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Genome-wide profiling of bZIP transcription factors in *Camelina sativa*: implications for development and stress response

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Abstract

Background The bZIP transcription factor family, characterized by a bZIP domain, plays vital roles in plant stress responses and development. While this family has been extensively studied in various plant species, its specific functions in *Camelina sativa* (False Flax) remain underexplored.

Methods and results This study identified 71 bZIP transcription factors in *C. sativa*, classified into nine distinct groups based on phylogenetic analysis. Subcellular localization predicted a nucleus-specific expression for these bZIPs. Analysis of GRAVY scores revealed a range from 0.469 to -1.256, indicating a spectrum from hydrophobic to hydrophilic properties. Motif analysis uncovered 10 distinct motifs, with one motif being universally present in all CsbZIPs. Conserved domain analysis highlighted several domains beyond the core bZIP domain. Protein-protein interaction predictions suggested a robust network involving CsbZIPs. Moreover, promoter analysis revealed over 60 types of *cis*-elements, including those responsive to stress. Expression studies through RNA-seq and Real-time RT-qPCR demonstrated high expression of CsbZIPs in roots, leaves, flowers, and stems. Specifically, *CsbZIP01*, *CsbZIP02*, *CsbZIP44*, and *CsbZIP60* were consistently up-regulated under cold, salt, and drought stresses, whereas *CsbZIP34* and *CsbZIP35* were down-regulated.

Conclusion This study presents the first comprehensive genome-wide profiling of bZIP transcription factors in *Camelina sativa*, providing novel insights into their roles in plant development and stress response mechanisms. By identifying and characterizing the bZIP gene family in *C. sativa*, this research offers new opportunities for improving stress tolerance and crop resilience through targeted genetic approaches, addressing key challenges in agriculture under changing environmental conditions.

Keywords Crop resilience, Flax, Stress response, Transcriptomics, Phylogenetic analysis

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Introduction

In Brassicaceae family, *Camelina sativa* is an oil-yielding flowering plant that produces pale yellow cross-shaped flowers. In addition to the common names "Gold of Pleasure," "False Flax," "Wild Flax," and "German Sesame," it is sometimes called "Siberian Oilseed" [1]. Various regions of North America, Europe, and Central Asia cultivate this plant for oilseeds. Its oil is used for cooking, fuel, and burning in lamps and is well known for its high omega-3 fatty acid content (up to 45%), oil content (up to 43%) with antioxidant properties, protein content (up to 32%), erucic acid (up to 3%), and almost 110 mg of Vitamin E per 100 g [2]. It is also known for its unique almond-like aroma and flavor, and the European Union protects it under the trademark name "Olej Rydzowy Tradycyjny". This plant grows on light or medium soils in temperate climate zones and has a short cultivation period [3]. Global climate change is significantly affecting the overall performance of *C. sativa* [4].

Abiotic stress can be caused by several factors including salinity, drought, cold or high temperatures, which are often linked to global climate change [5]. Plants have developed sophisticated regulatory mechanisms to cope with such environmental factors [6]. At the cellular level, transcription factors (TF) are master regulators for these processes. The basic leucine zipper (bZIP) transcription factors are a large family of genes and have been shown to play a critical role in response to various stresses and development of different plant organs [7–10]. The bZIP proteins have a conserved domain consisting of 60 to 80 amino acid residues [11]. It is further divided into an N-terminal leucine zipper domain and a C-terminal alkaline amino acid domain. Almost 16 to 20 basic conserved amino acid residues constitute the N-terminal that can specifically recognize the ACGT motif in promoter region of gene. The C-terminal is relatively variable and contains single or multiple repeat regions (heptapeptide) creating an α helix [12]. The bZIPs play a crucial role in various functions in plants, including the accumulation of anthocyanins in *Malus domestica* [13], and sucrose in *Arabidopsis thaliana* [14]. Moreover, bZIPs are also involved in stress responses including abiotic factors (drought, salt, ROS, temperature) [8, 15–17], biotic factors (like *Sclerotinia sclerotiorum* and *Pseudomonas syringae*) [18, 19] and the regulation of growth and development of flower in *Litsea cubeba* [10], and seed in *A. thaliana* [21]. These are also involved in managing responses to environmental stresses and modulating plant hormone signaling pathways in *Helianthus annuus* and *A. thaliana* [22–24]. Moreover, a potential association was observed between the expression of six bZIP TFs and oil accumulation in *Chlamydomonas reinhardtii* under stress conditions [25]. Overexpression of *NsbZIP21* enhanced biomass and lipid productivity in *Nannochloropsis salina* [26]. In *Arabidopsis thaliana*, *bZIP52* modulates the biosynthesis of seed oil through interaction with other genes like WRINKLED1 [27]. Similarly, *PfbZIP85* in *Perilla frutescens* mediates ω-3 fatty acid-enriched biosynthesis of oil [28]. Additionally, bZIP TFs are mostly studied for their involvement in plant stress response. It was reported that *AtbZIP11* can regulated the expression of IAA3/SHY2, which is a negative regulator of root development and growth [29]. The *VdAtf1* is involved in the regulation of virulence by modulation of nitrogen metabolism in *Verticillium dahlia* [30, 31]. Similarly, bZIP TFs are involved in abiotic stress responses [16, 20, 22, 24, 25, 32]. Altogether, bZIPs are integral to plants' ability to adapt to environmental stresses, regulate growth, and modulate key biosynthetic pathways, highlighting their potential in agricultural and biotechnological applications. Therefore, an understanding of the role of bZIPs in *C. sativa* is expected to guide novel avenues for improvement of this crop.

In this research study, we conducted an in-depth analysis of bZIP transcription factors in *C. sativa* involving CsbZIP classification, Phylogenetics, gene structure analysis, *cis*-elements prediction, and expression analysis using NGS and qPCR.

Materials and methods

Plant materials, treatments, and growth conditions

Plants were grown under controlled environmental conditions for three weeks in a growth room (at Government College University Faisalabad, Pakistan), with a 16-hour light and 8-hour dark cycle at a temperature of 20 °C ± 5 °C and a humidity level of 60% [33]. Seeds (Camelinanaheel variety) were obtained from the local market. To provide preliminary treatment, the seedlings were watered daily with the Hoagland solution, which contains essential nutrients for plant growth and development. After two weeks of growth, one group of plants was treated with a 1 M NaCl solution to induce salt stress, while the other group served as the control and was watered daily with the Hoagland solution. For drought stress, no water was given to a separate group of plants, and for cold stress, the plants were exposed to a temperature near the freezing point (0 °C) in the growth chamber. Following the treatments, the plants were watered every other day with NaCl for the salt stress treatment, while the control group continued to receive the Hoagland solution. One week after the treatments were applied, the samples were collected, quickly frozen with liquid nitrogen, and stored at -80 °C until analysis.

Identification of bZIP genes in *C. Sativa*

Members of the bZIP transcription factor gene family in *A. thaliana* were identified in the TAIR database ("https://www.arabidopsis.org/"), and their protein

sequences were retrieved from the NCBI (https://www.ncbi.nlm.nih.gov/) database using the accession numbers provided in the database. The sequences were annotated using the text editor Notepad++, and homologs in *C. sativa* were identified using NCBI BLASTp (score value≥100, e value≤1e-10) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein family database PFAM was employed to generate a Hidden Markov Model (HMM) profile with the *bZIP* domain in HMMER 3.0 [34]. Later on, PFAM (https://www.ebi.ac.uk/interpro/entry/pfam/PF00170/) and SMART program (http://smart.embl-heidelberg.de/) was used to confirm *bZIP* domains.

Sequence alignment and phylogenetic analysis of CsbZIP gene family

For multiple sequence alignment, protein sequences from *Cameline sativa*, *Arabidopsis thaliana*, *Helianthus annuus*, *Sinapis alba*, and *Lactuca sativa* were uploaded to EMBL Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) following default parameters and Pearson/FASTA as output format. Sequence alignment file was then used to generate phylogenetic tree in IQ-TREE (http://iqtree.cibiv.univie.ac.at/) [35]. The tree was built through 1000 bootstrapping maximum likelihood with default parameters. As a last step, the derived tree was annotated utilizing an online server-based tool called iTOL (https://itol.embl.de/) [36].

Physiochemical properties of *CsbZIP* proteins

The physicochemical properties of the bZIP transcription factor proteins were predicted using an online server-based tool called ProtParam (https://web.expasy.org/protparam/), hosted by the Swiss Institute of Bioinformatics. To predict the subcellular localization of proteins, an online server-based tool called ProtComp 9.0 (hosted by Softberry) was used (http://www.softberry.com/berry.phtml?topic=protcomppl&group=programs&subgroup=proloc).

Gene structure and motif analysis of *CsbZIP* proteins

Gene Structure Display Server (GSDS) Tool v2.0 (https://gsds.gao-lab.org/) [37] was used to predict bZIP transcription factor exon and intron arrangements. To predict the structures of genes, this tool used both their coding and genomic sequences. Moreover, to identify motifs within the bZIP transcription factor family proteins, the Multiple EM for Motif Elicitation (MEME) tool (https://meme-suite.org/meme/) [38] was employed. For MEME, parameters were adjusted such that the size distribution was limited to zero or one occurrence per sequence, and the number of motifs was adjusted to 10, with protein sequence input as the only input [39].

Protein-protein interaction and CsbZIP protein domain analysis

The NCBI conserved domain database also called the web CD-Search Tool (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi), was used to identify protein domains present in the bZIP transcription factors. Using a protein batch as input, this tool returns tabular data describing the domains present in the proteins. These domains were then visualized using TBtools [40], which is an open-source tool hosted on GitHub. This allowed us to easily interpret and compare the different domains present in the bZIP transcription factors, providing insight into their potential functional roles. To analyze the protein-protein interactions among the bZIP transcription factors, the STRINGdb [41] was utilized, which is a web-based tool that automatically generates protein interaction networks based on the input sequences. A bZIP transcription factor protein interaction network was generated by parsing all the protein sequences in the search box and clicking the search button. However, some manual adjustments were made to improve the visual representation of the network, such as adjusting the arrangement of protein nodes. Additionally, the strength of the interactions was visually represented by setting the lines between nodes as either dotted or straight, which represented weak and strong interactions, respectively [42].

Cis-regulatory elements and gene enrichment analysis

Promoter and UTR regions (2000 bp upstream) of every bZIP transcription factor gene were retrieved from NCBI nucleotide database [43]. PLANTCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to predict the *cis*-regulatory elements of these genes [44], and the results were compiled and visualized using the open-source software TBtools. To perform the gene enrichment analysis, we utilized the ShinyGO [45] database, a web-based tool for gene ontology and KEGG pathway enrichment analysis. We obtained a list of CsbZIP gene IDs from the NCBI database and used it as the input for the analysis.

Gene expression analysis using NGS data

Transcriptomic data from the NCBI Sequence Read Archive (SRA) database were used to study gene expression in the family of bZIP transcription factors. We obtained transcriptomic data from the NCBI Sequence Read Archive (SRA) database about root development (SRR935368), leaf development (SRR935362), stem development (SRR935365), and flower development (SRR935369) [46] (accession number SRP136191). A tool from the Galaxy toolkit called BOWTIE2 was used to map all reads to the *C. sativa* genome [47]. We determined the expression levels of the bZIP genes by first

calculating FPKM values using Galaxy's Cufflinks tool. These values were then used by TBtools to generate a heatmap of bZIP gene expression.

Real-time RT-qPCR analysis

RNA was isolated from *C. sativa* leaves [48] using Trizol reagent method and was quantified using Nanodrop 2000 (Thermo Fischer Scientific, Waltham, MA, USA). Subsequently, cDNA was synthesized from 1 µg of RNA using the First-Strand Synthesis kit and stored at -20 °C. Real-time RT-qPCR was performed with iTaq Universal SYBR Green Super-Mix (Bio-Rad Labs, Hercules, CA, USA) and a Real-time RT-qPCR detection system (CFX96 Touch Real-Time PCR Detection System) [49]. Oligo Calculator was used to design gene-specific primers, and Primer-BLAST from the NCBI confirmed their specificity (accessed on 17 July 2023). The *actin-2* gene (*Csa15g026420*) was used as the housekeeping gene, and each sample was tested three times for gene expression in triplicate [50].

Results

Identification and characterization of *bZIP* genes in *C. Sativa*

A total of 71 bZIP genes were identified in *C. sativa*. These bZIPs were named after the genes they were found based on blast results, as the protein homolog was obtained for all the reference bZIP proteins of *A. thaliana*. After analyzing the CsbZIP proteins, it was found that most of these proteins were predicted to be located inside the nucleus of the cell. The pI values of these proteins ranged from 4.5 to 11.41, indicating their acidic to basic nature. The GRAVY scores, which is a measure of the hydrophobicity of the protein, ranged from −0.469 to -1.256. The CsbZIP proteins with the highest GRAVY scores were the ones with the most hydrophobic nature, while the ones with the lowest scores were the most hydrophilic. In terms of GRAVY scores, the CsbZIP protein with the highest value was CsbZIP64 with a score of -1.256, while the protein with the lowest value was CsbZIP28 with a score of -0.469. The amino acid counts of the CsbZIP proteins showed that they were predominantly composed of small, polar, and charged amino acids such as Serine, Threonine, Asparagine, and Glutamine. The percentage of small amino acids in these proteins was higher than that of large amino acids, indicating their compact nature. The CsbZIP proteins with the highest and lowest molecular weight were CsbZIP49 and CsbZIP08, with values of 78912.36 and 16267.56 respectively. The physicochemical properties of CsbZIP proteins are presented in tabular form in Table 1.

Phylogenetic analysis

To explore the evolutionary relationship, bZIP proteins from *A. thaliana*, *H. annuus*, *S. alba*, *L. sativa* and *C. sativa* were used to generate phylogenetic tree (Fig. 1). The CsbZIPs were grouped into nine groups based on the phylogenetic analysis. Group 1 contained three bZIPs from *C. sativa* (CsbZIP02, CsbZIP44, and CsbZIP11). Similarly, 7, 4, 13, 13, 5, 14, 6, 6 CsbZIPs were classified into group 2, 3, 4, 5, 6, 7, 8, and 9, respectively.

A total of 41 orthologs pairs of CsbZIPs and AtbZIPs were identified based on their placement in the phylogenetic tree. In G1, there was only one pair as CsbZIP44/AtbZIP44. In group 7, there were nine orthologous pairs involving bZIP19, 20, 21, 26, 46, 47, 50, 57 and 65. Similarly, variable number of orthologous pairs were identified in G2 (bZIP03, 08, 42, 43,70), G3 (bZIP09, 10, 25, 63), G4 (bZIP13, 27, 39, 63), G5 (bZIP18, 30, 51, 52, 59, 61, 71), G6 (bZIP28, 56), G8 (bZIP16, 41, 54, 55, 68), and G9 (bZIP04, 06, 07, 53). Presence of a high number of orthologous pairs indicates that these proteins are likely to have similar functions in both species. Only two paralogs of CsbZIP were also identified as CsbZIP29/CsbZIP71 and CsbZIP31/CsbZIP33. The phylogenetic analysis provides valuable insights into the evolutionary relationship between bZIPs from different plant species and highlights the relationship between bZIPs from *C. sativa* and other plants. The results could be useful for understanding the functional roles of bZIP proteins and for identifying potential targets for genetic engineering in crop improvement.

Gene structure and motif analysis of CsbZIP proteins

Gene Structure Display Server 2.0 was used to analyze the intron and exon structures of CsbZIP genes to gain insight into their structural features. The analysis revealed that the Intron count in CsbZIP genes varies from 0 to 19. There were genes with only one intron and others with as many as 20 introns (Fig. 2A). *CsbZIP01*, *02*, *03*, *04*, *05*, *06*, *07*, *08*, *11*, *40*, *42*, *43*, *44*, *48*, *53*, *58*, and *70* represent intron-less bZIPs. Using the MEME program, conserved motifs were predicted for CsbZIP proteins to investigate their divergence. A total of 10 motifs were selected, with zero to one occurrence in each sequence. Among CsbZIPs, motif 1 was conserved in all sequences. This 29 residue long sequence (EKRQKRMJSNRESARRSRLRKQAYVEELE) represents conserved region of bZIP domain. Similarly, motif 7 was present in all CsbZIPs except for CsbZIP31, 33, 49, 62, and 72. This 19 residue long motif (EVEKLKZENQELQKQLSEL) also represents part of bZIP domain. In all of the CsbZIP proteins, motif 10 was the least conserved (Fig. 2B). The conserved motifs in CsbZIP proteins suggest their distinct functions in various cellular processes.

Table 1 Physiochemical properties of CsbZIP proteins

Sr. No.	Gene Locus ID in NCBI	Protein Name	Protein ID	No of Amino acids	Molecular weight	Theoretical PI	Gravy	Exon	Chromosome No
1	LOC104723851	CsbZIP01	XP_010440575.1	150	17020.08	9.11	-1.034	1	2
2	LOC104747650	CsbZIP02	XP_010467622.1	170	18832.72	5.32	-0.694	1	15
3	LOC104735607	CsbZIP03	XP_010453732.1	184	21386.43	5.73	-1.133	1	13
4	LOC104787209	CsbZIP04	XP_019101051.1	148	17114.45	5.61	-0.572	1	5
5	LOC104791204	CsbZIP05	XP_010515340.1	183	21317.96	7.74	-0.769	1	6
6	LOC104702820	CsbZIP06	XP_010417044.1	229	26255.59	6.61	-0.707	1	7
7	LOC104716349	CsbZIP07	XP_019086601.1	302	35079.21	8.31	-0.641	1	10
8	LOC104701508	CsbZIP08	XP_010415508.1	138	16267.56	9.76	-0.87	1	7
9	LOC104736502	CsbZIP09	XP_010454805.1	293	32172.5	5.65	-0.691	6	13
10	LOC104737499	CsbZIP10	XP_010456003.1	430	47150.9	5.61	-0.868	7	13
11	LOC104721332	CsbZIP11	XP_010437587.1	154	17446.56	5.38	-0.636	1	11
12	LOC104785286	CsbZIP12	XP_010508770.1	261	29453.58	8.52	-0.751	3	5
13	LOC104725049	CsbZIP13	XP_010441945.1	329	35802.89	5.27	-0.658	5	11
14	LOC104721180	CsbZIP14	XP_010437402.1	322	35816.98	9.75	-0.798	3	11
15	LOC104725184	CsbZIP15	XP_010442101.1	352	39484.85	9.91	-0.76	3	11
16	LOC104786001	CsbZIP16	XP_010509603.1	407	42887.19	5.7	-0.936	13	5
17	LOC104785300	CsbZIP17	XP_010508785.1	727	78778.13	5.56	-0.592	2	5
18	LOC104782545	CsbZIP18	XP_010505811.1	369	40922.01	6.25	-0.922	4	4
19	LOC104721282	CsbZIP19	XP_010437532.1	264	28803.87	5.52	-0.739	4	11
20	LOC104768895	CsbZIP20	XP_010491277.1	330	36626.15	8.63	-0.585	10	20
21	LOC104755012	CsbZIP21	XP_010475624.1	487	54383.21	8.29	-0.584	12	17
22	LOC104740906	CsbZIP22	XP_010459934.1	381	43496.68	5.98	-0.664	9	14
23	LOC104791854	CsbZIP23	XP_010516149.1	256	28045.41	6.08	-0.646	3	1
24	LOC104711744	CsbZIP24	XP_010426784.1	231	26327.68	6.94	-0.744	5	9
25	LOC104791444	CsbZIP25	XP_010515641.1	425	46983.94	6.91	-0.943	5	6
26	LOC104768900	CsbZIP26	XP_010491292.1	330	36928.44	8.58	-0.615	9	20
27	LOC104699826	CsbZIP27	XP_010413527.1	241	26750.69	9.49	-0.759	3	1
28	LOC104769802	CsbZIP28	XP_010492436.1	675	73490.49	5.89	-0.469	2	1
29	LOC104716168	CsbZIP29	XP_010431838.1	565	61734.24	6.72	-0.81	5	10
30	LOC104702667	CsbZIP30	XP_010416864.1	612	67030.14	8.98	-0.592	5	7
31	LOC104710162	CsbZIP31	XP_010425022.1	298	33375.57	6.33	-0.765	4	1
32	LOC104767940	CsbZIP33	XP_010490201.1	276	31167.3	5.89	-0.689	5	19
33	LOC104782746	CsbZIP34	XP_019100580.1	301	33934.23	6.26	-0.931	4	4
34	LOC104758304	CsbZIP35	XP_010479442.1	418	45712.8	8.74	-0.719	5	17
35	LOC104757994	CsbZIP36	XP_010479095.1	428	45747.34	9.36	-0.622	5	17
36	LOC104721407	CsbZIP37	XP_010437681.1	457	50059.72	9.49	-0.556	7	11
37	LOC104765793	CsbZIP38	XP_010507592.1	443	47594.01	9.62	-0.735	5	1
38	LOC104785906	CsbZIP39	XP_010509494.1	445	47289.11	9.18	-0.864	5	5
39	LOC104761492	CsbZIP40	XP_010482885.1	286	32199.99	5.75	-0.88	1	3
40	LOC104721103	CsbZIP41	XP_010437315.1	315	34102.54	6.27	-1.126	12	11
41	LOC104790606	CsbZIP42	XP_010514686.1	172	20005.13	5.73	-0.992	1	6
42	LOC104773617	CsbZIP43	XP_010496567.1	162	18961.13	6.38	-0.894	1	6
43	LOC104702281	CsbZIP44	XP_010416418.1	181	19902.06	6.42	-0.604	2	7
44	LOC104764990	CsbZIP45	XP_010486922.1	356	39782.69	7.87	-0.63	11	19
45	LOC104750682	CsbZIP46	XP_010470818.1	450	50553.64	5.77	-0.513	11	16
46	LOC104762691	CsbZIP47	XP_010484330.1	369	41999.58	6.93	-0.484	9	18
47	LOC104790163	CsbZIP48	XP_010514166.1	166	19469.64	6.33	-0.989	1	6
48	LOC104782582	CsbZIP49	XP_010505855.1	729	78912.36	5.61	-0.567	2	4
49	LOC104702575	CsbZIP50	XP_010416756.1	377	42701.98	6.17	-0.535	9	7
50	LOC104757924	CsbZIP51	XP_019095363.1	338	37203.27	6.69	-0.885	5	17
51	LOC104739247	CsbZIP52	XP_010457837.1	337	37197.17	5.27	-0.838	4	14

Table 1 (continued)

Sr. No.	Gene Locus ID in NCBI	Protein Name	Protein ID	No of Amino acids	Molecular weight	Theoretical PI	Gravy	Exon	Chromosome No
52	LOC104788562	CsbZIP53	XP_010512632.1	145	16637.8	5.65	-0.901	1	5
53	LOC104707997	CsbZIP54	XP_010422771.1	366	39335.48	7.83	-0.881	19	8
54	LOC104784689	CsbZIP55	XP_010508044.1	383	41151.74	9.27	-0.968	12	5
55	LOC104769347	CsbZIP56	XP_010491847.1	169	18517.31	9.47	-1.205	4	20
56	LOC104769200	CsbZIP57	XP_010491653.1	364	41731.24	6.91	-0.554	11	20
57	LOC104772504	CsbZIP58	XP_010495411.1	196	22855.98	5.55	-1.109	1	3
58	LOC104749718	CsbZIP59	XP_010469701.1	391	43789.46	5.89	-0.974	6	16
59	LOC104779156	CsbZIP60	XP_010501843.1	366	41305.41	4.73	-0.54	3	3
60	LOC104792108	CsbZIP61	XP_010516459.1	329	36806.15	6.23	-1.029	4	6
61	LOC104756295	CsbZIP62	XP_010477168.1	468	52312.31	5.47	-0.918	6	17
62	LOC104771303	CsbZIP63	XP_010494111.1	326	35354.39	9.02	-0.633	6	20
63	LOC104746104	CsbZIP64	XP_010465808.1	154	17396.16	8.73	-1.256	3	15
64	LOC104768888	CsbZIP65	XP_010491265.1	465	51542.03	6.72	-0.521	11	20
65	LOC104781579	CsbZIP66	XP_010504594.1	301	32786.87	9.25	-0.774	3	4
66	LOC104710907	CsbZIP67	XP_010425862.1	340	38773.48	7.14	-0.84	5	9
67	LOC104757586	CsbZIP68	XP_010478633.1	390	40768.74	6.27	-0.838	13	17
68	LOC104739155	CsbZIP69	XP_010457722.1	424	46920.82	5.84	-0.908	5	14
69	LOC104738273	CsbZIP70	XP_010456776.1	189	22047.74	8.82	-0.866	1	2
70	LOC104716168	CsbZIP71	XP_010431838.1	565	61734.24	6.72	-0.81	5	10
71	LOC104736828	CsbZIP72	XP_010455186.1	150	17276.01	9.61	-0.706	4	13

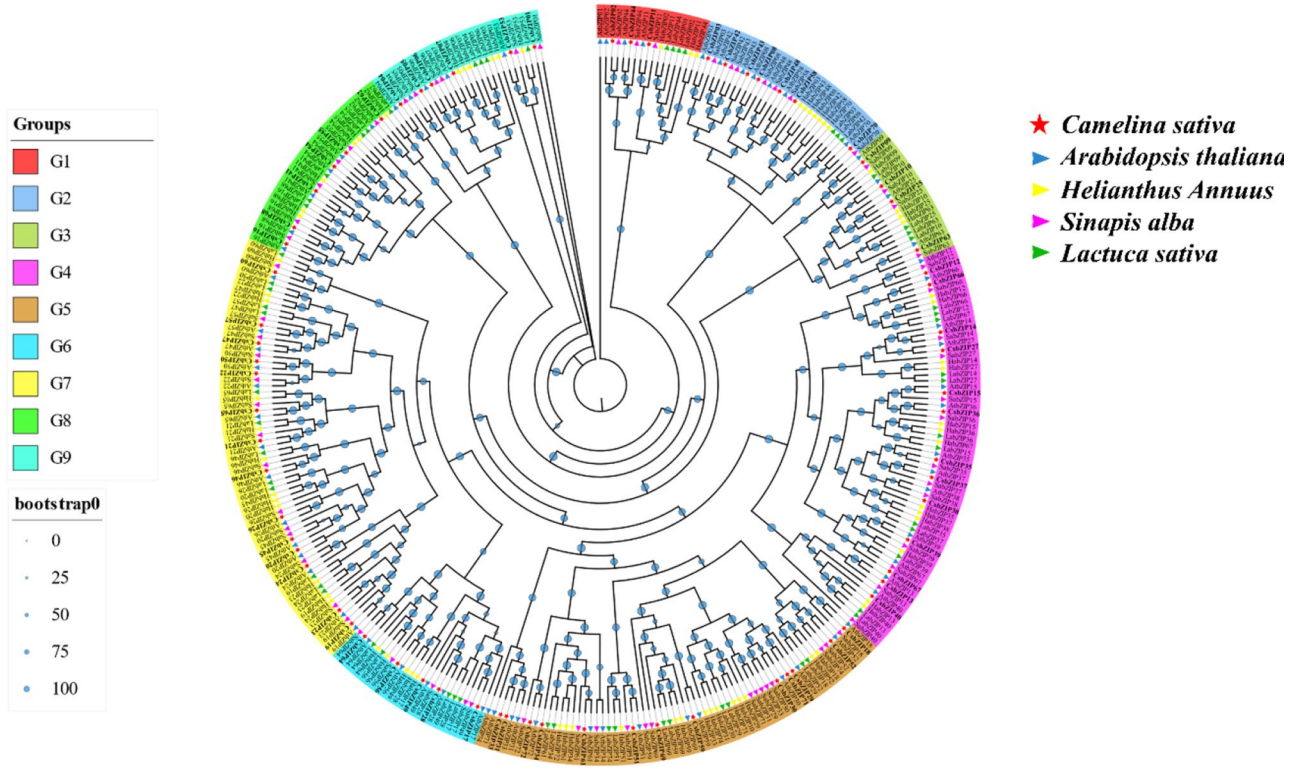

Fig. 1 Phylogenetic analysis of bZIP proteins from various plant species, including *C. sativa*, *A. thaliana*, *H. annuus*, *S. alba*, *and L. sativa*. Clustering (Group 1 to Group 9) identifies related CsbZIPs. Orthologs and paralogs indicate functional similarities and gene duplications. Valuable insights for crop improvement

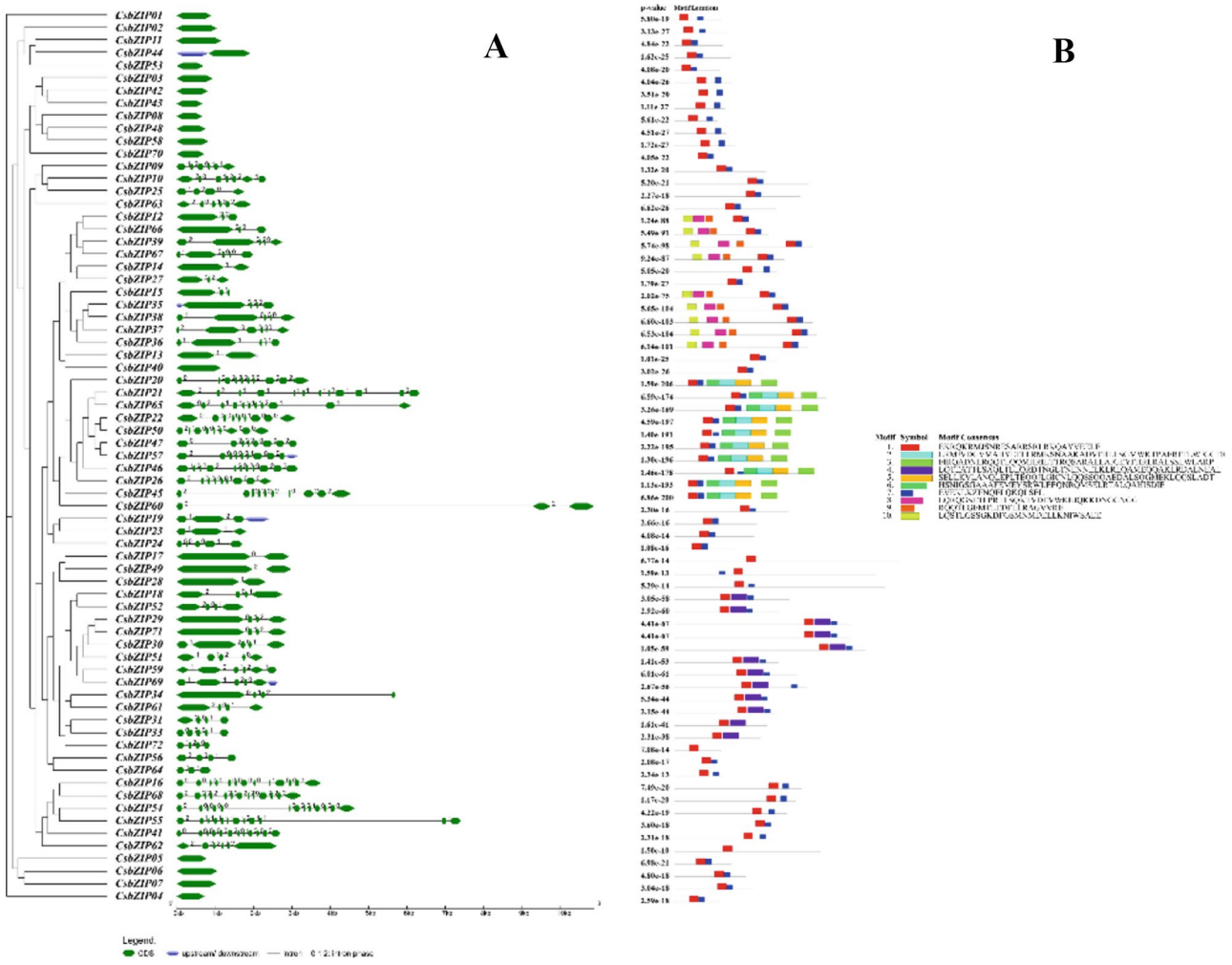

Fig. 2 Gene structure and conserved motif analysis. **A**) Variation in intron-exon structures of CsbZIP genes. **B**) Motif analysis predicted conserved motifs in CsbZIP proteins

Protein-protein interaction and CsbZIP protein domain analysis

Domain analysis of CsbZIP genes revealed that multiple domains were present in most of these proteins, but the bZIP and related domains were present in almost all of the proteins, these domains include bZIP domain, bZIP_1, bZIP_C, bZIP_plant_RF2, bZIP_HBP1b-like, bZIP_plant_GBF1, bZIP_plant_BZIP46, bZIP_HY5-like, bZIP_C superfamily and bZIP superfamily domains (Fig. 3A). Several CsbZIP proteins were found to have domains in addition to the ones discussed earlier, these domains include MFMR domain, BRLZ domain, DOG1 domain, and ZapB domain, indicating the multifunctionality of CsbZIPs and their potential involvement in more than one function within the cell.

The protein-protein interaction analysis of CsbZIP proteins showed the interaction of these proteins among each other in the cell, all the proteins of this family showed very strong interactions among one another indicating the potential complementary action of these proteins for one another (Fig. 3B). Some proteins like CsbZIP17, CsbZIP49, CsbZIP33, CsbZIP72, and CsbZIP04 did not show any interaction indicating their potential involvement in processes other than the ones other proteins are involved in and their potential function as the activator for other genes of the family.

Cis-regulatory elements and gene enrichment analysis

In the context of regulating gene expression and determining an organism's development and functioning, *cis*-regulatory elements such as enhancers and promoters play a crucial role. Mutations in these regulatory elements have been found in various species, leading to changes in phenotypic expression. For the current research, the promoter sequences of 2000 base pairs (bp) of 71 bZIP genes in *C. sativa* were analyzed, revealing the presence of sixty-one different types of *cis*-regulatory elements. These regulatory elements were found to be

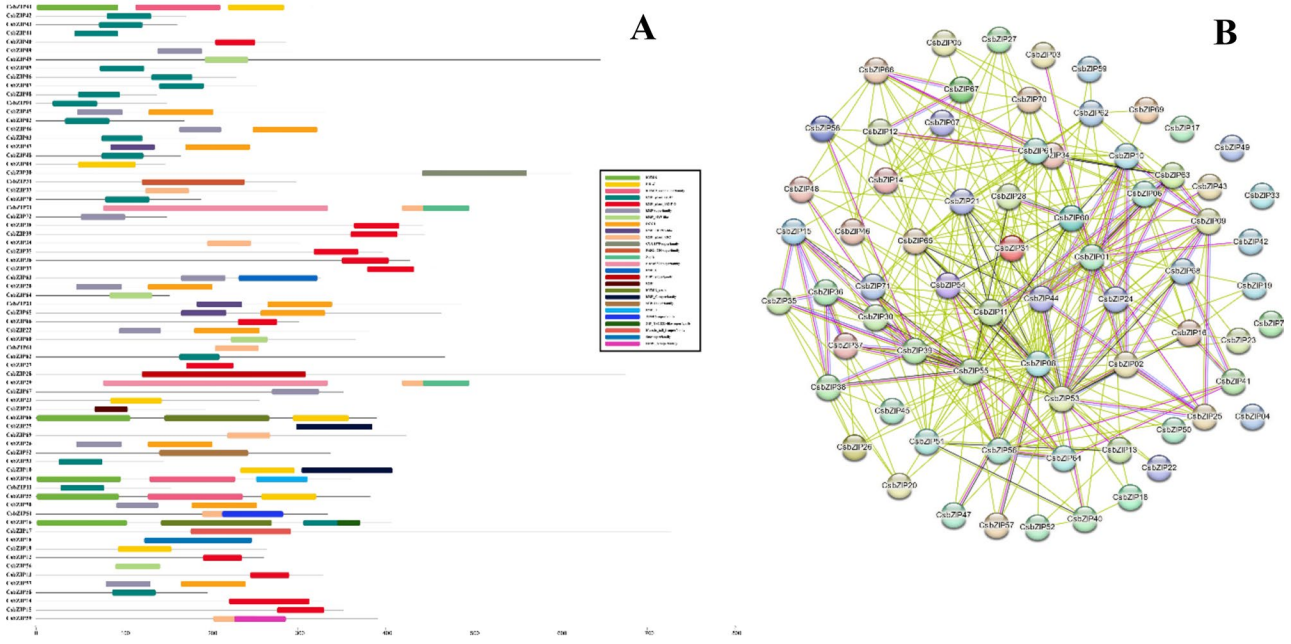

Fig. 3 The bZIP domains and protein-protein interaction. **A**) Domain analysis of CsbZIP genes in *C. sativa* showed the presence of bZIP and related domains in almost all proteins, along with additional domains (MFMR, BRLZ, DOG1, ZapB) indicating multifunctionality. **B**) Protein-protein interaction analysis of CsbZIP proteins revealed strong interactions among all family members

enriched in the promoter sequences, with the most prevalent element being the TATA-box, which helps to position RNA polymerase and initiate transcription. Other commonly found regulatory elements include ARE, ABRE, Box-4, G-box, TGACG-motif, CGTCA-motif, TCT-motif, TG1-motif, LTR, MBS, and MRE. Additionally, a few regulatory elements, such as O2-site, CAT-box, AT-rich element, I-box, ACE, and A-box, were present in fewer numbers. In contrast, elements like CAG-motif, AuxRE, L-box, LS7, and HD-Zip 1 were rare (Fig. 4A).

Gene enrichment analysis revealed that the CsbZIP genes are involved in various processes in plants, including seed dormancy control and signaling pathways. These genes function as activators for other genes and more (Fig. 4B). The figure shows the number of genes from this gene family in *C. sativa* involved in particular functions. The length of each bar indicates the number of genes, while the color of the bars represents the log fold change.

Gene expression analysis using transcriptomic data

Transcriptomic data were analyzed to understand the potential functional importance of CsbZIPs. In tissue-specific expression profiling, it was observed that several CsbZIP genes were highly expressed in *C. sativa* root tissues (including *CsbZIP01*, *CsbZIP02*, *CsbZIP47*, *CsbZIP51*, and *CsbZIP58*) as compared to flower, leaf or stem. Interestingly, most of these CsbZIPs were not highly expressed in other tissues (except for *CsbZIP02*, which is also expressed in stem). It indicated that these bZIPs play an important role in roots (Fig. 5A). Similarly, CsbZIPs were also expressed in other parts of plant including *CsbZIP44* in flowers, *CsbZIP35/CsbZIP61* in leaves, and *CsbZIP 37* in stem. It was noticeable that *CsbZIP02* was highly expressed in all samples (Fig. 5A).

Transcriptomic data was also analyzed to study response of CsbZIPs against cold, drought and NaCl (salt) stress (Fig. 5B). A diverse response was observed where CsbZIPs expression changes was observed in distinct groups. In response to cold stress, *CsbZIP02* and *CsbZIP44* were significantly upregulated, while *CsbZIP08*, *CsbZIP60 and CsbZIP61* were downregulated. In response to drought stress, *CsbZIP01*, *CsbZIP02*, and *CsbZIP37* were upregulated, while *CsbZIP08*, *CsbZIP60*, and *CsbZIP61* were downregulated. In response to salt (NaCl) stress, *CsbZIP02*, *CsbZIP37*, and *CsbZIP60* were upregulated, while *CsbZIP35* and *CsbZIP60* were downregulated (Fig. 5B).

qRT-PCR analysis

Real time RT-qPCR was used to determine the transcript abundance of nine CsbZIP genes from *C. sativa* leaves. The genes analyzed were *CsbZIP01*, *CsbZIP02*, *CsbZIP34*, *CsbZIP35*, *CsbZIP37*, *CsbZIP44*, *CsbZIP51*, *CsbZIP60*, and *CsbZIP61*. Under cold, drought, and salt stresses, all CsbZIP genes showed differential regulation. Specifically, *CsbZIP01*, *CsbZIP02*, *CsbZIP44*, and *CsbZIP60* were upregulated in response to all three stresses (Fig. 6). Conversely, *CsbZIP34* and *CsbZIP35* were downregulated

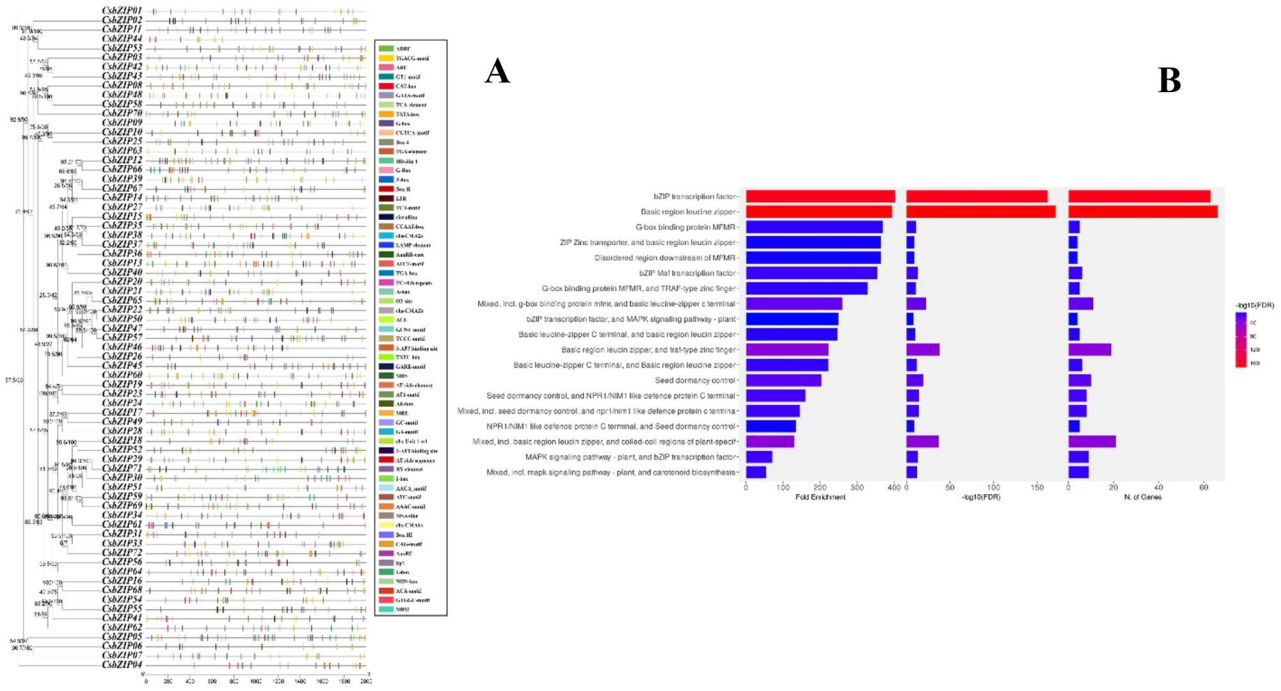

Fig. 4 Promoter analysis and enrichment analysis. **A**) The arrangement of CsbZIP genes' cis-acting regulatory elements. **B**) Gene enrichment analysis of CsbZIP genes in *C. sativa*, indicating their involvement in diverse plant processes. Each bar represents a specific function, with bar length reflecting the number of associated genes, and color indicating the log fold change of gene expression

under all stress conditions. A very significant upregulation of CsbZIP37 was observed during drought stress, but not much of a change was observed during cold and salt stress. The expression of CsbZIP61 did not change significantly in response to the given stresses, while the expression of CsbZIP51 increased during drought and decreased during cold and salt stress (Fig. 6).

Discussion

Understanding the genetic basis of stress responses and developmental processes in plants is essential for improving crop resilience and productivity [51]. In current study, we focused on *Camelina sativa*, an important oil-yielding plant known for its high nutritional value and unique aroma. By investigating the bZIP transcription factor family, we aimed to shed light on the molecular mechanisms underlying stress tolerance and organ development in this species. Current findings provide valuable insights that can inform future research and breeding programs to enhance the resilience and productivity of *C. sativa* under changing environmental conditions [52, 53]. Previously, bZIP gene family has been studied in a diverse range plant species including *vitis vinifera* [54], *Zea mays* [55], Legumes [56], *Manihot esculenta* [57], *Ginkgo biloba* [58], and many others [20, 31, 59–64]. Therefore, understanding the role of bZIPs in C. sativa is particularly important as it contributes to the broader knowledge of plant stress responses and development.

The phylogenetic analysis of bZIP proteins from *C. sativa*, *A. thaliana*, *H. annuus*, *S. alba*, and *L. sativa* provided valuable insights into the evolutionary relationships between these genes. The classification of bZIPs was consistent with previous studies [46–48, 50]. The grouping of CsbZIPs revealed distinct clades, indicating functional divergence within the bZIP gene family [65]. Some CsbZIPs showed orthology with specific bZIPs from *A. thaliana*, suggesting functional conservation between these genes in different species [66]. Additionally, paralogs resulting from gene duplication events were also identified, implying potential functional redundancy or specialization within the bZIP family [67]. The physiochemical properties analysis of CsbZIP proteins revealed their diverse nature. These proteins were predicted to be predominantly located in the nucleus, indicating their potential role as transcription factors [68]. The variation in pI values indicated a range of acidic to basic properties among CsbZIPs, which could influence their interactions with other biomolecules. The GRAVY scores reflected the hydrophobicity of the proteins, suggesting their possible roles in membrane-associated processes [58]. The high proportion of small, polar, and charged amino acids in CsbZIP proteins indicated their compact nature, potentially influencing their protein-protein interactions and overall structure [62].

The gene structure analysis revealed variations in the intron-exon organization among CsbZIP genes,

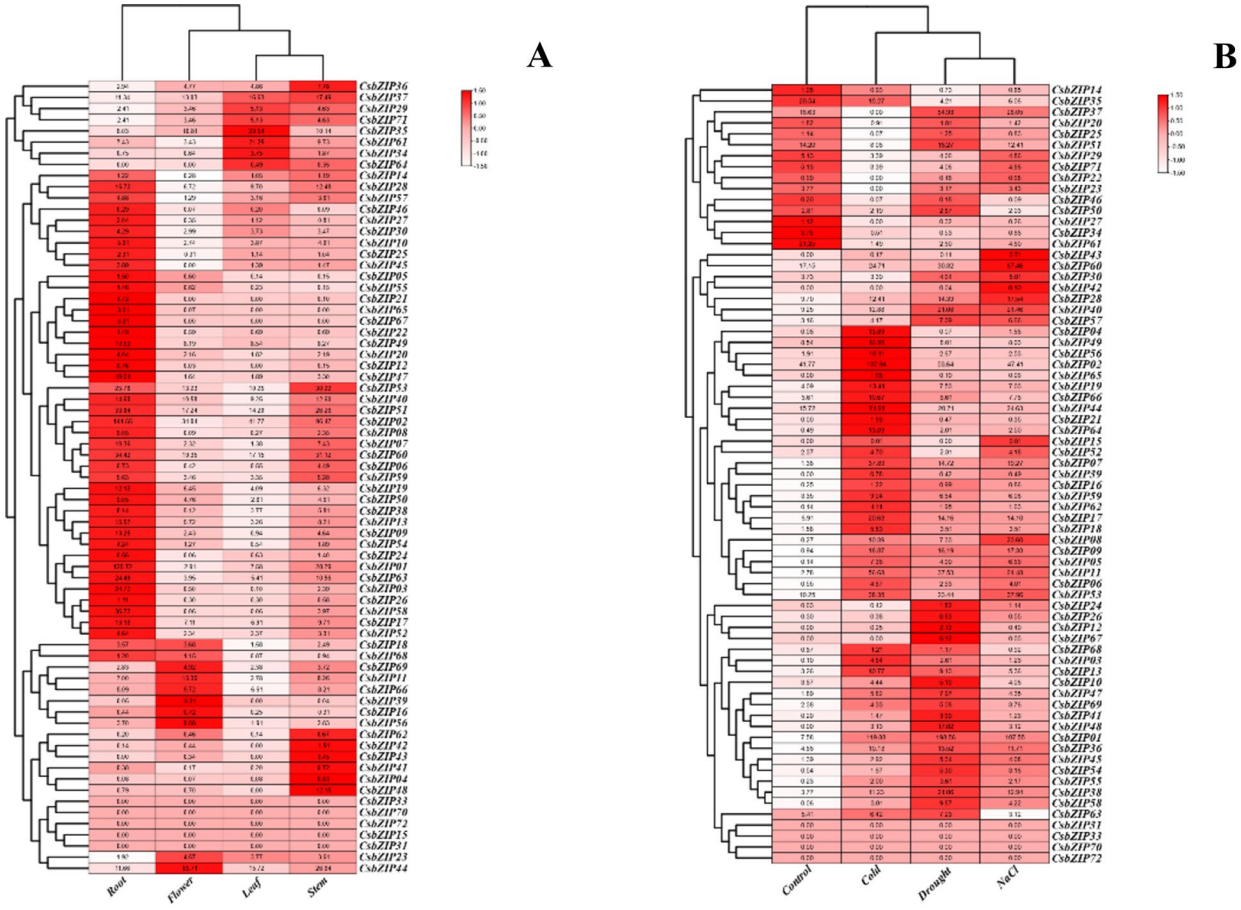

Fig. 5 Transcriptomic analysis. **A**) The expression patterns of CsbZIP genes across different tissues of C. sativa. **B**) CsbZIP gene expression under cold, drought, and salt stresses in *C. sativa* is visualized in a TBtools-generated heatmap. Rows represent genes, columns show control or stress experiments, and colors indicate changes in expression levels

indicating their diverse genomic organization [61]. Motif analysis identified conserved motifs, with some motifs present in all CsbZIPs, suggesting functional importance. Other motifs were specific to certain CsbZIP proteins, hinting at potential functional divergence within the gene family [58]. The domain analysis revealed the presence of various bZIP-related domains in CsbZIP proteins, suggesting their involvement in transcriptional regulation. Additionally, the presence of other domains in some CsbZIPs indicated their potential multifunctionality, possibly participating in different cellular processes [11]. The *cis*-regulatory element analysis of CsbZIP gene promoters identified multiple regulatory motifs associated with transcriptional regulation. The presence of diverse cis-regulatory elements suggested that CsbZIP genes may be involved in the control of various biological processes, including seed dormancy, signaling pathways, and other cellular functions.

Gene expression analysis using RNA-seq data provided insights into the tissue-specific and stress-responsive expression patterns of CsbZIP genes [69]. The higher expression of certain CsbZIP genes in roots indicated their potential role in root development. It was discovered that certain CsbZIP genes respond differentially to abiotic stresses, suggesting that they play an important role in stress responses and adaptation to stress [70]. In addition, qRT-PCR analysis revealed distinct responses to cold, drought, and salt stress in *C. sativa* leaves. It was observed that under different stress conditions, these CsbZIPs were regulated differently. As a result of all three stressors, *CsbZIP01*, *CsbZIP02*, *CsbZIP44*, and *CsbZIP60* were significantly upregulated. Conversely, *CsbZIP34* and *CsbZIP35* were downregulated in all stress conditions. Notably, *CsbZIP37* exhibited a unique pattern, being upregulated during drought stress but under cold and salt stresses showed very little upregulation and downregulation as compared to the control group, and *CsbZIP61* showed the slightest change in expression in response to these stresses. Additionally, *CsbZIP51* showed upregulation during drought stress but downregulation under cold and salt stresses.

Differential expression patterns of these CsbZIPs suggest they play specific roles in stress-responsive pathways, contributing to the plant's adaptive mechanisms

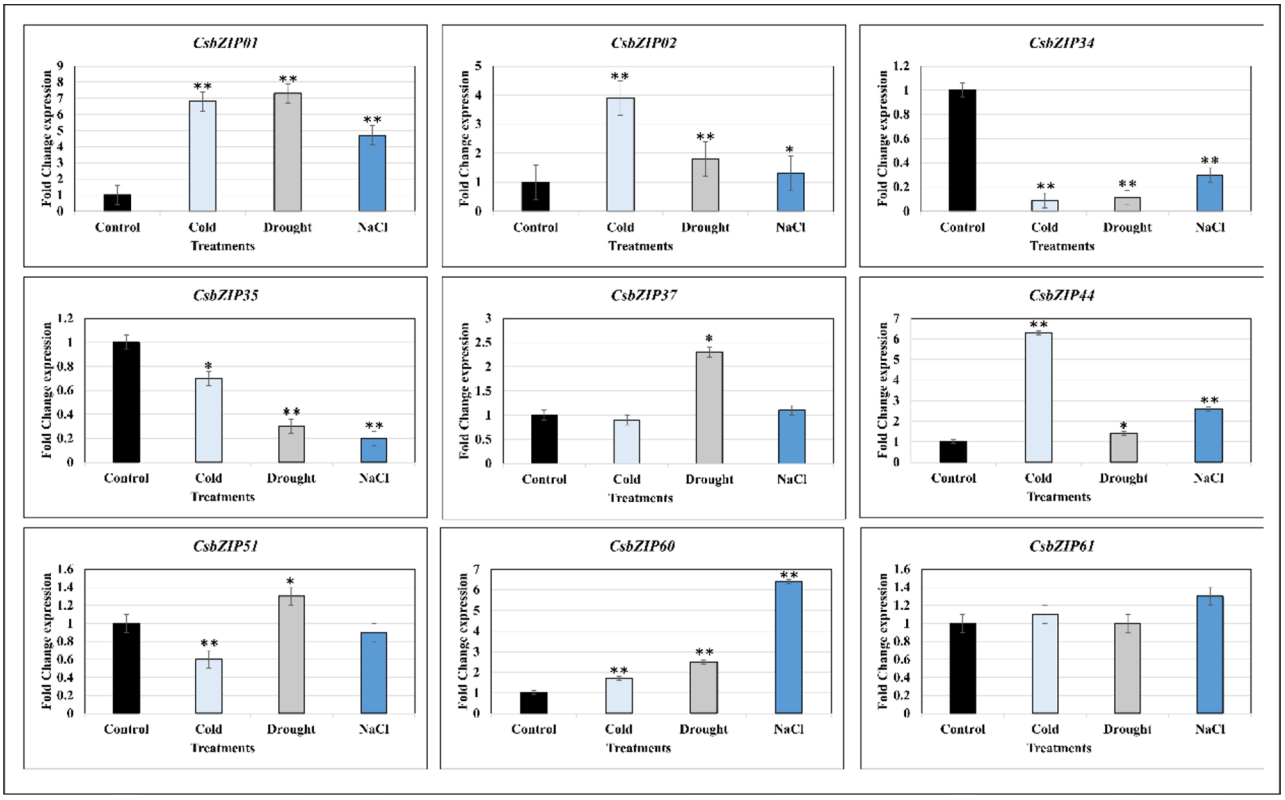

Fig. 6 Real-time RT-qPCR analysis of bZIPs in *C. sativa* leaves under cold, drought, and salt stresses. Each bar represents the mean ± SE from three replicates. (*) and (**) on error bars indicate significant differences ($P < 0.0001$) between control and stressed samples

[8]. Identifying and characterizing these CsbZIP genes will help us to understand the molecular mechanisms that cause *C. sativa* to tolerate cold, drought, and salinity [22, 23]. By enhancing our understanding of how these bZIP transcription factors function, we can improve the resilience and yield of crops under adverse conditions [71]. Overall, this study highlights the diversity and functional complexity of the bZIP gene family in *C. sativa*. The identification and characterization of CsbZIP genes, along with their role in development of *C. sativa* and response to various stresses in the plant.

Conclusion

This study identified and characterized the bZIP transcription factor family in *C. sativa* and analyzed their expression patterns under different stress conditions and during development. The results showed that some genes were significantly upregulated in response to specific stresses, while others were downregulated or showed no alteration in expression. The study also demonstrated that the bZIP transcription factors play a crucial role in regulating gene expression, response to abiotic stresses, and regulation of plant growth and development in *C. sativa*. The findings of this research provide valuable insights into the expression pattern of bZIP genes in stress tolerance and plant growth in this important crop species, which can be used to develop strategies for improving crop yields and ensuring food security. Additionally, this study serves as a foundation for future research on the bZIP transcription factor family in *C. sativa* and other plant species.

Abbreviations

C. sativa	Camelina sativa
bZIP	Basic Leucine Zipper
NGS	Next-Generation Sequencing
qPCR	Quantitative Polymerase Chain Reaction
TF	Transcription Factor
A. thaliana	Arabidopsis thaliana
H. annuus –	Helianthus annuus
S. alba	Sinapis alba
L. sativa	Lactuca sativa
pI	Isoelectric Point
GRAVY	Grand Average of Hydropathy
RNA-seq	RNA Sequencing
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
SRA	Sequence Read Archive

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Author contributions

SR and ARI devised the main idea and supervised the research. SR, ARI, and MUR performed analysis. MAA, FA, and SR wrote the manuscript. SR, ARI, MUR, and FA re-analyzed, the literature review and editing of the article. SF, KAA, LH, and FA provided technical expertise to improve the revised article. FA, SR, SF, MDFA and ARI helped during revision, editing and proof-reading of the

revised article. All authors made substantial efforts to improve the article and agreed to submit it.

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Data availability
Data will be made available on reasonable request with the corresponding author.

Declarations

Ethics approval and consent to participate
The experiments did not involve endangered or protected species. No specific permits were required for these locations/activities moreover, all methods were carried out by relevant guidelines and regulations, under ethical approval and consent to participate.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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