP2/ERFs on hap1 chromosomes of *M. sativa*.

To further elucidate the specific regulatory mechanisms of AP2/ERFs in drought resistance, we performed KEGG functional annotation on the target genes of differentially expressed MsAP2/ERFs under drought stress. The results (Figure 4C) showed that these target genes were highly concentrated in "Global and overview maps" under Metabolism, as well as in "Carbohydrate metabolism" and "Amino acid metabolism." Within Genetic Information Processing, pathways related to translation, folding, sorting and degradation, and transcription were prominently enriched. Additionally, significant enrichment was observed in signal transduction pathways and environmental adaptation pathways under Environmental Information Processing and Adaptation.

3.5. Expression Response and Regulatory Patterns of the MsAP2/ERF Gene Family Under Drought Stress

To characterize the response of *MsAP2/ERF* genes to drought stress, we analyzed differentially expressed genes (DEGs) under three levels of drought treatment (D1, D2, D3). The results (Figure 5A–C) showed that as drought intensity increased, the number of differentially expressed *AP2/ERF* genes rose from 9 under D1 to 49 under D3. Notably, under all drought treatments, the number of upregulated genes was significantly greater than that of downregulated genes, suggesting that upregulation of *MsAP2/ERF* expression may be beneficial for alfalfa adaptation to drought stress. Further analysis revealed that among all differentially expressed *MsAP2/ERFs* under drought stress, a total of 36 *MsAP2/ERF* genes

were upregulated after drought treatment (Figure 5D), with most reaching their highest expression levels under D3. In particular, *MsERF210* exhibited strongly induced expression under drought stress, with its expression level progressively increasing as drought intensified; the expression level under D3 was 3.05-fold higher than that of CK (Figure 5E, $P < 0.05$).

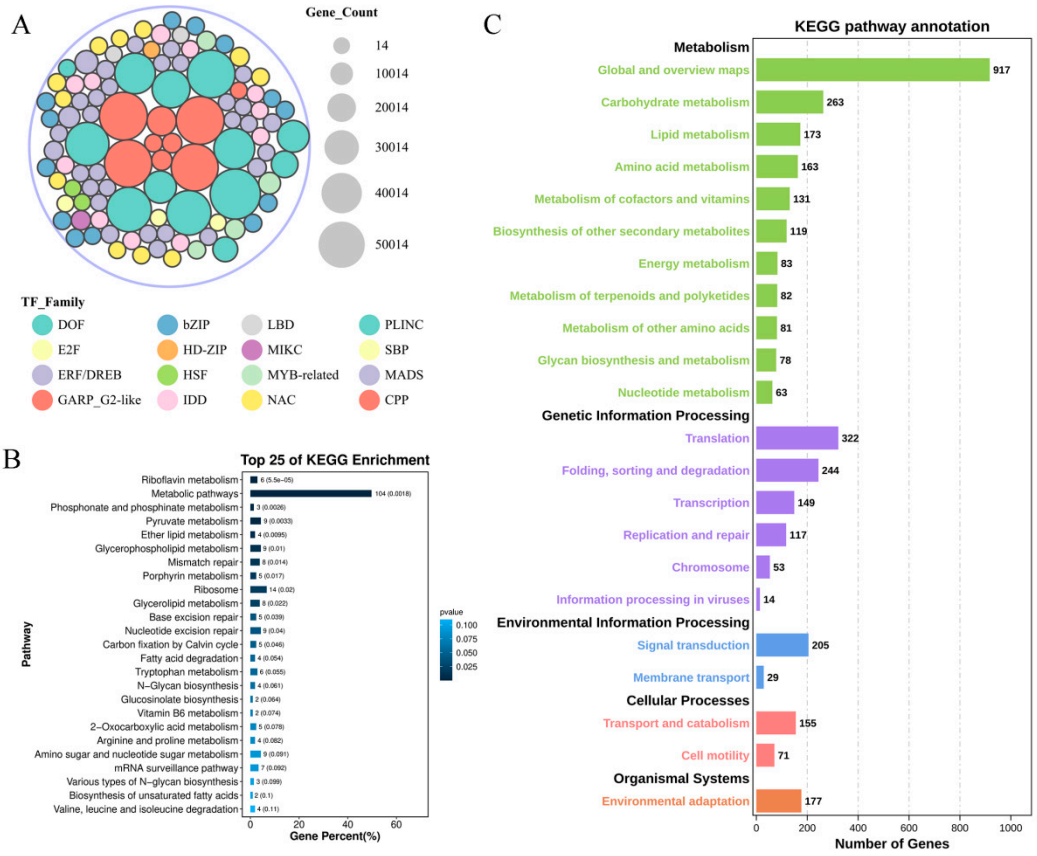


Figure 4. Prediction of TFBS in the promoter regions of *M. sativa* protein-coding genes and pathway enrichment analysis of *MsAP2/ERF* target genes. (**A**) Prediction results of TFBS in the promoter regions of *M. sativa* protein-coding genes. (**B**) Pathway enrichment analysis of *MsAP2/ERF* target genes. (**C**) Pathway enrichment analysis of target genes of differentially expressed *MsAP2/ERFs* under drought stress.

To further explore the potential regulatory function of *MsERF210*, we constructed a co-expression network using weighted gene co-expression network analysis (WGCNA) (Figure 5F,G). The results showed that the brown and turquoise modules exhibited significant positive correlations with *MsERF210* expression ($P < 0.05$), with correlation coefficients of 0.90 and 0.83, respectively. KEGG enrichment analysis of genes in the brown and turquoise modules (Figure 5H) revealed that these genes were significantly enriched in "Ribosome," "RNA degradation," and various amino acid and nucleotide metabolism pathways. Notably, three antioxidant-related pathways were enriched, namely "Glutathione metabolism," "Peroxisome," and "Ascorbate and aldarate metabolism" (Table S3), suggesting that *MsERF210* may coordinate protein homeostasis and antioxidant defense systems to maintain physiological functions under drought stress. Based on the WGCNA results, we further examined differentially expressed genes under drought stress within the Glutathione metabolism, Peroxisome, and Ascorbate and aldarate metabolism pathways. The results (Figure 5I) showed that six members from four gene families exhibited significant differential expression under drought stress: *MsIDH1*, *MsSOD24*, *MsSOD25*, *MsALDH3*, *MsALDH5*, and *MsGST18*. Compared with CK, their expression levels under D3 were upreg-

ulated by 2.08-, 2.02-, 2.11-, 3.03-, 2.39-, and 3.50-fold, respectively ($P < 0.01$). We predicted the binding conformation of MsERF210 on the promoter regions of *MsIDH1*, *MsSOD24*, *MsSOD25*, *MsALDH3*, *MsALDH5*, and *MsGST18*. The results (Figure S1) showed that the promoter regions of all six genes were predicted to be bound by *MsERF210*, indicating that *MsERF210* may exert transcriptional regulation on these six genes.

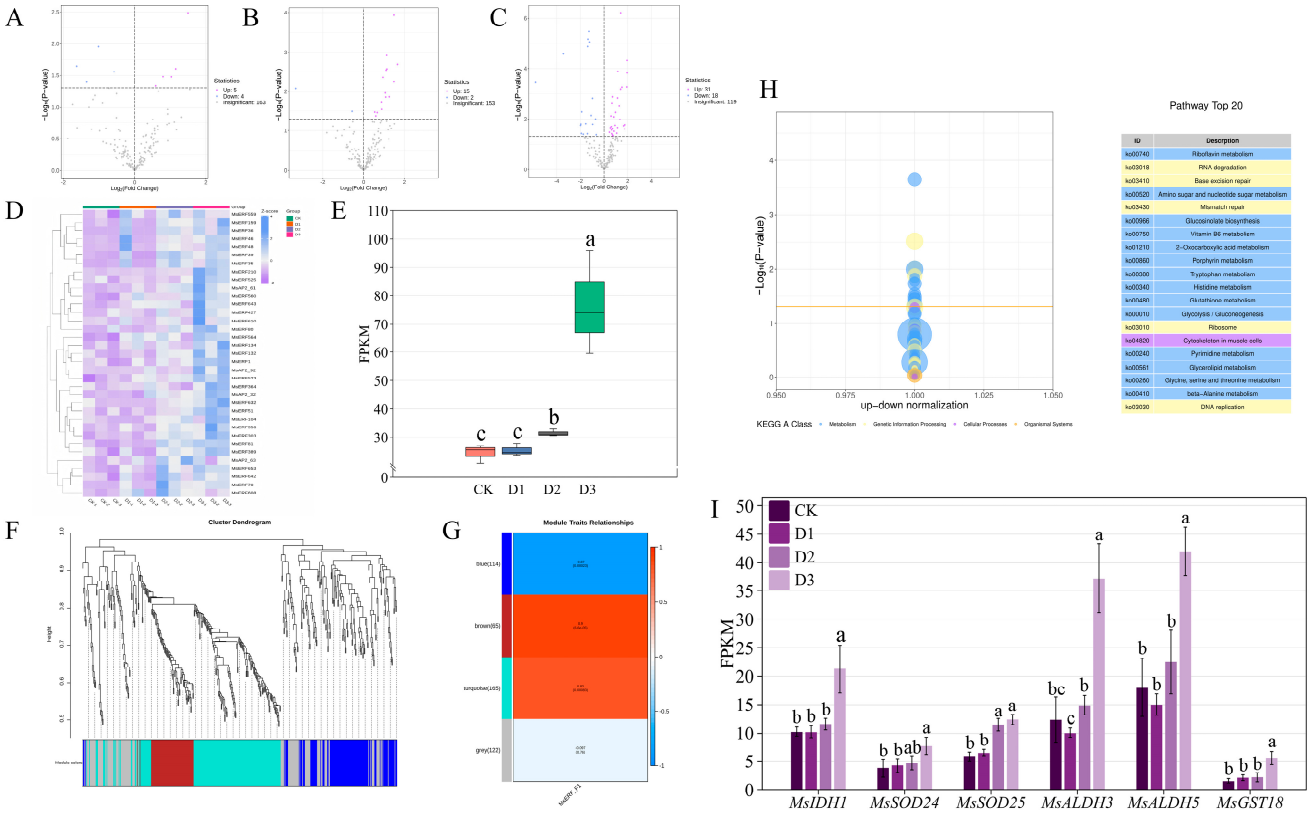


Figure 5. Differential expression analysis of *MsAP2/ERFs* under drought stress. (**A**) Volcano plot of differentially expressed *MsAP2/ERFs* under D1 stress. The dashed lines indicate the significance threshold ($P = 0.05$), consistent with the yellow line described in the KEGG enrichment bubble plot. (**B**) Volcano plot of differentially expressed *MsAP2/ERFs* under D2 stress. (**C**) Volcano plot of differentially expressed *MsAP2/ERFs* under D3 stress. (**D**) Heatmap of expression levels of differentially expressed *MsAP2/ERFs*. (**E**) Bar chart showing the expression difference of *MsERF210* under drought stress. (**F**) Module partitioning by WGCNA of differentially expressed *MsAP2/ERF* target genes and *MsERF210* under drought stress. (**G**) Correlation between co-expression modules and *MsERF210*. (**H**) KEGG enrichment pathways of positively correlated modules, The yellow line in the KEGG enrichment bubble plot indicates the significance threshold ($P = 0.05$). The size of the bubble scales the count of the enriched genes. (**I**) Bar chart of differentially expressed genes in the antioxidant modules. Expression differences in (**E**,**I**) were assessed using one-way ANOVA followed by Tukey's HSD test, with different letters indicating significant differences ($P < 0.05$).

3.6. Analysis of Antioxidant Physiological Indicators

To further investigate whether additional members of the *IDH*, *SOD*, *ALDH*, and *GST* gene families are involved in the drought stress response, we analyzed the transcriptional expression profiles of these four gene families. The results (Figure 6A–D) showed that among the *ALDH*, *IDH*, *GST*, and *SOD* families, 18, 7, 37, and 25 members were expressed (FPKM > 1), respectively. In the *ALDH* family, 15 *MsALDHs* genes were upregulated after drought stress, with 11 reaching their highest expression levels under D3. In the *IDH* family, all seven expressed members were upregulated after drought stress, and six of them reached maximum expression under D3. In the *GST* family, 36 members were upregulated

under drought stress, with 26 *MsGSTs* genes showing the highest expression levels under D3. In the *SOD* family, 15 *MsSODs* genes were upregulated after drought stress, and 14 of them reached peak expression under D3. These results indicate that members of the *IDH*, *SOD*, *ALDH*, and *GST* gene families participate in the antioxidant defense process of alfalfa under drought stress.

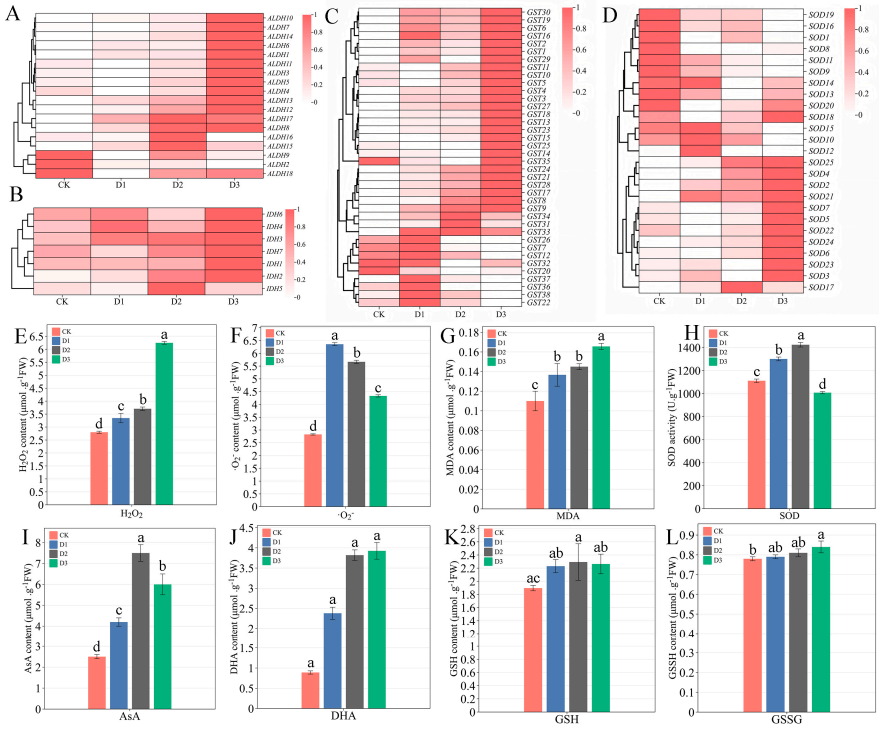


Figure 6. Changes in expression levels of antioxidant gene family members and related physiological indicators under drought stress. (**A**) Expression heatmap of the *ALDH* gene family under drought stress. (**B**) Expression heatmap of the *IDH* gene family under drought stress. (**C**) Expression heatmap of the *GST* gene family under drought stress. (**D**) Expression heatmap of the *SOD* gene family under drought stress. (**E**) Changes in H_2O_2 content under drought stress. (**F**) Changes in O_2^- content under drought stress. (**G**) Changes in MDA content under drought stress. (**H**) Changes in SOD enzyme activity under drought stress. (**I**) Changes in AsA content under drought stress. (**J**) Changes in DHA content under drought stress. (**K**) Changes in GSH content under drought stress. (**L**) Changes in GSSG content under drought stress. Expression differences in (**E**–**L**) were assessed using one-way ANOVA followed by Tukey's HSD test, with different letters indicating significant differences ($P < 0.05$).

To verify the association between gene expression levels and physiological resistance, we measured physiological and biochemical indicators under different drought gradients. The results showed that drought stress significantly induced ROS accumulation. Specifically, H_2O_2 content increased markedly with increasing drought severity (Figure 6E), reaching 2.23–fold that of CK under D3. O_2^- content peaked under D1 stress, then decreased under D2 and D3, but remained higher than CK overall (Figure 6F). MDA content increased with drought intensity, reaching 1.51-fold that of CK under D3 (Figure 6G). SOD enzyme activity increased significantly under D1 and D2 stress but decreased significantly under D3 stress (Figure 6H), suggesting that plants actively initiate defense under mild to moderate drought but may suffer damage under severe drought. Meanwhile, the contents of AsA and DHA (Figure 6I,J) as well as GSH (Figure 6K) and GSSG (Figure 6L) all accumulated significantly after drought stress ($P < 0.05$). Notably, AsA and DHA contents increased by 200% and 341.2%, respectively, under D2 and D3 compared with CK.

3.7. Quantitative qRT-PCR Validation

To validate the roles of *AP2/ERF* and antioxidant enzyme families under drought stress, we selected six highly differentially expressed genes and measured their relative expression levels under drought stress. The results (Figure 7) showed that, except for *MsALDH5*, which was downregulated under D1 stress, the expression levels of *MsERF610*, *MsERF210*, *MsGST18*, *MsIDH1*, and *MsSOD24* all increased with increasing drought severity and reached their maximum values under D3 stress. Correlation analysis (Figure S2) revealed that, except for *MsERF610* (with a correlation coefficient of 0.57 between FPKM and relative expression), the correlation coefficients for the remaining genes were all greater than 0.60, indicating that the transcriptome data are reliable and suggesting that these genes may play important roles under drought stress.

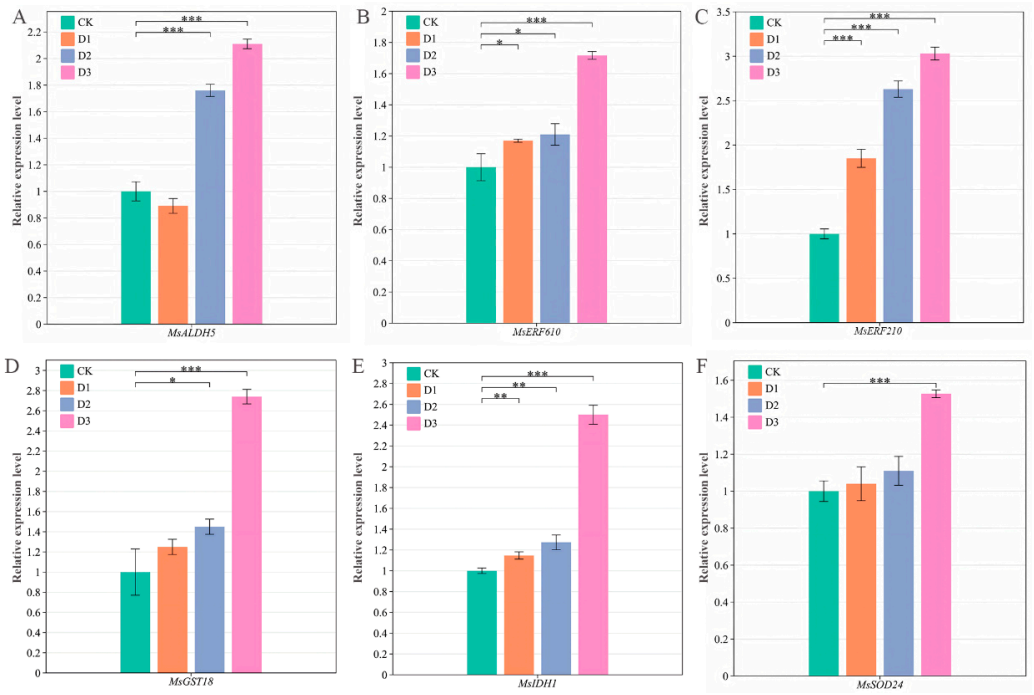


Figure 7. Relative expression levels of key differentially expressed genes under drought stress. (**A**) MsALDH5, (**B**) MsERF610, (**C**) MsERF210, (**D**) MsGST18, (**E**) MsIDH1, (**F**) MsSOD24. Significant differences in the expression of each gene between CK and different drought treatments were assessed using independent-samples *t*-test. *, **, and *** indicate significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

4. Discussion

4.1. Expansion and Evolutionary Characteristics of the Alfalfa AP2/ERF Gene Family

AP2/ERF play important roles in plant growth and development, hormone signal transduction, and abiotic stress responses [46]. Previous studies have shown that the AP2/ERF family is extensively involved in responses to environmental stresses such as drought, salt, and low temperature. A hallmark feature of this family is the presence of a highly conserved AP2 domain, which enables recognition and binding to specific cis-acting elements in the promoters of downstream target genes, thereby regulating the expression of stress-responsive genes [10,46–48]. To date, the *AP2/ERF* gene family has been systematically identified in numerous plant species. Overall, AP2/ERF represents a relatively large transcription factor family in plants, with most plant genomes containing more than 100 members. For example, 148 *AP2/ERF* genes have been identified in soybean [49], and 322 *AP2/ERF* genes in common wheat (*Triticum aestivum*) [50]. In the present study, a total of 756 *AP2/ERF* gene family members were identified in the autotetraploid alfalfa genome

and classified into four subfamilies: *ERF*, *DREB*, *AP2*, and *RAV*, comprising 390 *ERF*, 267 *DREB*, 92 *AP2*, and 7 *RAV* members. This indicates a significant expansion of the *AP2/ERF* family in alfalfa, particularly the *ERF* and *DREB* subfamilies, suggesting that these two subfamilies may play more prominent roles in alfalfa's adaptation to complex environments. Regarding the compositional ratios of different subfamilies, our results are generally consistent with those reported in other plants: the *ERF* subfamily typically contains the largest number of members, followed by *DREB* and *AP2*, while the RAV subfamily has relatively few members. For instance, among the 148 AP2/ERF members in soybean, 120 belong to the ERF subfamily [49]; among the 123 AP2/ERF members in *M. truncatula*, 98 are ERF members [19]. The larger sizes of the ERF and DREB subfamilies may be closely related to their functional diversity in plant adaptation to changing environments. Previous studies have demonstrated that DREB transcription factors can enhance plant tolerance to drought, salt, and low-temperature stresses by regulating osmotic adjustment, reactive oxygen species (ROS) scavenging, cold protection, and ABA-related signaling genes [50–53]; ERF are widely involved in ethylene signaling, pathogen defense, drought stress, and root and organ development [54,55]. Therefore, the expansion of the *ERF* and *DREB* subfamilies may provide a more complex and flexible transcriptional regulatory basis for alfalfa in terms of perennial growth, root development, post-cutting regeneration, and abiotic stress adaptation.

Notably, the identification of the *AP2/ERF* family in *M. truncatula* was based on a haploid genome, whereas the present study utilized an autotetraploid alfalfa genome, which likely accounts for the substantially larger number of AP2/ERF members identified here. Polyploidization is a major driving force for plant genome evolution and gene family expansion. Following whole-genome duplication (WGD), numerous duplicated genes can influence plant morphogenesis, environmental adaptation, and species differentiation through retention, loss, subfunctionalization, or neofunctionalization [56]. In cotton, the number of AP2/ERF family members in tetraploid genomes is typically about twice that in diploid genomes [57], further supporting the significant contribution of polyploidization to AP2/ERF family expansion. As an autotetraploid species, alfalfa possesses four sets of haplotypes in its genome, theoretically retaining more homologous gene copies derived from polyploidization or segmental duplication. Thus, the much higher number of AP2/ERF members identified in this study compared with *M. truncate* likely reflects the combined effects of gene dosage effects, duplicate gene retention, and subsequent functional divergence under polyploidization.

Furthermore, previous studies did not identify RAV subfamily members in tetraploid alfalfa, whereas the present study detected seven RAV members in its genome. The RAV subfamily typically contains both AP2 and B3 domains and plays important roles in plant growth and development, hormone responses, and stress resistance [58,59]. For example, some RAV genes have been reported to participate in ABA, ethylene, and brassinosteroid signaling and are associated with plant senescence, flowering, and drought and salt stress responses [60]. The identification of RAV members in this study indicates that this subfamily is not entirely absent from the tetraploid alfalfa genome. Discrepancies between previous and current findings may stem from differences in genome versions, annotation completeness, and identification strategies. In particular, given the small number of RAV subfamily members, reliance on a single database annotation or overly stringent domain screening thresholds may lead to the omission of some members. Therefore, combining methods such as HMM search, conserved domain validation, and redundant sequence filtering can improve the completeness and accuracy of AP2/ERF family member identification.

Synteny analysis further revealed extensive homologous relationships among *ERF* genes across the four alfalfa haplotypes, as well as clear syntenic conservation with the

closely related species *M. truncatula*, indicating that the *ERF* family is highly conserved during the evolution of the *Medicago* genus. This cross-species syntenic conservation suggests that some *ERF* members may originate from common ancestors prior to the divergence of *Medicago* species and have been retained under strong purifying selection over long-term evolution. On the other hand, some ERF members exhibit complex syntenic relationships across different chromosomes or haplotypes, suggesting that the family may have experienced chromosomal rearrangements, segmental duplications, gene losses, and local amplifications following polyploidization. Polyploid genomes typically undergo a "diploidization" process after formation, gradually restoring a relatively stable genetic structure through chromosomal rearrangements, homologous gene loss, and expression divergence [61]. Therefore, the coexistence of conserved syntenic blocks and complex non-one-to-one relationships in the alfalfa ERF family likely reflects the outcomes of genome remodeling and differential retention of duplicated genes after polyploidization.

This study also found that WGD/segmental duplication, dispersed duplication, and tandem duplication all contributed to the expansion of the alfalfa *ERF* family. Among these, WGD/segmental duplication was the predominant duplication type, indicating that whole-genome duplication or large-scale segmental duplication events may be the main drivers of *ERF* family expansion. For transcription factor families, WGD-derived duplicate genes tend to be preferentially retained because transcription factors are often involved in complex protein interaction networks and regulatory pathways, where copy number changes may be subject to dosage balance constraints [62,63]. Under such circumstances, retention of duplicated transcription factors not only helps maintain the stability of regulatory networks but may also provide a genetic basis for subsequent functional divergence. Meanwhile, tandem duplication and dispersed duplication also accounted for considerable proportions, suggesting that local gene amplification and genomic positional reorganization likewise contributed to the increase in ERF member numbers. Tandem duplication can lead to rapid expansion of certain gene clusters, resulting in strong functional redundancy or dosage effects under specific stress conditions or in particular tissues, thereby acquiring distinct expression patterns and regulatory relationships [64,65]. After duplication, *ERF* genes may participate in regulatory processes in different tissues, developmental stages, or stress conditions through changes in promoter cis-elements, expression pattern divergence, or altered protein functions. Therefore, the expansion of the *AP2/ERF* family, especially the *ERF* subfamily, may represent an important genetic basis for alfalfa's adaptation to variable ecological environments and agricultural production conditions.

4.2. ERF Possesses Significant Regulatory Potential in Alfalfa Drought Response

Drought stress is a critical environmental factor limiting alfalfa growth and yield formation. Upon perceiving drought signals, plants rapidly initiate a series of processes involving transcriptional regulation, osmotic adjustment, antioxidant defense, and metabolic reprogramming. AP2/ERF TF, as key regulators of ethylene signaling and abiotic stress responses, play essential roles in this process [10]. This study found that AP2/ERF TF exhibit high potential binding coverage across the alfalfa genome, suggesting that these transcription factors may not act solely on a few typical stress-responsive genes but are instead broadly embedded in the global transcriptional regulatory network of alfalfa. Their potential target genes are primarily enriched in metabolic pathways, ribosomes, lipid metabolism, nucleic acid repair, and amino acid metabolism, indicating that the AP2/ERF family may participate in plant growth and environmental adaptation by coordinating basal metabolism, protein synthesis, membrane structural stability, and genomic stability. Previous studies have shown that drought stress significantly alters plant carbon metabolism, nitrogen metabolism, amino acid synthesis, lipid remodeling, and antioxidant

metabolism, collectively determining energy supply, osmotic balance, and cell protection capacity under water deficit conditions [66,67]. Therefore, the enrichment of AP2/ERF potential target genes in basal metabolism-related pathways may reflect their role in regulating the "growth-defense" balance during drought adaptation.

Under drought stress conditions, the target genes of differentially expressed AP2/ERFs were further enriched in pathways related to carbohydrate metabolism, amino acid metabolism, energy metabolism, signal transduction, and environmental adaptation. These processes are all closely associated with plant drought tolerance. Carbohydrate metabolism not only provides energy for stress responses but also enhances cellular osmotic adjustment through the accumulation of soluble sugars, trehalose, and related sugar metabolites, and acts as signaling molecules to regulate stress-responsive gene expression [68]. Amino acid metabolism, particularly changes in pathways involving proline, glutamate, and branched-chain amino acids, helps maintain cellular osmotic potential, stabilize proteins and membrane structures, and buffer the damage caused by ROS accumulation [69]. Reprogramming of energy metabolism aids plants in reallocating limited carbon sources and energy under stress conditions to maintain cellular homeostasis, repair damage, and initiate defense responses [66]. Additionally, drought-induced ROS accumulation can lead to DNA damage and membrane lipid peroxidation; thus, the enrichment of target genes related to nucleic acid repair, antioxidant defense, and lipid metabolism also suggests that ERFs may participate in maintaining genomic stability and membrane system integrity [70]. Collectively, these findings indicate that MsAP2/ERFs may be involved in drought stress adaptation by regulating multiple downstream functional modules: on the one hand, they may directly regulate signal transduction and typical stress-responsive genes to rapidly initiate drought-resistance transcriptional programs; on the other hand, they may enhance cellular homeostatic maintenance under water deficit conditions by influencing fundamental biological processes such as carbohydrate metabolism, amino acid metabolism, protein synthesis, lipid homeostasis, and DNA damage repair.

4.3. MsERF210 May Be a Key Candidate Gene for Alfalfa Drought Stress Response

Different AP2/ERF members exhibit varying degrees of responsiveness to drought stress. This study found that the number of differentially expressed *AP2/ERF* genes gradually increased with increasing drought severity, indicating a significant intensity-dependent response characteristic of the *MsAP2/ERF* family to drought stress. Under mild drought conditions, only a few *MsAP2/ERF* members were induced, potentially representing early or sensitive stress-responsive factors; under moderate and severe drought, the number of upregulated genes increased markedly, suggesting that more *MsAP2/ERF* members are recruited into stress signal transduction, transcriptional reprogramming, and defense response networks as water deficit intensifies. This graded response pattern aligns with the "early perception–signal amplification–defense execution" regulatory logic of plant stress adaptation, whereby plants dynamically adjust transcription factor activity and downstream defense gene expression according to stress intensity to balance growth maintenance and stress tolerance [66,71].

MsERF210 belongs to the *ERF* subclade and exhibited prominent induced expression under drought treatment, with its expression level significantly increasing as drought severity intensified and reaching a high level under severe drought. This expression pattern indicates that *MsERF210* is highly sensitive to water deficit signals and may represent an important candidate node in the alfalfa drought resistance regulatory network. Previous studies have shown that ERF subclade members are an important branch of the AP2/ERF family involved in abiotic stress responses, capable of regulating target gene expression in dehydration response, antioxidant defense, osmotic adjustment, ethylene

signaling, and ABA-related pathways by binding to cis-acting elements such as GCC-box or DRE/CRT [55,72,73]. In plants such as Arabidopsis, rice, wheat, tomato, and alfalfa, multiple ERF members have been confirmed to participate in drought, salt, and oxidative stress responses, functioning either as positive regulators of stress tolerance or by influencing growth, development, and hormonal balance during stress adaptation [47,74]. Therefore, the significant induction of MsERF210 under severe drought suggests that it may be involved in defense reinforcement processes under strong water deficit conditions.

WGCNA further revealed that *MsERF210* exhibited significant co-expression relationships with multiple antioxidant-related modules, which contained members of gene families closely associated with redox homeostasis, including *ALDH*, *IDH*, *GST*, and *SOD*. Drought stress typically disrupts electron transport and metabolic balance within plant cells, leading to massive accumulation of ROS-including superoxide anions, hydrogen peroxide, and hydroxyl radicals—in chloroplasts, mitochondria, and peroxisomes [75]. Moderate levels of ROS can act as signaling molecules involved in calcium signaling, MAPK cascades, hormone signaling, and transcriptional regulation, thereby activating defense responses; however, when ROS accumulation exceeds cellular scavenging capacity, it causes membrane lipid peroxidation, protein oxidation, nucleic acid damage, and disruption of cellular structures, ultimately inhibiting plant growth or even inducing cell death [70]. Therefore, maintaining a dynamic balance between ROS production and scavenging is fundamental for plant adaptation to drought stress.

From the perspective of co-expressed gene functions, SOD is a key enzyme in the ROS scavenging system, converting superoxide anions into hydrogen peroxide, which is subsequently eliminated by CAT, APX, GPX, or peroxidases; GST participates in glutathione-dependent detoxification and antioxidant processes, helping to reduce cellular damage from lipid peroxidation products and electrophilic toxic substances; ALDH can scavenge aldehyde toxic products accumulated under drought and oxidative stress, mitigating cell damage caused by membrane lipid peroxidation; IDH is involved in the tricarboxylic acid cycle, NADPH supply, and maintenance of cellular reducing power, providing necessary metabolic support for the antioxidant system [75]. The significant co-expression relationships between these genes and *MsERF210* suggest that *MsERF210* may not only participate in the transcriptional response to drought signals but also enhance alfalfa's ROS scavenging capacity and cell protection ability under water deficit conditions by regulating redox homeostasis-related genes. In summary, MsERF210 may be an important ERF member involved in alfalfa drought stress response. Its expression significantly increases with drought intensity, indicating that this gene may participate in transcriptional regulation amplification under severe stress conditions; its co-expression relationships with antioxidant-related genes such as ALDH, IDH, GST, and SOD further suggest that MsERF210 may be involved in alfalfa's adaptive regulation to drought stress by coordinating ROS signaling, antioxidant defense, and metabolic homeostasis. Future studies employing promoter binding analysis, yeast one-hybrid assays, dual-luciferase reporter experiments, or transgenic functional validation could further clarify whether MsERF210 directly regulates antioxidant-related target genes and its biological function in drought resistance.

4.4. ALDH, IDH, GST, and SOD Synergistically Participate in Redox Homeostasis Regulation Under Drought Stress

To further validate the role of antioxidant modules in drought response, this study analyzed the expression patterns of members from four gene families—ALDH, IDH, GST, and SOD—under different drought treatments. Some members of these families were upregulated under drought stress, particularly under moderate or severe drought conditions, indicating that they may constitute important components of alfalfa's defense against drought-induced oxidative stress. The ALDH family primarily participates in the oxidative

detoxification of aldehydes. Under drought, high salinity, low temperature, and oxidative stress conditions, membrane lipid peroxidation generates a large number of cytotoxic aldehyde compounds, such as MDA, 4-hydroxynonenal (4–HNE), and acetaldehyde. These aldehydes can react with proteins, nucleic acids, and membrane lipids, further exacerbating cellular damage [76,77]. ALDH can oxidize toxic aldehydes into corresponding carboxylic acids, thereby reducing their reactivity and cytotoxicity, and plays important roles in plant tolerance to drought, salt, and oxidative stress [77]. In this study, multiple ALDH members were induced under drought stress, suggesting that alfalfa may mitigate drought-induced membrane lipid peroxidation damage by enhancing aldehyde detoxification capacity. This finding is consistent with the physiological results showing elevated H_2O_2 and MDA contents, indicating that ALDH-mediated aldehyde scavenging may be an important component of alfalfa's drought resistance mechanism. The IDH family comprises key enzymes in the tricarboxylic acid cycle and cellular reducing power supply, catalyzing the conversion of isocitrate to α-ketoglutarate while producing NADPH. NADPH is not only an important source of reducing power for cellular anabolic metabolism but also a crucial basis for maintaining the reduced state of the glutathione cycle and the ascorbate–glutathione cycle [78]. Under stress conditions, plant demand for NADPH increases significantly, as GR, thioredoxin systems, and various antioxidant reactions rely on NADPH for reducing power. In this study, some IDH members showed increased expression under drought conditions, suggesting that they may support the regeneration of antioxidant molecules such as GSH and AsA by enhancing NADPH supply, thereby improving cellular capacity to scavenge H_2O_2 and maintain redox balance. Additionally, α-ketoglutarate produced by IDH catalysis is closely linked to amino acid metabolism and carbon–nitrogen balance, indicating that IDH may not only participate in antioxidant defense but also connect energy metabolism, nitrogen metabolism, and stress adaptation processes. The GST family is primarily involved in glutathione conjugation reactions, playing important roles in detoxification, antioxidant defense, hormone responses, and secondary metabolism. GST can catalyze the conjugation of GSH with toxic electrophilic compounds, facilitating their transformation, transport, and elimination; meanwhile, some GSTs also possess glutathione peroxidase-like activity and can participate in the scavenging of lipid peroxidation products [55]. Under drought stress, GST-mediated GSH-dependent detoxification processes help reduce damage to cell membranes and proteins caused by ROS and their derived toxic products. This study found that some GST genes were upregulated under severe drought, consistent with changes in physiological indicators related to glutathione metabolism, suggesting that GST may enhance alfalfa's cell protection capacity under severe water deficit by promoting GSH-participated detoxification and antioxidant processes. The SOD family serves as the first line of defense in the ROS scavenging system, dismutating superoxide anions into H_2O_2, which is then further eliminated by CAT, APX, POD, or the AsA-GSH cycle. Enhanced SOD activity typically indicates a rapid response to ROS accumulation and is an important marker of antioxidant defense system activation [55]. In this study, some SOD members showed induced expression under drought stress, indicating that SOD-mediated superoxide anion scavenging may participate in alfalfa's early or moderate drought response. However, under D3, SOD enzyme activity was significantly lower than that under D2 and CK, despite the fact that most *SOD* genes reached their highest transcriptional levels at D3. This paradox may be attributed to post-translational modifications, protein instability, or enzyme inactivation under excessive ROS accumulation, wherein the overwhelming oxidative pressure exceeds the translational and folding capacity of SOD isozymes, leading to a decoupling between SOD transcript abundance and actual enzyme activity. However, after SOD converts O_2^- to H_2O_2, downstream systems such as APX and the AsA-GSH cycle must work synergistically; otherwise, H_2O_2 may continue to

accumulate and cause oxidative damage. Therefore, SOD, GST, ALDH, and IDH may form an interconnected antioxidant network: SOD is responsible for initial superoxide anion scavenging, IDH provides NADPH for the antioxidant cycle, GST utilizes GSH to eliminate toxic electrophilic compounds and lipid peroxidation products, and ALDH further detoxifies aldehydes. This synergistic mechanism may jointly maintain redox homeostasis in alfalfa under drought stress.

5. Conclusions

This study systematically elucidated the evolutionary characteristics of the alfalfa ERF gene family and its regulatory patterns under drought stress. The results showed that the ERF family underwent significant expansion in alfalfa, with WGD/segmental duplication, tandem duplication, and dispersed duplication jointly contributing to the increase in family members. Under drought stress, multiple ERF members were significantly induced, among which *MsERF210* emerged as a core family member potentially responsible for drought stress response. *MsERF210* was significantly associated with Glutathione metabolism, Peroxisome, and Ascorbate and aldarate metabolism modules. Further gene expression and physiological/biochemical validation demonstrated that members of antioxidant-related gene families, including *ALDH*, *IDH*, *GST*, and *SOD*, were induced under drought stress, accompanied by significant changes in indicators such as H_2O_2, MDA, SOD activity, AsA, DHA, GSH, and GSSG. Collectively, these results suggest that *MsERF210* may enhance alfalfa's adaptability to drought stress by regulating the antioxidant defense system and redox homeostasis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy16171682/s1, Table S1: MsAP2ERF gene information. Table S2: Tetraploid allele set. Table S3: Enriched pathways of differentially expressed genes. Table S4: Primer sequences used for RT-qPCR. Figure S1. Predicted binding models of MsERF210 with the promoter regions of target genes. Figure S2. Correlation between FPKM and relative expression levels of key differentially expressed genes.

Author Contributions: Conceptualization, X.W., Y.L., Y.K., L.Z. and X.Z.; Methodology, X.W., J.Q., Y.K., X.Z. and S.L.; Software, X.W., J.Q., Y.K., L.Z. and S.L.; Validation, X.W., J.Q. and Y.K.; Formal analysis, Y.L. and Y.K.; Investigation, X.W. and J.Q.; Resources, J.Q.; Data curation, X.W. and X.Z.; Writing—original draft, X.W. and J.Q.; Writing—review & editing, Y.L., Y.K., L.Z., X.Z. and S.L.; Visualization, L.Z. and S.L.; Supervision, Y.L. and X.Z.; Project administration, Y.L.; Funding acquisition, Y.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (32160409) and the Outstanding Doctoral Project of Gansu Provincial Science and Technology Program (25JRRA387).

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Wahab, A.; Abdi, G.; Saleem, M.H.; Ali, B.; Ullah, S.; Shah, W.; Mumtaz, S.; Yasin, G.; Muresan, C.C.; Marc, R.A. Plants' Physio-Biochemical and Phyto-Hormonal Responses to Alleviate the Adverse Effects of Drought Stress: A Comprehensive Review. *Plants* **2022**, *11*, 1620. [CrossRef] [PubMed]
2. Yang, X.; Lu, M.; Wang, Y.; Wang, Y.; Liu, Z.; Chen, S. Response Mechanism of Plants to Drought Stress. *Horticulturae* **2021**, *7*, 50. [CrossRef]

3. Joshi, R.; Wani, S.H.; Singh, B.; Bohra, A.; Dar, Z.A.; Lone, A.A.; Pareek, A.; Singla-Pareek, S.L. Transcription Factors and Plants Response to Drought Stress: Current Understanding and Future Directions. *Front. Plant Sci.* **2016**, *7*, 1029. [CrossRef] [PubMed]
4. Hrmova, M.; Hussain, S.S. Plant Transcription Factors Involved in Drought and Associated Stresses. *Int. J. Mol. Sci.* **2021**, *22*, 5662. [CrossRef] [PubMed]
5. Zong, W.; Tang, N.; Yang, J.; Peng, L.; Ma, S.; Xu, Y.; Li, G.; Xiong, L. Feedback Regulation of ABA Signaling and Biosynthesis by a bZIP Transcription Factor Targets Drought-Resistance-Related Genes. *Plant Physiol.* **2016**, *171*, 2810–2825. [CrossRef] [PubMed]
6. Kong, L.; Song, Q.; Wei, H.; Wang, Y.; Lin, M.; Sun, K.; Zhang, Y.; Yang, J.; Li, C.; Luo, K. The AP2/ERF transcription factor PtoERF15 confers drought tolerance via JA-mediated signaling in *Populus*. *New Phytol.* **2023**, *240*, 1848–1867. [CrossRef] [PubMed]
7. Hu, H.; Dai, M.; Yao, J.; Xiao, B.; Li, X.; Zhang, Q.; Xiong, L. Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 12987–12992. [CrossRef] [PubMed]
8. Abe, H.; Yamaguchi-Shinozaki, K.; Urao, T.; Iwasaki, T.; Hosokawa, D.; Shinozaki, K. Role of arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. *Plant Cell* **1997**, *9*, 1859–1868. [CrossRef] [PubMed]
9. Khoso, M.A.; Hussain, A.; Ritonga, F.N.; Ali, Q.; Channa, M.M.; Alshegaihi, R.M.; Meng, Q.; Ali, M.; Zaman, W.; Brohi, R.D.; et al. WRKY transcription factors (TFs): Molecular switches to regulate drought, temperature, and salinity stresses in plants. *Front. Plant Sci.* **2022**, *13*, 1039329. [CrossRef] [PubMed]
10. Xu, Z.S.; Chen, M.; Li, L.C.; Ma, Y.Z. Functions of the ERF transcription factor family in plants. *Botany* **2008**, *86*, 969–977. [CrossRef]
11. Nakano, T.; Suzuki, K.; Fujimura, T.; Shinshi, H. Genome-Wide Analysis of the ERF Gene Family in Arabidopsis and Rice. *Plant Physiol.* **2006**, *140*, 411–432. [CrossRef] [PubMed]
12. He, Y.; Li, R.; Li, D.; Fan, X.; Chen, X.; Zhuang, C.; Li, J. Research Advances in AP2/ERF Transcription Factors in Rice Growth and Development. *Plants* **2025**, *14*, 2673. [CrossRef] [PubMed]
13. Li, M.; Zou, J.; Cheng, Q.; Fu, R.; Zhang, D.; Lai, Y.; Chen, Y.; Yang, C.; Hu, W.; Ding, S. Genome-Wide Identification and Expression of the *ERF* Gene Family in *Populus trichocarpa* and Their Responses to Nitrogen and Abiotic Stresses. *Life* **2025**, *15*, 550. [CrossRef] [PubMed]
14. Quan, R.; Hu, S.; Zhang, Z.; Zhang, H.; Zhang, Z.; Huang, R. Overexpression of an ERF transcription factor *TSRF1* improves rice drought tolerance. *Plant Biotechnol. J.* **2010**, *8*, 476–488. [CrossRef] [PubMed]
15. Maruyama, K.; Takeda, M.; Kidokoro, S.; Yamada, K.; Sakuma, Y.; Urano, K.; Fujita, M.; Yoshiwara, K.; Matsukura, S.; Morishita, Y.; et al. Metabolic Pathways Involved in Cold Acclimation Identified by Integrated Analysis of Metabolites and Transcripts Regulated by DREB1A and DREB2A. *Plant Physiol.* **2009**, *150*, 1972–1980. [CrossRef] [PubMed]
16. Zhang, G.; Chen, M.; Li, L.; Xu, Z.; Chen, X.; Guo, J.; Ma, Y. Overexpression of the soybean GmERF3 gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco. *J. Exp. Bot.* **2009**, *60*, 3781–3796. [CrossRef] [PubMed]
17. Singer, S.D.; Hannoufa, A.; Acharya, S. Molecular improvement of alfalfa for enhanced productivity and adaptability in a changing environment. *Plant Cell Environ.* **2018**, *41*, 1955–1971. [CrossRef] [PubMed]
18. Feng, Y.; Shi, Y.; Zhao, M.; Shen, H.; Xu, L.; Luo, Y.; Liu, Y.; Xing, A.; Kang, J.; Jing, H.; et al. Yield and quality properties of alfalfa (*Medicago sativa* L.) and their influencing factors in China. *Eur. J. Agron.* **2022**, *141*, 126637. [CrossRef]
19. Zhang, X.; Zhu, W.; Liu, Z.; Ren, X.; Li, Y.; Li, G.; Wang, J.; Zhu, X.; Shi, Y.; Wang, C.; et al. Transcriptomic analysis of melatonin-mediated drought stress response genes in alfalfa during germination period. *BMC Plant Biol.* **2025**, *25*, 637. [CrossRef] [PubMed]
20. Chen, F.; Ha, X.; Ma, T.; Ma, H. Comparative analysis of the physiological and transcriptomic profiles reveals alfalfa drought resistance mechanisms. *BMC Plant Biol.* **2024**, *24*, 954. [CrossRef] [PubMed]
21. Santoro, D.F.; Anderson, A.W.; Alavi, S.N.; Pierleoni, V.A.M.; Rosellini, D. Whole genome duplication drives transcriptome reprogramming in response to drought in alfalfa. *Plant Cell Rep.* **2025**, *44*, 209. [CrossRef] [PubMed]
22. Shu, Y.; Liu, Y.; Zhang, J.; Song, L.; Guo, C. Genome-Wide Analysis of the AP2/ERF Superfamily Genes and their Responses to Abiotic Stress in Medicago truncatula. *Front. Plant Sci.* **2016**, *6*, 1247. [CrossRef] [PubMed]
23. Qiu, R.; Wang, J.; Fan, W.; Mu, Y.; Zhang, Y.; Zhao, L.; Shi, F. Genome-wide analysis of Alfalfa AP2/ERF gene family and abiotic stress response. *Front. Plant Sci.* **2026**, *17*, 1776336. [CrossRef] [PubMed]
24. Chen, T.; Yang, Q.; Gruber, M.; Kang, J.; Sun, Y.; Ding, W.; Zhang, T.; Zhang, X. Expression of an alfalfa (*Medicago sativa* L.) ethylene response factor gene MsERF8 in tobacco plants enhances resistance to salinity. *Mol. Biol. Rep.* **2011**, *39*, 6067–6075. [CrossRef] [PubMed]
25. Jin, X.; Yin, X.; Ndayambaza, B.; Zhang, Z.; Min, X.; Lin, X.; Wang, Y.; Liu, W. Genome-Wide Identification and Expression Profiling of the *ERF* Gene Family in *Medicago sativa* L. Under Various Abiotic Stresses. *DNA Cell Biol.* **2019**, *38*, 1056–1068. [CrossRef] [PubMed]
26. Qi, J.; Luo, Y.; Lu, S.; Liu, H.; Huang, H.; Qiu, Y.; Zhou, X.; Ma, C. Multi-omics integration analysis reveals the molecular mechanisms of drought adaptation in homologous tetraploid alfalfa (*Medicago sativa* 'Xinjiang-Daye'). *Physiol. Plant.* **2024**, *176*, e14476. [CrossRef] [PubMed]

27. Kim, D.; Paggi, J.M.; Park, C.; Bennett, C.; Salzberg, S.L. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat. Biotechnol.* **2019**, *37*, 907–915. [CrossRef] [PubMed]
28. Liao, Y.; Smyth, G.K.; Shi, W. feature Counts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **2013**, *30*, 923–930. [CrossRef] [PubMed]
29. Nguyen, L.-T.; Schmidt, H.A.; Von Haeseler, A.; Minh, B.Q. IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol. Biol. Evol.* **2015**, *32*, 268–274. [CrossRef] [PubMed]
30. Capella-Gutiérrez, S.; Silla-Martínez, J.M.; Gabaldón, T. trimAl: A tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* **2009**, *25*, 1972–1973. [CrossRef] [PubMed]
31. Xie, J.; Chen, Y.; Cai, G.; Cai, R.; Hu, Z.; Wang, H. Tree Visualization By One Table (tvBOT): A web application for visualizing, modifying and annotating phylogenetic trees. *Nucleic Acids Res.* **2023**, *51*, W587–W592. [CrossRef] [PubMed]
32. Chen, C.; Wu, Y.; Li, J.; Wang, X.; Zeng, Z.; Xu, J.; Liu, Y.; Feng, J.; Chen, H.; He, Y. TBtools-II: A "one for all, all for one" bioinformatics platform for biological big-data mining. *Mol. Plant* **2023**, *16*, 1733–1742. [CrossRef] [PubMed]
33. Wang, Y.; Tang, H.; DeBarry, J.D.; Tan, X.; Li, J.; Wang, X.; Lee, T.-H.; Jin, H.; Marler, B.; Guo, H.; et al. *MCScanX*: A toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Res.* **2012**, *40*, e49. [CrossRef] [PubMed]
34. Grant, C.E.; Bailey, T.L.; Noble, W.S. FIMO: Scanning for occurrences of a given motif. *Bioinformatics* **2011**, *27*, 1017–1018. [CrossRef] [PubMed]
35. Aramaki, T.; Blanc-Mathieu, R.; Endo, H.; Ohkubo, K.; Kanehisa, M.; Goto, S.; Ogata, H. KofamKOALA: KEGG Ortholog assignment based on profile HMM and adaptive score threshold. *Bioinformatics* **2020**, *36*, 2251–2252. [CrossRef] [PubMed]
36. Graham, F. Daily briefing: AlphaFold3 is now open source. *Nature* **2024**. [CrossRef] [PubMed]
37. PyMOL. *The PyMOL Molecular Graphics System*; Version 3.0; Schrödinger, LLC: New York, NY, USA, 2024.
38. Love, M.I.; Huber, W.; Anders, S. Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2. *Genome Biol.* **2014**, *15*, 550. [CrossRef] [PubMed]
39. Zhang, R.; Chen, Y.; He, J.; Gou, H.-Y.; Zhu, Y.-L.; Zhu, Y.-M. WGCNA combined with GSVA to explore biomarkers of refractory neocortical epilepsy. *IBRO Neurosci. Rep.* **2022**, *13*, 314–321. [CrossRef] [PubMed]
40. Heath, R.L.; Packer, L. Photoperoxidation in isolated chloroplasts: I. kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* **1968**, *125*, 189–198. [CrossRef] [PubMed]
41. Wang, X.K. *Principles and Techniques of Plant Physiological and Biochemical Experiments*; Higher Education Press: Beijing, China, 2006. (In Chinese)
42. Monostori, P.; Wittmann, G.; Karg, E.; Túri, S. Determination of glutathione and glutathione disulfide in biological samples: An in-depth review. *J. Chromatogr. B* **2009**, *877*, 3331–3346. [CrossRef] [PubMed]
43. Giannopolotis, C.N.; Ries, S.K. Superoxide dismutases: I. Occurrence in higher plants. *Plant Physiol.* **1977**, *59*, 309–314. [CrossRef] [PubMed]
44. Patterson, B.D.; MacRae, E.A.; Ferguson, I.B. Estimation of hydrogen peroxide in plant extracts using titanium(IV). *Anal. Biochem.* **1984**, *139*, 487–492. [CrossRef] [PubMed]
45. Wang, A.G.; Luo, G.H. Quantitative Relation between the Reaction of Hydroxylamine and Superoxide Anion Radicals in Plants. *Plant Physiol. Commun.* **1990**, *6*, 55–57. (In Chinese)
46. Feng, K.; Hou, X.; Xing, G.; Liu, J.; Duan, A.; Xu, Z.; Li, M.; Zhuang, J.; Xiong, A. Advances in AP2/ERF super-family transcription factors in plant. *Crit. Rev. Biotechnol.* **2020**, *40*, 750–776. [CrossRef] [PubMed]
47. Ma, Z.; Hu, L.; Jiang, W. Understanding AP2/ERF Transcription Factor Responses and Tolerance to Various Abiotic Stresses in Plants: A Comprehensive Review. *Int. J. Mol. Sci.* **2024**, *25*, 893. [CrossRef] [PubMed]
48. Su, Z.-L.; Li, A.-M.; Wang, M.; Qin, C.-X.; Pan, Y.-Q.; Liao, F.; Chen, Z.-L.; Zhang, B.-Q.; Cai, W.-G.; Huang, D.-L. The Role of AP2/ERF Transcription Factors in Plant Responses to Biotic Stress. *Int. J. Mol. Sci.* **2025**, *26*, 4921. [CrossRef] [PubMed]
49. Zhang, G.; Chen, M.; Chen, X.; Xu, Z.; Guan, S.; Li, L.-C.; Li, A.; Guo, J.; Mao, L.; Ma, Y. Phylogeny, gene structures, and expression patterns of the ERF gene family in soybean (*Glycine max* L.). *J. Exp. Bot.* **2008**, *59*, 4095–4107. [CrossRef] [PubMed]
50. Riaz, M.W.; Lu, J.; Shah, L.; Yang, L.; Chen, C.; Mei, X.D.; Xue, L.; Manzoor, M.A.; Abdullah, M.; Rehman, S.; et al. Expansion and Molecular Characterization of AP2/ERF Gene Family in Wheat (*Triticum aestivum* L.). *Front. Genet.* **2021**, *12*, 632155. [CrossRef] [PubMed]
51. Liu, Q.; Kasuga, M.; Sakuma, Y.; Abe, H.; Miura, S.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Two Transcription Factors, DREB1 and DREB2, with an EREBP/AP2 DNA Binding Domain Separate Two Cellular Signal Transduction Pathways in Drought- and Low-Temperature-Responsive Gene Expression, Respectively, in Arabidopsis. *Plant Cell* **1998**, *10*, 1391–1406. [CrossRef] [PubMed]
52. Sakuma, Y.; Maruyama, K.; Osakabe, Y.; Qin, F.; Seki, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. Functional Analysis of an *Arabidopsis* Transcription Factor, DREB2A, Involved in Drought-Responsive Gene Expression. *Plant Cell* **2006**, *18*, 1292–1309. [CrossRef] [PubMed]
53. Shinozaki, K.; Yamaguchi-Shinozaki, K. Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.* **2006**, *58*, 221–227. [CrossRef] [PubMed]

54. Wang, M.; Du, P.; Xi, L.; Lin, H.; Zhang, S. Dynamic Coordination: How ERF Transcription Factors Coordinate Plant Development and Adaptive Stress Responses. *Biomolecules* **2026**, *16*, 466. [CrossRef] [PubMed]
55. Müller, M.; Munné-Bosch, S. Ethylene Response Factors: A Key Regulatory Hub in Hormone and Stress Signaling. *Plant Physiol.* **2015**, *169*, 32–41. [CrossRef] [PubMed]
56. Van de Peer, Y.; Mizrachi, E.; Marchal, K. The evolutionary significance of polyploidy. *Nat. Rev. Genet.* **2017**, *18*, 411–424. [CrossRef] [PubMed]
57. Zafar, M.M.; Rehman, A.; Razzaq, A.; Parvaiz, A.; Mustafa, G.; Sharif, F.; Mo, H.; Youlu, Y.; Shakeel, A.; Ren, M. Genome-wide characterization and expression analysis of Erf gene family in cotton. *BMC Plant Biol.* **2022**, *22*, 1–18. [CrossRef] [PubMed]
58. Kagaya, Y.; Ohmiya, K.; Hattori, T. RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants. *Nucleic Acids Res.* **1999**, *27*, 470–478. [CrossRef] [PubMed]
59. Matías-Hernández, L.; Aguilar-Jaramillo, A.E.; Marín-González, E.; Suárez-López, P.; Pelaz, S. RAV genes: Regulation of floral induction and beyond. *Ann. Bot.* **2014**, *114*, 1459–1470. [CrossRef] [PubMed]
60. Sohn, K.H.; Lee, S.C.; Jung, H.W.; Hong, J.K.; Hwang, B.K. Expression and functional roles of the pepper pathogen-induced transcription factor RAV1 in bacterial disease resistance, and drought and salt stress tolerance. *Plant Mol. Biol.* **2006**, *61*, 897–915. [CrossRef] [PubMed]
61. Cheng, F.; Wu, J.; Cai, X.; Liang, J.; Freeling, M.; Wang, X. Gene retention, fractionation and subgenome differences in polyploid plants. *Nat. Plants* **2018**, *4*, 258–268. [CrossRef] [PubMed]
62. Freeling, M. Bias in Plant Gene Content Following Different Sorts of Duplication: Tandem, Whole-Genome, Segmental, or by Transposition. *Annu. Rev. Plant Biol.* **2009**, *60*, 433–453. [CrossRef] [PubMed]
63. Edger, P.P.; Pires, J.C. Gene and genome duplications: The impact of dosage-sensitivity on the fate of nuclear genes. *Chromosom. Res.* **2009**, *17*, 699–717. [CrossRef] [PubMed]
64. Qiao, X.; Li, Q.; Yin, H.; Qi, K.; Li, L.; Wang, R.; Zhang, S.; Paterson, A.H. Gene duplication and evolution in recurring polyploidization–diploidization cycles in plants. *Genome Biol.* **2019**, *20*, 38. [CrossRef] [PubMed]
65. Panchy, N.; Lehti-Shiu, M.; Shiu, S.-H. Evolution of Gene Duplication in Plants. *Plant Physiol.* **2016**, *171*, 2294–2316. [CrossRef] [PubMed]
66. Zhang, H.; Zhu, J.; Gong, Z.; Zhu, J.-K. Abiotic stress responses in plants. *Nat. Rev. Genet.* **2021**, *23*, 104–119. [CrossRef] [PubMed]
67. Sewelam, N.; Kazan, K.; Schenk, P.M. Global Plant Stress Signaling: Reactive Oxygen Species at the Cross-Road. *Front. Plant Sci.* **2016**, *7*, 187. [CrossRef] [PubMed]
68. Saddhe, A.A.; Manuka, R.; Penna, S. Plant sugars: Homeostasis and transport under abiotic stress in plants. *Physiol. Plant.* **2020**, *171*, 739–755. [CrossRef] [PubMed]
69. Szabados, L.; Savouré, A. Proline: A multifunctional amino acid. *Trends Plant Sci.* **2010**, *15*, 89–97. [CrossRef] [PubMed]
70. Waszczak, C.; Carmody, M.; Kangasjärvi, J. Reactive Oxygen Species in Plant Signaling. *Annu. Rev. Plant Biol.* **2018**, *69*, 209–236. [CrossRef] [PubMed]
71. Chen, K.; Li, G.; Bressan, R.A.; Song, C.; Zhu, J.; Zhao, Y. Abscisic acid dynamics, signaling, and functions in plants. *J. Integr. Plant Biol.* **2019**, *62*, 25–54. [CrossRef] [PubMed]
72. Phukan, U.J.; Jeena, G.S.; Tripathi, V.; Shukla, R.K. Regulation of Apetala2/Ethylene Response Factors in Plants. *Front. Plant Sci.* **2017**, *8*, 150. [CrossRef] [PubMed]
73. Xie, Z.; Nolan, T.M.; Jiang, H.; Yin, Y. AP2/ERF Transcription Factor Regulatory Networks in Hormone and Abiotic Stress Responses in Arabidopsis. *Front. Plant Sci.* **2019**, *10*, 228. [CrossRef] [PubMed]
74. Licausi, F.; Ohme-Takagi, M.; Perata, P. APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factors: Mediators of stress responses and developmental programs. *New Phytol.* **2013**, *199*, 639–649. [CrossRef] [PubMed]
75. Hasanuzzaman, M.; Bhuyan, M.B.; Zulfiqar, F.; Raza, A.; Mohsin, S.M.; Al Mahmud, J.; Fujita, M.; Fotopoulos, V. Reactive Oxygen Species and Antioxidant Defense in Plants under Abiotic Stress: Revisiting the Crucial Role of a Universal Defense Regulator. *Antioxidants* **2020**, *9*, 681. [CrossRef] [PubMed]
76. Hussain, S.; Rao, M.J.; AnjumM, A.; Ejaz, S.; Zakir, I.; Ali, M.A.; Ahmad, N.; Ahmad, S. Oxidative Stress and Antioxidant Defense in Plants Under Drought Conditions. In *Plant Abiotic Stress Tolerance*; Hasanuzzaman, M., Hakeem, K., Nahar, K., Alharby, H., Eds.; Springer: Cham, Switzerland, 2019. [CrossRef]
77. Tola, A.J.; Jaballi, A.; Germain, H.; Missihoun, T.D. Recent Development on Plant Aldehyde Dehydrogenase Enzymes and Their Functions in Plant Development and Stress Signaling. *Genes* **2020**, *12*, 51. [CrossRef] [PubMed]
78. Noctor, G.; Reichheld, J.-P.; Foyer, C.H. ROS-related redox regulation and signaling in plants. *Semin. Cell Dev. Biol.* **2018**, *80*, 3–12. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.