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Research Article

Genome-wide Characterization of *AP2/ERF* Transcription Factors in Spinach Reveals Candidate Regulators for Nitrogen-use Efficiency

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ABSTRACT

Spinach (*Spinacia oleracea* L.) is a nutrient-dense leafy vegetable characterized by its rapid growth rate, high antioxidant content, and adaptability to various environmental conditions. The elucidation of the genetic regulatory networks of spinach, particularly the transcription factor families, are important for stress responses and are essential for improving adaptability and sustainability. A genome-wide analysis identified 42 member families of the Ethylene Response Factor family gene product. Within each clade, they were classified into three major subclades (*AP2, ERF,* and *RAV*) consistent with the composition of structural domains. Gene mapping showed an uneven number of *SpoERF* genes on six chromosomes, many of which were clustered together for an unclear purpose and suggests that tandem duplication is an important mechanism responsible for gene expansion. Gene structure and motif analysis revealed highly conserved *AP2* domains but varied intron–exon patterns, which suggest functional diversification. Synteny analysis identified phylogeographically strongly related genes between spinach and Arabidopsis thaliana, probably due to ancient duplication events and the conserved functional elements of the essential *ERF* regulators. Promoter analysis showed extensive enrichment for many cis-acting elements (*Abre, DRE, LTR, W-box, G-box*) and revealed involvement of *SpoERF*s in hormone, developmental, and environmental stress responses among *SpoERF* family. The findings of this study systematically identify and characterize the *AP2/ERF* gene family in spinach, revealing their chromosomal organization, evolutionary relationships, conserved structural features, and nitrogen-responsive expression patterns, thereby providing candidate regulatory genes for future functional studies and molecular improvement of nitrogen-use efficiency in spinach.

Keywords: Abiotic stress, *AP2/ERF* gene family, Expression analysis, Genome-wide analysis, Nitrogen-use efficiency, Phytogenic relationship, *Spinacia oleracea* L.

INTRODUCTION

Spinacia oleracea L., also known as spinach, is a perennial herbaceous green vegetable of the family *Amaranthaceae* and subfamily *Chenopodioideae*. It is endemic to ancient Persia (modern-day Iran) and is cultivated worldwide as a cool-season crop, valued for its high nutritional value (Nešković and Ćulafić, 1988; Ramaiyan *et al.*, 2020). Spinach is a diploid (dioecious) species (2n = 12) and displays dioecious plants in which the male plants are distinct from the female plants.

The genome has been sequenced from roots to tips in attempts to gain insight into sex determination and responses to stress (Janick, 1954; Razumova *et al.*, 2023). The plant grows well in temperate climates, grows best in loamy soils rich in organic matter, and contains many vitamins (A, C, and K) and minerals (iron, calcium, magnesium, and potassium). The leaves contain carotene, lutein, and flavonoids, which are thought to have antioxidant properties. Through its short growth cycle, high-yielding potential, and nutritional value, spinach has become a financially important crop in recent times. It is being increasingly considered in health and nutrition and molecular breeding research (Senesi and Loffredo, 2018; Hilgard, 2025). The spinach crops have emerged as a useful model crop in research on abiotic stress tolerance, plant physiology, and nutritional metabolism because of the low genome size and availability of genetic resources, and the genetic variety this provides (Bhattarai and Shi, 2021). Studies in molecular breeding and biotechnology are being carried out on spinach in order to discover cultivars that will contribute to achieving high production rates whilst being stress-tolerant and benefiting from nutrients. *Spinacia oleracea*, therefore, has great economic, nutritional, and scientific value worldwide (Ribera *et al.*, 2020). The largest producer by far is China, which produces more than 90% of the world's spinach. Other notable top spinach producers include the United States (mainly California and Arizona), Turkey, Japan, and Indonesia, while Europe has important growers in Italy, France, and the Netherlands, particularly in the fresh-market industry and processing industries (Simko *et al.*, 2014). South Asia spinach is cultivated in India, Pakistan, and Bangladesh in both commercial gardens as well as at home, growing under irrigated conditions throughout the year. In general, spinach is grown in soils that are well-drained, fertile, and cooler than freezing (35-60°C), so it is best grown in spring and autumn in most parts of the world (Bihari *et al.*, 2024). The increasing global demand for spinach, driven by its high nutritional value and expanding commercial production, highlights the need for sustainable strategies to improve yield stability and stress resilience. However, spinach productivity is strongly influenced by environmental factors such as nutrient availability, temperature fluctuations, and water stress, which can significantly limit growth and quality. Understanding the molecular mechanisms that regulate stress adaptation and nutrient responsiveness is, therefore, essential for maintaining consistent production under diverse cultivation conditions. In this context, transcription factor families such as *AP2/ERF* play pivotal roles in coordinating developmental and stress-responsive gene networks. A systematic investigation of the *AP2/ERF* gene family in spinach is thus directly relevant to bridging agricultural performance with molecular regulation (Nakano *et al.*,2022; Wang *et al.*,2022).

The gene family *ERF/AP2* (Ethylene-Responsive Factor/APETALA2) is one of the largest and most important families of transcription factors in plants. Members of the family are characterized by one or two *AP2/ERF* DNA-binding domains (60-70 amino acids) specific to *GCC-box* (*AGCCGCC*) or DRE/*CRT* (Dehydration Responsive Element/C-repeat) cis-elements located in the promoters of target genes. These transcription factors play crucial regulatory roles in plant growth, development, and responses to various abiotic and biotic stresses (Zhai *et al.*, 2022; Feng *et al.*, 2020). The *ERF/AP2* superfamily is broadly divided into three major subfamilies based on the number and structure of their *AP2* domains. The *AP2* subfamily, which contains two *AP2* domains, is mainly involved in key developmental processes such as flower and seed formation, organ identity, and embryogenesis. A well-known example is the *APETALA2 (AP2)* gene, which plays a central role in defining floral organ identity in plants (Sharma *et al.*, 2020; Zumajo-Cardona and Pabón-Mora, 2016). The *ERF* subfamily contains only a single *AP2* domain and is primarily associated with regulating plant responses to environmental stresses. It plays key roles in managing stress signals triggered by ethylene, drought, salinity, and various pathogens. Members of this family include *ERF* and *DREB*, which act as regulators of the ethylene and abscisic acid (*ABA*) signaling pathways (Ma *et al.*, 2024; Xie *et al.*, 2019). *RAV* subfamily: characterized by the presence of one *AP2* domain (and optionally an additional *B3* domain), *RAV* genes are involved in hormone signaling and developmental regulation, particularly leaf senescence and stress adaptation (Kandeel *et al.*, 2023). The *ERF/AP2* family shows a diverse biological function in regulating the expression of genes under normal and stress conditions. Members of the *DREB* (Dehydration-Responsive Element-Binding) subgroup activate downstream genes responsible for cold, drought, and salinity tolerance, while *ERF* genes regulate responses to biotic stresses such as pathogen attack through the jasmonic acid (*JA*) and ethylene (*ET*) pathways (Li *et al.*, 2022a; Su *et al.*, 2025). For several decades, the *AP2/ERF* transcription factor gene family has been extensively studied across various plant species. Functional and evolutionary investigations of this important gene family in higher plants have been facilitated by valuable information generated through diverse genomic and molecular analyses. The first *AP2/ERF* gene was identified in *Arabidopsis thaliana and Oryza sativa* during studies on floral development, which have approximately 139 and 122 members (Nakano *et al.*, 2006). The populus identified 200 *AP2/ERF* members (Zhuang Jing *et al.*, 2008), grapevine has 132 members (Zhuang *et al.*, 2009), 103 in cucumbers (Hu and Liu, 2011), 117 in wheat (Zhuang *et al.*, 2011), 292 Zeamays (Zhou *et al.*, 2012), 118 in barley (Guo *et al.*, 2016), 220 in upland cotton (Zafar *et al.*, 2022), 301 in soybean (Wang *et al.*, 2022), and lettuce have 220 members (Park *et al.*, 2023). However, in this study, all expressed genes of the *AP2/ERF* gene family were detected from the genomic database and investigated for their phylogenetic, conserved domain, gene structure, and cis-regulatory element analyses. We provide a thorough overview of the evolution and structural transformation of the *AP2/ERF* transcription factor family of plant species. We also demonstrate their genome-wide identification and classification. Furthermore, physicochemical properties and synteny analysis were utilized to predict the potential functional roles of the discovered *AP2/ERF* family genes. The expression patterns of the proposed genes were under abiotic stress conditions, including drought and salinity. Our findings offer insights into the genomic organization, expression dynamics, and functional roles of the *AP2/ERF* gene family, providing a deeper understanding of their involvement in stress adaptation and regulatory mechanisms in plants.

MATERIALS AND METHODS

Identification of *SpoERF* Gene Family Members

Spinacia oleracea genomic and protein sequences were extracted from the phytozome database (https://phytozome-next.jgi.doe.gov/info/Soleracea_Spov3). To identify members of the *AP2/ERF* gene family in spinach, *AP2* protein sequences from Arabidopsis thaliana were used as queries for local *BLASTP* searches with an E-value threshold of >e-10 (Park *et al.*, 2023). Those sequences were subsequently validated using Pfam ID (http://pfam.xfam.org/) to confirm the presence of the *AP2/ERF* domain (*PF00847*) (Su *et al.*, 2025). Based on confirmation of the domain, the putative members of the *AP2/ERF* family were identified and characterized in the genome of spinach for further characterization.

Comparative Synteny Analysis of *ERF* in Spinach and Arabidopsis on the Protein and Chromosomal Level

The Circletto web server (https://bat.infspire.org/circoletto/) is used for synteny analysis to investigate the evolutionary relationships and similarities among the subject protein and query sequences of the *ERF* protein (Chen *et al.*, 2025). All default settings were used for this purpose. Collinearity analysis of *AP2/ERF* genes was performed by the use of the One Step (MCScanX) function (Wang *et al.*, 2024), to explore the evolutionary relationship among *AP2/ERF* genes across spinach and Arabidopsis (Zhao *et al.*, 2019). The result of the syntenic relationships was graphically visualized using TBtools.

Physicochemical Properties and Chromosomal Localization of *SpoERF* Members

The physicochemical properties, isoelectric point (pI), molecular weight (MW), Aliphatic Index, and Instability Index of *AP2/ERF* proteins were calculated using the ExPASy Compute pI/Mw tool (http: //www. expasy. org/resources/compute-pi-mw) (Azimi *et al.*, 2024). Subcellular localization of the identified *AP2/ERF* proteins was predicted by the Bologna Unified Subcellular Component Annotator (*BUSCA*) program (http: //busca. biocomp. unibo. it/) (Prakash *et al.*, 2023).

The Sequence Manipulation Suite server (https://www.bioinformatics.org/sms2) of the function Protein *GRAVY* was utilized in bioinformatics to calculate the numerical values of the Grand Average of Hydropathy (*GRAVY*) (Abdala-Roberts *et al.*, 2019). Information on chromosome numbers, gene locations, strand orientations and gene and CDS lengths was extracted from the GFF annotation files of *Spinacia oleracea* and the distribution of the *AP2/ERF* genes was visualized using MG2C v2. 1 (http: //mg2c.iask. in/mg2c_v2. 1/index. html) to display their positional organization across the spinach genome (Hildebrand and Dekker, 2020).

Multiple Sequence Alignment and Phylogenetic Interference of *SpoERF* Members

To examine evolutionary relationships among *SpoERF* genes in spinach, multiple sequence alignment was performed using Muscle5 available on the EMBL-EBI server (Wohlgemuth *et al.*, 2023; Rahmani and Mirakabad, 2021). In the MEGA12 software, phylogenetic trees were generated using the maximum likelihood method using a bootstrap value of 1000 (Abaza, 2020; Wang *et al.*, 2020). The generated circular developmental tree was landscaped by iTOL v7 (https://itol.embl.de/) (Letunic and Bork, 2024).

Cis-acting Elements, Motifs, Domains, and Intron/Exon Distribution of *SpoERF* Gene Members

The promoter sequences (1500 bp upstream of the start codon) of the *AP2/ERF* gene family were obtained from the spinach genome using TBtools (Chen *et al.*, 2023). The upstream sequences were then assessed for the presence of cis-acting regulatory elements using the PlantCARE database (http://webtools/plantcare/html/) (Hernandez-Garcia and Finer, 2014). To identify and characterize conserved protein motifs, the MEME Suite v5.03 (http://meme-suite.org/meme/) was employed (Nystrom and McKay, 2021), providing insight into the structural heterogeneity and functional conservation of *AP2/ERF* protein family members in spinach. The intron-exon structures of identified *SpoERF* genes were visualized using the Gene Structure Display Server (*GSDS*) v2.0 (http://gsds.gao-lab.org/) (Hu *et al.*, 2015), a method similar to that recently employed by Ahmad *et al.* (2024) and Ali *et al.* (2017).

Table 1. Physicochemical properties of *SpoERF* (Protein length, Exon count, Strand, Molecular weight, theoretical pI, and Subcellular location).

TF ID	Name	Chr No.	Protein length (aa)	Start	End	Exon Count	Strand	Mol. Weight	pI	GRAVY	Subcellular location
Spov3_C0007.00031	*SpoERF*R1	C7	587	966134	969263	9	R	64729.62	6.28	-0.901	nucleus
Spov3_C0007.00050	*SpoERF*R2	C7	171	1328459	1328974	1	R	19189.42	8.53	-0.798	nucleus
Spov3_C0010.00105	*SpoERF*R3	C10	178	1878799	1879335	1	R	19742.99	4.85	-0.519	nucleus
Spov3_C0014.00035	*SpoERF*R4	C14	415	1200980	1202227	1	F	47007.35	6.47	-0.896	nucleus
Spov3_C0092.00003	*SpoERF*R5	C92	1342	37025	48119	20	R	148779.4	4.83	-0.185	endomembrane system
Spov3_C0336.00001	*SpoERF*R6	C336	399	1229	4298	1	R	44194.73	5.5	-0.37	nucleus
Spov3_C0336.00003	*SpoERF*R7	C336	159	15325	15804	1	R	17636.41	6.84	-0.899	nucleus
Spov3_C0960.00001	*SpoERF*R8	C960	273	2417	3238	1	R	31160.68	5.7	-0.827	nucleus
Spov3_chr1.00040	*SpoERF*R9	Chr1	379	441742	447240	1	R	42150.99	6.07	-0.688	chloroplast
Spov3_chr1.00084	*SpoERF*R10	Chr1	600	909371	914916	7	R	64502.99	5.71	-0.729	nucleus
Spov3_chr1.00229	*SpoERF*R11	Chr1	457	2541944	2550021	1	F	51713.27	6.21	-0.927	chloroplast
Spov3_chr1.00321	*SpoERF*R12	Chr1	745	3691111	3698401	8	R	80209.19	5.92	-0.714	nucleus
Spov3_chr1.02113	*SpoERF*R13	Chr1	452	52127747	52132805	6	R	51262.62	5.15	-0.925	nucleus
Spov3_chr1.04023	*SpoERF*R14	Chr1	176	1.04E+08	1.04E+08	2	F	19856.65	9.45	-0.551	chloroplast
Spov3_chr2.00432	*SpoERF*R15	Chr2	273	4851988	4852809	1	R	29514.36	8.86	-0.349	nucleus
Spov3_chr2.00433	*SpoERF*R16	Chr2	349	4879656	4880705	1	R	38296.8	7.68	-0.524	nucleus
Spov3_chr2.00460	*SpoERF*R17	Chr2	247	5224599	5225342	1	F	27395.17	5.9	-0.74	nucleus
Spov3_chr2.01077	*SpoERF*R18	Chr2	207	13818837	13819499	1	F	22845.07	5	-0.767	nucleus
Spov3_chr2.01078	*SpoERF*R19	Chr2	217	13823754	13824407	1	F	24301.65	4.79	-0.815	nucleus
Spov3_chr2.01559	*SpoERF*R20	Chr2	306	23227421	23228425	2	F	34654.5	5.27	-0.837	nucleus
Spov3_chr2.04537	*SpoERF*R21	Chr2	354	1.16E+08	1.16E+08	1	R	40564.68	8.5	-0.775	nucleus
Spov3_chr3.00214	*SpoERF*R22	Chr3	385	7469242	7470399	1	R	42659.47	9.03	-0.472	nucleus
Spov3_chr3.00974	*SpoERF*R23	Chr3	759	32310524	32315938	1	R	84501.09	6.62	-0.956	nucleus
Spov3_chr3.01547	*SpoERF*R24	Chr3	560	50509719	50514774	9	R	61726.08	6.32	-0.789	nucleus
Spov3_chr3.04777	*SpoERF*R25	Chr3	229	1.44E+08	1.44E+08	1	R	25387.26	5.31	-0.739	nucleus
Spov3_chr4.00045	*SpoERF*R26	Chr4	455	1507584	1508951	2	F	50162.61	8.58	-0.606	nucleus

Spov3_chr4.00739	*SpoERF*R27	Chr4	387	24528231	24530158	1	R	42747.57	4.9	-0.87	nucleus
Spov3_chr4.03097	*SpoERF*R28	Chr4	189	95751962	95752642	2	F	21265.05	6.52	-0.56	chloroplast
Spov3_chr4.03893	*SpoERF*R29	Chr4	371	1.08E+08	1.08E+08	1	R	41002.64	7.16	-0.205	nucleus
Spov3_chr4.04525	*SpoERF*R30	Chr4	541	1.16E+08	1.16E+08	8	F	59859.1	6.37	-0.836	nucleus
Spov3_chr4.04587	*SpoERF*R31	Chr4	387	1.17E+08	1.17E+08	2	R	42764.17	4.76	-0.702	nucleus
Spov3_chr5.00340	*SpoERF*R32	Chr5	379	3657956	3659095	1	F	41238.31	5.52	-0.649	nucleus
Spov3_chr5.00712	*SpoERF*R33	Chr5	185	7436546	7437103	1	F	20552.82	5.45	-0.648	nucleus
Spov3_chr5.02833	*SpoERF*R34	Chr5	345	54349058	54353131	7	R	39417.49	5.58	-0.873	nucleus
Spov3_chr5.03065	*SpoERF*R35	Chr5	432	63103409	63106022	1	R	47828.68	6.18	-0.655	nucleus
Spov3_chr5.03279	*SpoERF*R36	Chr5	358	69857114	69862330	1	R	38494.85	5.45	-0.307	nucleus
Spov3_chr5.04012	*SpoERF*R37	Chr5	502	92769884	92778087	8	R	54982.2	6.15	-0.759	nucleus
Spov3_chr6.00492	*SpoERF*R38	Chr6	139	5155087	5155506	1	R	15259.53	7.84	-0.186	nucleus
Spov3_chr6.01320	*SpoERF*R39	Chr6	315	16625504	16634237	2	F	35139.12	6.12	-0.973	nucleus
Spov3_chr6.01439	*SpoERF*R40	Chr6	225	19320129	19320946	2	R	24262.93	8.99	-0.678	nucleus
Spov3_S14.00015	*SpoERF*R41	S14	580	488377	492060	1	F	64172.73	7.37	-0.737	nucleus
Spov3_S16.00001	*SpoERF*R42	S16	223	24895	25566	1	F	24583.81	4.57	-0.596	nucleus

Whereas: R=Reverse strand, F= Forward strand, C= Chromosome scaffolds, S= Singleton scaffolds, Chr= Chromosome, pI= Isoelectric point, GRAVY=Grand Average of Hydropathicity.

Expression profiling of *AP2/ERF* genes under abiotic stresses was performed using RNA-seq datasets

In silico evaluation of transcriptomic data was carried out by extracting gene expression data from the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). in search terms matching relevant to the expressed function of genes, such as "Spinach" or corresponding Bio-project number PRJNA1150981 (Zhang *et al.*, 2015), annotated with normalized expression values of genes subjected to abiotic stress treatments. Normalized data were then annotated, transformed, and visualized using the TBtools software to provide a comparative analysis of differential expression patterns of the *AP2/ERF* gene family under stress conditions (Van den Berge *et al.*, 2019).

RESULTS AND DISCUSSION

Identification and molecular characterization of *AP2/ERF* gene family in spinach

The genome sequence of *Spinacia oleracea* was retrieved from the Phytozome v3 genome database to identify *SpoERF genes* within the spinach genome. The *AP2* domain (PF00847) was used as a query for *Arabidopsis thaliana ERF* proteins in a BLASTP search to detect potential members of the *AP2/ERF* gene family (Wang *et al.*, 2022). A total of 42 genes (*SpoERF1-SpoERF42*) were identified, and their sequences were further validated using Pfam, InterPro, and the Net-genomic database to confirm the presence of the conserved *AP2/ERF* domain. This suggests that the *AP2/ERF* family in spinach is relatively smaller than in *Arabidopsis thaliana* (139 members) and *Oryza sativa* (180 members) (Nakano *et al.*, 2006). It likely retains core functional diversity and stress-responsive roles through conserved domain structures and sequence motifs.

Furthermore, the molecular properties shown in Table 1, *SpoERF* proteins have a significant length range from 171 to 1342 amino acids, with the equivalent molecular weights ranging from 19.1 kDa to 148.7 kDa, carrying an average of 38.1 kDa. This is consistent with the diverse functional roles of the different members of this family: short proteins, in general, may play regulatory transcription factors and longer proteins may serve as additional domains for signal transduction or protein-protein interactions (Zhou *et al.*, 2012). The isoelectric point (pI) ranges from 4.57 to 9.45, indicating that some proteins are acidic and others basic, potentially influencing their interaction with DNA and other proteins under different pH conditions (Li *et al.*, 2022b).

The GRAVY values range from -0.185 to -0.901indicate that all *SpoERF* proteins are hydrophilic, favoring nucleus localization and interaction with aqueous environments, consistent with their function as transcription factors. In upland cotton, Liu *et al.* (2025) found that most *ERF* proteins were hydrophilic, nuclear-localized, and moderately stable. Most *SpoERF* proteins are predicted to localize in the nucleus, aligning with their role as transcription factors that regulate gene expression. Three members located in the cytoplasm (*SpoERF-9, 14, 28*) and one protein (*SpoERF-5*) were localized at the endomembrane system.

Chromosomal localization of *ERF* genes in Spinach

There were 42 *SpoERF* genes represented in Figure 1; the greatest expression in most strains was on chromosomes 1, 2, 4, and 5, but a smaller number of genes were found on chromosomes 3 and 6. As well, a few extant *ERF* loci have been observed on unplaced scaffolds. C-Scaffolds cover 1 to 8 genes, and S-Scaffolds have 41 and 42 members, probably as constituent genomic segments not yet assembled or of late duplication remnants. With the observed distribution pattern, it can be concluded that several *ERF* genes have been clustered within each other, indicating that the development of this family has likely been heavily driven by tandem duplication in spinach. One common feature of clustering multiple *ERF* genes in proximity is localized diversification, and single-copy *ERFs* scattered across chromosomes are likely to represent ancestral members conserved by purifying selection (Nakano *et al.*, 2006).

Phylogenetic relationships between *SpoERF protein* members

The nomenclature of protein sequences was followed by Khalid *et al*. (2025); Sharif *et al*. (2025). Figure 2 demonstrates phylogenetic tree organization of the *AP2/ERF/RAV* gene family of *Spinacia oleracea*. The 42 *SpoERF* members were neatly subdivided into three subfamilies (*AP2, ERF*, and *RAV*), shown in Figure 2. This is compatible with the classical classification of the *AP2/ERF* superfamily of other plants. The *AP2* clade (blue bars) comprises genes with two *AP2* domains, indicating that they are conserved in functions related to developmental processes, such as floral organ identity, leaf differentiation, and growth regulation. The *ERF* clade (pink bars) represents the largest group of members, containing gene sets with one *AP2* domain, primarily related to roles in abiotic and biotic stress signaling pathways. The *RAV* subfamily (green bars) contains members with one *AP2* domain plus one B3 domain. These data suggest that the biological functions of these genes are involved in integrating hormone signaling and stress responses. The bootstrap values across the tree support these relationships and also suggest that the diversification of these groups occurred early in their evolution and has been conserved by plant lineages. The phylogenetic clustering observed in spinach is highly consistent with earlier studies in *Arabidopsis*, rice, wheat, and tomato, where *AP2/ERF/RAV* members also form three distinct evolutionary branches (Ma *et al.*, 2024; Guo *et al.*, 2016; He *et al.*, 2021).

Cis-acting Elements of the *ERF* gene family at the promoter region of Spinach

The promoter analysis of spinach *AP2/ERF/RAV* genes revealed a wide distribution of cis-acting regulatory elements associated with stress responses (Figure 3A), hormone signaling, and light-mediated developmental regulation. Cis-elements such as *DRE* core**,** *MBS, LTR***,** *W-box***,** *WUN*-motif**,** and *ARE* were abundant, indicating that many *SpoERF* genes are strongly regulated under abiotic stresses such as drought, salinity, cold, and low oxygen. Hormone-responsive elements, including *ABRE***,** *ERE***,** *TGACG/CGTCA* motifs (*MeJA*)**,** and TCA elements (SA) were also prevalent, suggesting extensive hormonal control involving ABA, ethylene, jasmonic acid, and salicylic acid. Light-responsive motifs such as *G-box***,** *Box 4***,** *ACE***,** *Sp1***,** and *AE-box* were particularly enriched in *AP2* genes, highlighting their role in photomorphogenesis, leaf development, and circadian-mediated gene expression (Figure 3B). Together, these elements indicate that spinach *AP2/ERF/RAV* genes are driven by a complex regulatory network that integrates environmental cues and developmental signals. Overall, the cis-element composition in spinach suggests evolutionary conservation of regulatory mechanisms within the *AP2/ERF* superfamily and highlights their importance in mediating both growth and stress adaptation (Ma *et al.*, 2024; Nakano *et al.*, 2006; Song *et al.*, 2013).

Motif, Domain, and Gene Structure Analysis of *ERF* genes

Gene-level functional divergence in Motifs 1-10 is attributed to different subfamily-specific regulatory functions. The conserved motif variants (1, 2, and 4) were found to be predominant within the 42 homologous members of the *SpoERF* family (Figure 4). Motifs 1-4 were found to be expressed in nearly all members of the Family. Thus, their presence at the core of the DNA-binding activity of the *ERF* family may be related to divergence in their functions or subfamily-specific regulators**.**

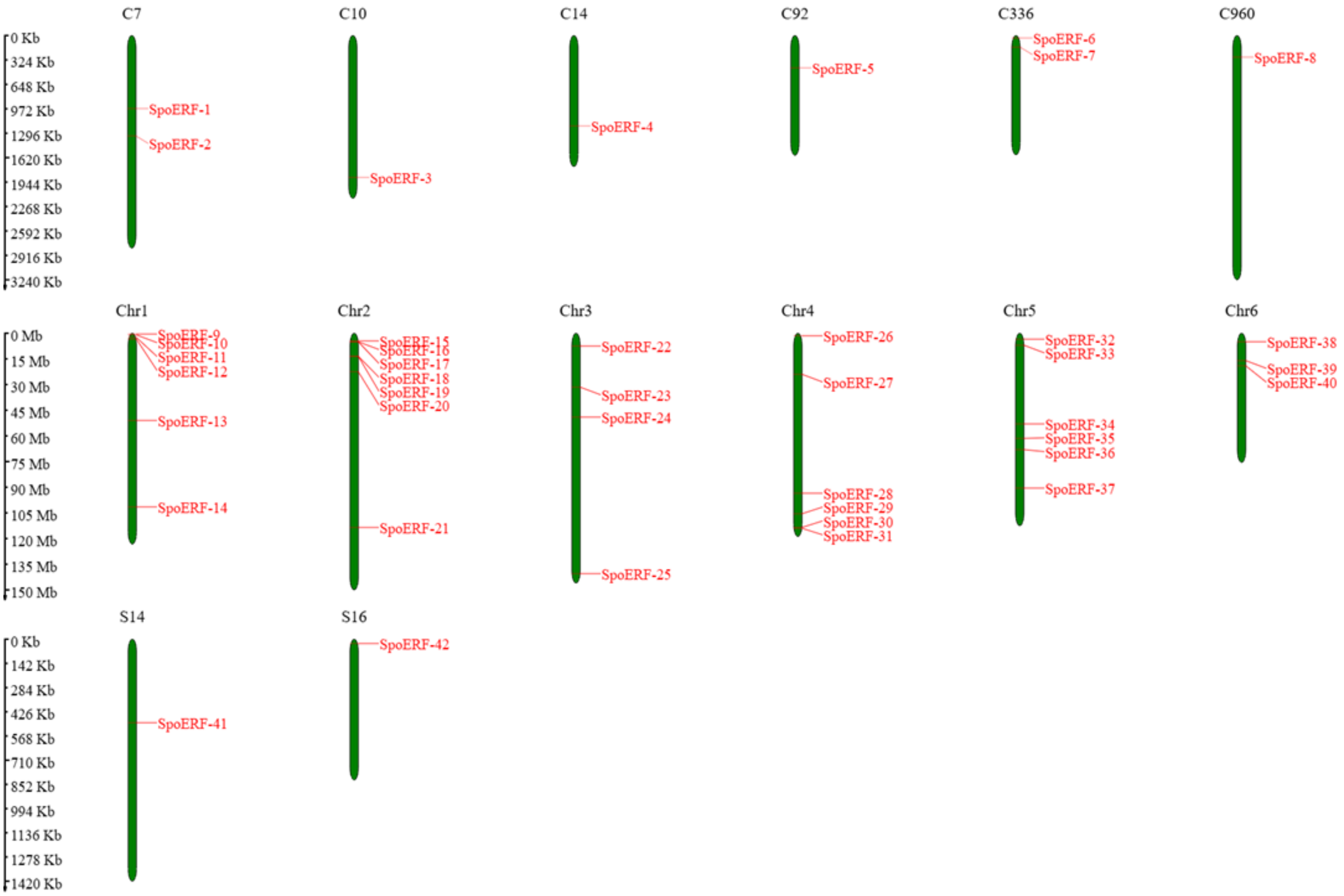

Figure 1. Chromosomal distribution of *AP2/ERF* gene family in Spinach. The black lines on chromosomes represent the distributed locations of *AP2/ERF* genes.

All identified *SpoERF* proteins showed at least one *AP2* domain with a typical 60-70 amino acid size and four-stranded sheet followed by a -α-helix, which is essential for recognition of GCC-box or DRE elements in promoter regions of stress-responsive genes. Based on the composition and identity of their domains, the *SpoERF* proteins we identified were grouped into three structural subfamilies. The *AP2* subfamily includes proteins that contain two tandem *AP2* domains and mainly function in plant growth, organ formation, and overall development. The *ERF* subfamily consists of proteins with a single *AP2* domain that are largely involved in regulating plant responses to both abiotic and biotic stresses.

The *RAV* subfamily is characterized by proteins that carry one *AP2* domain along with an additional B3 DNA-binding domain. Structural roles in hormone signaling and stress adaptation. In *Beta vulgaris*, another member of the Chenopodiaceae family, motif variation was linked to gene expression under salinity stress (Zhai *et al.*, 2024), suggesting a comparable adaptation mechanism in spinach. Therefore, the observed motif diversity among *SpoERF* members likely reflects sub-functionalization to meet the species-specific regulatory demands for stress tolerance and growth modulation (Su *et al.*, 2025).

In order to better understand the structure of the *ERF* gene family in spinach, we performed exon-intron analysis of 42 *SpoERF* genes using GSDS 2.0 software (Figure 5). Exon–intron compositions of the *ERF* gene family for spinach varied significantly between family members, and most *SpoERF* genes contain one to three exons, but a small group (*SpooERF-1, SpoERF-5, SpoERF-12, SpoERF-24, and SpoERF-30*) had more introns. Several *SpoERF* genes - *SpoERF-2, SpoERF-18, SpoERF-23* and *SpoERF-38* - are intronless. This suggests an evolutionarily simple gene structure that may be inherited by loss of introns. The presence of very few introns in the majority of genes suggests that the genome is compact, and it may thus be possible to respond faster under stress conditions by utilizing smaller gene bundles (Su *et al.*, 2025; He *et al.*, 2021; Ma *et al.*, 2024). Despite the absence of upstream and downstream untranslated regions across most genes, it is obvious that conserved regulatory sequences exist within the genome, which might affect the stability of transcripts and translational efficiency (Park *et al.*, 2023; Feng *et al.*, 2020).

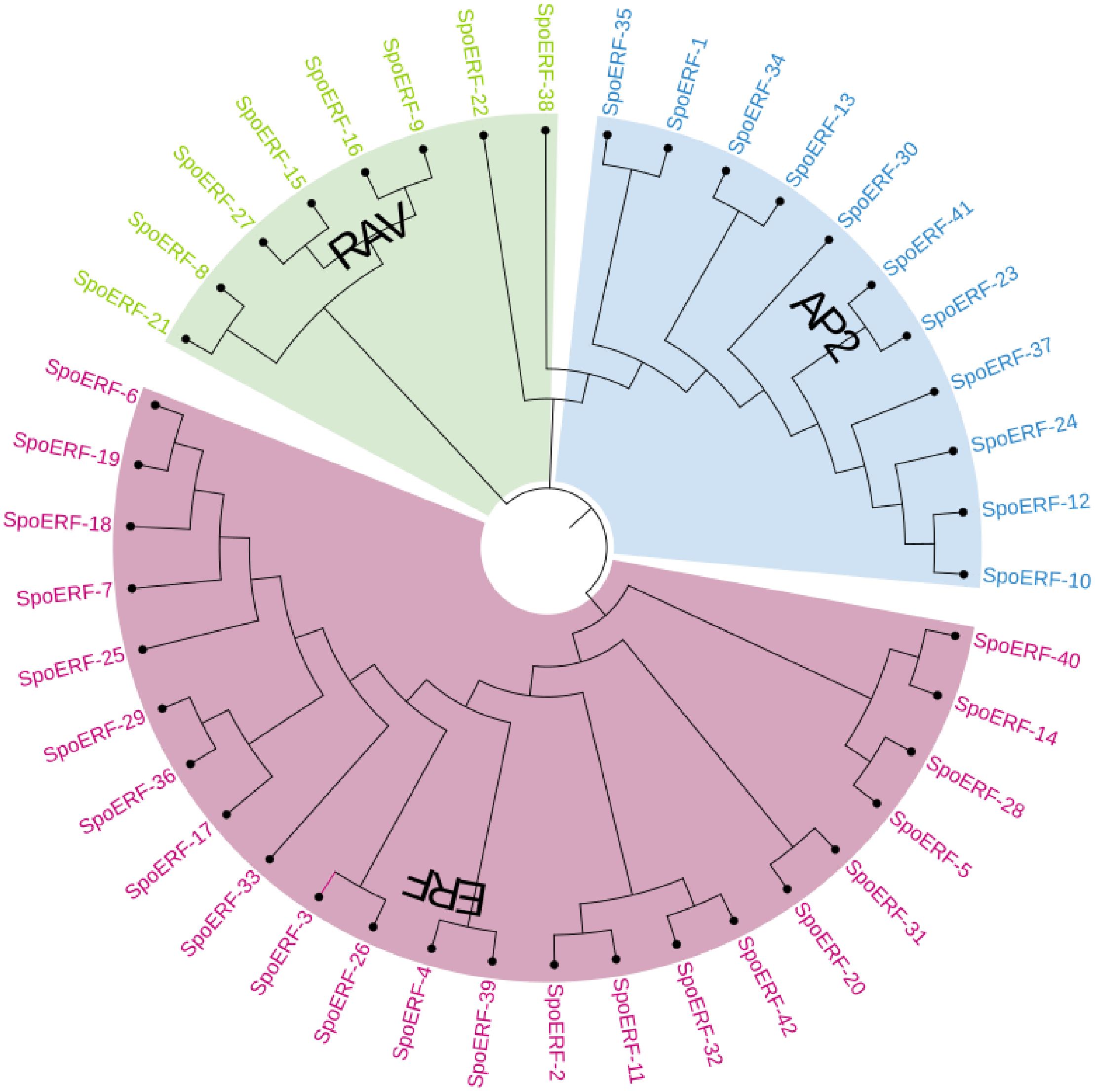

Figure 2. Phylogenetic analysis of *SpoERF* with division of three clades *AP2*, *ERF* and RAV.

Collinear Relationship between Arabidopsis and Spinach at the protein and chromosome levels

The Circos synteny analysis, as Figure 6, reveals strong collinearity relationships between *Spinacia oleracea* and Arabidopsis thaliana, highlighting that many *AP2/ERF* genes in spinach have conserved orthologous relations to those in Arabidopsis. The connecting lines (blue, green, and red) represent orthologous gene pairs across the two genomes, highlighting several segmental duplication events that likely contributed to the expansion of the *AP2/ERF* gene family in spinach. Such conservation across species implies that the core regulatory functions in growth, development, and stress responses may have remained conserved throughout evolution. The identification of 42 *SpoERF* compared to 139 *AtERF* genes, it has likely retained the essential functional modules needed for hormonal signaling. Previous studies have also reported similar evolutionary conservation and diversification of *AP2/ERF* gene families in other crops, including cotton (Zafar *et al.*, 2022) and tomato (Zhu *et al.*, 2020)**.** These findings collectively suggest that gene duplication and selective retention have played crucial roles in shaping the diversity and specialization of *AP2/ERF* transcription factors across plant lineages. The linear synteny analysis is shown in Figure 7, between Arabidopsis (chr 1-5) and spinach (chr 1-6) at the chromosome level. The spinach was aligned with Arabidopsis to better infer the evolutionary orthologous gene linkage, comparative homology, and interspecific genomic collinearity (Shahzaib *et al.*, 2024). Spinach showed 34 collinear gene pairs with A. thaliana at the chromosome level. The chromosome no. 6 in spinach shows one orthologous pair linkage with Arabidopsis, and other chromosomes have more than 7 pairs in linkage.

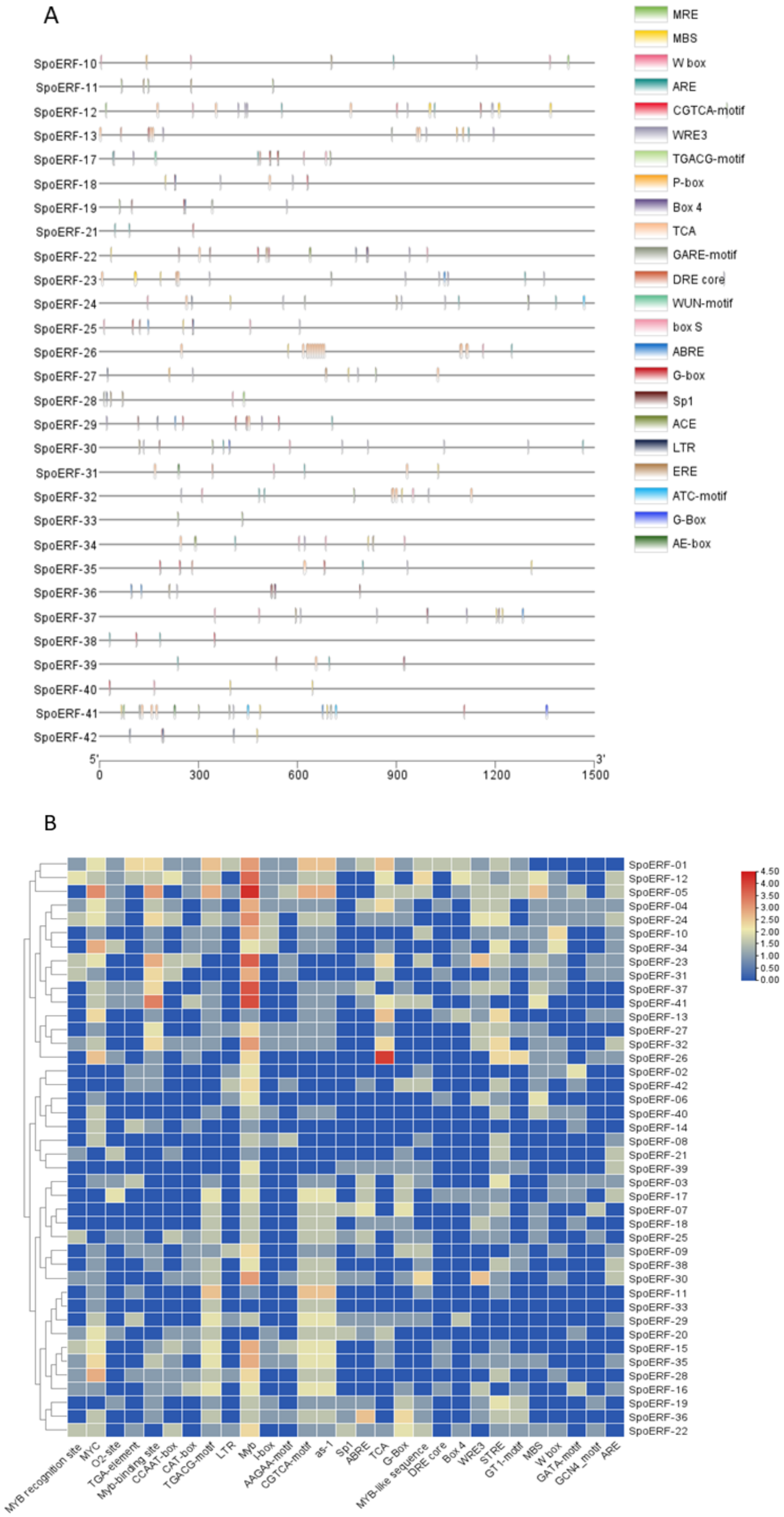

Figure 3. Promoter analysis of *SpoERF*. A) Cis-acting Regulatory Elements of *SpoERF* genes are visualized in the figure, while in the ligand figure, the colors of cis-acting regulatory elements are shown. B) Heatmap shows Cis-acting Elements of *SpoERF* members. The colors indicate the number of cis regulatory elements according to the legend given on right side of the figure.

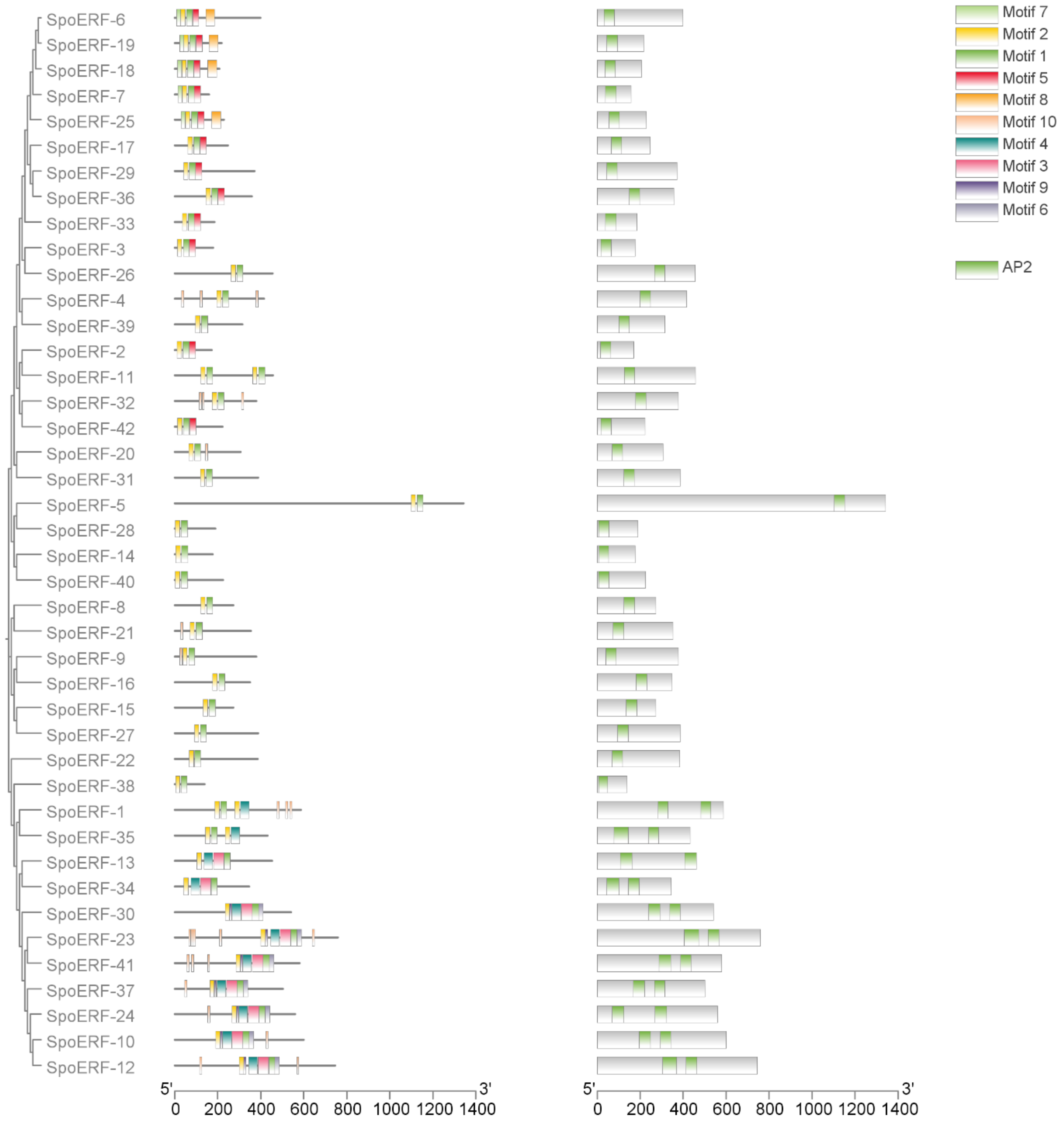

Figure 4. Conserved motifs and Domain confirmation of *SpoERF* (In the image, two panels show, the left panel shows motif structure, and the right shows the domain of *ERF* proteins.

These syntenic relationships highlight that, despite evolutionary diversification, the core regulatory network of *AP2/ERF* genes remains conserved between spinach and *Arabidopsis*, underscoring their crucial role in plant growth and environmental response mechanisms (Nakano *et al.*, 2006). These findings collectively suggest that gene duplication and selective retention have played essential roles in shaping the diversity and specialization of *AP2/ERF* transcription factors across plant lineages (Ma *et al.*, 2024; Wang *et al.*, 2022; Zhu *et al.*, 2020).

Expression Analysis of *SoERF* Proteins in Response to Different Nitrogen Levels

To investigate how *SpoERF* transcription factors respond to nitrogen availability and circadian timing, RNA-seq data generated from 48-day-old spinach leaves (GSE275461) were used to quantify the expression of 42 *SpoERF* genes (Figure 8). The RNA-seq dataset consisted of leaf samples collected from plants grown under high nitrogen (HN) and low nitrogen (LN) conditions at four distinct time points; T1: End of dark cycle, T2: Middle of light cycle, T3: End of light cycle, and T4: Middle of dark cycle.

The heatmap shows differential expression profiles of *SpoERF* genes over nitrogen treatment and time points, highlighting several important patterns. Multiple *SpoERF* genes (e.g., *SpoERF-12, SpoERF-19, SpoERF-28*) showed distinct upregulation in the presence of low nitrogen (LN), at particular times during T2 and T3. Thus, these genes may act as positive regulators of the nitrogen-stress signaling. Some genes (e.g., *SpoERF-4, SpoERF-22, SpoERF-37*) expressed in sequence patterns rhythmic (peak expression at different times) regardless of nitrogen supply, indicating that *ERF* transcriptic activity may be related to an internal clock of a plant. More members (e.g., *SpoERF-7, SpoERF-14*) were more highly expressed in high nitrogen conditions, implying roles in nitrogen sufficiency or growth-promoting metabolic pathways. *ERF* genes are clustered into similar signature expression profiles. Clusters triggered by LN suggest activation of *AP2/ERF* stress response pathways consistent with the previously reported roles of *ERF* members in nutrient deficiency and abiotic stress responses. The RNA-seq-based expression analysis clearly demonstrates that the *SpoERF* gene family is highly responsive to nitrogen availability and diurnal signals. Many *ERF* members were strongly induced under limited nitrogen. These results highlight the dynamic transcriptional behavior of the Spo*ERF* family and suggest potential roles for specific members in nitrogen-stress tolerance, metabolic regulation, and signaling pathways in spinach. Moreover, the differential expression of several spinach *AP2/ERF* genes under contrasting nitrogen levels aligns with earlier findings that *AP2/ERF* factors participate in nutrient-responsive regulatory networks. These findings are consistent with previous literature and indicate that *AP2/ERF* genes contribute to the regulation of abiotic and nutrient stress in spinach (Nakano *et al*., 2006; Feng *et al*., 2020).

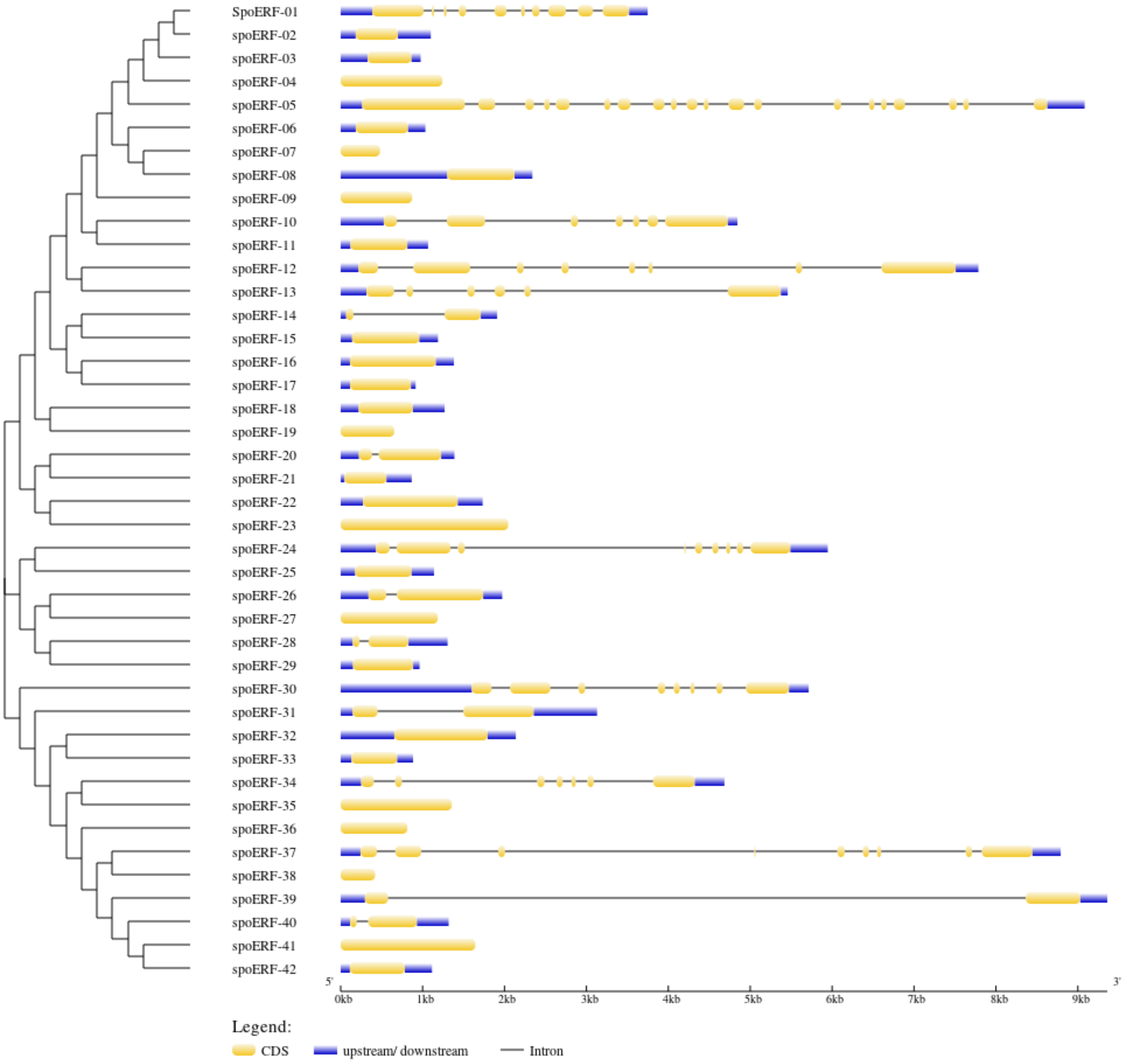

Figure 5. Gene structures of *SpoERF* with phylogenetic tree develop with 1000 bootstrap replicates. The yellow, blue, and green colors represent CDS, up/down streams, and introns, respectively.

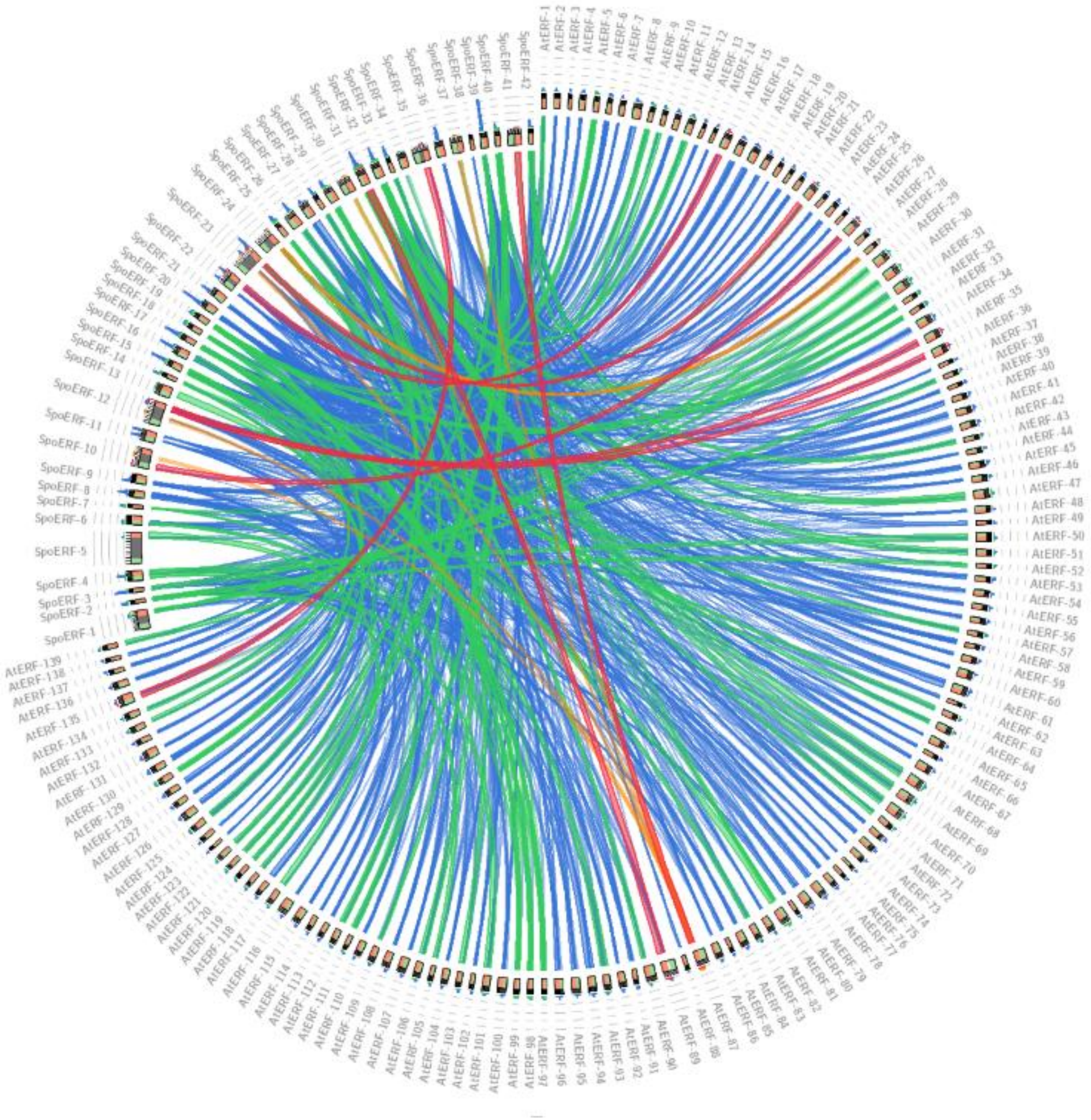

Figure 6. The syntenic regions in selected *AP2/ERF* proteins were identified by taking the *AtERF* proteins as the query sequence. blue, green, orange, and red are 50, 75, 99.99, and 100% similar, respectively

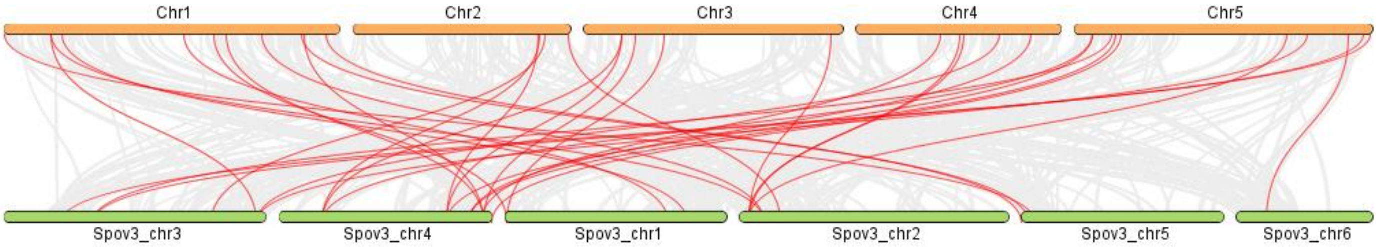

Figure 7. The linear synteny relationship between the *AP2/ERF* gene family of Arabidopsis (1-5 chromosomes) and Spinach (1-6 chromosomes).

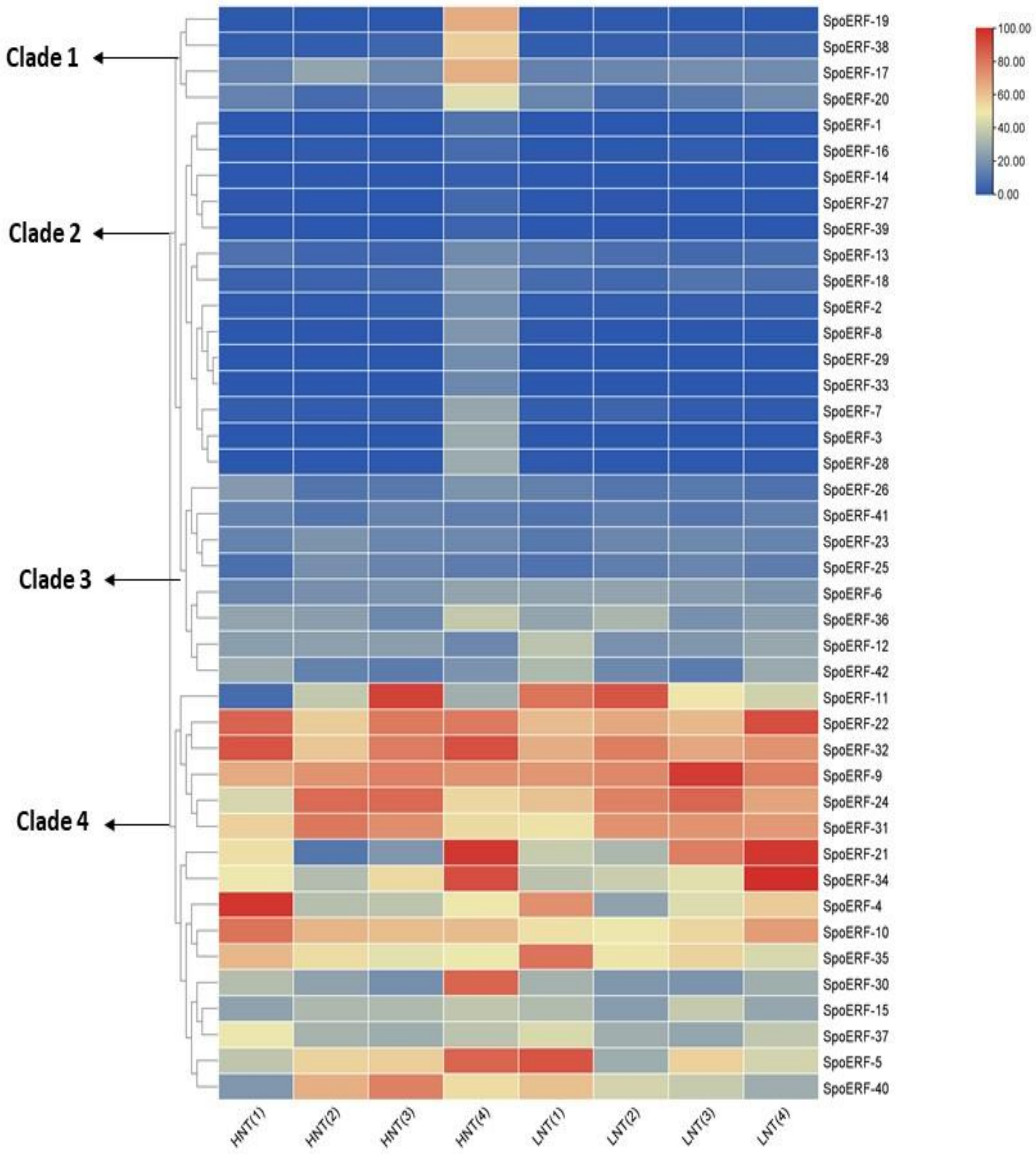

Figure 8. Heatmap of expression analysis of *SpoERFs* based on RNA-seq data available on SRA database in response to different nitrogen levels.

CONCLUSION

This study is the first comprehensive analysis of the *ERF* gene family from spinach. The 42 non-redundant genes were unevenly distributed across the chromosomes. Comprehensive analyses, including phylogenetic relationships, ortholog identification, gene structure, conserved motifs, synteny, promoter regions, and expression profiling, revealed substantial diversity among *SpoERF* genes and proteins. Their differential expression patterns suggest a potential regulatory role in improving nitrogen use efficiency. These findings provide a valuable foundation for future studies and support the development of stress-resistant crops against abiotic conditions.

AUTHOR CONTRIBUTIONS

Abdullah Shakeel: Writing – original draft, Writing – review & editing, Formal analysis. Rabia Irshad: Validation, Resources, Supervision. Muhammad Abdullah Hussain: Writing – review & editing. Zahid Khan: Writing – review & data analysis. Fatima Tu Zahra: Writing – review & editing. Aqib Ali: Validation, Visualization. Syed Din Muhammad: Writing – review & editing. Ameer Hamza Aslam: Conceptualization, Methodology, Supervision, Resources, Validation.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest related to this research.

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