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Research Article

Genome-wide Identification and Expression Analysis of ERF (Ethylene Response Factor) Gene Family in *Beta vulgaris* under Salt Stress

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ABSTRACT

The ERF (ethylene response factor) gene family constitutes one of the most significant families of transcription factors present in plants. These factors play a pivotal role in regulating various biological and physiological responses, including development, plant growth, and biotic and abiotic stresses. In order to explore the characteristics of sugar beet (*Beta vulgaris* L.), a series of analyses were employed. These included genome-wide identification, gene structure, phylogenetic, and promoter analysis of the ERFs. The objective of these analyses was to predict the potential functions of the ERFs. Among the 73 genes examined, 67 were identified as non-redundant, and the chromosomal distribution pattern revealed that all genes were mapped across nine different chromosomes. A subsequent analysis of conserved motifs identified ten distinct motifs, with *BvERF07* and *BvERF51* demonstrating the least motif complexity. A subsequent analysis, employing a phylogenetic approach and comparing the genes with those of A. thaliana, resulted in the delineation of nine distinct clades. This finding indicates not only functional conservation but also divergence over time. A thorough examination of the promoter sequence revealed the presence of a multitude of cis-regulatory elements, which have been associated with stress and hormonal responses. Synteny and gene duplication analyses further supported the evolutionary conservation of the ERF gene family. Transcriptomic profiling under salt stress revealed several differentially expressed genes, notably *BvERF02*, *BvERF50*, and *BvERF34*, indicating their potential involvement in salinity tolerance. This study presents foundational visions into the structure, evolution, and stress-responsive roles of *BvERFs*, offering candidate genes for genetic improvement of salt tolerance in sugar beet.

Keywords: *Beta vulgaris*, ERF gene family, Expression analysis, Phylogenetic, Salt stress

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INTRODUCTION

In plants, the *AP2/ERF* transcription factor (TF) superfamily is one of the most sizable gene families, containing at least one *APETALA2* (*AP2*) domain with 60 amino acid residues. The *AP2* domain has been demonstrated to play a regulatory role in plant development and to respond to various biotic and abiotic stresses (Jofuku *et al.*, 2005). *AP2/ERF* TFs can be classified into *RAV*, *AP2*, and *ERF* gene subfamilies according to the rule of conserved domains' factors (Cao *et al.*, 2001; Sakuma *et al.*, 2002; Nakano *et al.*, 2006). The majority of *AP2/ERF* TFs belonging to the ERF subfamily are characterized by the presence of a conserved *AP2* domain and play a role in the encoding of proteins that contain two *AP2* domains. Furthermore, the single *AP2* domain contains a B3 DNA-binding domain, which

is also conserved in different transcription factors (TFs) of plants (Hu et al., 2011). Nevertheless, the AP2 domain exhibits a high degree of conservation in its sequence. However, it should be noted that each subfamily comprises distinct DNA-binding elements. The ERF subfamily can be further categorized into two groups on the basis of DNA binding motifs i.e., the DREB group (containing A1 to A6 subgroups) and the ERF group (containing B1 to B6 subgroups) (Wu *et al.,* 2008; Licausi *et al.,* 2013). The AP2 subfamily is unable to bind the CCGA/CC core element recognized by the ERF subfamily; instead, it binds to the GCAC(A/G)N(A/T)TCCC(A/G)ANG(C/T) motif (Nole-Wilson *et al.,* 2000; Gong *et al.,* 2008). The ERF family is generally categorized in two subfamilies (ERF and DREB/CBF), and both are subdivided into I to X groups (Nakano *et al.,* 2006).

In different plant species, proteins encoded by ERF family regulate various biological events like, brassinosteroid, transduction of hormonal signals mediated by ethylene and cytokinin (Hu *et al.,* 2004; Rashotte *et al.,* 2006), abiotic and biotic stress responses (Stockinger *et al.,* 1997; Liu *et al.,* 1998), beneficial symbiotic interaction (Vernie *et al.,* 2008), cell differentiation (Iwase *et al*, 2011) and metabolism regulation (Aharoni *et al.,* 2004; Zhang *et al.,* 2005). These proteins are also involved in developmental processes, such as flower development (Elliott *et al.,* 1996), embryo development (Boutilier *et al.,* 2002) and leaf epidermal cell density (Moose and Sisco, 1996).

Until now, in various plant species ERF family proteins have been reported such as, *Arabidopsis thaliana* (Sakuma *et al.,* 2002), *Gossypium hirsutum* (Jin and Liu, 2008), *Vitis vinifera* (Zhuang *et al.,* 2009), *Glycine max* (Li *et al.,* 2005), *Populus trichocarpa* (Zhuang *et al.,* 2008), *Oryza sativa* (Sharoni *et al.,* 2011) and *Solanum lycopersicum* (Sharma *et al.,* 2010). There are 147 genes reported in *Arabidopsis* genome which encode AP2/ERF-type proteins from which 122 genes belong to the ERF family (Nakano *et al.,* 2006). In *Arabidopsis*, expression of *DREB1A* gene (with two homologs in group III) and *DREB2A* gene (with single homolog in group IV) regulate temperature and drought stresses, respectively (Gilmour *et al.,* 2000), suggesting their functions as members within the same group of ERF family, likely relate bHLH and MADS-box families which have been similarly reported (Toledo-Ortiz *et al.,* 2003). However, evaluation of structural relationships among proteins of ERF family as each TF function's part can provide a foundation to understand the related genes functions.

Genetics of sugar beet needs to be improved mainly against biotic (Biancardi *et al*., 2005) and abiotic stresses (Vastarelli *et al.,* 2013; Lv *et al*., 2019). Shape and quality of roots mainly influenced by breeding in which molassigenic elements such as sodium, potassium and alpha-amino nitrogen increase under stress conditions, throughout plant life. *B. vulgaris* is tolerant to salt stress (Lv *et al*., 2019), contains traits tolerant to salt, and can remain viable up to seven days in high salt concentration (500 mM) (Yang *et al*., 2012). To the date, no study has been reported on proteins of ERF domain in sugar beet. It is probably due to late release of genome as compared with other species. Current research explores 62 ERF genes of the sugar beet genome, which were identified via genome-wide study, and then different analyses i.e., evolutionary relationship, gene structure, chromosomal localization of genes, synteny analysis, conserved protein motifs and domains, promoter analysis and *in silico* gene expression were executed for these identified genes to indicate their functional and evolutionary relationships.

MATERIALS AND METHODS

Retrieval of sequences

B. vulgaris with different protein characters and sequences of the ERF family were collected from Plant Transcription Factor Database (PlantTFDB) (https://planttfdb.gaolab.org/) (Jin *et al*., 2016). The characteristics of *BvERF* proteins, including isoelectric points (pI) and amino acid lengths (aa), were obtained from the PlantTFDB database. (https://planttfdb.gao-lab.org/). To justify the predicted ERF protein sequences of *BvERFs*, the Basic Local Alignment Search Tool (BLAST) was used via the National Center for Biotechnology Information (NCBI) platform (https://www.ncbi.nlm.nih.gov/) (Johnson *et al*., 2008). Additionally, the complete genome sequence was also obtained from the NCBI database.

Chromosome mapping, intron–exon structure, and conserved motif analyses

The positions of *BvERF*s were retrieved from NCBI, and for visualization of genes on their respective chromosomes, they were visualized by MapChart software (v.2.32) (Voorrips, 2002). The intron/exon formations of *BvERF* genes were evaluated using the Gene Structure Display Server (GSDS) (https://gsds.gao-lab.org/) (Hu *et al*., 2015). The conserved protein motifs within *BvERF* proteins were linked and visualized using the MEME Suite (Multiple Em for Motif Elicitation; v5.03) (https://meme-suite.org/meme/) (Bailey *et al*., 2009).

Comparative analysis of phylogenetic relationships and conserved domains of *BvERF* genes

To investigate the evolutionary relationships of the ERF gene family in *B. vulgaris* the protein sequences of *BvERF* and *AtERF* were retrieved from the PlantTFDB database. The nomenclature of *BvERF* protein sequences was

appointed according to the chromosomal locations of the corresponding genes. All protein sequences were exposed to multiple sequence alignment (MSA) using ClustalW under default parameters, with a gap-opening drawback set at 10.00, a pairwise alignment gap extension penalty of 0.10, and 0.20 for multiple alignments (Thompson *et al*., 2003). The negative matrix option was deactivated, and a divergence cutoff of 30% was applied. To construct a comparative phylogenetic tree aligned sequences were employed using the Neighbor-Joining (NJ) method with 1000 bootstrap replicates using MEGA v11.0 software (Tamura *et al*., 2021). For enhanced visualization and annotation, the resulting tree was further refined using the Interactive Tree of Life (iTOL) web server (https://itol.embl.de/) (Letunic and Bork, 2024). For identification of conserved domains lying within *B. vulgaris* proteins of ERF transcription factor NCBI Conserved Domain Database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cg/) was used (Marchler-Bauer *et al.,* 2015). For domain visualization, TBtools-II software was utilized (Chen *et al.,* 2023).

Analysis of syntenic blocks and gene duplication patterns

Syntenic blocks between *BvERF*s and *AtERF*s were conducted using TBtools-II, employing One Step MCScanX and Dual Synteny Plot functions (Wang *et al*., 2012). Intraspecific duplication events of *BvERF* genes were visualized using the Advanced Circos module.

Identification of cis-regulatory elements within the promoter regions of ERF genes

To find cis-regulatory elements in the promoters of *BvERF* genes, a 1-kb upstream region was manually retrieved for each gene from the NCBI database. PlantCARE database were used to analyze these promoter sequences (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot *et al*., 2002) to predict potential cis-acting regulatory elements. The identified elements were visualized using TBtools-II software for comprehensive interpretation.

Utilization of RNA-seq data for expression profiling of genes under abiotic stress conditions

The bio-project PRJNA936097 (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP423168&o=acc_s%3Aa/) has 12 runs carrying 19.84Gb based on experimental data available in the Sequence Read Archive (SRA) under the assigned accession number SRP423168, respectively. The reference genome of B. vulgaris was used for read mapping using Bowtie2 on the Galaxy server platform (https://usegalaxy.org/) with default settings (De Almeida *et al*., 2016). Gene expression levels were quantified from RNA-Seq data by calculating fragments per kilobase of transcript per million mapped reads (FPKM) using the Feature Counts tool (Liao *et al*., 2013) available on the Galaxy platform. Heatmaps were subsequently generated to visualize the expression patterns of *BvERF* genes under abiotic stress conditions in TBtools-II software (Chen *et al*., 2023; Ali *et al*., 2024)

RESULTS

Identification and comprehensive characterization of the ERF gene family in the *B. vulgaris* genome

The availability of the complete *B. vulgaris* genome enabled the identification of ERF gene family members, resulting in the prediction of 73 *BvERF* genes. The presence of ERF-specific conserved domains within the encoded proteins was assessed to confirm gene family membership. Identification of a complete ERF domain served as the primary criterion for classifying proteins as members of the ERF gene family. Six *BvERF* proteins were excluded from consideration due to the presence of incomplete ERF domains in their amino acid sequences. These proteins likely underwent evolutionary modifications that resulted in the loss or duplication of segments within their functional domains, leading to incomplete or altered amino acid sequences.

These genes are considered redundant and have been observed in other species and referred to as paralogs (Nadeem *et al*., 2025). The other 67 non-redundant genes of *BvERF* whose nomenclature was developed (*BvERF*01 to *BvERF*67) as described by Waqas *et al*. (2019) and Shafique *et al*. (2023) by following their ascending order. Protein lengths ranged from 126 amino acids (*BvERF*14) to 267 amino acids (*BvERF*37), indicating substantial size diversity within the family. The isoelectric point (pI) values spanned from 4.8575 (*BvERF*37) to 8.1905 (*BvERF*14), while the molecular weights varied between 14,358 Da (*BvERF*14) and 30,071.2 Da (*BvERF*37). These findings suggest considerable diversity in the structural and biochemical properties of *BvERF* proteins, which may reflect their functional specialization.

Mapping of *BvERF* on respective chromosomes

All of the identified 67 *BvERF* genes were distributed across all 9 chromosomes (Figure 1). Chromosome 5 had the largest number of *BvERF* genes, 12 members, followed by chromosome 6, which had 11 genes. Chromosome 3 contained 10 genes and Chromosome 8 contained 9 genes. Chromosomes 1 and 7 had 6 *BvERF* genes each, while Chromosome 2 had 5 *BvERF* genes. Chromosomes 4 and 9 had the least, with 4 *BvERF* genes per chromosome.

Table 1: Different characteristics of ERF genes and proteins from *B. vulgaris*.

TF ID	Given names	Locus ID	Chr. No.	Gene start	Gene end	Protein length (aa)	Isoelectric point (pI)	Molecular weight (Da)
Bv1_011970_tsyx.t1	*BvERF01*	LOC104896121	1	35521355	35526542	457	6.5204	49765
Bv1_009470_kyxu.t1	*BvERF02*	LOC104892628	1	45800093	45802214	383	5.8579	41581
Bv1_018210_djqs.t1	*BvERF03*	LOC104905311	1	53835489	53836959	308	5.7837	34225
Bv1_003570_quwj.t1	*BvERF04*	LOC104886648	1	60381695	60383404	322	4.785	36493.4
Bv1_002890_zepi.t1	*BvERF05*	LOC104908760	1	61099931	61100879	183	5.8824	20414.6
Bv1_000090_pnxw.t1	*BvERF06*	LOC104887356	1	64165614	64166893	263	6.0064	28553.8
Bv2_032900_urkh.t1	*BvERF07*	LOC104892802	2	11808640	11809071	141	10.8619	16044.5
Bv2_032310_xjoh.t1	*BvERF08*	LOC104885368	2	45484251	45485492	276	4.8416	30274.6
Bv2_031130_cghd.t1	*BvERF09*	LOC104883082	2	47581108	47582344	189	8.6657	21399.1
Bv2_030370_nmyr.t1	*BvERF10*	LOC104908848	2	48734874	48736627	216	8.0677	23913.6
Bv2_044600_drzk.t1	*BvERF11*	LOC104905955	2	56764279	56766620	299	4.2847	32765.7
Bv3_049250_daej.t1	*BvERF12*	LOC104888082	3	1489955	1490935	243	6.7232	26968.8
Bv3_049280_qgjt.t1	*BvERF13*	LOC104887960	3	1520112	1520827	142	6.5274	16390
Bv3_049290_kacx.t1	*BvERF14*	LOC104887961	3	1540956	1541722	126	8.1905	14358
Bv3_052580_wurf.t1	*BvERF15*	LOC104888301	3	5252007	5253986v	391	4.5213	43178.7
Bv3_066590_ignp.t1	*BvERF16*	LOC104906454	3	9395969	9396866	221	5.7294	24655.8
Bv_001670_stnf.t1	*BvERF17*	LOC104883557	3	37523897	37525342	326	4.6521	36400.3
Bv_006850_gcqz.t1	*BvERF18*	LOC104884212	3	40744274	40746838	263	5.0692	29625
Bv3_068470_jamk.t1	*BvERF19*	LOC104906769	3	45768781	45770160	307	7.6614	34155.2
Bv3_061260_uzyk.t1	*BvERF20*	LOC104889346	3	49647418	49648652	289	6.5236	33071.2
Bv3_065890_nand.t1	*BvERF21*	LOC104889810	3	57102235	57111752	425	8.6951	47183.7
Bv4_088950_cddu.t1	*BvERF22*	LOC104891730	4	2630349	2632233	242	7.1006	26689.7
Bv4_086670_khde.t1	*BvERF23*	LOC104891540	4	12881663	12882562	183	6.5289	19596.8
Bv4_073920_ryre.t1	*BvERF24*	LOC104890278	4	61835027	61836519	233	7.6922	26565.2
Bv4_073910_eape.t1	*BvERF25*	LOC104890277	4	61844011	61845106	231	7.5498	26302.1
Bv5_098930_sojm.t1	*BvERF26*	LOC104892162	5	2442282	2443811	337	8.1844	37225.4
Bv5_099430_qqci.t1	*BvERF27*	LOC104892198	5	2829737	2831606	357	6.377	39874.4
Bv_017190_rxhh.t1	*BvERF28*	LOC104886234	5	4881974	4887608	205	4.8859	22052.9
Bv5_101120_supk.t1	*BvERF29*	LOC104892685	5	4905515	4909720	748	7.6892	82943.7
Bv5_103360_emgs.t1	*BvERF30*	LOC104892557	5	8264264	8265908	327	4.7901	36589.3
Bv5_103370_napz.t1	*BvERF31*	LOC104892560	5	8269399	8271870	291	6.5195	32585
Bv5_104600_ssrc.t1	*BvERF32*	LOC104892777	5	10874497	10876182	241	7.0167	26700.6
Bv5_104890_chgw.t1	*BvERF33*	LOC104892795	5	11570464	11571852	348	6.7898	38691.1
Bv5_104900_ghsa.t1	*BvERF34*	LOC104892796	5	11631785	11632953	273	8.4752	29293
Bv_006010_ajds.t1	*BvERF35*	LOC104884113	5	50513256	50516499	313	6.7012	34892
Bv5_121050_fnik.t1	*BvERF36*	LOC104894654	5	66167702	66168403	134	6.5243	15130.4
Bv5_121060_wnjc.t1	*BvERF37*	LOC104894655	5	66188428	66189821	267	4.8575	30071.2
Bv_003330_fefj.t1	*BvERF38*	LOC104883763	6	231696	232860	233	6.6342	26728.1
Bv_003320_epdu.t1	*BvERF39*	LOC104883780	6	235077	236979	225	9.3435	26298.3
Bv_003310_ipmt.t1	*BvERF40*	LOC104883779	6	248075	249161	245	6.0981	28117.1
Bv6_127190_stch.t1	*BvERF41*	LOC104894865	6	979503	981324	177	6.0501	20216.2

Bv6_133340_zkhw.t1	*BvERF42*	LOC104895475	6	8603337	8607856	336	6.3164	36923.6
Bv6_135390_gwrr.t1	*BvERF43*	LOC104895700	6	11235160	11236940	283	5.5999	31757.6
Bv6_141990_jfsa.t1	*BvERF44*	LOC104896506	6	29858720	29861164	340	4.828	37831.1
Bv6_146520_achh.t1	*BvERF45*	LOC104897104	6	54889920	54891100	323	5.8335	36527
Bv6_148380_saoc.t1	*BvERF46*	LOC104897347	6	60388520	60390387	389	6.6418	44491.5
Bv6_149630_ammr.t1	*BvERF47*	LOC104897454	6	64716682	64724340	290	9.244	31837.7
Bv6_152840_fucw.t1	*BvERF48*	LOC104897803	6	69043512	69044897	200	4.4878	22187.6
Bv7_160130_ygqf.t1	*BvERF49*	LOC104898606	7	5540984	5542170	205	4.4508	22059.2
Bv7_163350_ryod.t1	*BvERF50*	LOC104898899	7	32485365	32486663	288	5.1427	33236.6
Bv7_178910_dohw.t1	*BvERF51*	LOC104908811	7	44062919	44067792	249	9.8067	27911.1
Bv7_169290_jndd.t1	*BvERF52*	LOC104899692	7	51780418	51782983	390	4.4448	42899.3
Bv7_176570_fgjx.t1	*BvERF53*	LOC104900477	7	60834461	60835668	257	5.8526	28840.4
Bv7_176580_eaea.t1	*BvERF54*	LOC104900478	7	60843718	60845123	318	9.959	35184.8
Bv8_182210_zdgz.t1	*BvERF55*	LOC104900685	8	2357075	2358102	258	5.1173	29589.1
Bv8_182220_noqg.t1	*BvERF56*	LOC104900686	8	2359875	2367196	148	10.1468	16558.6
Bv8_182660_gzjr.t1	*BvERF57*	LOC104900638	8	2938530	2939878	263	8.5136	28140.2
Bv8_182680_iuha.t1	*BvERF58*	LOC104900640	8	2996621	2997678	165	10.5	18052.5
Bv8_190680_oogs.t1	*BvERF59*	LOC104901646	8	50333866	50334923	225	4.4163	25407.7
Bv8_190700_iajz.t1	*BvERF60*	LOC104901648	8	50362264	50365325	227	4.4536	25996.4
Bv8_194420_hkxp.t1	*BvERF61*	LOC104902033	8	56253273	56255865	217	7.9846	24463.7
Bv8_197150_tysy.t1	*BvERF62*	LOC104902271	8	59691395	59693331	283	4.8118	31024.9
Bv8_198510_ekky.t2	*BvERF63*	LOC104902404	8	61478038	61485812	304	6.099	33926.5
Bv9_208590_puum.t1	*BvERF64*	LOC104903353	9	21653680	21656091	415	6.1163	47044.2
Bv9_225840_iyth.t1	*BvERF65*	LOC104883290	9	25564814	25565840	211	8.2395	23079
Bv9_219670_amqj.t1	*BvERF66*	LOC104904567	9	50685222	50686792	177	10.5088	20003.8
Bv9_221880_kecu.t1	*BvERF67*	LOC104904762	9	53112447	53113779	236	4.5967	25946.5

This non-random distribution suggests that there were likely multiple episodes of gene duplication and divergence during the evolution of the ERF gene family in *B. vulgaris*. The AP2/ERF transcription factors take part in regulating a variety of biological processes in plants; for example, plant growth, development, and responses to numerous environmental stresses (Nakano *et al*., 2006). As of our study, Salicylic acid, Jasmonic acid, and cellular response to heat or freezing all contain contributions from *BvERF* genes. In particular, *BvERF*06 (Bv1_000090_pnxw.t1) and *BvERF*02 (Bv1_009470_kyxu.t1) display co-moderate acidic to near neutral profiles and may impact DNA binding affinity related to stress. Additionally, *BvERF*31 (Bv5_103370_napz.t1), especially considering *BvERF*31, indicates a stable profile across a variety of physiological conditions. Collectively, these genes may contribute mammoth regulatory roles in stress signaling pathways, having implications of impacts against salinity or drought, or in coordination with hormonal signaling, similar to patterns of function in those members of the ERF family from other species.

Comparative evolutionary analysis of *BvERF* and *AtERF* genes

To clarify the evolutionary relationships of the ERF (Ethylene Response Factor) gene family within *B. vulgaris* and *A. thaliana*, we generated a phylogenetic tree based on the full-length amino acid sequences of the ERFs. We usually assigned ERF genes to one of nine subgroups based on the classification in Arabidopsis. Groups I, III, and VIII tended to contain a relatively high number of *BvERF* genes, possibly due to a process of gene duplication. In contrast, groups II, VI, and VII contained fewer representatives, possibly due to loss of genes, evolutionary divergence, or specific functions.

Clustering of *BvERF* genes with their Arabidopsis counterparts in the same subgroups implies conserved evolutionary origins and functional similarities. However, several clades exhibited a clear enrichment of *B. vulgaris*-specific genes, pointing toward species-specific duplication or neofunctionalization events during the evolutionary history of the ERF family in sugar beet.

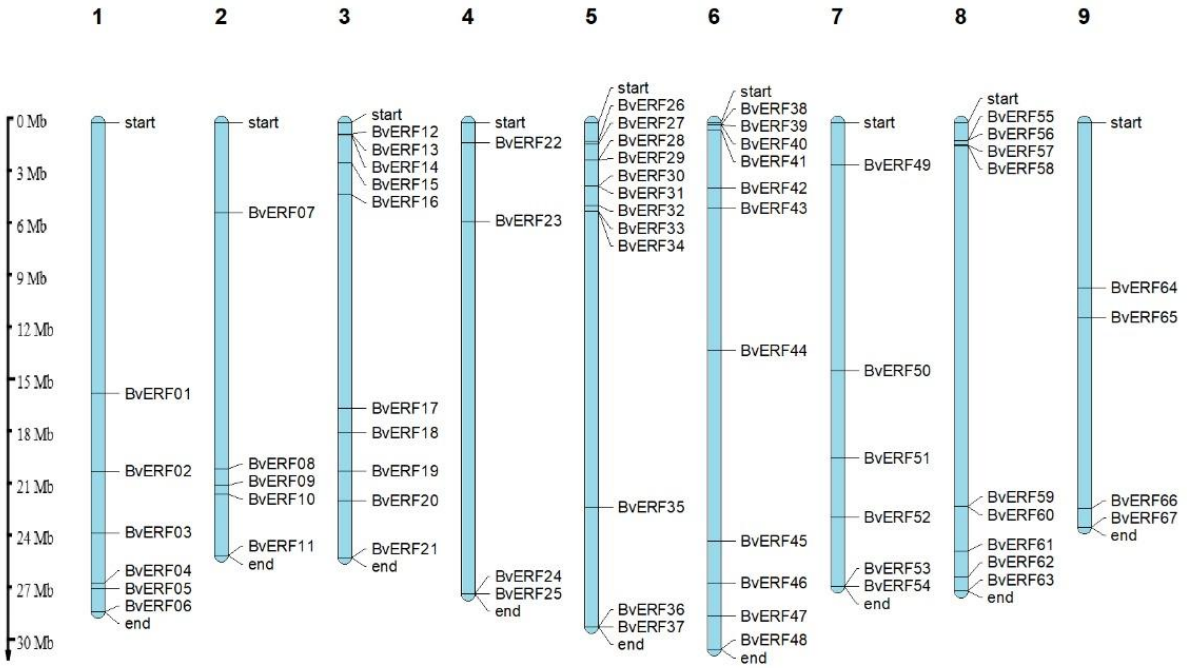

Figure 1. Chromosomal distribution of *BvERF* genes. The black lines on the chromosomes indicate the positions where ERF genes are located. Scale on the left side illustrating the length of chromosomes in Mb (million base pairs).

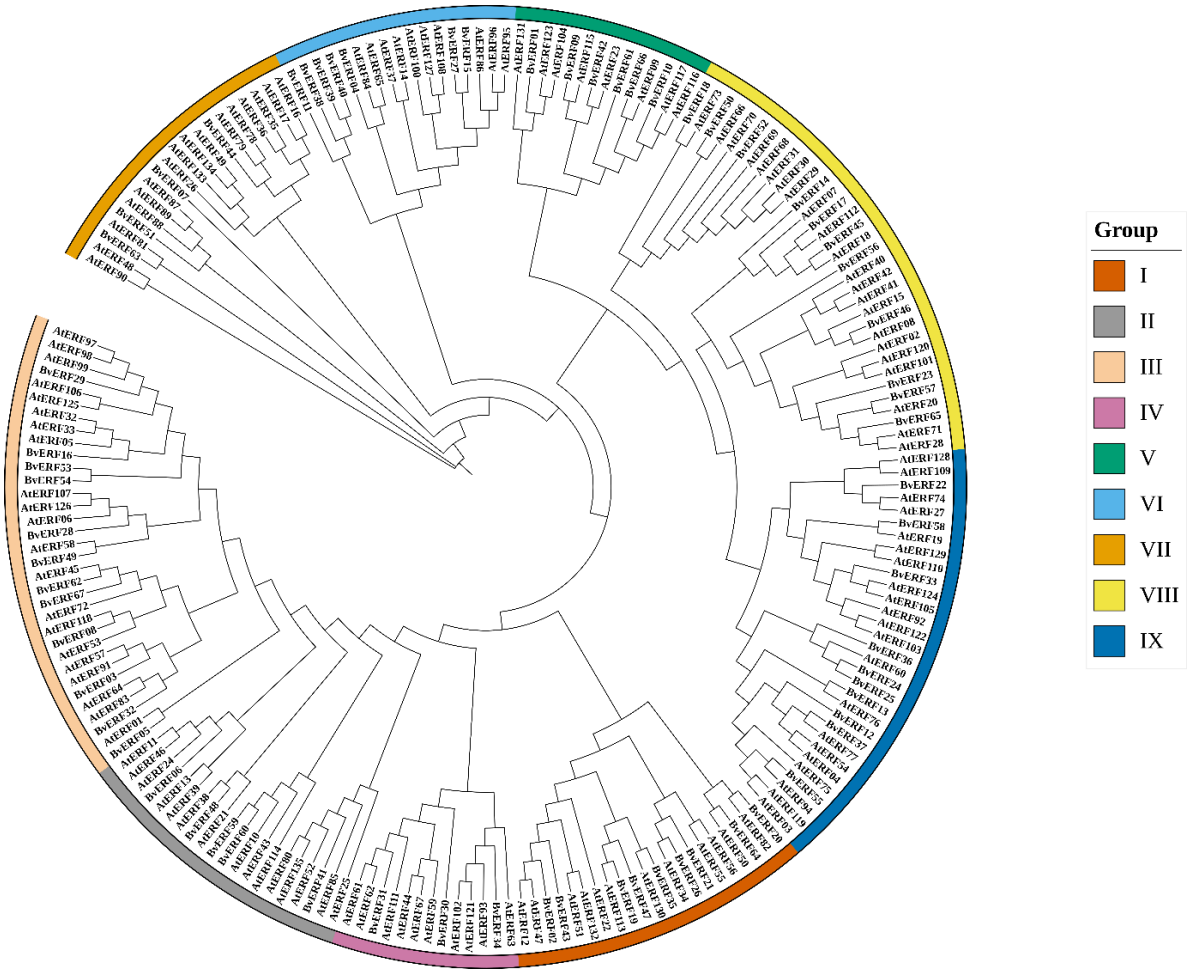

Figure 2. A phylogenetic tree of full-length amino acid sequences of *AtERF* and *BvERF* was constructed by the neighbor-joining (NJ) method by ClustalW in MEGA11 software. Various subfamilies of orthologous genes, as shown in the legend, are differentiated by different colors.

Gene structure analysis of *BvERF* genes

To understand the evolution of the ERF family in *B. vulgaris*, the gene structure and the intron-exon distribution pattern of every ERF gene were identified. There has been evidence of a rather consistent gene organization within the phylogenetic tree, despite the disparity in the sizes of their genomic areas. The genomic area of a gene's longitudinal position and intron-exon distribution pattern serve as supporting evidence for the gene family's expansion trend and evolutionary relationship to its ancestors, as shown in Figure 3. The number of introns in beetroot ERF genes varied, for example, ranging from 1-5. *BvERF*28 and *BvERF*62 have the maximum number of introns (five), while *BvERF*09 has three introns, and only one intron was observed in 16 *BvERF* genes. The remaining *BvERF* genes had no introns. The number of exons ranged from 1-6 in this family, with the maximum number of exons (six) present in *BvERF*28 and *BvERF*62 genes, while the rest of the genes had a single exon.

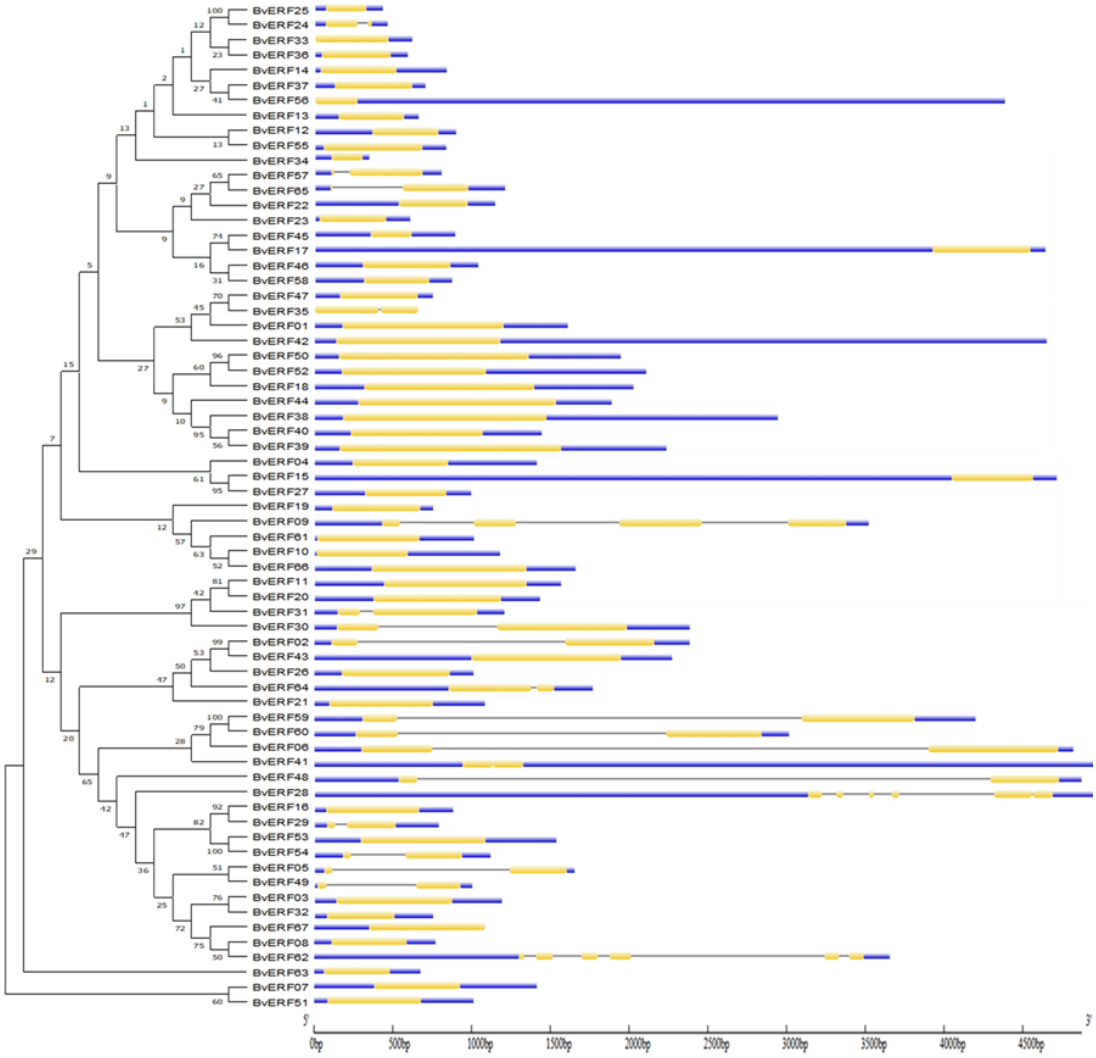

Figure 3. An unrooted neighbor-joining phylogenetic tree was constructed using *BvERF* protein sequences with 1000 bootstrap replicates that illustrate the intron/exon distribution of *BvERF* genes. Yellow, blue, and grey (line) colors illustrate the CDS, upstream/downstream UTRs and intron, respectively.

Conserved protein motifs and domain analyses of *BvERF* genes

Important information on the evolution and functional conservation of the *BvERF* proteins were gleaned from the conservation of conserved protein motifs. Since comparable motifs most likely had similar roles but conserved motifs can support additional classification. This analysis showed that ERF genes contained ten different conserved motifs. Motif 1 was observed in all *BvERF* proteins. *BvERF*07 and *BvERF*51 contained the least number of conserved motifs (only one), that are Motif 1 as shown in Figure 4. Conserved domains (AP2, MFS_STP, and AP2 superfamily) in all ERF genes of *B. vulgaris* are shown in Figure 5. The most conserved domain identified was AP2, while the least conserved domain is MFS_STP, present only in the *BvERF*29 protein. However, the AP2 superfamily domain was present in *BvERF*28, *BvERF*63, and *BvERF*51 proteins only.

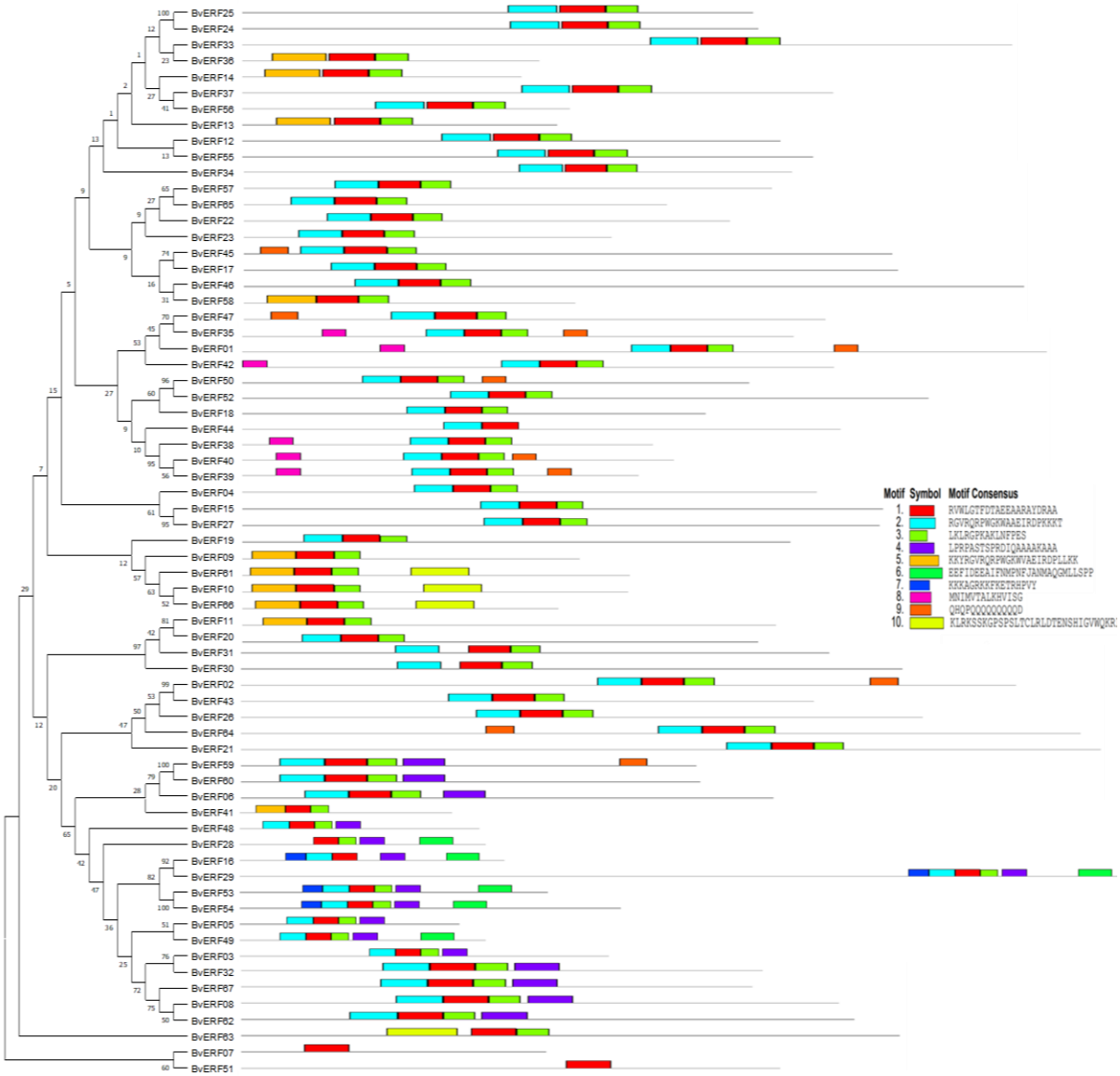

Figure 4. Conserved protein motifs of *BvERF* proteins along the phylogenetic tree. Different colors illustrate the presence of various conserved motif regions. Longer sequences tend to contain more highly conserved motifs compared to shorter ones.

Synteny and gene duplication analysis

Comparative synteny analysis between *B. vulgaris* and *A. thaliana* revealed a high degree of genomic collinearity, indicating strong evolutionary conservation within the *BvERF* genes. In Figure 6, the upper bars represent chromosomes of *B. vulgaris*, while the lower bars correspond to *A. thaliana*. The green connecting curves represented the conserved syntenic blocks and homologous gene pairs. Over 50 ERF homologs were identified between the two genomes, reflecting extensive preservation of genomic regions across both species. This conserved genomic architecture suggested that many ERF genes had retained their ancestral functions, underscoring their potential biological importance. The observed synteny not only highlights shared evolutionary origins but also provides insights into chromosomal rearrangements, gene retention, and the structural stability of ERF loci across divergent plant lineages. Analyses of segmental duplication and phylogenetic classification yield important information on the structural and evolutionary dynamics of the *BvERF* gene family in *B. vulgaris*. As depicted in the Circos plot (Figure 7), 14 gene pairs of 28 *BvERF* genes were identified as duplicated genes. The duplication events occurred on different chromosomes and were consistent with segmental or whole genome duplication; such events would have contributed to the expansion and functional diversification of the *BvERF* gene family. Most duplication events were found with pairs in groups I and VIII (three pairs each), followed by groups V and VII (two pairs each), and single duplication events in groups III, VI, IX, and II.

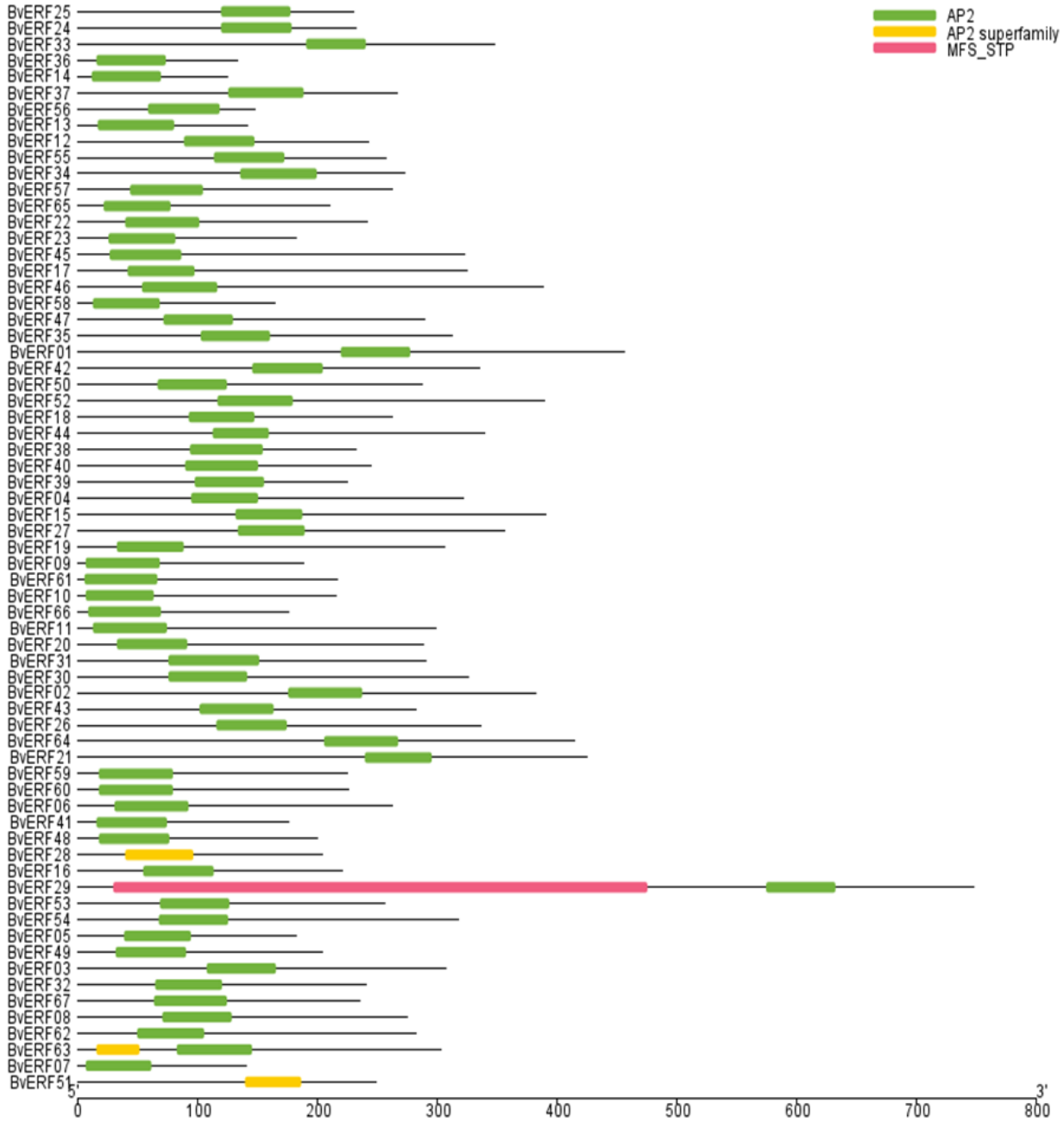

Figure 5. Conserved domain regions of *BvERF* genes are illustrated in green, yellow, and pink colors. Conserved domains represent MFS_STP, AP2, and AP2 superfamily, respectively.

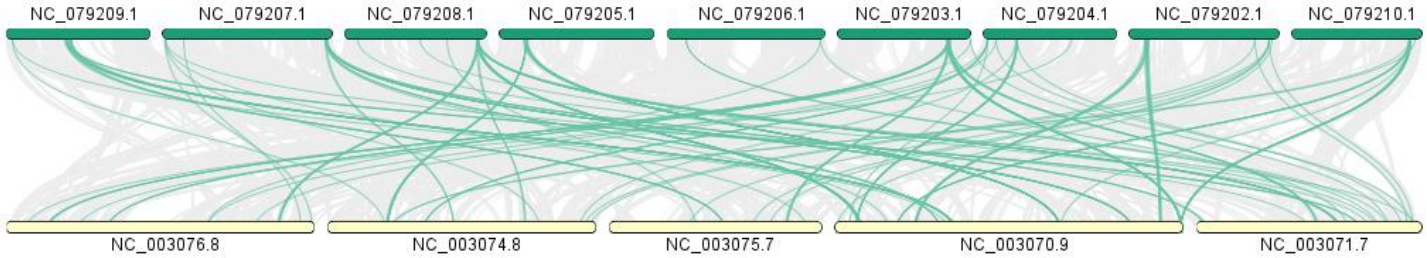

Figure 6. Synteny analysis illustrates that the syntenic sites of the ERF gene family between the *B. vulgaris* and *A. thaliana* genomes were conducted using the dual synteny plot function of TBtools-II software. The green lines connecting the chromosomal bars represent conserved syntenic blocks, illustrating homologous genomic regions shared between the two species.

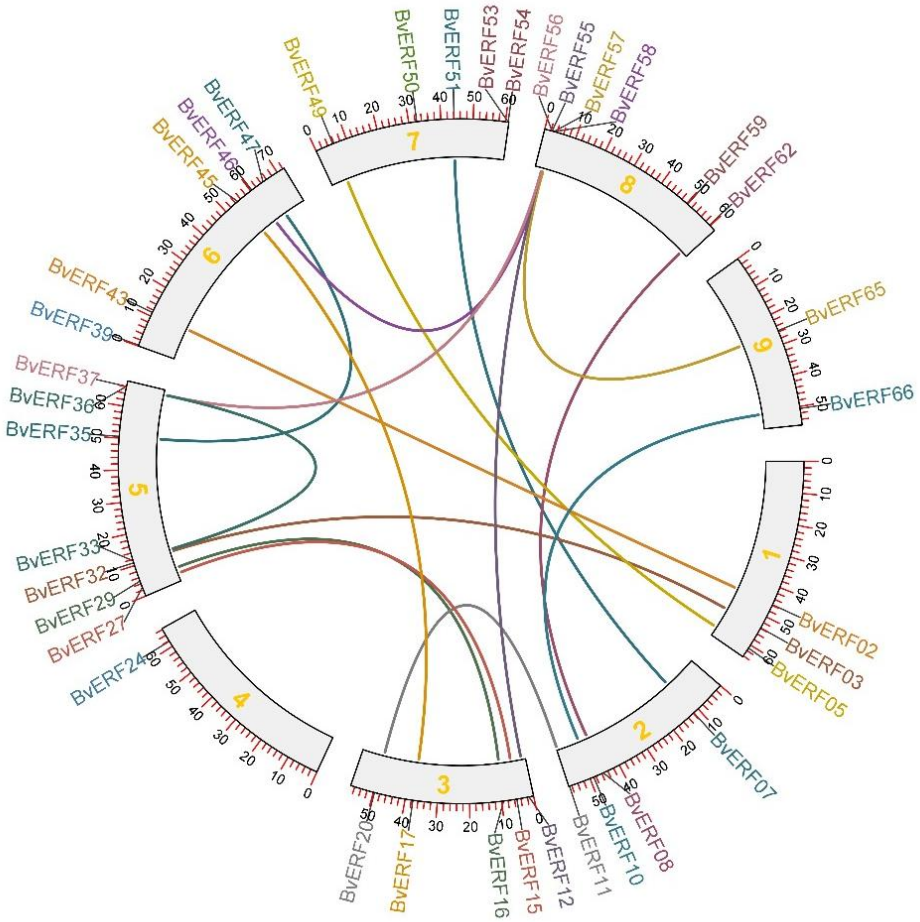

Figure 7. Collinear relationship among *BvERF* genes. Lines of different colors represent the members of various subfamilies as discussed in phylogenetic analysis.

Identification of cis-regulatory elements through promoter analysis

Cis-acting regulatory elements located in promoter regions function as enhancers, inhibitors, or insulators in regulating gene expression. They function as molecular regulators, in many cases, modulating transcription, as well as biological processes such as hormonal signaling, stress response, and development (Nadeem *et al*., 2025). A plethora of cis-acting regulatory elements were found distributed in the promoter regions of *BvERF* genes (Figure 8). Among these elements, the TATA-box, which is a core promoter element necessary for the initiation of transcription, was found across all *BvERF* genes. The CAAT-box element, which is a common enhancer and promoter element, was also abundant, indicating the CAAT-box is likely responsible for mediating basal transcriptional activities, as it was also found across all *BvERF* genes. Among other identified elements, the ARE (anaerobic response element) was present in high numbers specifically in the promoter region of *BvERF*65, indicating its role in mediating response under anaerobic or hypoxia stress. The presence of multiple stress- and hormone-responsive elements such as, ABRE, MBS, LTR, and G-box present in the promoter region, indicates the potential involvement of *BvERF* genes in mediating transcriptional responses to biotic and abiotic stresses in *B. vulgaris*.

Transcriptomic expression profiles of *BvERF* genes under NaCl stress

By identifying and characterizing the *BvERF* genes, we began to identify the molecular mechanisms by which *B. vulgaris* tolerates the stress associated with salt stress, since ERF transcription factors are often associated with abiotic stresses. Salt stress causes ionic imbalance, osmotic stress, and oxidative damage, which can change some physiological processes such as transpiration, stomatal conductance and photosynthesis (Munns and Tester, 2008). We used RNA-seq data to assess the expression pattern of *BvERF* genes expressed in *B. vulgaris* under 300 mM NaCl stress. Under NaCl stress, a substantial number of *BvERF* genes in tissues exhibited significant upregulation.

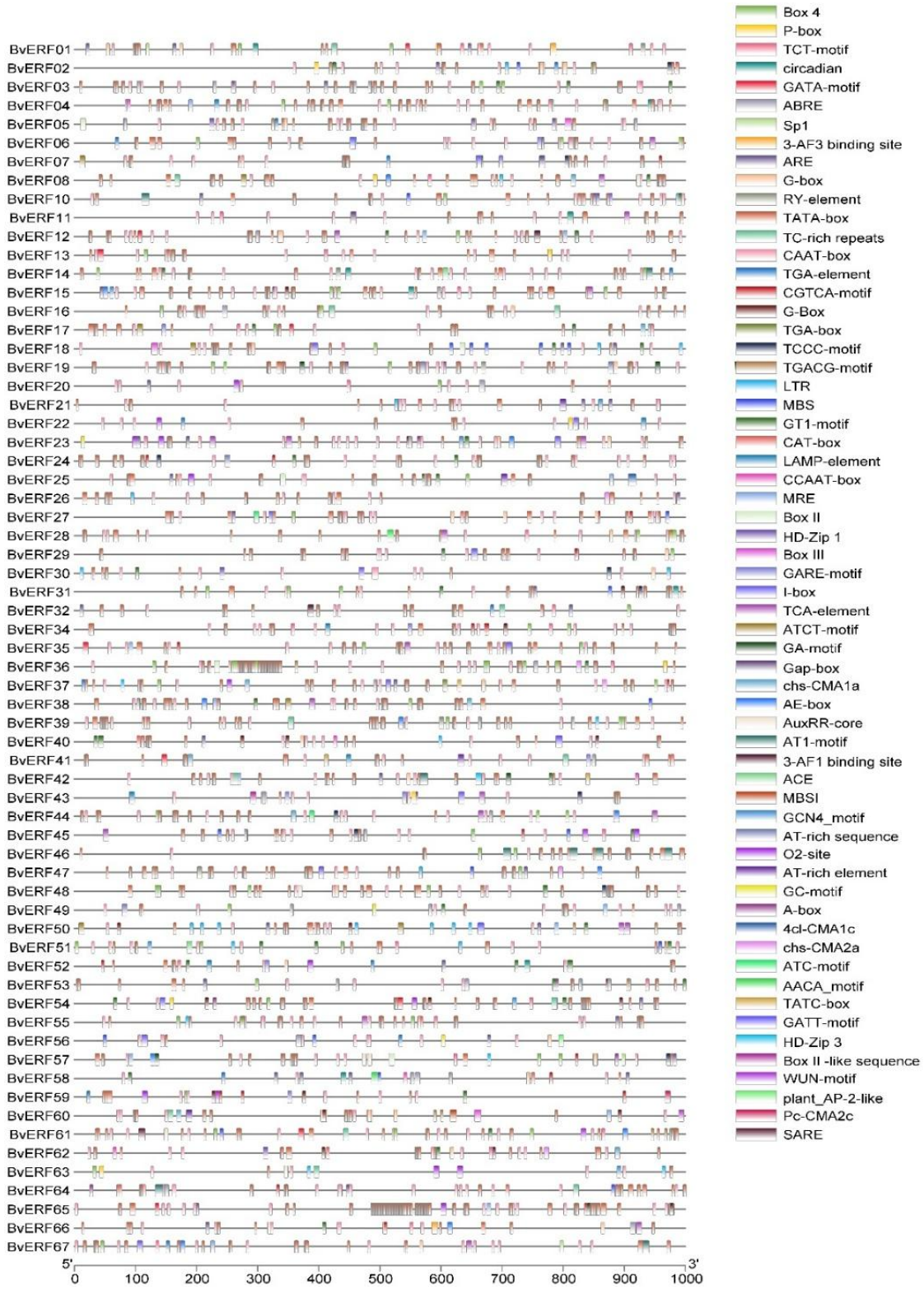

Figure 8. Distribution and visualization of cis-regulatory elements predicted in 1bp upstream regions of *BvERF* genes. Promoter regions are displayed in the figure on the right side with color-coded ligand mappings corresponding to each element type.

In contrast, several *BvERF* genes, including *BvERF*07*, BvERF*20*, BvERF*40*, BvERF*61*,* and *BvERF*66, showed notable downregulation under NaCl stress conditions. The differential expression pattern indicated that *BvERF* genes participate in both activating and suppressing the pathways during salt stress adaptation. These findings suggest that specific members of the *BvERF* genes contributed to salinity tolerance by modulating transcriptional networks involved in stress signaling, ion homeostasis, and osmotic adjustment.

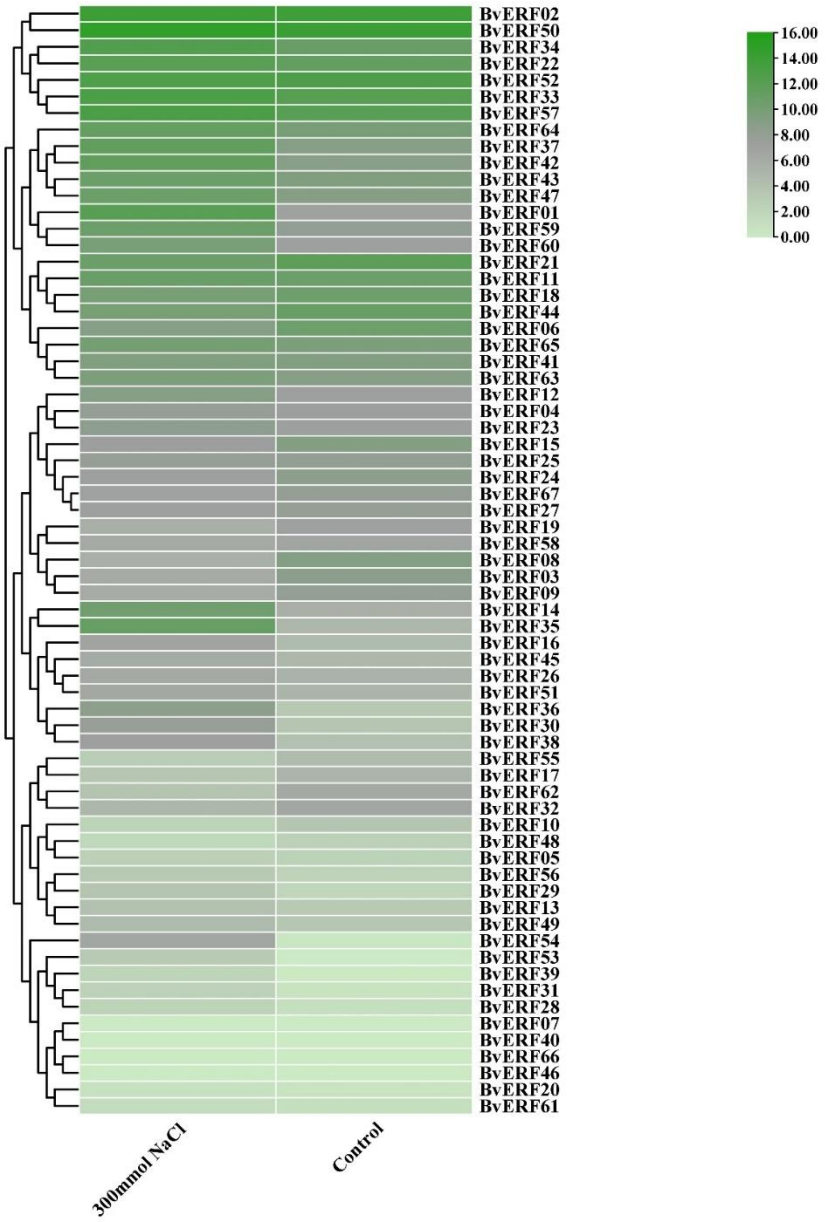

Figure 9. Transcriptomic expression patterns of *BvERF* genes under 300 mM NaCl stress. Rows represent genes, while columns are control or stress experiments, with different colors illustrating the regulation of genes in response to NaCl effect.

DISCUSSION

Plant genomes comprise a large number of ERF transcription factors (Yang *et al*., 2020). The ERF family are important transcription factors for abiotic stress in many plant species. According to Gu *et al*. (2017), this protein family is important for regulating different physiological and biological processes in plants including plant growth, stress responses, hormone signals, and production of metabolites. There are 122 ERF genes in Arabidopsis (Nakano *et al*., 2006), 105 in maize (Cheng *et al*., 2023), 139 in rice (Nakano *et al*., 2006) and 576 in wheat (Zhang *et al*., 2022). Despite its importance, a complete characterization of this family has not been done for beets. In this study, a search of the entire genome of beetroot was performed for ERF family genes and 73 were identified. 67 of the 73 were non-redundant and were found to be unevenly distributed across 9 chromosomes of *B. vulgaris*. It shows that even though *B. vulgaris* has bigger genome size (407 Mb) compared with Arabidopsis (145 Mb), it harbors a smaller number of ERF genes in the genome.

The promoter regions of *BvERFs* were found to appear a variety of cis acting elements associated with stress, phytohormones, growth, and development. Thus, there is a good possibility that *BvERF* genes have roles in regulating various responses to the environment, phytohormones, and development (Onishi *et al*. 2006; Yang *et al*. 2019). For example, Faraji *et al*. (2018), Zhang *et al*. (2020), and Anas *et al*. (2023) indicated that TATA-box, ARE, and CAT-box

are bioactive elements associated with phytohormones, stressors, and growth and development for resilience, respectively. Our overall findings lead us to hypothesize that the *BvERFs* genes most likely represent biotic and abiotic stressor response roles. Synteny analysis allows the identification of chromosomal structural changes, homologous gene evolutionary phylogenetics, and functional relationships among different genomes (Wang *et al*., 2012). In the current study, synteny analysis demonstrated that the syntenic gene pairs in *A. thaliana* and *B. vulgaris* are widely spread and in a one-to-one correspondence, suggesting that the genes are homologous, and the two species may have evolved simultaneously (Figure 6). Additionally, it is possible that members of the ERF family also underwent similar environmental selection during the evolution (Ahmed *et al*., 2021).

The examination of gene architecture is critical for estimating gene functionality (Huang *et al*., 2020). Our study found that 45% of *BvERF* genes exhibited introns. Additionally, some research showed a relationship between the number and spacing of introns and plant evolutionary history (Qiu *et al*., 1998), indicating that intron's may be eliminated as ERF family genes evolved in higher plants. Our study found that 55% of *BvERF* genes are intronless, which is consistent with rice, cucumber, and Arabidopsis (Nakano *et al*., 2006; Hu *et al*., 2011). The proportion of *BvERF* genes that are intronless suggests that multiple *BvERF* genes would respond rapidly to external environmental changes (Huang *et al*., 2020). *BvERFs* had a total of 10 conserved motifs. Variation in the sizes of these found motifs may affect functional diversity. The first motif was highly conserved and was found in all *BvERFs*.

Gene function can be inferred from a gene expression pattern analysis (Zhang *et al*., 2018). Plants go through different life cycles relative to animals, and need to acclimate to various biotic and abiotic complexities (Sakuma *et al*., 2006). Some transcription factors (TFs) were activated in these processes to regulate the target genes, which correlated with the plants developing certain resistance phenotypes. Some TFs, such as AP2/ERF TFs, play a key role in plants' ability to improve resilience to, and protect themselves from, external stimuli (Dietz *et al*., 2010; Mizoi *et al*., 2012; Shu *et al*., 2016; Tang *et al*., 2016). Here we employed transcriptomic expression profiling to investigate the potential role of *BvERF* genes against NaCl salt stress in *B. vulgaris*. We chose 67 *BvERFs* to conduct expression profiling analysis. Several *BvERF* genes showed significant differential expression under NaCl stress that suggest a role in salinity tolerance mechanisms. *BvERF* genes such as *BvERF*02, *BvERF*50, *BvERF*34, *BvERF*22, and *BvERF*52 were strongly upregulated while *BvERF*07, *BvERF*40, *BvERF*66, *BvERF*20, and *BvERF*61 were strongly downregulated compared to controls. These contrasting expression levels suggest *BvERF* genes regulate both activation and repression of transcriptional networks involved with stress signaling, ion homeostasis, and osmotic regulation during salt stress acclimatization.

CONCLUSION

This study represents the first genome-wide identification and comprehensive analysis of the ERF transcription factor gene family in *Beta vulgaris*. In total, we identified 67 non-redundant *BvERF* genes with a wide range of structural characteristics, conserved motifs, and chromosomal locations. Using phylogenetic trees and synteny analysis with *Arabidopsis thaliana*, we found evolutionary conservation and divergence although crops and other species in the Beta lineage did have some of subclades with the same number of genes or synteny patterns similar to *A. thaliana*. In order to better understand the roles of *BvERFs*, we performed a cis-element promoter analysis which uncovered important cis-elements associated with abiotic stress and hormone responses. Additionally, a transcriptome analysis of NaCl stress highlighted several *BvERFs* that were differentially expressed, specifically *BvERF*02, *BvERF*50 and *BvERF*34, suggesting a role for some of these *BvERFs* in tolerance to salinity stress. These findings provide fundamental information about functional and evolutionary aspects of *BvERFs*, which will contribute to future validation and possible use in genetic improvement of salt-stress resilience traits in *Beta vulgaris.*

AUTHOR CONTRIBUTIONS

Muhammad Anas: Writing – original draft, Writing – review & editing, Formal analysis. Muhammad Luqman Aleem: – original draft, Writing – review & editing, Formal analysis. Mahad Ur Rehman: Conceptualization, Validation, Supervision, Resources, Validation. Ayesha Khalid: Validation, Writing – review & editing. Muhammad Qasif Naeem: Writing–review & editing. Muhammad Awais: Writing – review & data analysis. Muhammad Zahid Ramzan: Writing – review & editing.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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