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The Role of the ADF Gene Family in Maize Response to Abiotic Stresses

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Abstract: The highly conserved actin depolymerizing factor (ADF) plays an important role in plant growth, development and responses to biotic and abiotic stresses. A total of 72 ADF genes in Arabidopsis, wheat, rice and sorghum can be divided into four groups. The multicollinearity analysis revealed that the maize ADF gene family exhibited more collinearity events with closely related gramineous plants. Fifteen ADF genes in maize were screened from the latest database, and bioinformatics analysis showed that these ADF genes were distributed across seven chromosomes in maize. The gene structure of the ADF gene family in maize exhibits significant conservation and cluster consistency. The promoter region contains rich regulatory elements that are involved in various regulations related to growth, development and adverse stresses. The drought-tolerant *ZmADF5* gene in maize was further studied, and it was found that the allelic variations in *ZmADF5* were mainly concentrated in its promoter region. A superior haplotype, with drought tolerance, was identified by candidate-gene association analysis of 115 inbred lines. By comparing the phenotypes of anthesis silking interval, grain yield and ear height, it was found that Hap2 performed better than Hap1 under drought stress. This study provides a theoretical reference for understanding the function of the ADF gene family and proposes further investigation into the role of *ZmADF5* in abiotic-stress tolerance.

Keywords: actin depolymerization factor; ADF gene family; candidate-gene association analysis; abiotic stresses

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1. Introduction

Maize (*Zea mays* L.) is now the highest yielding cereal crop worldwide, and its planting area and production are increasing year by year [1]. As one of the main food crops in China, the majority of maize is processed into feed and biofuels, in addition to food [2–4]. Maize is a monoecious and cross-pollination crop, which is susceptible to various abiotic stresses during growth and development. Environmental stress at the flowering stage directly leads to serious yield reduction, with drought being one of the most serious abiotic stresses [5,6]. Therefore, it is of great practical significance to explore the abiotic stress-tolerant genes and study their functional mechanism to further improve the production or keep a stable level under abiotic stresses.

The actin depolymerizing factor (ADF/cofilin) is the main binding protein of microfilaments in the cytoskeleton and exists in all eukaryotic cells [7]. The first ADF in animals was isolated from chicken embryos and named cofilin, and the first ADF in plants was identified in lily [8–10]. In maize, plant biochemical characterization confirmed the conservative activity of ADF-binding F-actin and G-actin, observed for the first time in

ZmADF3 [11]. More ADF genes were identified in plants than in animals, including 11 ADF genes in Arabidopsis, 11 ADF genes in rice, 26 ADF genes in wheat, 15 ADF genes in maize, 9 ADF genes in sorghum and so on [12,13]. The number of plant ADF genes participate in important life activities, with more functional characteristics, such as cell movement, cell migration, cell division, cytoplasmic circulation, cell expansion, cell structure maintenance, intracellular material transport, polar growth and biotic- and abiotic-stress response [14–16]. It has been found that ADF genes have a non-negligible contribution to plant growth and development, especially in response to stress [17].

ADF belongs to the actin-binding protein (ABP) regulatory gene family, which participates in the dynamic regulation of actin cytoskeleton in cells by shearing and depolymerizing actin filaments [18]. Under abiotic stresses, ADF can regulate the rate of actin depolymerization by rapidly dissociating into small fragments and polymerizing into microfilaments to avoid plant damage [19]. Plant ADF genes are generally involved in the process of pollen tube germination, root hair growth and other polar growth processes related with microfilament skeleton rearrangement, where a large number of active G-actins concentrate [20]. Multiple rearrangements will happen when the growth apex and subapex continuously transform in the microfilament skeleton [21]. The microfilament skeleton is depolymerized at high speed, providing a carrier, power and anchor point for cytoplasmic circulation and vesicle transport [22].

In 2020, 13 ADF genes were publicly identified in maize [23]. Soon afterwards, a total of 15 maize ADF genes were updated based on the latest published maize genome database of the Zm-B73-REFERENCE-NAM-5.0 (B73 RefGen_v5) reference, along with the improvement of genome sequencing technology. Tandem and segmental duplication events are the main reasons driving the evolution of plant genomes [24].

In this study the new member of the maize ADF family *ZmADF14* undergoes segmental duplication with *ZmADF3* and tandem duplication with *ZmADF10*, respectively. In this study, the 15 ADF genes in maize were analyzed in detail by bioinformatics, including chromosome location, physical and chemical properties, functional clustering, collinearity, gene structure, conserved sequence, promoter elements, responses to abiotic stresses and so on. *ZmADF5*, which shows potential drought tolerance, was selected for candidate-gene association analysis. Combining with phenotypic data acquired from the field, it was proved that excellent variations from *ZmADF5* could help improving the drought tolerance in maize. This study is the first time to update the member information of maize ADF gene family and its related bioinformatics analysis after Huang which further proves that ADF gene family plays an important role in maize response to abiotic stresses. Secondly, a drought-tolerant candidate-gene *ZmADF5* and excellent variation was proposed, which laid an important foundation for further study on the function of the maize ADF gene.

2. Materials and Methods

2.1. Download and Arrangement of ADF Gene Family in Maize

The ZmADF family sequences (Zm-B73-REFERENCE-NAM-5.0, B73 RefGen_v5) were downloaded from the MaizeGDB (https://www.maizegdb.org/, accessed on 10 March 2023) database. ADF domains were used as queries to identify representative ZmADF proteins using TBtools (v1.120) (score $\geq$ 100 and e-value $\leq 1 \times 10^{-10}$) [25]. Non-redundant ZmADF protein sequences were then additionally screened with the NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 10 March 2023) to confirm the presence of an ADF domain. The chromosomal locations of the 15 ZmADFs were determined according to maize reference genome B73 RefGen_v5 information. MapChart software (v 2.0) was used to plot their positions along the 7 chromosomes. ExPASy (https://web.expasy.org/, accessed on 10 March 2023) was used to analyze the biochemical parameters of ZmADFs family proteins, such as isoelectric point (pI), molecular weight (MW) and protein hydrophilic properties etc. [26].

2.2. Clustering, Collinearity, Gene Structure and Promoter Analysis of ADF Family

The species Arabidopsis, wheat, rice and sorghum sequences were downloaded from NCBI website (https://www.ncbi.nlm.nih.gov/, accessed on 10 March 2023). The aligned sequences were subjected to phylogenetic analysis by the maximum likelihood method using MEGA (v7.0) with 1,000 bootstrap replicates [27]. The online software tool iTOL (Interactive Tree of Life, v5) was applied to modify the phylogenetic tree [28]. Tandem and segmental duplication events for ZmADF genes were identified using MCScanX (https://github.com/wyp1125/MCScanX, accessed on 10 March 2023) and displayed through TBtools. Synteny analyses assessing the relationships among ADF genes encoded by maize and Arabidopsis, wheat, rice and sorghum bicolor were conducted with TBtools [29,30]. MEME (https://meme-suite.org/tools/meme, accessed on 10 March 2023) was applied to predict possible conserved motifs in the ZmADFs family, and the maximum number of motifs was set to 10. The intron and exon structures of the ZmADF gene were mapped by GSDS (http://GSDS.cbi.pku.edu.cn/, accessed on 10 March 2023). PlantCare online software (http://bioinformatics.psb.ugent.be/webtools/plancare/html/, accessed on 10 March 2023) was used to analyze the cis-acting elements in the promoter region of maize ADF genes.

2.3. Drought Stress Treatment in Seedling Stage of Maize

Fifty seeds of the inbred line Zheng 58 were soaked in 0.5% sodium hypochlorite for 10 min for surface disinfection, washed repeatedly with distilled water and soaked in saturated calcium persulfate for 12 h. The seeds were germinated on filter paper and then transferred to Hoagland nutrient solution for culture. The culture conditions were 28 °C, 16 h light and 8 h dark in a greenhouse. The treatment with 20% PEG6000 was applied to seedlings at the three-leaf stage. At each treatment point (0, 1, 3, 6 and 12 h), 3 seedlings were selected as 3 independent biological replicates.

2.4. Real-Time Fluorescence Quantitative PCR Detection

The total RNA of the sample was extracted with the FastPure Universal Plant Total RNA isolation kit (Nanjing Nuoweizan Biotechnology Co., Ltd., Nanjing, China), and the first-strand cDNA synthesis was performed using the FastQuant RT kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China). The Real Master Mix (SYBR Green I) kit (Tiangen Biochemical Technology Co., Ltd.) was used for qRT-PCR experiments. The real-time PCR fluorescence quantitative reaction system was 20 μL: 2 × SuperReal PreMix Plus 10 μL, forward (reverse) primer (10 μL) 0.6 μL, cDNA template 2 μL, RNase-free ddH_2O 6.8 μL. Quantitative PCR was implemented using the SYBR Premix Ex Taq II (Takara) on an ABI 7500 real-time detection system (Applied Biosystems), and three independent RNA samples were prepared for each biological replicate. *ZmUBI* was used as the reference gene (Table 1). The relative expression of the gene and its standard deviation were analyzed according to the $2^{-\Delta\Delta CT}$ method [31].

Table 1. Real-time quantitative PCR primer sequence.

Name	Registration Number
ZmUBI-Forward primer	TGGTTGTGGCTTCGTTGGTT
ZmUBI-Reverse primer	GCTGCAGAAGAGTTTTGGGTACA
ZmADF5-Forward primer	CAGGGCCAAGATCCTGTACG
ZmADF5-Reverse primer	ATGACGTCGAAGCCCATCTC

2.5. Analysis of Transcription Level of Maize ADF Family under Abiotic Stresses

The maize transcriptome sequencing data published in the SRA database was downloaded and converted into Fastq data using Fastq-dump.2.11.0. Then, FastQC software (v0.11.5) (https://github.com/s-andrews/FastQC, accessed on 10 March 2023) was used to determine the quality of Fastq data [32]. Trimmomatic software (v0.33) was used to

remove joints and low-quality sequences from the Fastq data, and ultimately to obtain filtered clean data [33]. The filtered data were compared with the maize B73 RefGen_v5 genome to generate a SAM file. SAM files were converted into sorted BAM files using SAMtools software (v1.17). StringTie software (v2.2.1) was used to estimate the expression data of each gene, and DESeq2 software (vR4.1.2) was used to analyze the differentially expressed genes [34].

2.6. Candidate-Gene Association Analysis in ZmADF5

In this study, we collected the phenotypic data of 188 maize inbred lines under drought stress (WS) and normal irrigation (WW) conditions in Hainan (HN) and Xinjiang (XJ) [35]. Subsequently, 115 maize NL inbred lines preserved in the laboratory were cultured to 1–2 leaf stage and genomic DNA was extracted by CTAB method. The plant genome database website was used to find the sequence of *ZmADF5* gene promoter region and gene region. The maize inbred line B73 genome was used as the reference sequence. Primer 5.0 software was used to design primers, including forward primer: 5′-TCTTCGGCAATCTCCAG-3′ and reverse primer: 5′-TCTACTCCACCCATCAACATC-3′. The PCR amplification reaction system was as follows: P520 mix 25 μL, upstream and downstream primers (10 μL) 1.5 μL, cDNA template 2 μL, RNase-free ddH_2O 20 μL. The PCR amplification program consisted of the following steps: 98 °C for 30 s, 35 cycles of 98 °C for 10 s, 60 °C for 5 s, 72 °C for 20 s, 72 °C for 1 min. Finally, the PCR product was sent to the biological company for sequencing.

3. Results

3.1. Chromosome Location and Physicochemical Properties of ADF Family in Maize

Based on the released genome information of maize (B73 RefGen_v5), 15 genes containing the ADF domain were screened by comparing them to the maize genome, and classified them as ADF genes. They were distributed on seven chromosomes in clusters or scattered conditions, with chromosome 1 containing the largest number of *ZmADFs* (Figure 1). Among them, *ZmADF7* and *ZmADF13*, *ZmADF1* and *ZmADF12*, *ZmADF6* and *ZmADF9*, *ZmADF5* and *ZmADF8*, *ZmADF14* and *ZmADF10* appeared as tandem duplications, respectively, and only *ZmADF3* and *ZmADF14* existed as segmental duplications. The protein sequence length of the maize ADF gene family ranged from 132 to 210 aa. The molecular weight ranged from 15,620.91 to 22,733.91 Da, with the isoelectric point values ranging from 4.81 to 9.51. The highest theoretical isoelectric point value was 9.51 for ZmADF8. Among the 15 ADF members, nine proteins were acidic (ZmADF1, 2, 3, 6, 7, 10, 11, 12, 14) and the other six proteins were alkaline (ZmADF4, 5, 8, 9, 13, 15). The prediction results showed that, except for ZmADF14, which exhibited hydrophobic properties, the remaining 14 proteins were hydrophilic proteins. The unstable parameter was between 37.04 and 64.63, and the aliphatic amino acid index ranged from 62.45 to 84.56. Subcellular localization prediction indicated that 15 genes were mainly located in the cytoplasm (Table 2).

Table 2. Physicochemical properties of maize ADF family members.

Name	Registration Number	Molecular Weight (Da)	Chromosome	Gene Location		Number of Amino Acids (aa)	Isoelectric Point	GRAVY	Unstable Parameter	Aliphatic Amino Acid Index	Subcellular Localization
ZmADF1	Zm00001eb321460_T001	16,554.63	7	155289254	155290450	144	6.32	−0.586	55.68	64.38	Cytoplasmic
ZmADF2	Zm00001eb105010_T001	16,083.09	2	206083396	206084459	139	5.57	−0.642	45.49	63.17	Cytoplasmic
ZmADF3	Zm00001eb062600_T001	15,899.93	1	300195447	300197511	139	5.46	−0.480	49.66	74.39	Cytoplasmic
ZmADF4	Zm00001eb266570_T001	15,855.13	6	43778235	43779873	139	7.66	−0.369	51.09	77.88	Cytoplasmic
ZmADF5	Zm00001eb010370_T001	16,413.96	1	32840219	32842834	143	8.41	−0.287	47.01	73.64	Cytoplasmic
ZmADF6	Zm00001eb213630_T001	16,833.10	5	5554015	5557587	145	6.15	−0.471	53.27	70.62	Cytoplasmic
ZmADF7	Zm00001eb186790_T001	15,855.09	4	158650414	158652626	139	6.31	−0.316	49.98	67.34	Cytoplasmic
ZmADF8	Zm00001eb398870_T001	20,038.07	9	149321956	149324583	172	9.51	−0.505	64.63	70.45	Cytoplasmic
ZmADF9	Zm00001eb059710_T001	15,620.91	1	291011670	291012909	132	7.78	−0.448	37.04	81.97	Cytoplasmic
ZmADF10	Zm00001eb211740_T001	15,913.96	5	2677695	2679728	139	5.47	−0.485	48.80	74.39	Cytoplasmic
ZmADF11	Zm00001eb021290_T002	22,733.91	1	80880988	80884908	210	5.64	−0.627	38.45	61.05	Cytoplasmic
ZmADF12	Zm00001eb074420_T001	15,981.05	2	20224012	20227127	139	5.27	−0.570	51.55	62.45	Cytoplasmic
ZmADF13	Zm00001eb249860_T001	15,893.23	5	197451938	197454933	139	7.56	−0.271	52.66	71.58	Cytoplasmic
ZmADF14	Zm00001eb062580_T001	15,698.58	1	300194049	300194984	149	4.81	0.019	38.49	84.56	Cytoplasmic
ZmADF15	Zm00001eb057310_T001	16,725.47	1	283186285	283187993	144	9.44	−0.274	59.98	75.90	Cytoplasmic

Note: Table 1 mainly shows gene location and the physicochemical characterization of the ZmADF genes family, in which the registration number is derived from (https://www.maizegdb.org/, accessed on 10 March 2023) based on the latest database of maize genome B73 RefGen_v5. Unstable parameter refers to chemical instability and physical instability. The isoelectric point, GRAVY value, aliphatic amino acid index and subcellular localization were all predicted by ExPASy software analysis.

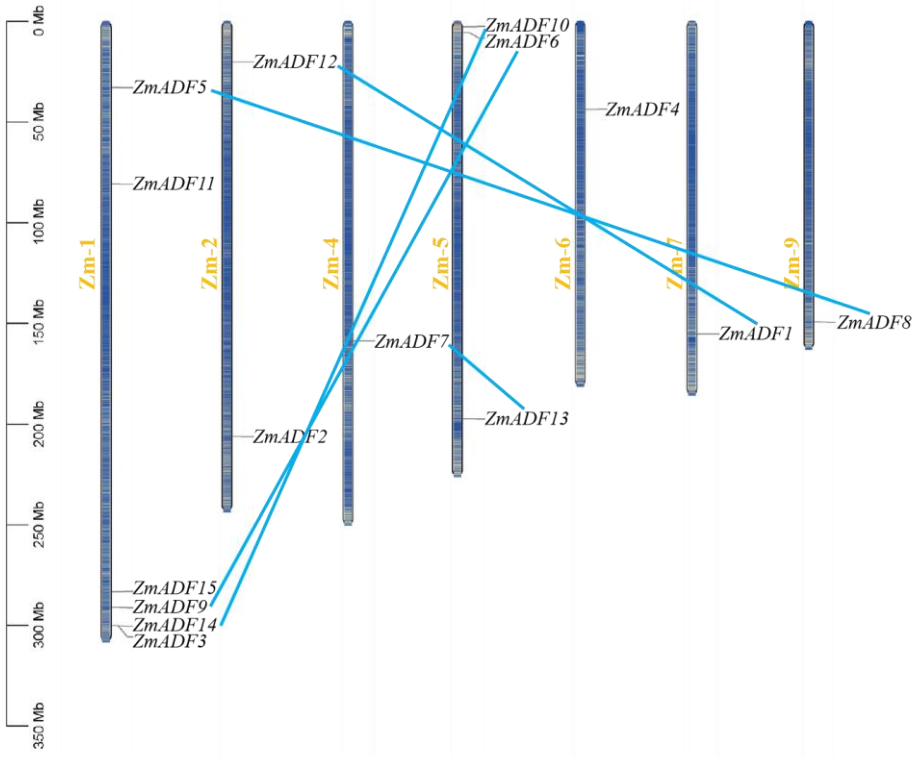

Figure 1. The distribution of ADF gene family on maize chromosomes. The figure shows 7 chromosomes of maize, namely chromosomes 1, 2, 4, 5, 6, 7 and 9, respectively. The left side of each chromosome is marked with yellow for chromosome number, and the right side is marked with red for chromosomal location distribution of 15 ZmADF genes. The blue line represents the gene pairs that undergo tandem duplication.

3.2. Phylogenetic Analysis of Maize ADF Family with Arabidopsis, Wheat, Rice and Sorghum

According to the classification results of the Arabidopsis ADF family, 72 ADF genes of these five species could be divided into four groups [36]. Group I contained the most genes, including *AtADF1*, *AtADF2*, *AtADF3* and *AtADF4*, which were constitutively expressed in all tissues except pollen. *AtADF7*, *AtADF8*, *AtADF10* and *AtADF11* were apical meristem-specific expression genes, which were expressed mainly in pollen and root. *ZmADF1*, *ZmADF2*, *ZmADF3*, *ZmADF4*, *ZmADF7*, *ZmADF10*, *ZmADF12*, *ZmADF13* and *ZmADF14* were found in the same group. Group II was a monocotyledon-specific ADF group, with the majority of its members belonging to ADF3. However, the monocotyledon plant *ZmADF3* was not clustered into this group. Protein sequence alignment analysis found that *ZmADF3* has low homology with *OsADF3*, *SbADF3* and *TaADF3*, with only 57.96% similarity, eventually leading to functional differentiation. According to the clustering results of *AtADF5* and *AtADF9*, group III was divided into several genes widely expressed in multiple tissues and organs, including meristem. *ZmADF5* and *ZmADF8* belong to this group. Represented by *ZmADF6*, group IV was speculated to be constitutively expressed in all tissues, including *ZmADF6*, *ZmADF9*, *ZmADF11* and *ZmADF15*, which exhibit high homology to each other (Figure 2).

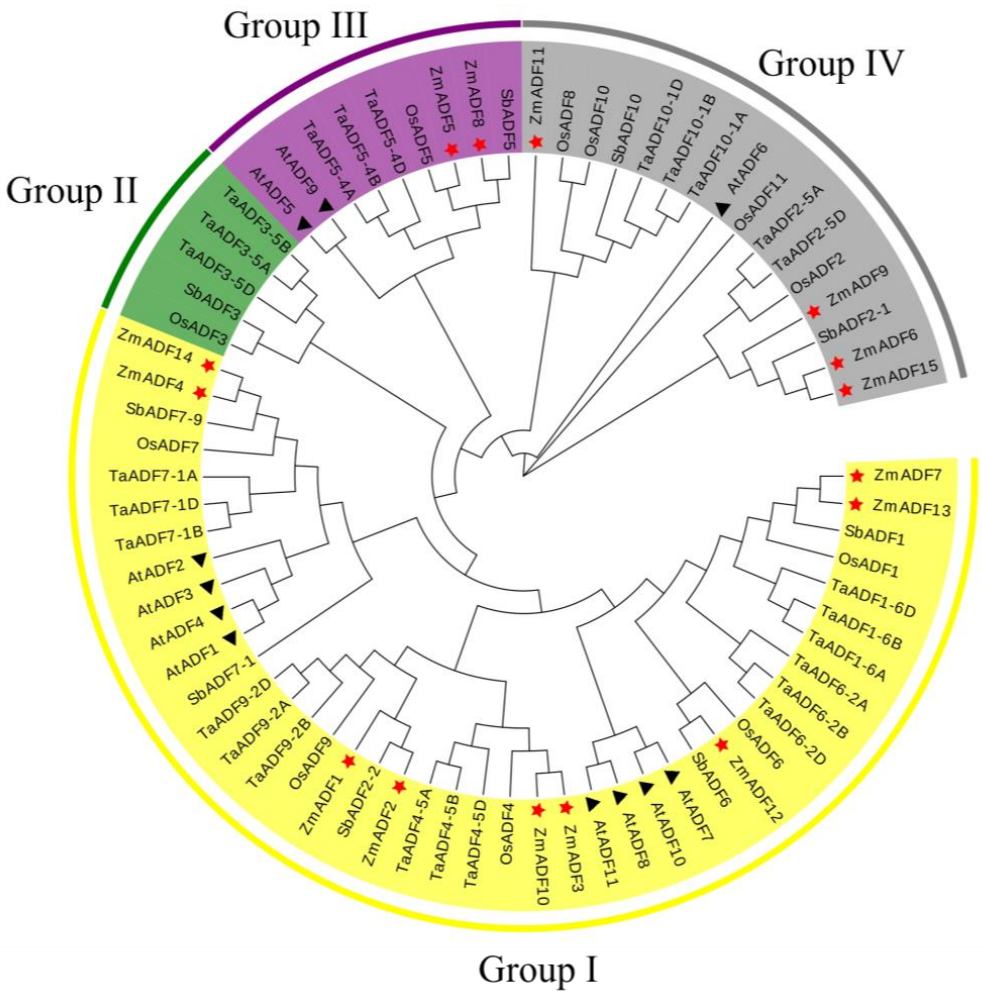

Figure 2. Phylogenetic analysis of ADF proteins from maize, Arabidopsis, wheat, rice and sorghum. The ADF genes were divided into four groups: yellow represents Group I, green represents Group II, purple represents Group III and gray represents Group IV. The four species are abbreviated as follows: maize (Zm), Arabidopsis (At), wheat (Ta), rice (Os) and sorghum (Sb). The black triangle marks the ADF genes of Arabidopsis, and the red pentagram marks the ADF genes in maize.

3.3. Collinearity Analysis of Maize ADF Gene Family with Arabidopsis, Wheat, Rice and Sorghum

Except for *ZmADF1*, the remaining 14 maize genes were collinear with Arabidopsis, wheat, rice and sorghum (Figure 3). *ZmADF4*, *ZmADF12* and *ZmADF13* had five collinear events with *AtADF1*, *AtADF6*, *AtADF7* and *AtADF11*. The collinearity logarithms of maize with wheat, rice and sorghum are 36, 15 and 10, respectively. Collinearity mainly exists between chromosomes 1, 2, 4, 5, 6, and 9. There are more collinearity events among wheat, rice, sorghum and maize, and fewer collinearity events with Arabidopsis. Combined with the results of cluster analysis, it was found that the genes with close genetic relationships within the same group were highly consistent with collinearity genes in other species, and the number of collinearity occurrences was also almost the same, exhibiting group-specific characteristics.

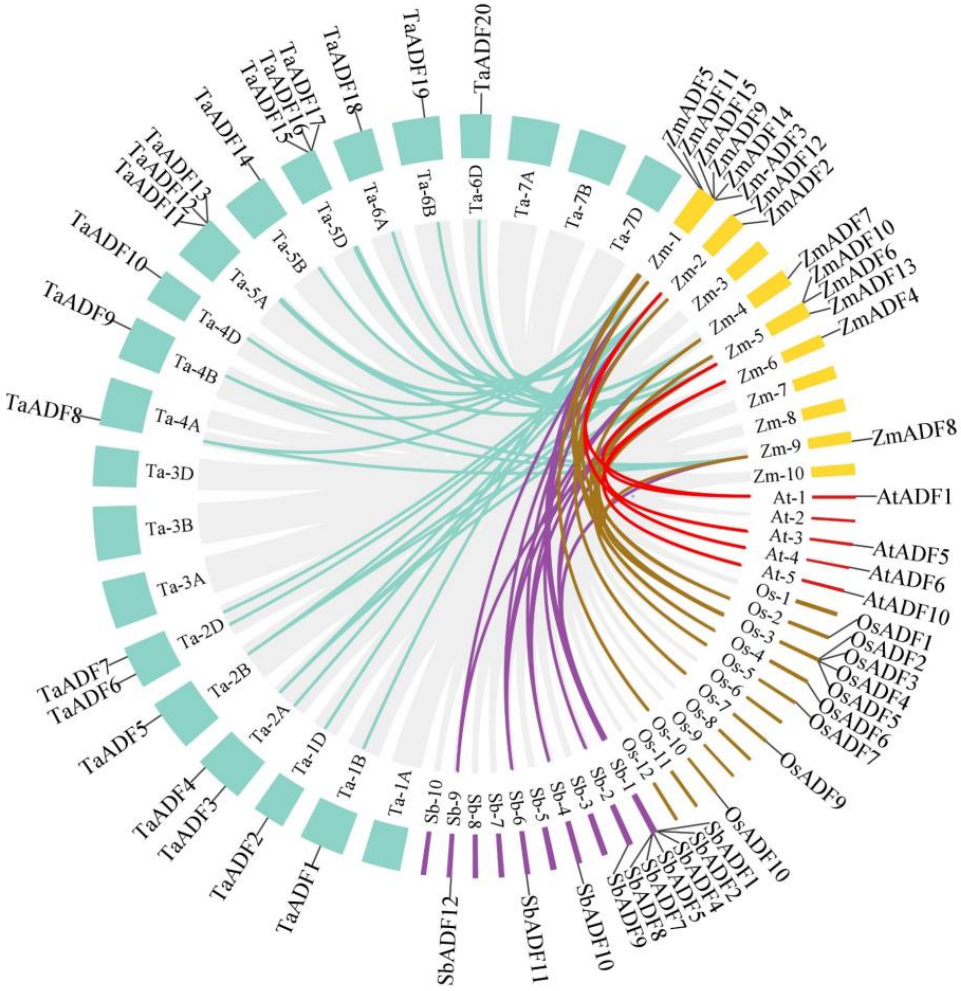

Figure 3. Collinearity analysis of maize ADF gene family in maize, Arabidopsis, wheat, rice and sorghum. The background of whole genome represents by gray, while the collinear gene pairs of ZmADF with different species are connected with different colors. Red represents Arabidopsis, yellow-green represents rice, purple represents sorghum, and blue-green represents wheat.

3.4. Gene Structure and Conserved Sequence of ADF Family in Maize

The 15 maize ADF gene families were individually clustered into four subfamilies, which was consistent with the clustering results in 2.2 (Figure 2). The conserved motifs of ADF proteins showed that the structures within the same group were similar each other. All 15 maize ADF gene families shared motif 3 (MAVADECKLKFVELKAKRSFRFIVFKIDE), which should be a highly conserved ADF domain (Figure 4A,C). The ADF gene family in maize exhibits relatively conserved characteristics, with a simple structure (Figure 4B).

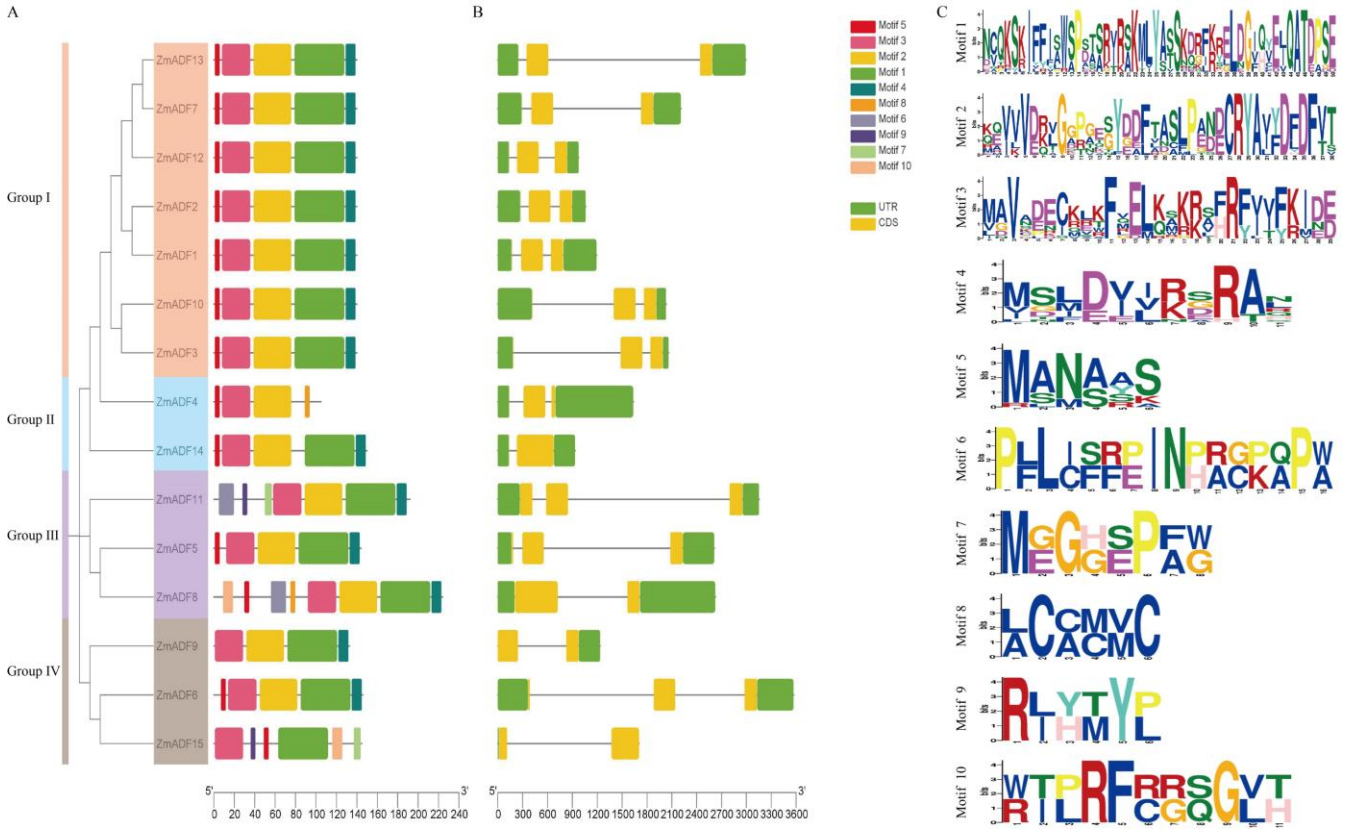

Figure 4. Exon-intron structures of ADF genes and a schematic diagram of the amino acid motifs of ADF proteins in maize. (**A**) The protein motif structure in ZmADFs. There are 10 main protein motifs displayed on the right side. (**B**) The gene structure in ZmADFs, including exons and introns. (**C**) Ten protein motif sequences are predicted in the ZmADFs protein.

3.5. Analysis of Cis-Acting Elements in the Promoter Regions of Maize ADF Family Genes

The promoter regions of the maize ADF gene family contained rich regulatory elements, of which 92 were light-responsive elements (LREs), 84 were jasmonic acid-responsive elements, 79 were ABA-responsive elements (ABRE) and 34 were anoxic inducibility elements (Figure 5A,B). On the whole, regulatory elements were divided into five categories according to their functions: hormone-responsive elements, abiotic stress-responsive elements, light-responsive elements, tissue-specific regulatory elements and other elements. Among them, the hormone-responsive elements constituted the highest proportion at 51.5%, while the proportion of abiotic stress-responsive elements was 25.2%. The light-responsive elements accounted for 16.16% (Figure 5C). Most elements play an important role in plant growth, development and responses to abiotic stresses. It has been reported that the ADF genes can participate in multiple functions at the same time, including growth and development [37].

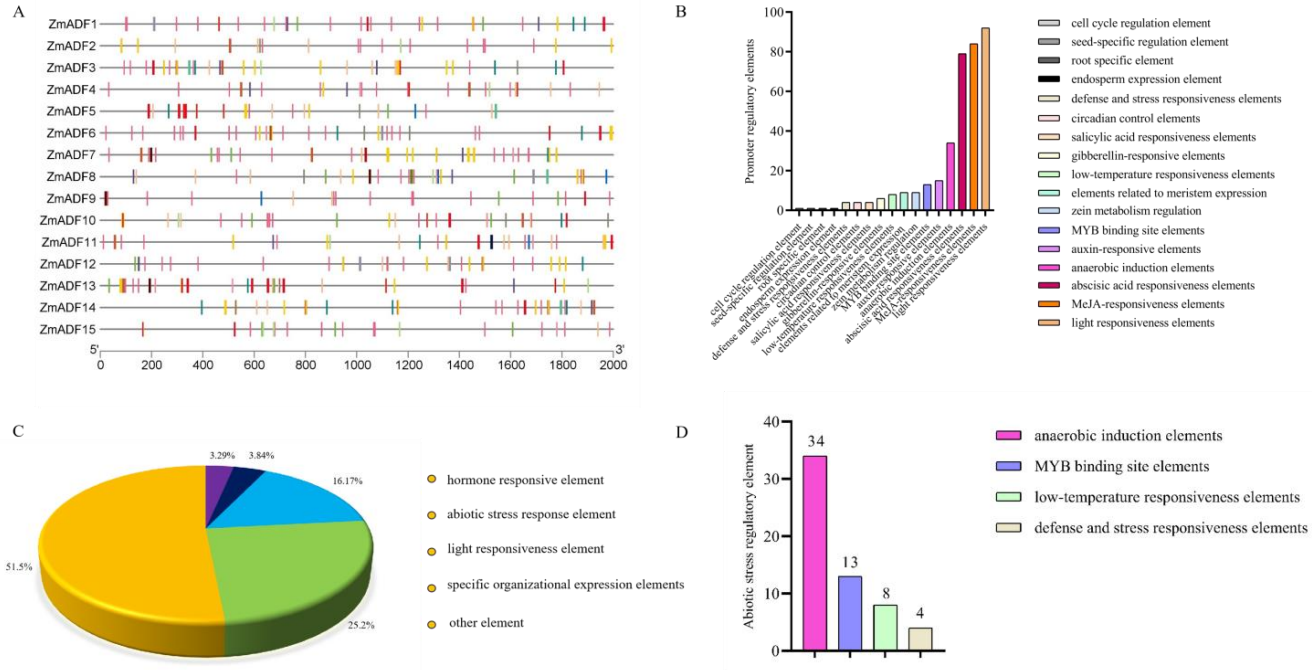

Figure 5. Cis-acting elements in the promoter regions of maize ADF family. (**A**) The distribution of cis-acting elements in the promoters of maize ADF gene family, where each color represents a cis acting element, and the same color in different genes represents the same cis acting element. (**B**) Quantitative statistics of different cis-acting elements in the promoters of maize ADF genes. (**C**) All elements can be divided into 5 categories, and the proportion of each type of component is showed. (**D**) Detailed information of 4 elements involved in abiotic-stress regulation.

3.6. Transcriptome Analysis of Maize ADF Gene Family under Abiotic Stresses

Based on the transcriptome sequencing data on maize under abiotic stress sourced from public databases and the transcriptome data on maize under drought stress in our laboratory, the expression heat maps of maize ADF gene families under four abiotic stresses, such as low temperature, drought, salt stress and high temperature, were drawn (Figure 6). Among them, *ZmADF1*, *ZmADF2*, *ZmADF7*, *ZmADF12* and *ZmADF13* are responsive to both drought and high temperature; *ZmADF3*, *ZmADF4*, *ZmADF5*, *ZmADF6*, *ZmADF10* and *ZmADF11* are mainly responsive to drought stress; and *ZmADF15* responds to low temperature. *ZmADF9* responds to low temperature and salt stress, *ZmADF8* responds to drought and high temperature, and *ZmADF14* responds to salt and high temperature (Figure 6A). The results showed that the up-regulated expression of 15 *ZmADF* genes after drought stress included *ZmADF3*, *ZmADF4*, *ZmADF5*, *ZmADF8*, *ZmADF11* and *ZmADF13* (Figure 6B). A comparison of both transcriptome results showed that most expression patterns of the ZmADF gene were the same, except for *ZmADF6*, *ZmADF8* and *ZmADF10*, which exhibited some differences in response to drought stress. From the FPKM value, the expression level of *ZmADF5* after drought was significantly different from that of the control (Figure 6C). *ZmADF5* had a strong response to drought stress both in the public database and the transcriptome results (Figure 6D).

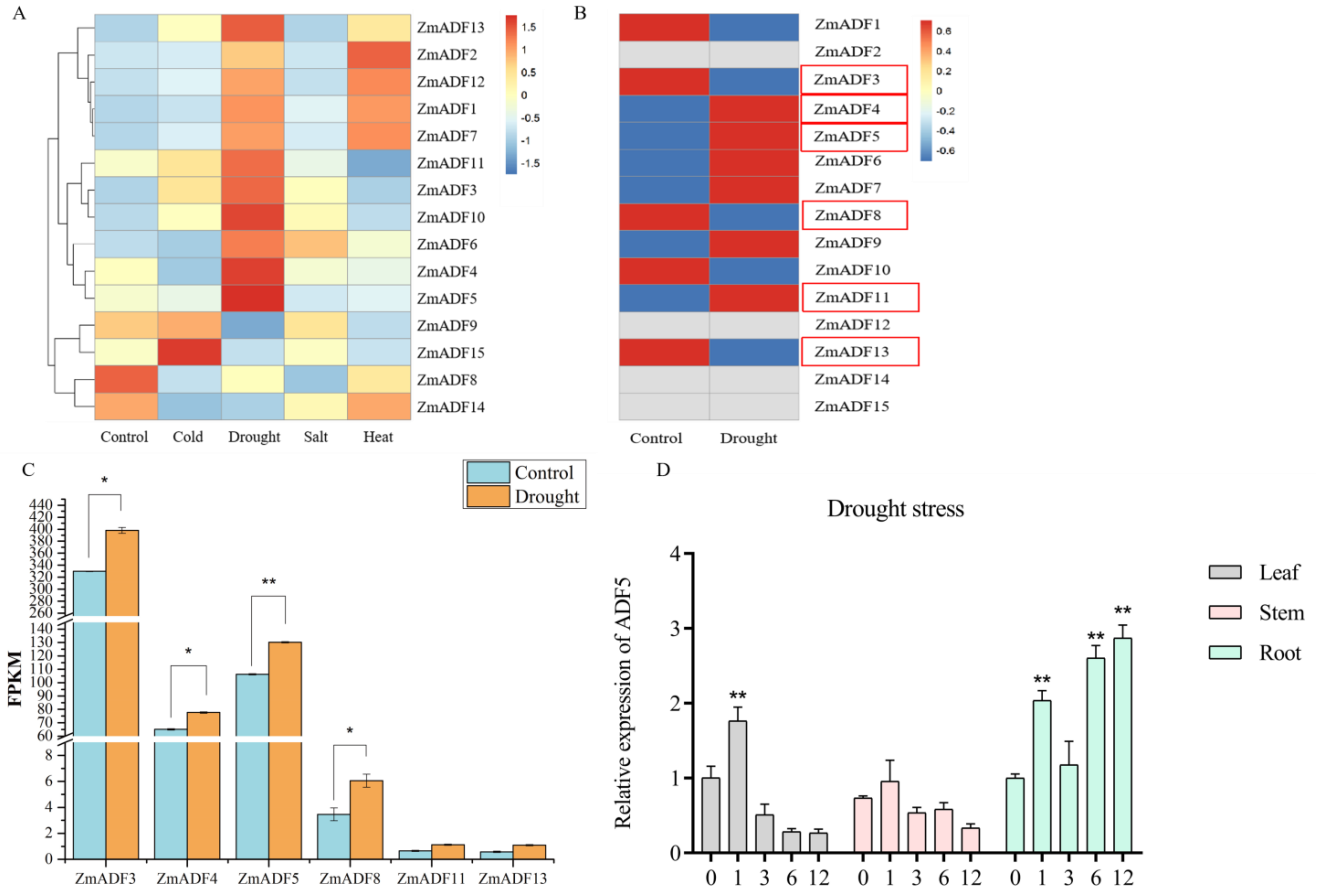

Figure 6. Transcriptional analysis of maize ADF gene under abiotic stresses. (**A**) Transcriptome data analysis on low temperature, drought, high temperature and salt stress from public databases. (**B**) Heat map analysis of ADF gene family expression in transcriptional data after drought treatment, sourced from RNA-seq, the red frame indicates genes that significantly respond to drought stress (**C**) FPKM value analysis of 6 drought resistance genes from transcriptome results. (**D**) Analysis of *ZmADF5* expression in leaves, stems and roots at 0, 1, 3, 6 and 12 h after drought treatment verified the response of *ZmADF5* to drought stress. Significant difference analysis in the figure: * represents $p < 0.05$, ** represents $p < 0.01$.

3.7. Association Analysis of ZmADF5 as a Candidate Gene for Drought Tolerance

To determine the allelic variations related to drought tolerance in *ZmADF5*, a total of 4521 bp length in the *ZmADF5* gene was sequenced, including the promoter, coding and non-coding regions, across 115 maize inbred lines (Figure 7). After candidate-gene association analysis, two indels and one single nucleotide polymorphism (SNP) variation loci in the promoter region of *ZmADF5* were identified that were significantly related to drought tolerance. These loci are located at positions ADF5-Indel-1511, ADF5-Indel-1435 and ADF5-SNP-206 (the first base of the start codon is marked as position 1, with positions being numbered negatively before it and positively after it) (Figure 7A). These three loci were in a complete linkage disequilibrium state and were significantly associated with ear height, grain yield and anthesis silking interval under drought stress. Based on these three loci, 115 inbred lines were divided into two haplotypes (Figure 7B). The anthesis silking interval of Hap2 was significantly shorter than that of Hap1, while the ear height and grain yield of Hap2 were significantly higher than those of Hap1 (Figure 7C).

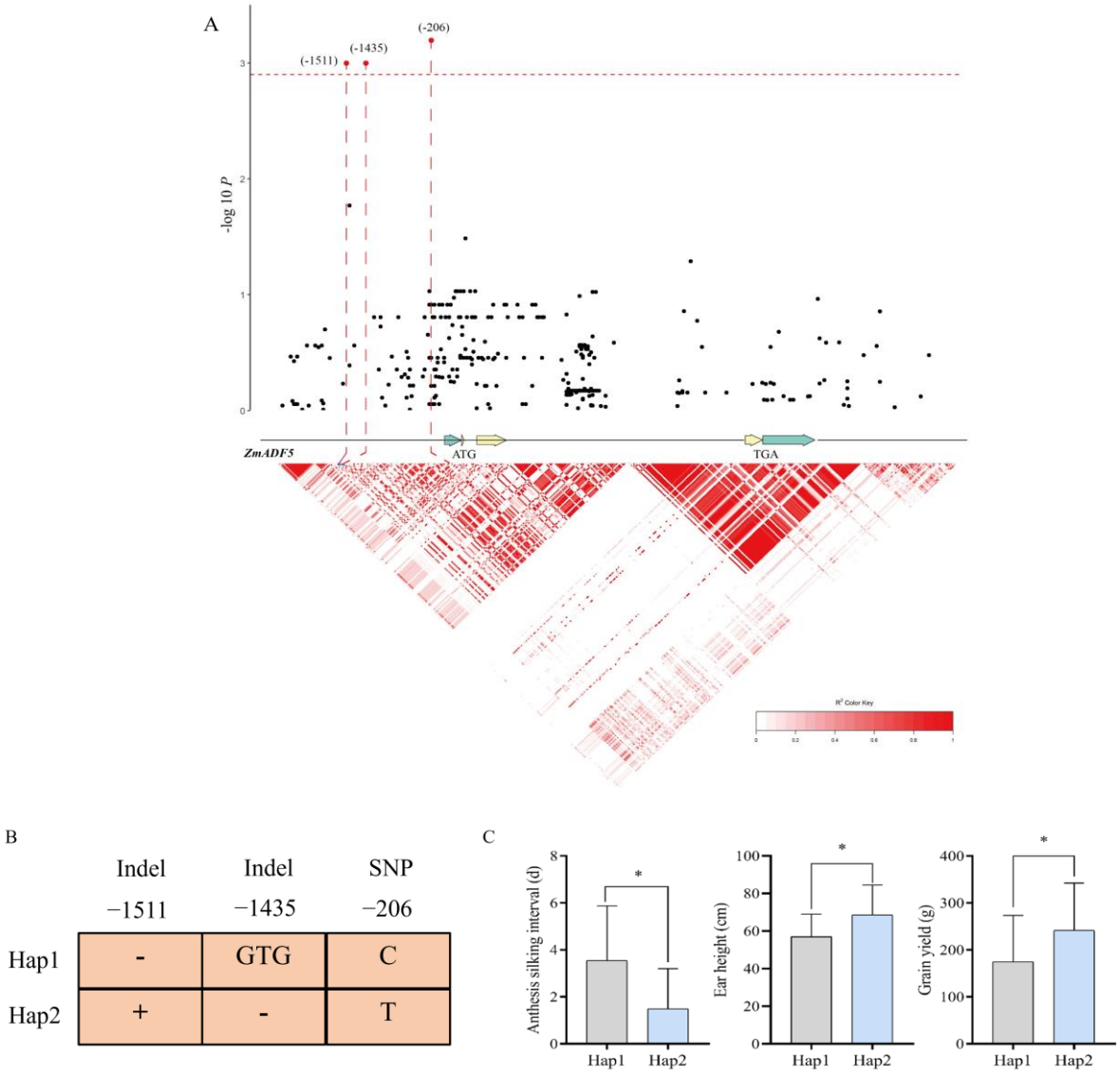

Figure 7. Candidate-gene association analysis in *ZmADF5*. (**A**) Linkage disequilibrium map for *ZmADF5* drawn based on phenotypic date of drought-tolerant-related traits by candidate-gene association analysis. The red dash line represents the screening threshold for achieving significant differences. The green arrow in the figure represents the 5′-UTR and 3′-UTR of the *ZmADF5* gene, and the yellow arrow represents the 3 exon regions. (**B**) Based on 2 Indel (−1511, −1435) and 1 SNP (−206), 115 inbred lines were divided into Hap1 and Hap2 haplotypes; "+" represents containing the corresponding mutation site, "−" represents not containing the corresponding mutation site. (**C**) Statistical analysis of anthesis silking interval, ear height and grain yield under Hap1 and Hap2 drought stress. Hap1 is represented in gray, Hap2 is represented in light blue. Significant difference analysis in the figure: * represents $p < 0.05$.

4. Discussion

4.1. Gene Duplication in ADF Gene Family and Its Possible Function in the Evolutionary Process of Genes

Tandem duplication and segmental duplication in genes are the main driving forces for the expansion and evolution of gene families [38]. The original genes provide raw materials for the formation of new genes, and the new genes promote functional diversification [39]. The ADF gene family has strong evolutionary conservation and relatively few gene duplication events. Only two genes have segmental duplication (*ZmADF3* and

ZmADF14), and five pairs of genes have tandem duplication (*ZmADF7* and *ZmADF13*, *ZmADF1* and *ZmADF12*, *ZmADF6* and *ZmADF9*, *ZmADF5* and *ZmADF8*, *ZmADF14* and *ZmADF10*) (Figure 1). It was found that gene duplication events were clustered in the same group and had similar protein and gene structures (Figures 2 and 4). Therefore, it is speculated that the biological functions of genes with gene duplication are similar or complementary. For example, it was found that *AtADF7* and *AtADF10* showed tandem replication in Arabidopsis (Figure S1). Both genes showed distinct intracellular localizations during pollen germination. However, they could cooperate with nonequivalent functions in promoting pollen cells to achieve exquisite control of the turnover of different actin structures, thereby meeting different cellular needs [40]. Recently, *ZmADF1* has been confirmed to negatively regulate pollen development [41]. *AtADF5* has been confirmed to be involved in drought and low temperature stress in Arabidopsis [42,43]. *ZmADF5* can improve the drought resistance of maize [44]. The function of *ZmADF8* has not been reported, but with a homology of 97.3% to *ZmADF5*, it is speculated that *ZmADF8* and *ZmADF5* may have similar functions or be complementary to each other. The newly identified gene, *ZmADF14*, is likely produced by the segmental duplication of *ZmADF3* and the tandem duplication of *ZmADF10*. *ZmADF15* exhibited high homology with *ZmADF6* and *ZmADF9*, but no gene duplication events occurred. Currently, there are relatively few studies on the maize ADF gene. According to evolutionary conservation and gene duplication within the ADF gene family, both the ADF gene of Arabidopsis and the reported maize ADF gene can be used as a reliable references for studying the biological function of maize ADF genes.

4.2. The Characterization of ADF Gene Family and Its Regulation in Response to Abiotic Stresses

The structure of the maize ADF gene family is relatively simple, but the promoter region contains rich regulatory elements, with the most abundant being hormone response elements. Hormone response can participate in plant growth and development and can also participate in plant stress response. The quantity of abiotic-stress response elements in the promoter region of the maize ADF gene family is also high, including drought, low temperature, stress response and so on. It can be seen that the maize ADF gene family has great potential in coping with abiotic stresses. For example, ADF1 in Group I can improve the heat tolerance of Arabidopsis and Chinese cabbage, as well as enhance the low-temperature tolerance of Arabidopsis [45–47]; *AtADF4* can respond to osmotic stress and drought [48]; *AtADF7* not only participates in pollen tube development but also positively regulates osmotic stress [49]. *OsADF3*, in Group II, can positively regulate drought tolerance in rice [19]. In Group III, *AtADF5* can promote stomatal closure and improve drought tolerance in Arabidopsis by regulating ABA and actin cytoskeleton remodeling under drought stress. It can also respond to low temperature stress by regulating the stomata, while *PeADF5* in Populus euphoretic is mainly responsive to drought [42,43,50]. To sum up, it can be seen that the ADF gene has multiple biological functions, which are associated with their numerous regulatory elements in the promoter region, as discussed in Section 2.5 (Figure 5). According to the evolutionary conservation of ADF genes, it is speculated that the maize ADF gene family might participate in a variety of abiotic-stress responses.

4.3. Excellent Allelic Variations Associated with Drought Tolerance in ZmADF5

ZmADF5 is a drought-tolerant gene identified by genome-wide association analysis, which has been verified through overexpression in maize. The main three excellent variation sites are concentrated in its promoter region. The promoter region of *ZmADF5* contains a large number of hormone-responsive elements (MeJA, GA, ABA), followed by growth regulatory elements (light response, diurnal regulation) and stress-responsive elements (drought, low temperature and anoxic conditions) (Figure 5). According to the promoter elements, it is speculated that *ZmADF5* might participate in plant growth, development and stress response. Through candidate-gene association analysis of *ZmADF5*, 115 inbred lines were divided into two haplotypes, of which Hap2 was significantly better compared to Hap1, with a grain yield 18.77% higher than that of Hap1. As a screened and confirmed

drought-tolerant candidate gene, understanding the mechanism behind these variations in drought tolerance will help us to study the biological function of the maize ADF gene family, indirectly promoting the process of marker-assisted breeding in maize.

5. Conclusions

In this study, 15 ADF family genes were identified and characterized in the whole maize genome, updating it with new members of *ZmADF14* and *ZmADF15*. These ADF genes were distributed across seven chromosomes and phylogenetically divided into four groups, with conservation and gene duplication trajectory captured in their gene structure. Nonetheless, the rich regulatory elements in their promoter region endow them with multiple biological functions, especially in response to abiotic stresses. Candidate-gene association analysis revealed that the promoter region in *ZmADF5* contained three excellent variations associated with drought resistance. Overall, ADF genes are expected to participate in various abiotic stresses with high potential, and their excellent variations related to drought resistance could be used for marker-assisted breeding in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14040717/s1, Figure S1 The chromosome distribution and gene duplication map of the ADF gene family in Arabidopsis, which show five chromosomes, namely chromosomes 1, 2, 3, 4, and 5. The left side of each chromosome is marked with black chromosome number, and the right side is marked with red chromosome location distribution of 11 AtADF genes. The red line represents gene pairs undergoing tandem duplication.

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