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Article

Analysis of AP2/ERF Domain of DREB Transcription Factor in Several Wheat Species

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Abstract: The Apetala2/ethylene response factor superfamily refers to a group of transcription factors that share a conserved AP2 DNA binding domain. These factors have been found to have different roles in plant responses to both biotic and abiotic stresses. Samples of hexaploid wheat, tetraploid pasta (or durum wheat), and diploid wheat progenitors were selected. The 29 dehydration-responsive element binding transcription factors in these samples were downloaded from NCBI GenBank for six different countries: Iran, China, Italy, France, Afghanistan, and Azerbaijan. The AP2 domain sequences were identified from the dehydration-responsive element binding transcription factors using PROSITE, ProDom, and SMART software. Next, all sequences were aligned using Multalin and Jalview software. The aligned sequences were then analyzed to identify amino acid locations, types, and frequencies. The multiple alignments showed that approximately 76% of the amino acid residues in the AP2 domains are conserved. According to the amino acid analysis, alanine, serine, and glutamic acid are the most abundant amino acids found in three motifs. The performed phylogenetic analysis illustrates the role of geographical effects on the transcription factor sequences of bread wheat in the Middle East. Significant differences were found between Iranian and Chinese transcription factor sequences. Moreover, genetic variation was observed in the transcription factors of Italian sequences found in pasta and wheat progenitors. Motif structures play a critical role in the domain organization of wheat proteins to enhance the characteristics of assorted metabolic pathways. The structure of the AP2 domain was analyzed by several programs, I-TASSER for instance, to identify the α -helix, β -sheets, and the regions of some significant amino acids in the 3-D model.

Keywords: AP2/ERF; amino acid analysis; motif analysis; structure of AP2 domain; transcription factor; dehydration-responsive element; wheat

1. Introduction

The AP2/ERF superfamily consists of transcription factors (TF) with a conserved DNA-binding domain called AP2. This superfamily plays important roles in plant responses to biotic and abiotic stresses. The domain consists of 57-66 amino acids [1–3]. The DREB proteins belong to the ERF family of transcription factors and are involved in the ABA-independent signal transduction pathway. These proteins bind to dehydration-responsive elements (DRE) in the promoter region of genes to regulate their expression. The AP2/ERF gene family consists of five major groups named AP2, DREB, ERF, RAV, and Soloist. AP2 ERF transcription factors (TFs) possess one or two AP2 domains, while DREB, ERF, and RAV groups have a single AP2/ERF domain [4]. The Soloist group is a small group of transcription factors that have a structure similar to an AP2 domain, but deviate considerably [5]. The AP2/ERF gene family has a wide range of transcription factor genes that play essential roles in plant growth and development, signal transduction, and abiotic stresses [1]. They are also involved in developmental processes and plant morphogenesis [6]. Additionally, these genes have roles in determining leaf epidermal cells, floral organ patterning, spike-let meristem differentiation [7], seed yield, and seed mass [8,9]. These AP2/ERF transcription factors have been found in various plants such as Arabidopsis, rice [10], maize [3], wheat, and soybean. Furthermore, studies have

demonstrated their existence in ciliates and protists [11,12]. AP2/ERF is abundantly present in wheat. This transcription factor has various functions and responses [9]. For instance, AP2 domain genes play a vital role in the development of reproductive and vegetative organs. However, a missense mutation in this gene reduces spike density [13–16].

There are two main species of wheat: common or hexaploid wheat (*Triticum aestivum* L.), which is grown and consumed worldwide for the production of bread, pasta, and cakes; the other species are pasta or durum wheat (*Triticum turgidum* L. subsp. durum Desf.), which is tetraploid ($4n = 28$) and originated in the Middle East thousands of years ago. One of the ancestors of wheat is *Aegilops speltoides*, which is a diploid grass with seven pairs of chromosomes ($2n = 14$) [17].

The alignment analysis, amino acid dissection, and structural analysis have the crucial potential to make the character of AP2 domains appear. From this point, in some wheat species to create genetically enhanced crops resistant to multiple abiotic stresses, AP2/ERF domain analysis of DREB transcription factors might suggest changes in the gene and amino acid composition for better knowledge.

2. Results

2.1. Sequence Analysis of DREB Proteins

Analysis of the AP2 domains of the DREB-TS proteins was performed using several alignment tools. The alignment results of the DREB sequences suggest that they contain 58 conserved amino acid residues. When the AP2 domain sequences were aligned, the result showed 44 (about 76 percent) highly conserved amino acid residues, namely Tyr-2, Gly-4, Val-5, Arg-6, Gln-7, Arg-8, Thr-9, Trp-10, Gly-11, Lys-12, Trp-13, Val-14, Ala-15, Ile-17, Arg-18, Glu-19, Pro-20, Asn-21, Arg-22, Arg-25, Leu-26, Leu-28, Gly-29, Phe-31, Pro-32, Thr-33, Ala-34, Ala-38, Arg-39, Ala-40, Tyr-41, Asp-42, Ala-44, Ala-45, Arg-46, Ala-47, Met-48, Tyr-49, Gly-50, Ala-51, Ala-53, Arg-54, Asn-56, and Phe-57 (Figure 1). All 29 protein sequences with their AP2 domain regions were considered to determine the conserved residues and some other data such as quality, consensus, and occupancy (Figure 2). Alanine (A), glutamine (Q), lysine (K), valine (V), isoleucine (I), glutamic acid (E), serine (S), and methionine (M) in conserved amino acid residues were highlighted (Figure 3).

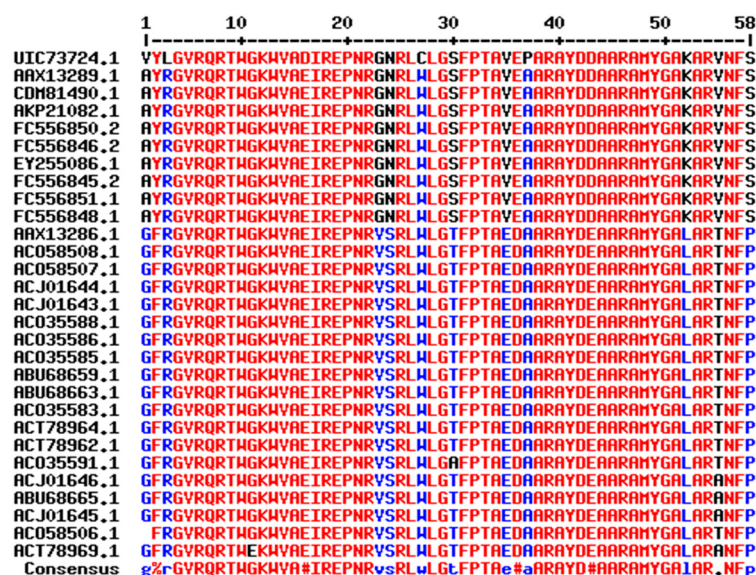


Figure 1. 29 AP2 domains were subjected to Multalin alignment. The alignment was performed on the segment of the domains possessing the desired functional domain, which includes 58 conserved amino acid residues.



Figure 2. Analysis of multiple sequence alignment. The sequences are aligned and edited using Jalview. Residues are shaded based on their conservation level.



Figure 3. Fifty-eight conserved amino acid residues were identified from the Multalin alignment. Alanine (A): Green; Valine (V), and Glutamic Acid (E): Orange; Glutamine (Q), Lysine (K), Isoleucine (I), Serine (S), and Methionine (M): The yellow residues are important amino acids.

2.2. Conserved Motif Analysis

MEME analysis identified three conserved motifs. Table 1 shows that motif 1 shares significant similarities with the AP2 domain. Table 1 shows that in some instances, two other motifs are present with different sequences compared to motif 1. All three motifs were present in 18 out of 29 protein sequences. Motifs 2 and 3 were not present in any protein sequences. Motif 1 was observed solely in 11 sequences, which included UIC73724.1, AAX13289.1, CDM81490.1, AKP21082.1, ABU68663.1, FC556850.2, FC556846.2, FC556845.2, FC556851.1, FC556848.1, and EY255086.1. A total of 18 sequences were detected through the combination of three motifs (Figure 4).

Table 1. The default settings of the MEME software identified three motif sequences.

Motif Number	Amino Acid Sequence
1	FRGVRQRTWGWVAEIREPNRVSRLWLGTFPTAEDAARAYDEAARAMYGA
2	IEGVVRGASASCESTTTSTNHSDVASSLPRQAQALEIYSQPDVLESTESV
3	STSEEDVFEPLEPISSLPDGEADGFDIEELLRLMEADPIEVEPVTGGSWN

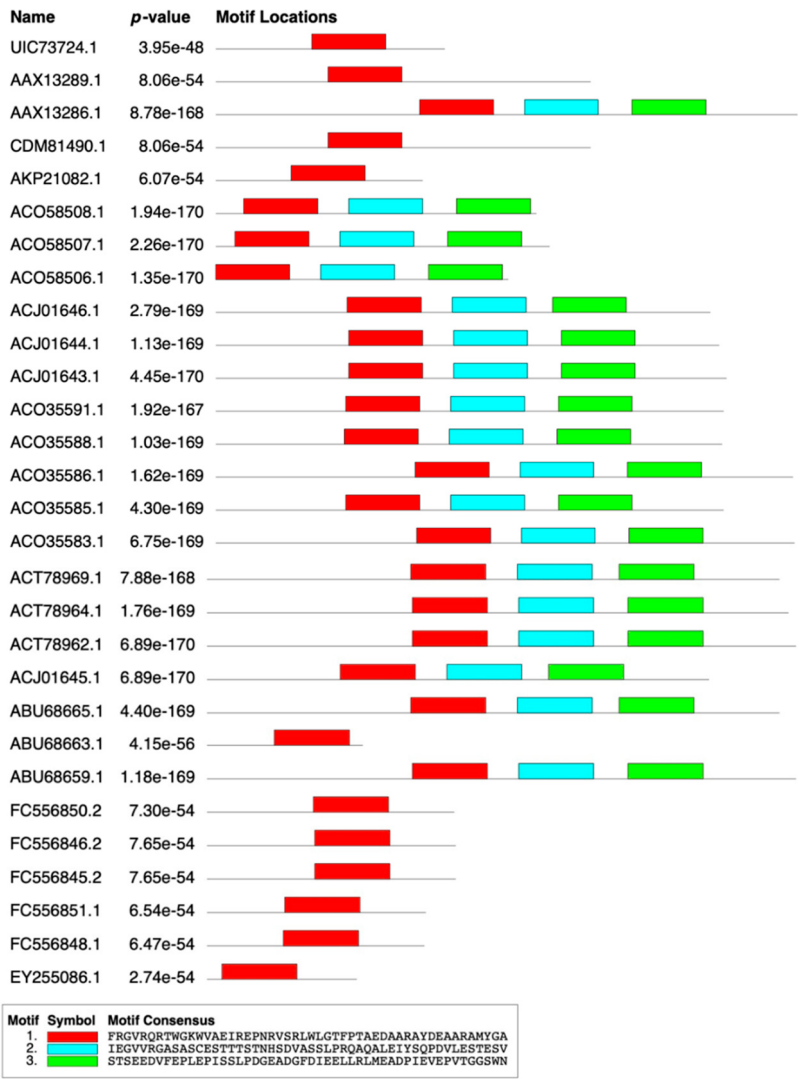


Figure 4. Distribution of motifs for transcription factors in a select wheat sample. Three motifs that are conserved and identified by MEME are denoted by differently colored boxes, namely, red for motif 1, cyan for motif 2, and green for motif 3. Relative positions of these motifs are also provided.

According to PortParam analysis, alanine constituted 18.0% of motif 1, while serine and glutamic acid were the most prevalent amino acids in motif 2 and 3, respectively, both accounting for 20.0% (Table 2).

Table 2. Three identified motifs were subjected to amino acid analysis. Motif 1 contains the highest percentage of alanine, motif 2 has the highest percentage of serine, and motif 3 has the highest percentage of glutamic acid compared to other amino acids.

Amino acid	Motif 1	Motif 2	Motif 3	Amino acid	Motif 1	Motif 2	Motif 3
Ala (A)	18.0%	10.0%	4.0%	Lys (K)	2.0%	0.0%	0.0%
Arg (R)	16.0%	4.0%	2.0%	Met (M)	2.0%	0.0%	2.0%
Asn (N)	2.0%	2.0%	2.0%	Phe (F)	4.0%	0.0%	4.0%
Asp (D)	4.0%	4.0%	10.0%	Pro (P)	4.0%	4.0%	10.0%
Cys (C)	0.0%	2.0%	0.0%	Ser (S)	2.0%	20.0%	10.0%

Gln (Q)	2.0%	6.0%	0.0%	Thr (T)	6.0%	10.0%	4.0%
Glu (E)	8.0%	10.0%	20.0%	Trp (W)	6.0%	0.0%	2.0%
Gly (G)	8.0%	4.0%	8.0%	Tyr (Y)	4.0%	2.0%	0.0%
His (H)	0.0%	2.0%	0.0%	Val (V)	6.0%	10.0%	6.0%
Ile (I)	2.0%	4.0%	6.0%	Pyl (O)	0.0%	0.0%	0.0%
Leu (L)	4.0%	6.0%	10.0%	Sec (U)	0.0%	0.0%	0.0%

2.3. Phylogenetic Analysis of AP2 Domains

To accurately demonstrate the evolutionary relationships among the AP2 domains, we performed a phylogenetic analysis. As a result, all 29 AP2 domains were segregated into two groups. Eleven AP2 domains were found in Group 1 while Group 2 contained eighteen domains (Figure 5). Transcription Factors (TFs) from *Lolium arundinaceum* (Schreb.) Darbysh (Poaceae) were used. They were utilized as an out-of-group protein and separated from the other transcription factors.

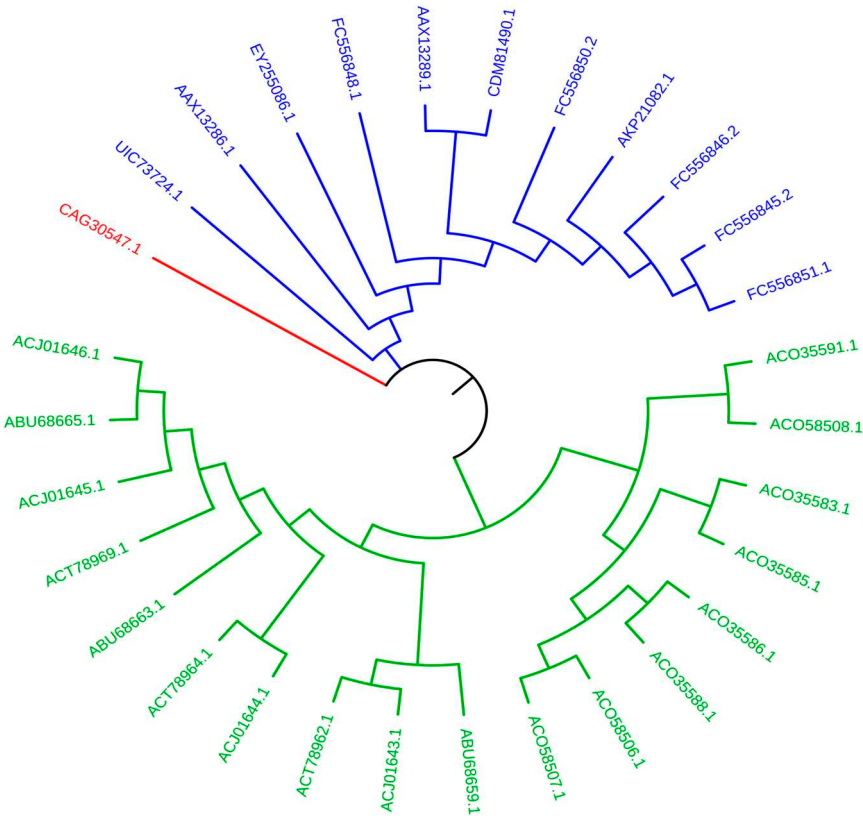


Figure 5. An analysis was conducted on the phylogeny of transcription factors. The dendrogram was built using the MEGA package with the maximum likelihood method and 100 bootstrap replicates. The out-of-group (CAG30547.1), group 1, and group 2 were assigned the colors red, blue, and red, respectively.

2.4. Structural Analysis of AP2 Domain

The C-score and TM-score (i.e., Template Modeling score) of the modeled AP2 domain (Figure 6) suggested a high degree of confidence in the model, with a C-score of 1.36, which ensured that the correct topology was attained [18–20]. We used the Ramachandran plot, a tool for assessing the quality of protein structures, to select and validate the model with the highest C-score (Figure 7). Our analysis of the Ramachandran plot revealed that the majority of residues were located in the core

region (as shown in red), which indicates an acceptable 3-D model. The statistics provided in the Ramachandran plot section illustrated the detailed structure of the AP2 domain, which comprised 25% helix, 34% beta, 39% coil, and 13% turn. Furthermore, the Z score (i.e., a measure of confidence in the quality of a structural model) for the AP2 domain was -3.44, which provided additional evidence supporting the accuracy of the model (Figure 8) [21,22]. The I-TASSER program predicted that the AP2 domain functions as a DNA-binding transcription factor, with a high Gene Ontology (GO) Score of 0.75.

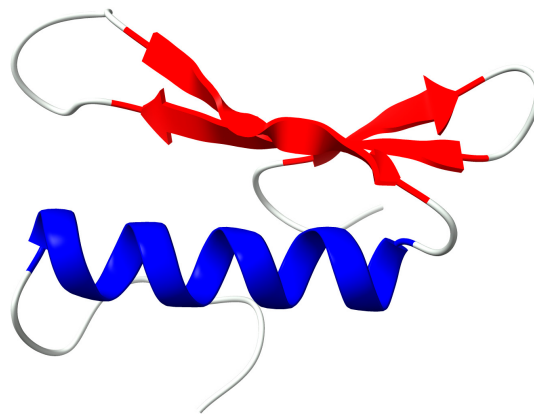


Figure 6. Three-dimensional model of the AP2 domain. Specifically, this model showcases three β -sheets (VRQR, KWVAEIR, and RLWLGTG) and an α -helix (AEDAARAYDEAARAMY) shown in red and blue colors, respectively.

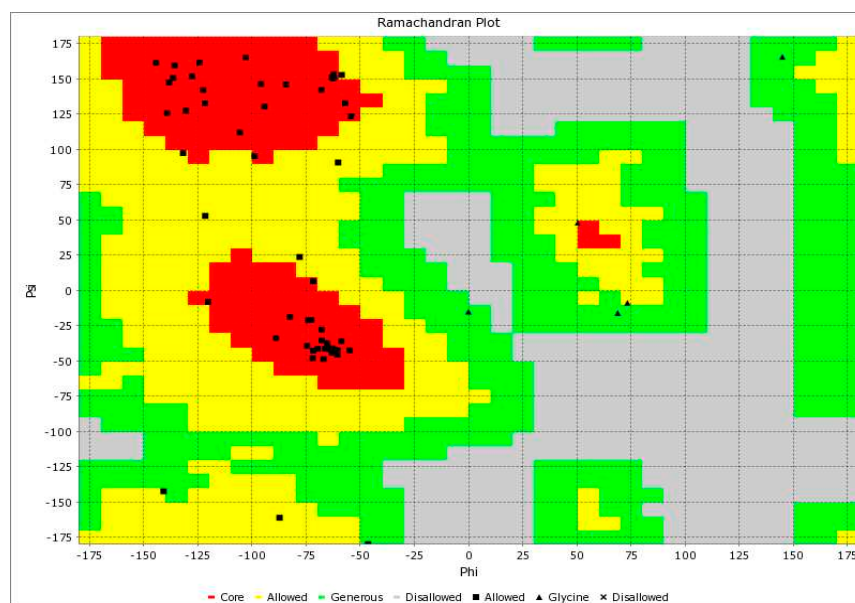


Figure 7. The Ramachandran plot for the AP2 domain. The red section represents the core region, the yellow section represents the allowed region, and the green section represents the generous region. The model is valid as most of the residues were located in the core region of the plot.

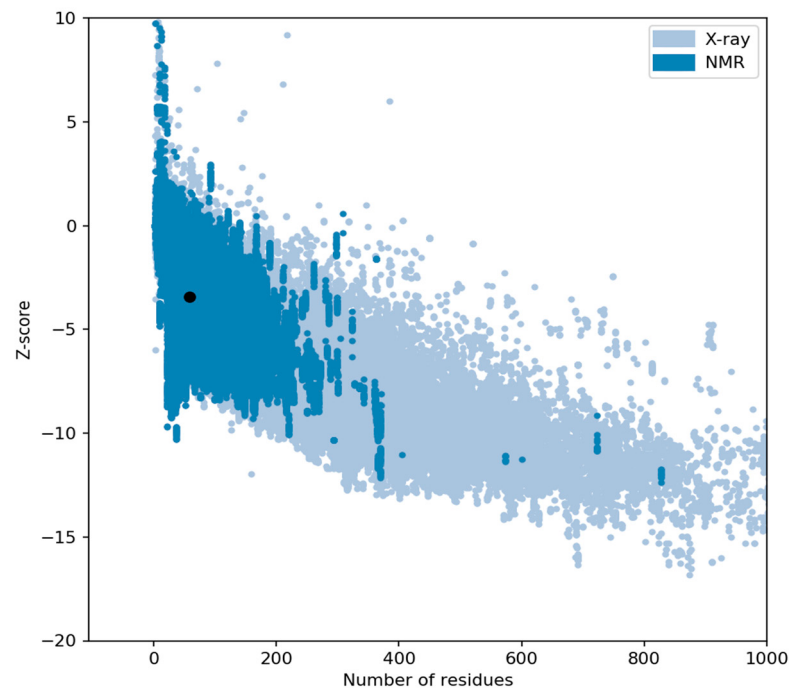


Figure 8. ProSA-web z-score of the PDB model determined by X-ray crystallography (light blue) or NMR spectroscopy (dark blue). The z-score is -3.44 and marked as a dot.

3. Discussion

Dehydration-responsive element binding (DREB) transcription factors increase the expression of abiotic tolerance pathway genes by binding to DNA upstream of genes. Most DREB genes coding for the AP2 domain in wheat have one AP2 domain, as shown in [23]. AP2/ERF is one of the largest families of plant transcription factors, playing a crucial role in abiotic stress responses in wheat. Gene structure analysis has revealed that approximately 85% of AP2/ERF genes lack introns, and the evolution of the wheat genome is the leading cause of this phenomenon [16]. Additionally, six partial DNA genomic sequences of Iranian AP2/ERF transcription factors previously submitted to NCBI GenBank showed similarity to their cDNA counterparts [24,25], as revealed by a BLASTX search conducted in this study.

The selected transcription factor sequences, including six Iranian, one Afghan, and one Azerbaijani cultivar of bread wheat (*T. aestivum*), are part of group 1 in the phylogenetic analysis. However, the transcription factor sequences from two sequences of bread wheat from China, one from France, and five from Italy are in group 2 (Table 4). This indicates the role of geographical location on transcription factor sequences of bread wheat in the Middle East. There is a genetic variation in the transcription factors of Italian sequences found in pasta and wheat progenitors. Specifically, one sequence of the transcription factor from Italian *T. turgidum* subsp. *durum* is in group 1, and four are in group 2. Additionally, two sequences of Italian *A. speltoides* var. *speltoides* are in group 1, and six are in group 2. The distinction between the sequences from Iran and China in the phylogenetic analysis of TS sequences (Table 4) has been reported in another study on the DREB partial gene sequences (500 bp long) of eight Iranian and ten Chinese bread wheat cultivars [26]. It was explained that the phylogenetic analysis of the Chinese sequences was completely distinct from the Iranian sequences. The Iranian sequences are divided into seven groups; in contrast, the Chinese sequences are divided into three groups only. This indicates that the Iranian DREB sequences exhibit greater biodiversity. However, the AP2 domains of the chosen DREB transcription factors, as

presented in the Multalin alignment of AP2 domains (Figure 1), demonstrate a greater degree of conservation (approximately 76 percent).

Transcription factors (TFs) in the AP2/ERF superfamily possess one or more AP2/ERF domains that contain conserved amino acids at positions 14 (alanine, A) and 19 (aspartic acid, D). In contrast, the DREB superfamily TFs have different conserved amino acids at the same positions, with valine (V) at position 14 and glutamic acid (E) at position 19 [27]. Table 1 shows that all AP2 domain sequences of the 29 DREB transcription factors in this study have conserved valine (V) and glutamic acid (E) amino acids at positions 14 and 19, respectively, as indicated by italicized and bolded V and E letters. Val14 is crucial for DREB binding to the dehydration-responsive element (DRE) cis-acting elements, whereas Glu19 plays a relatively less significant role [4].

The MEME software reveals three protein motifs with distinct sequences, and their amino acid composition is presented in Table 3. In the chosen wheat species, DREB transcription factors contained all three motifs in 18 out of 29 sequences, and the first motif represented the AP2 domain. Therefore, gene rearrangement may cause variations in the structure of motifs, which have significant roles in the domain arrangements of wheat proteins that enhance the properties of diverse metabolic pathways [28].

The analysis of three motifs revealed that alanine, serine, and glutamic acid are the most abundant amino acids in motifs 1, 2, and 3, respectively (Table 3). Glutamic acid and serine among hydrophilic amino acids have a considerable positive effect, especially in cases of high net charge; hence, the presence of serine and glutamic acid has the potential to improve protein solubility [29]. Out of 22 amino acids presented in Table 3, motif 1 has more amino acid diversity (18) than motif 2 (16) and motif 3 (15).

The 3-D structure of the AP2 domain used for DNA binding consists of three β -sheets and one α -helix. The conserved Ala37 in the α -helix is crucial for binding to the GCC box. According to a study [30], Ala38 is essential for maintaining the stability and structure of the hydrogen bonds between strand 2 and strand 3 of the β -sheet as depicted in Figure 3. The function of ERF proteins' AP2 domain depends on the α -helix, which may play a role in protein-protein interactions [31,32].

4. Materials and Methods

4.1. Database Search of DREB and AP2s in Wheat

We obtained 66 protein and 6 DNA sequences of the DREB transcription factor in fasta format from the NCBI, originating from six different countries: Iran (Shahi 1 & 2, Bayat, Al-vand, Zarrin, and Omid cultivars of bread wheat, submitted by one of the authors of this article), China (*Triticum aestivum*), Italy (*T. aestivum*, *T. turgidum*, and *Aegilops speltoides*), France (*T. aestivum*), Afghanistan (*T. aestivum*), and Azerbaijan (*T. aestivum*). We translated six DNA sequences into protein sequences by conducting a BLASTX search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 16 March 2022). Then, we checked all 72 protein sequences for the presence of the AP2 domain sequence using PROSITE (<https://prosite.expasy.org>, accessed on 4 April 2022), ProDom (<http://prodom.prabi.fr/prodom/current/html/home.php>, accessed on 13 April 2022), and SMART (<http://smart.embl-heidelberg.de>, accessed on 27 April 2022). As a result, we chose 29 protein samples that contained AP2 domains.

4.2. Multiple Sequence Alignment and Biochemical Characteristics

Table 3 lists twenty-nine protein samples that contain AP2 domains. The alignment of all AP2 domain sequences was done using Multalin (<http://multalin.toulouse.inra.fr/multalin/>, accessed on 19 May 2022) [33]. The analysis of twenty-nine AP2 domains was done based on their amino acid locations, types, and frequencies. To verify the data regarding the conserved region, Jalview (v2.11.2.6) with Clustal color [34] was used for further protein sequence analysis.

Table 3. 29-transcription factors and their AP2 domain sequences were analyzed using SMART.

Accession number of TS sequence	AP2 Domain Sequence	Country	Species
EY255086.1	AYRGVRQRTWGKWVAEIREPNRGNRLWLGSFPTAVEAARAYDDAARAMYGAKARVNFSEQSPDA	Iran	<i>Triticum aestivum</i> cv. Shahi1
ABU6866.3.1	GFRGVRQRTWGKWVAEIREPNRVSRWLGLTFPTAEDAARAYDEAARAMYGALARTNFPVH	Italy	<i>Triticum turgidum</i> subsp. <i>Durum1</i>
AKP2108.2.1	AYRGVRQRTWGKWVAEIREPNRGNRLWLGSFPTAVEAARAYDDAARAMYGAKARVNFSEQSPDA	Afghanistan	<i>Triticum aestivum</i> cv. Kalak Afghani
FC556848.1	AYRGVRQRTWGKWVAEIREPNRGNRLWLGSFPTAVEAARAYDDAARAMYGAKARVNFSEQSPDA	Iran	<i>Triticum aestivum</i> cv. Omid
FC556851.1	AYRGVRQRTWGKWVAEIREPNRGNRLWLGSFPTAVEAARAYDDAARAMYGAKARVNFSEQSPDA	Iran	<i>Triticum aestivum</i> cv. Zarrin
UIC73724.1	VYLGVRQRTWGKWVADIREPNRGNRLCLGSFPTAVEPARAYDDAARAMYGAKARVNFSEQSPDA	Azerbaijan	<i>Triticum aestivum</i> cv. Azerbaijan
FC556850.2	AYRGVRQRTWGKWVAEIREPNRGNRLWLGSFPTAVEAARAYDDAARAMYGAKARVNFSEQSPDA	Iran	<i>Triticum aestivum</i> cv. Shahi2
FC556846.2	AYRGVRQRTWGKWVAEIREPNRGNRLWLGSFPTAVEAARAYDDAARAMYGAKARVNFSEQSPDA	Iran	<i>Triticum aestivum</i> cv. Bayat
FC556845.2	AYRGVRQRTWGKWVAEIREPNRGNRLWLGSFPTAVEAARAYDDAARAMYGAKARVNFSEQSPDA	Iran	<i>Triticum aestivum</i> cv. Alvand
ACO5850.6.1	FRGVRQRTWGKWVAEIREPNRVSRWLGLTFPTAEDAARAYDEAARAAMYGALARTNFPVHPAQA	Italy	<i>Aegilops speltoides</i> var. <i>speltoides</i>
ACO5850.8.1	GFRGVRQRTWGKWVAEIREPNRVSRWLGLTFPTAEDAARAYDEAARAMYGALARTNFPVHPAQA	Italy	<i>Aegilops speltoides</i> var. <i>speltoides</i>
ACO5850.7.1	GFRGVRQRTWGKWVAEIREPNRVSRWLGLTFPTAEDAARAYDEAARAMYGALARTNFPVHPAQA	Italy	<i>Aegilops speltoides</i> var. <i>speltoides</i>
AAX1328.9.1	AYRGVRQRTWGKWVAEIREPNRGNRLWLGSFPTAVEAARAYDDAARAMYGAKARVNFSEQSPDA	China	<i>Triticum aestivum</i> cv. China
CDM8149.0.1	AYRGVRQRTWGKWVAEIREPNRGNRLWLGSFPTAVEAARAYDDAARAMYGAKARVNFSEQSPDA	France	<i>Triticum aestivum</i> cv. France
ACJ01646.1	GFRGVRQRTWGKWVAEIREPNRVSRWLGLTFPTAEDAARAYDEAARAMYGALARANFPVHPAQA	Italy	<i>Triticum turgidum</i> subsp. <i>durum2</i>
ACJ01645.1	GFRGVRQRTWGKWVAEIREPNRVSRWLGLTFPTAEDAARAYDEAARAMYGALARANFPVHPAQA	Italy	<i>Triticum turgidum</i> subsp. <i>durum3</i>
ACJ01644.1	GFRGVRQRTWGKWVAEIREPNRVSRWLGLTFPTAEDAARAYDEAARAMYGALARTNFPVHPAQA	Italy	<i>Triticum aestivum</i> cv. Italy1

ACO3558	GFRGVRQRTWGWVAEIREPNRVSRWLGTFTPTAEDAARAYDEAAR	Italy	<i>Aegilops speltoides</i> var. <i>speltoides</i>
8.1	AMYGALARTNFPVHPAQA		
ACO3559	GFRGVRQRTWGWVAEIREPNRVSRWLGTFTPTAEDAARAYDEAAR	Italy	<i>Aegilops speltoides</i> var. <i>speltoides</i>
1.1	AMYGALARTNFPVHPAQA		
ACO3558	GFRGVRQRTWGWVAEIREPNRVSRWLGTFTPTAEDAARAYDEAAR	Italy	<i>Aegilops speltoides</i> var. <i>speltoides</i>
5.1	AMYGALARTNFPVHPAQA		
ACJ01643	GFRGVRQRTWGWVAEIREPNRVSRWLGTFTPTAEDAARAYDEAAR	Italy	<i>Triticum aestivum</i> cv. Italy2
.1	AMYGALARTNFPVHPAQA		
ACT7896	GFRGVRQRTWGWVAEIREPNRVSRWLGTFTPTAEDAARAYDEAAR	Italy	<i>Triticum aestivum</i> cv. Italy3
9.1	AMYGALARTNFPVHPAQA		
ABU6866	GFRGVRQRTWGWVAEIREPNRVSRWLGTFTPTAEDAARAYDEAAR	Italy	<i>Triticum aestivum</i> cv. Italy4
5.1	AMYGALARTNFPVHPAQA		
ACT7896	GFRGVRQRTWGWVAEIREPNRVSRWLGTFTPTAEDAARAYDEAAR	Italy	<i>Triticum aestivum</i> cv. Italy5
4.1	AMYGALARTNFPVHPAQA		
ACO3558	GFRGVRQRTWGWVAEIREPNRVSRWLGTFTPTAEDAARAYDEAAR	Italy	<i>Aegilops speltoides</i> var. <i>speltoides</i>
6.1	AMYGALARTNFPVHPAQA		
ACO3558	GFRGVRQRTWGWVAEIREPNRVSRWLGTFTPTAEDAARAYDEAAR	Italy	<i>Aegilops speltoides</i> var. <i>speltoides</i>
3.1	AMYGALARTNFPVHPAQA		
ACT7896	GFRGVRQRTWGWVAEIREPNRVSRWLGTFTPTAEDAARAYDEAAR	Italy	<i>Triticum turgidum</i> subsp. <i>Durum</i> 4
2.1	AMYGALARTNFPVHPAQA		
ABU6865	GFRGVRQRTWGWVAEIREPNRVSRWLGTFTPTAEDAARAYDEAAR	Italy	<i>Triticum turgidum</i> subsp. <i>Durum</i> 5
9.1	AMYGALARTNFPVHPAQA		
AAX1328	GFRGVRQRTWGWVAEIREPNRVSRWLGTFTPTAEDAARAYDEAAR	China	<i>Triticum aestivum</i> cv. China
6.1	AMYGALARTNFPVHPAQA		

4.3. Composition and Motif Analysis

Multiple Em for Motif Elicitation Suite (MEME) was employed to identify additional protein motifs in the DREB transcription factors of selected wheat species (i.e., 17 cultivars of *Triticum aestivum*, 8 varieties of *Aegilops speltoides*, and 4 subspecies of *Triticum turgidum*). By using the default settings and profiles, MEME (v5.5.1) (<https://meme-suite.org/meme/meme 5.5.1/tools/meme>, accessed on 22 February 2023) identified novel motifs in the TS protein sequences. The amino acid composition analysis of the identified motifs was carried out by using the PortParam tool (<https://web.expasy.org/protparam/>, accessed on 29 February 2023) [35], and it provided information about the abundance and frequency of amino acids in the sequences.

4.4. Phylogenetic Analysis

The quality of the sequences was assessed and then manually aligned using the MUSCLE MEGA package (v11.0.13) [36] with the maximum likelihood method and default settings. For further validation of the tree, we constructed a Bayesian phylogenetic tree using MrBayes version 3.2 [37], following the methodology described in [38]. We followed the methodology from [38] to develop the

Bayesian phylogenetic tree using MrBayes version 3.2 [37]. Finally, we used the Interactive Tree of Life (iTOL) to design the final format of the phylogenetic tree.

4.5. Structure and Modeling of AP2 Domain

The Iterative Threading Assembly Refinement (I-TASSER) program (<https://zhanggroup.org/I-TASSER/>, accessed on 17 March 2023) was used to determine the secondary and tertiary structures of the AP2 domain [18–20]. The Ramachandran plot was depicted using the model's PDB file in VADAR (v1.8.) (<http://vadar.wishartlab.com>, accessed on 5 April 2023) [39]. The Protein Structure Analysis program (ProSA) (<https://prosa.services.came.sbg.ac.at/prosa.php>, accessed on 20 April 2023) was used to report the Z-score, further confirming the overall quality of the model [21,22].

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