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Genome-Wide Identification and Characterization of Q-Type C2H2 Zinc Finger Proteins in Rapeseed (*Brassica napus* L.) and Their Expression Patterns Across Tissues and Under Abiotic Stress

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Abstract

Q-type C2H2 zinc finger protein (ZFP) transcription factors, a plant-specific subfamily of C2H2 ZFP, have been implicated in regulating abiotic stress responses, growth, and developmental processes in plants. Rapeseed (*Brassica napus* L.) is a crucial oil crop widely used for the production of high-quality vegetable oil, animal feed, and biodiesel. Compared with studies on *Q-type C2H2-ZFP* genes in other plant species, systematic research has not been performed in *B. napus*. In this study, a comprehensive genome-wide analysis of *Q-type C2H2-ZFPs* in *B. napus* was conducted. A total of 216 *Q-type C2H2-ZFP* genes were identified, exhibiting extensive and uneven distribution across the 19 chromosomes. Phylogenetic analysis, based on homologs from *Arabidopsis*, classified these genes into eight distinct subfamilies, with each containing one to three conserved "QALGGH" motifs. Each subfamily exhibited similar motif compositions and gene structures. Evolutionary studies revealed that segmental duplication events played a crucial role in the expansion of the *BnaQ-type C2H2-ZFP* gene family. Expression pattern analysis in different tissues and under abiotic stress identified *BnaA03g09250D*, *BnaC09g35160D*, *BnaC03g11570D*, and *BnaA10g25850D* as candidate genes involved in the response to freezing stress. Overexpression of *BnaC09g35160D* provided preliminary evidence that it enhances freezing tolerance in plants. This comprehensive study of Q-type C2H2-ZFPs in *B. napus* will enhance our understanding of the *BnaQ-type C2H2-ZFP* gene family and provide valuable insights for further functional investigations of *BnaC09g35160D*.

Keywords: *Brassica napus* L.; Q-type C2H2 zinc finger proteins; expression pattern; abiotic stress

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1. Introduction

Brassica napus L. (2n = 4x = 38, A_nC_n), an allotetraploid of the *Brassica* genus, was formed by the natural hybridization of two progenitor species, *Brassica rapa* L. (2n = 20, A_r) and *Brassica oleracea* L. (2n = 18, C_o) [1,2]. *B. napus*, as a crucial oil crop worldwide, is grown for the production of high-quality vegetable oil which has low levels of saturated fatty acids and high microelement content, and also as a feed crop for animal feeding stuffs and a source of biodiesel. However, its yield is seriously limited by multiple abiotic stresses, including drought, salinity, cold, heavy metal stress, fungal pathogen stress, and so on. In Northwest China, winter rapeseed has already become an essential oilseed crop

and winter cover crop, but cold stress seriously restricts its production. In our previous transcriptome analysis of *B. napus*, the *ZAT10* and *ZAT12* genes, which belong to the Q-type cysteine-2/histidine-2 zinc finger protein (C2H2-ZFP) subfamily, were highly expressed in cold-tolerance strain 2016TS(G)10 under continuous freezing stress [3], so these genes made us interested in the Q-type C2H2 zinc finger protein (ZFP) subfamily transcription factors.

C2H2-ZFPs represent the largest and most extensively studied class among all ZFP types [4,5]. These proteins feature a conserved structural motif comprising two cysteine (Cys) and two histidine (His) residues that coordinate a zinc ion, thereby stabilizing the characteristic three-dimensional structure. This characteristic zinc finger domain typically folds into a two-stranded antiparallel β-sheet and an α-helix [6–8]. Previous studies have shown that plant C2H2 ZFPs typically contain one to five zinc finger domains. Among these, Q-type C2H2-ZFPs are characterized by a conserved domain with the consensus sequence "$CX_{2–4}CX_3FX_3QALGGHX_{3–5}H$", which has been identified as the plant-specific subfamily of C2H2 ZFPs. This approximately 25-amino-acid motif includes two invariant cysteine (Cys) residues and two histidine (His) residues, which are essential for zinc binding and structural stability [8].

Several Q-type C2H2-ZFP transcription factors have been implicated in mediating abiotic stress responses across multiple plant species [9–14]. In *AtZAT12*-overexpressing transgenic plants, tolerance to high light stress, osmotic stress, and oxidative stress was enhanced; in contrast, knockout of *AtZAT12* increased the sensitivity to osmotic and salt stresses [15]. In addition, *AtZAT10* has been shown to play a dual role in plant abiotic stress resistance. Both gain-of-function and loss-of-function mutants exhibited enhanced tolerance to drought, osmotic, and salt stresses [16]. The *AtZAT6*-activated CBF pathway is essential for melatonin-mediated freezing stress response [10–12]. Transgenic plants overexpressing *ZAT7* exhibited inhibited growth but enhanced tolerance to salinity stress [17]. Overexpression of *ZAT11* enhances primary root elongation but reduces the tolerance of transgenic plants to Ni^{2+} [18]. Overexpression of *ZAT18* improved seed germination following mannitol treatment and enhanced drought tolerance under water-deficit conditions [19]. As mentioned above, *Q-type C2H2-ZFPs* are involved in plant stress responses. *B. napus LATE FLOWERING* (*BnLATE*), which acts as a negative regulator of lignin biosynthesis in yellowing silique walls and reduces silique shattering in *B. napus* by inhibiting the polymerization of monolignols and lignin [20]. *BnaA04G.ZAT4* showed a negative correlation with seed oil content and a positive correlation with seed coat content [21]. Knockout of the *JAG* gene (*BnJAG.A02*, *BnJAG.C02*, *BnJAG.C06*, *BnJAG.A07*, and *BnJAG.A08*) drastically affected the development of lateral organs involved in determining pod shape and size. These mutants were unable to produce viable seeds for subsequent generations [22]. Knockout of the *Bna.TAC1* genes (*BnaA5.TAC1* and *BnaC4.TAC1*) produced two mutants with varying degrees of compact plant architecture, characterized by significantly reduced branch angles, without affecting plant height or yield-related traits [23]. In conclusion, they play crucial roles in the plant's response to multiple abiotic stresses and development.

Compared with studies on *Q-type C2H2-ZFP* genes in other plant species, systematic research has not yet been conducted in *B. napus*. As an important oil crop, *B. napus* therefore warrants a genome-wide identification and expression analysis of the *Q-type C2H2-ZFP* gene family. The resequencing of the *B. napus* genome has provided valuable resources for the systematic identification of the *BnaQ-type C2H2-ZFP* gene family and its potential functions. In this study, we conducted a genome-wide analysis to identify *Q-type C2H2-ZFP* genes using bioinformatics methods. We analyzed the physiochemical properties, chromosomal localization, gene structure, conserved motif compositions, gene duplication events, and genomic collinearity. The expression patterns of *BnaQ-type C2H2-ZFPs* in different tissues and under abiotic stresses were also investigated. Additionally, we

examined and compared the expression patterns of ten genes significantly induced under low temperature in different cold-tolerant winter-type *B. napus* varieties. Furthermore, we preliminarily validated that the gene *BnaC09g35160D* enhances cold resistance in plants. This study provides the first systematic analysis of the *BnaQ-type C2H2-ZFP* gene family, laying a foundation for further functional studies on stress tolerance regulatory mechanisms in *B. napus* and offering valuable insights.

2. Materials and Methods

2.1. Sequence Retrieval and Q-Type C2H2-ZFPs Identification in B. napus

The genomic library, cDNA library, and protein database of *B. napus* were obtained from the BnIR Database (https://yanglab.hzau.edu.cn/BnIR, accessed on 20 July 2025). Initially, we identified C2H2 gene family members in *B. napus* using two complementary approaches. The 176 previously characterized C2H2 ZFPs from *Arabidopsis* [5] were retrieved from the TAIR database (http://www.arabidopsis.org/, accessed on 12 June 2025) and used as query sequences for BLASTp searches against the complete *B. napus* genome annotation data available in the BnIR Database. Subsequently, a hidden Markov model (HMM) profile of the C2H2-ZFPs was constructed based on the 176 known *Arabidopsis* C2H2-ZFP sequences using HMMER software (http://hmmer.org/download.html, Version 3.1, accessed on 12 June 2025), which was then employed to perform BLASTp searches against the entire *B. napus* genome available in the BnIR Database. The identified members were filtered for both cases to remove duplicates, then submitted to the SMART database (http://smart.embl-heidelberg.de/, accessed on 13 June 2025) to verify the presence of the C2H2-ZFP domain in these sequences. Sequences lacking the complete conserved domain were subsequently excluded. Q-type C2H2-ZFPs were defined by the motif "X_2-C-X_2-C-X_3-F-X_3-QALGGH-X_3-H" [6,24]. The *B. napus* Q-type C2H2-ZFP members containing the "QALGGH" domains were selected for further analysis. The molecular weight and isoelectric point (pI) of the deduced polypeptides were calculated using the ExPasy ProtParam tool (http://web.expasy.org/protparam/, accessed on 14 June 2025) [25]. The subcellular localization of each Q-type C2H2-ZFP was predicted using CELLO online software (http://cello.life.nctu.edu.tw/, Version 2.5, accessed on 14 June 2025) [26].

2.2. Conserved Motifs and Gene Structural Analysis

The Multiple Expectation Maximization for Motif Elicitation suite (MEME, https://meme-suite.org/meme/tools/meme, Version 5.5.8, accessed on 15 June 2025) [27] was used to predict conserved motifs in *B. napus* Q-type C2H2-ZFPs. The parameters were set as follows: the maximum number of motifs was set to 10, and the optimal motif width ranged from 6 to 50 residues (inclusive). To graphically display the exon–intron structure of all *B. napus Q-type C2H2-ZFP* genes, the Gene Structure Display Server (https://gsds.gao-lab.org/Gsds_help.php, Version 2.0, accessed on 17 June 2025) [28] was utilized for generating structural diagrams.

2.3. Chromosomal Localization and Gene Duplication

Each *B. napus Q-type C2H2-ZFP* gene was mapped to its corresponding chromosome based on chromosomal location information retrieved from the BnIR Database, and the chromosomal localization map was constructed using MapChart software (Version 2.32) [29].

The Multiple Collinearity Scan toolkit (MCScanX) was employed to analyze gene duplication events using default parameters [30]. Tandem duplicated genes were highlighted with red boxes in the chromosomal localization map, while segmentally duplicated genes were visualized with a Circos plot, generated using Circos Version 0.69 [31]. To further assess the duplication events of *BnaQ-type C2H2-ZFP* genes, the Ka (synonymous) and Ks

(non-synonymous) of each duplicated *Q-type C2H2-ZFP* gene pair were calculated using the Ka/Ks Calculator tool Version 2.0 [32] to better analyze gene duplicates.

2.4. Phylogenetic Relationship Analysis

The full-length amino acid sequences of *Arabidopsis* and *B. napus* Q-type C2H2-ZFPs were obtained from the TAIR database and the BnIR Database. Multiple sequence alignment of the Q-type C2H2-ZFPs domains in *Arabidopsis* and *B. napus* was performed using ClustalX. Unrooted maximum likelihood phylogenetic trees were constructed using MEGA software Version 7.0 with a bootstrap test with 1000 iterations [33,34].

2.5. Expression Profile Analysis of BnaQ-Type C2H2-ZFP Genes in Different Tissues and Under Abiotic Stress Conditions

To investigate the transcriptional patterns of *BnaQ-type C2H2-ZFP* genes across various tissues, including 4 mm bud length, filament, petal, pollen, sepal, cotyledon, vegetative rosette, leaf (at the 1st, 8th, and 20th leaf positions), root, 30-day post-flowering seed, 30-day post-flowering silique, and stem peel (lower, middle, and upper), and the expression profiles of *BnaQ-type C2H2-ZFP* genes under abiotic stress conditions (salt, drought, freezing, cold, heat, and osmotic stress) in *B. napus* cultivar "ZS11", the FPKM values were obtained from BnIR (https://yanglab.hzau.edu.cn/BnIR, accessed on 25 June 2025) [35]. In the expression analysis of *BnaQ-type C2H2-ZFPs* in different tissues, the thresholds for "no expression" and "expression" were defined as FPKM < 1 and FPKM $\geq$ 1. In the expression analysis of *BnaQ-type C2H2-ZFPs* under different abiotic stresses, the thresholds for upregulation and downregulation were defined as $\log_2$FC > 1 and $\log_2$FC < 1, respectively. The heatmap representing the gene expression profiles was generated using the TBtools software (Version 2.225) [36].

2.6. RNA Extraction, Reverse Transcription, and qPCR Analysis of Freezing-Stress-Responsive Genes in Different Cold-Tolerant Winter B. napus

Winter-type *B. napus* varieties "Longyou 88" and "Tianyou2288" were selected as plant materials. "Longyou 88" is a cold-tolerant *B. napus* cultivar bred by Gansu Agricultural University (Gansu, China), and "Tianyou2288" is a cold-sensitive cultivar bred by Tianshui Institute of Agricultural Sciences (Gansu, China). Seedlings were grown under controlled environmental conditions at a day/night temperature regime of 22 °C/18 °C, with a 16 h photoperiod and a light intensity of 6000 Lx. After 50 days (approximately at the five-true-leaf stage), the seedlings were transferred to a pre-cooling chamber maintained at −4 °C (16 h photoperiod, light intensity of 6000 Lx) for freezing treatment. To minimize potential variations induced by circadian rhythms, freezing treatments were initiated at 8:00 AM under light conditions and maintained continuously for 24 h.

The samples, corresponding to the second leaf position from the inner part of the plant, were collected at 0 h (non-frozen), 1 h, 24 h, and 48 h, respectively, and immediately frozen in liquid nitrogen for RNA extraction. All timepoint measurements were conducted with three biological replicates.

The total RNA was extracted using the Plant RNA Extraction Kit (Tiangen DP419, Beijing, China). Agarose gel electrophoresis was conducted to assess RNA integrity, and RNA concentration was measured using a Nanodrop ND-2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Reverse transcription of RNA into cDNA was performed using the Tiangen KR116 FastKing RT Kit containing gDNase (Beijing, China). qPCR primers were designed using Primer Premier 6.0 software. qPCR was performed using the Takara SYBR Premix Ex Taq.II kit (Beijing, China) and run on the Bio-Rad CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). The assay was conducted independently in triplicate. Data for each sample were normalized to the reference gene *SAND*

analyzed using the $2^{-\Delta\Delta CT}$ method [37]. Data were analyzed by one-way ANOVA in SPSS software R27.0.1.0 (IBM, Armonk, NY, USA).

2.7. Data Processing and Statistical Analysis

Data processing and statistical analysis were conducted using Microsoft Excel (Microsoft, Redmond, WA, USA) and SPSS software (IBM, Armonk, NY, USA), respectively, while graphical representations were generated using Origin software (OriginLab, Northampton, MA, USA).

3. Results

3.1. Identification and Characterization of Q-Type C2H2-ZFP in B. napus

To comprehensively identify all Q-type C2H2-ZFP members in *B. napus*, three approaches were employed to characterize the *C2H2-ZFP* gene family. First, the 176 previously identified C2H2-ZFPs from *Arabidopsis* were used as query sequences to search the *B. napus* genomic database, resulting in the identification of 1044 candidate protein sequences. Second, an HMM profile was constructed based on the 176 AtC2H2-ZFP sequences and utilized to screen for proteins containing the C2H2-ZFP domain, yielding a total of 383 candidate members. Third, redundant sequences were removed, and the remaining candidates were submitted to the SMART database to verify the presence of conserved C2H2-ZFP domains, ultimately confirming 548 C2H2-ZFP members.

Based on variations in the "QALGGH" motif and the spatial distances between metal ligands, C2H2-ZFP domains were categorized into four types: M-type, Q-type, Z-type, and D-type [6,24]. The Q-type C2H2-ZFP domain was specifically characterized by the consensus sequence "X_2-C-X_2-C-X_3-F-X_3-QALGGH-X_3-H". A total of 216 genes encoding Q-type C2H2-ZFP were identified in *B. napus* and designated as *BnaQ-type C2H2-ZFP*.

The lengths of the 216 BnaQ-type C2H2-ZFP sequences ranged from 135 amino acids for both BnaA06g36670D and BnaC07g17180D to 948 amino acids for BnaC04g45560D, with molecular weights varying between 14.98 kDa and 106.74 kDa. Theoretical isoelectric point (pI) exhibited a wide range, from 4.42 (BnaA09g03910D) to 10.03 (BnaCnng48130D and BnaA03g17210D). Subcellular localization prediction using CELLO indicated that nearly all BnaQ-type C2H2-ZFP members were localized exclusively in the nucleus. However, seven proteins were predicted to have non-nuclear localization: one in the cytoplasm (BnaA06g19880D), three in the extracellular space (BnaC03g11570D, BnaA10g12780D, and BnaC09g35160D), and three in the mitochondria (BnaA03g09250D, BnaA02g06780D, and BnaA02g06790D). Detailed information including gene ID, chromosomal distribution, amino acid length, molecular weight, theoretical pI, GRAVY value (grand average of hydropathicity), exon count, and predicted subcellular localization is provided in Table S1.

3.2. Chromosomal Localization and Gene Structure Analysis of BnaQ-Type C2H2-ZFP Genes in B. napus

The 216 *BnaQ-type C2H2-ZFP* genes were nearly equally distributed between the An subgenome (95 genes) and the Cn subgenome (93 genes), but showed extensive and uneven distribution across the 19 chromosomes (Chr) of *B. napus* (Figure 1). The number of *BnaQ-type C2H2-ZFP* genes per Chr. ranged from four (ChrA01 and ChrC08) to 20 (ChrC03). A high gene density was observed on ChrA03 (18 genes), ChrA07 (15 genes), ChrC03 (20 genes), ChrC04 (16 genes), and ChrC07 (13 genes). Notably, *BnaQ-type C2H2-ZFP* genes on ChrA09 (10 genes), ChrA10 (10 genes), ChrC02 (11 genes), ChrC03 (20 genes), and ChrC09 (nine genes) were predominantly localized at the proximal or distal ends of the respective Chrs.

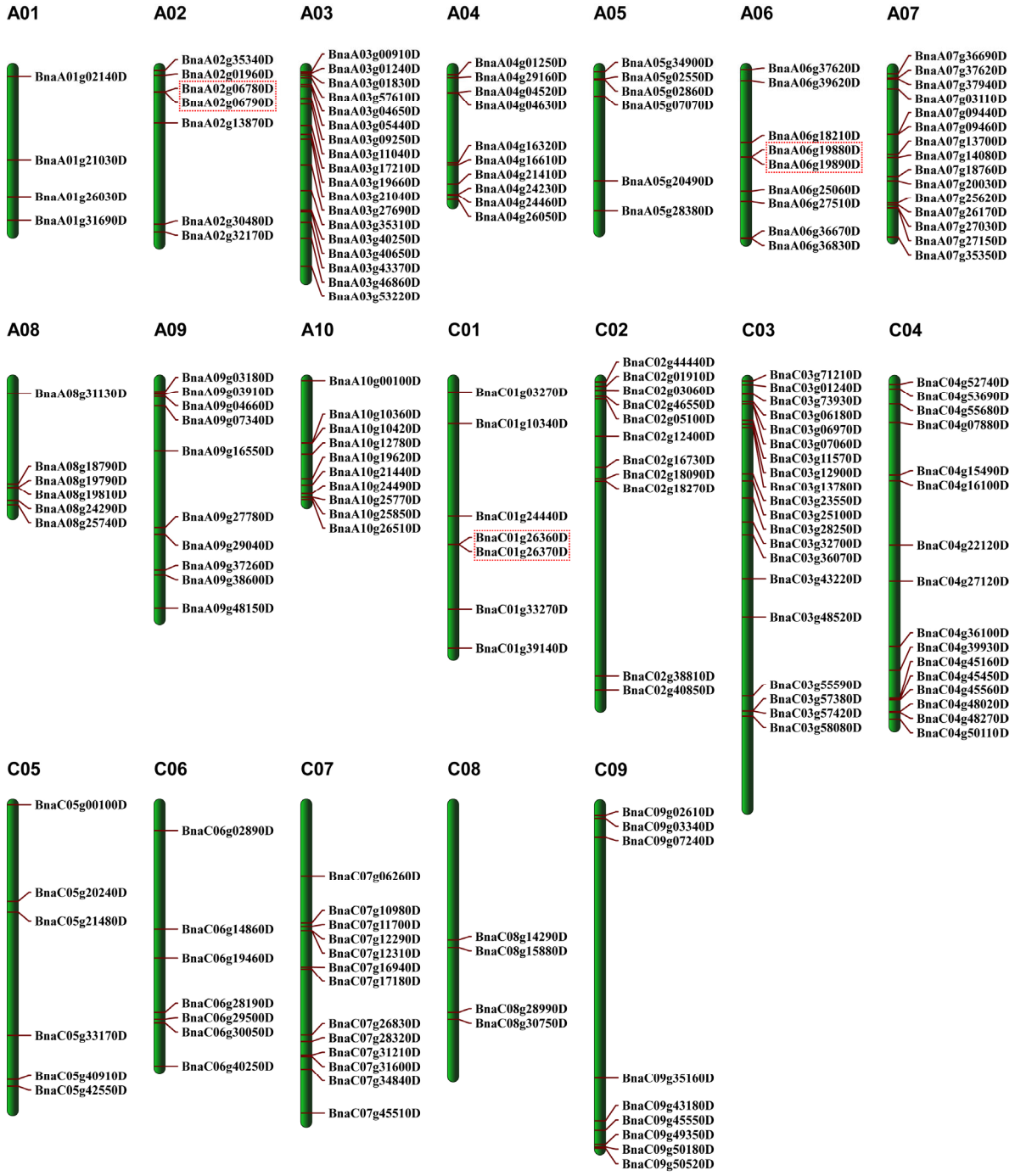


Figure 1. Chromosomal locations of 216 *BnaQ-type C2H2-ZFPs* in *B. napus*. Note: Tandem duplicated genes are marked by red rectangles.

The exon–intron structures of all 216 identified *BnaQ-type C2H2-ZFP* genes were analyzed to obtain deeper insights into the structural characteristics of the Q-type C2H2-ZFP subfamily. The number of exons per gene ranged from two to eight. Notably, more than 70% of the members (154 genes) contained a single exon, while 37 genes had two exons, 12 genes had three exons, three genes had four exons, three genes had five exons, and five genes had six exons. Only one gene (*BnaC01g03270D*) contained seven exons, and a single gene (*BnaC05g20240D*) contained eight exons (Figure S1).

3.3. Conservative Motif Analysis of BnaQ-Type C2H2-ZFPs

To investigate variations in conserved protein motifs among Q-type C2H2-ZFPs, the protein sequences were submitted to the MEME online website for identification of the predicted conserved motifs. Conserved motifs were identified, and sequence logos were generated to visualize the amino acid composition and positional distribution within domains. Motif 1 was present in each BnaQ-type C2H2-ZFP sequence, and motifs 1, 2, and 5 all contained the C2H2-ZFP conserved domain. Among these motifs, motifs 1 and 2 exhibited the invariant amino acid sequence "QALGGH", which is a distinctive feature specific to Q-type C2H2-ZFP motifs. Among the Q-type C2H2-ZFPs, 50 members contained two Q-type domains (motifs 1 and 2) along with one motif 5. A total of 105 members possessed both motif 1 and motif 2. Six members contained three Q-type domains, consisting of one copy of motif 1 and two copies of motif 2. Nearly half of all Q-type C2H2-ZFPs (104 members) included motif 3, also known as the "L-box" motif, and among these, 96 members also contained both motifs 1 and 2 (Figure S1, Tables S2 and S3).

Among the 216 Q-type C2H2-ZFP members, 114 members were found to contain motif 4, a conserved "L/FDLNL/F(x)P" motif, which is predominantly localized at the C-terminus of the protein sequences, with only five exceptions. Similarly, motif 7, characterized by the "LxLxL" conserved amino acid signature, was predominantly localized at the C-terminus in 100 members. However, among these 100 members, four members contained two copies of motif 7, with one copy localized at the C-terminus and the other at the N-terminus. Additionally, 18 members were identified that contained a single copy of motif 4 and either one or two copies of motif 7. Motif 4 was positioned at the N-terminal side of motif 7 or between the two motif 7 domains. Motifs 6 and 8 contained the consensus sequences "KG/RKRT/SKR" and "MALEA/T," respectively. The BnaQ-type C2H2-ZFPs possessing motif 6 (36 members) or motif 8 (26 members) were localized to the C-terminal region (Figure S1).

3.4. Gene Duplication and Genomic Collinearity Analysis of BnaQ-Type C2H2-ZFPs

Gene duplication plays a significant role in the formation of gene families, contributing to the expansion of novel gene members during plant genome evolution and serving as a key driving force in genomic evolutionary processes. In the *BnaQ-type C2H2-ZFP* genes, a total of three pairs (6/216) of tandemly duplicated genes were identified, located on chromosomes A02, A06, and C01 (Figure 1 and Table S4). A total of 232 pairs (160/216) were identified as segmental duplication genes (Figure 2), with 22 pairs originating from the C1-i subgroup, 33 pairs from the C1-ii subgroup, 13 pairs from the C2-i subgroup, 43 pairs from the C2-ii subgroup, 36 pairs from the C2-iii subgroup, three pairs from the C3-i subgroup, 41 pairs from the C3-ii subgroup, and 41 pairs from the C3-iii subgroup. Analysis of gene duplication events reveals that segmental duplication has played a predominant role in the expansion, evolution diversification, and functional differentiation of the *BnaQ-type C2H2-ZFP* transcription factor family. Ka/Ks analysis reveals that the Ka/Ks values of nearly all duplicated genes are below 1, indicating that purifying selection has predominantly shaped their evolutionary trajectories. Only two pairs of fragment-derived duplicated genes have Ka/Ks values exceeding 1, suggesting the potential for positive or adaptive selection acting on these genes (Table S5 and Figure S2).

Comparative genomic analysis indicates the homology of the *Q-type C2H2-ZFP* genes in *B. napus* with those in *B. rapa* and *B. oleracea*. Among them, there are 80 pairs of homologous genes between *B. napus* and *B. rapa*, and 136 pairs between *B. napus* and *B. oleracea*. The results of the whole-genome alignment show that the *Q-type C2H2-ZFP* genes in *B. napus* maintain collinearity with all chromosomes of *B. rapa* and *B. oleracea*. This extensive collinearity indicates their genetic relationship (Figure S3).

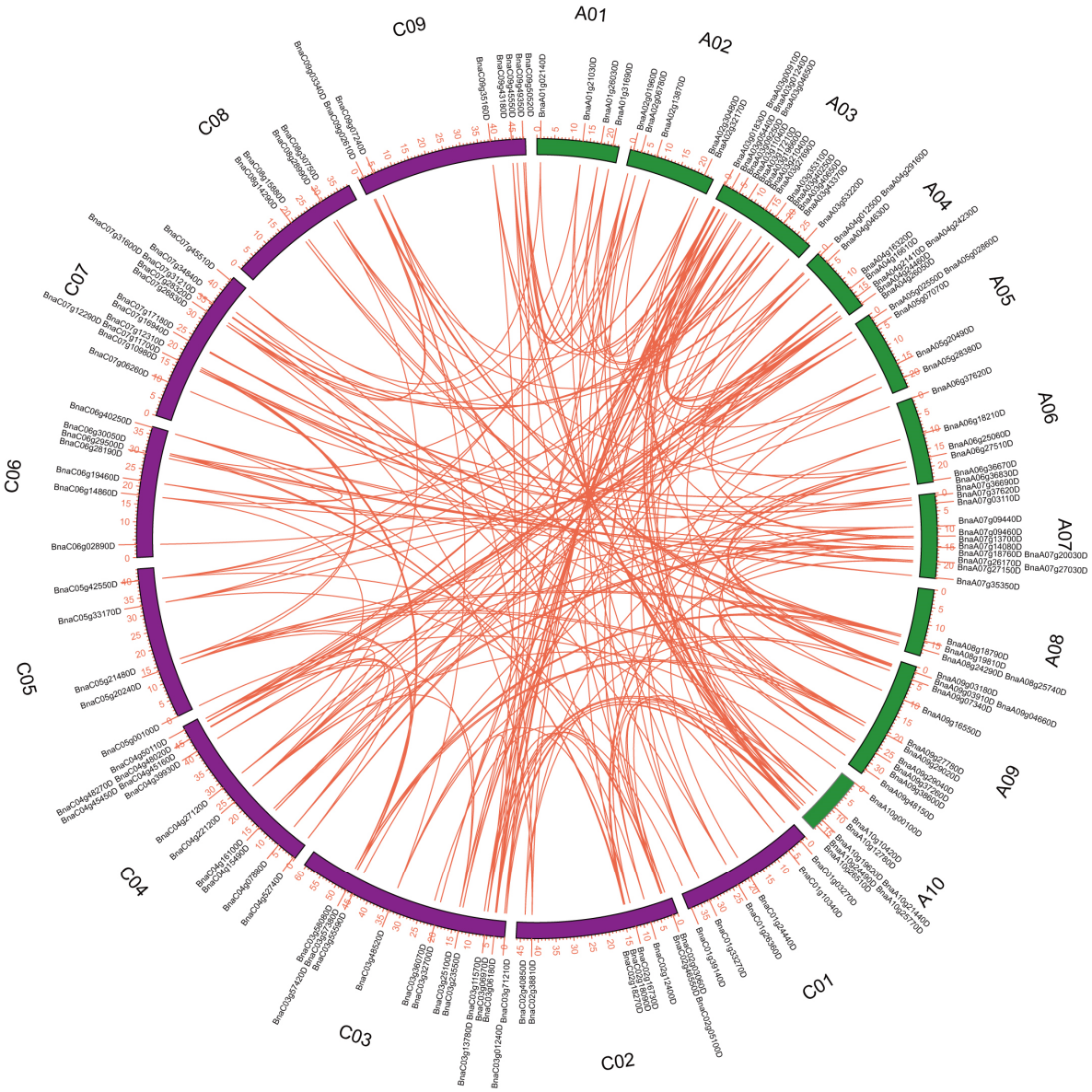


Figure 2. Collinearity analysis of *BnaQ-type-C2H2-ZFP* genes in the *B. napus* genome. Note: Purple and green boxes represent the An subgenome and Cn subgenome, respectively. Red lines out of boxes indicate the chromosomal locations of *BnaQ-type C2H2-ZFP* genes. Collinear gene pairs are highlighted by red lines in the Circos plot.

3.5. Phylogenetic Analysis of BnaQ-Type C2H2-ZFPs

To analyze the evolutionary relationships of BnaQ-type C2H2-ZFPs in *B. napus* and *Arabidopsis*, an unrooted phylogenetic tree was constructed based on the full-length amino acid sequences of 59 *Arabidopsis* proteins and 216 proteins from the *B. napus* genome, using MEGA 7.0 software. The *B. napus* and *Arabidopsis* members were classified into eight clusters: C1-i, C1-ii, C2-i, C2-ii, C2-iii, C3-i, C3-ii, and C3-iii. Cluster 1 (C1) contained 48 BnQ-type C2H2-ZFPs, which were further divided into two subclusters: C1-i (23 members) and C1-ii (22 members). C2-i (16 members), C2-ii (28 members), and C2-iii (28 members) belonged to Cluster 2 (C2), which contained a total of 72 BnaQ-type C2H2-ZFP members. Cluster 3 (C3), comprising 96 members, was further divided into three subclusters: C3-i, which contains six members; C3-ii, with 34 members; and C3-iii, with 56 members (Figure 3).

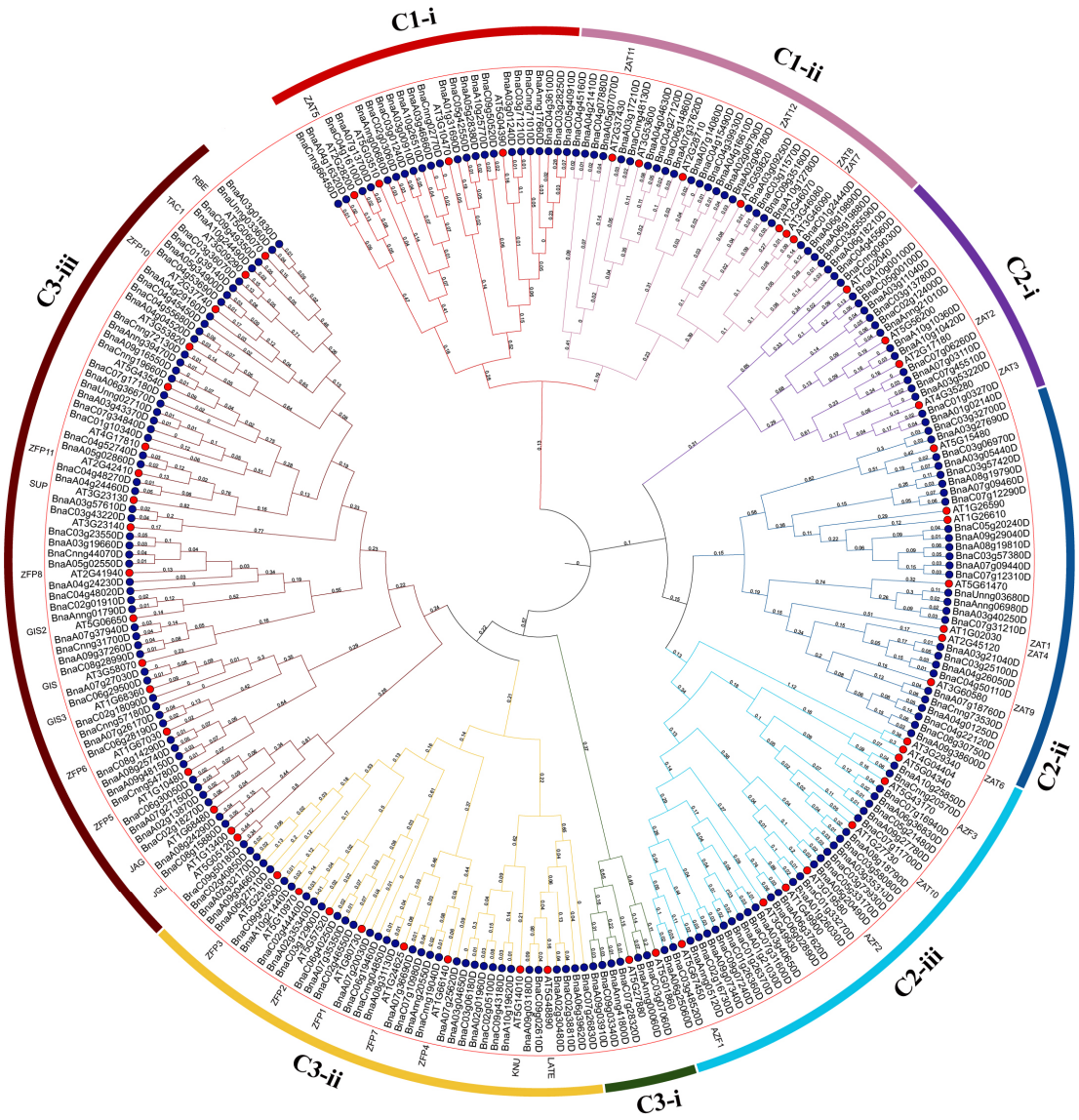


Figure 3. Phylogenetic tree of all Q-type C2H2-ZFPs from *B. napus* and *Arabidopsis*. Blue dots represent *B. napus* Q-type C2H2-ZFPs, red dots represent *Arabidopsis* Q-type C2H2-ZFPs. According to the classification of 176 *Arabidopsis* Q-type C2H2-ZFPs, the 216 *B. napus* Q-type C2H2-ZFPs were divided into eight groups and distinguished by different colors.

As shown in Figure 3, clusters C1 and C2 contained two or three Q-type domains, whereas cluster C3 possessed only one. Based on their domain compositions, members belonging to subgroup C1-ii predominantly contained two Q-type domains (motifs 1 and 2). In addition to two Q-type domains, C1-i contained motifs 3, 4, and 5. Subclusters C2-i to C2-iii each possessed two Q-type domains: C2-i was characterized by motif 4, C2-ii was specific to motif 6, and C2-iii was associated with motifs 5 and 8. C3-i contained a single Q-type domain, and in contrast, C3-ii exhibited the presence of motif 7, thereby distinguishing it from C3-i.

3.6. Expression Analysis of BnaQ-Type C2H2-ZFPs in Different Tissues of B. napus

To investigate the tissue-specific expression patterns of *BnaQ-type C2H2-ZFPs*, a heatmap illustrating the expression profiles of 216 genes across 16 tissues of *B. napus* was constructed. Among the 16 tissues, 78 *BnaQ-type C2H2-ZFPs* were not expressed in the 16 tissues (FPKM < 1) and eight genes (*BnaA01g26030D*, *BnaA04g16320D*, *BnaA07g13700D*, *BnaA10g12780D*, *BnaC01g33270D*, *BnaC04g16100D*, *BnaC05g33170D*, and *BnaC09g35160D*)

were expressed in all 16 tissues (FPKM < 1). The remaining 130 genes showed varying degrees of expression levels in the 16 different tissues. The roots exhibited the highest number of expressed genes, with 82 genes showing FPKM values > 1, whereas the pollen tissue had the lowest number of expressed genes, with only 29 expressed genes showing FPKM values > 1. The expression level of *BnaA03g35310D* in floral organs is the highest among all genes in all tissues. The floral organs include the filament (FPKM = 346), petal (FPKM = 453), and sepal (FPKM = 373) (Figure 4 and Table S6).

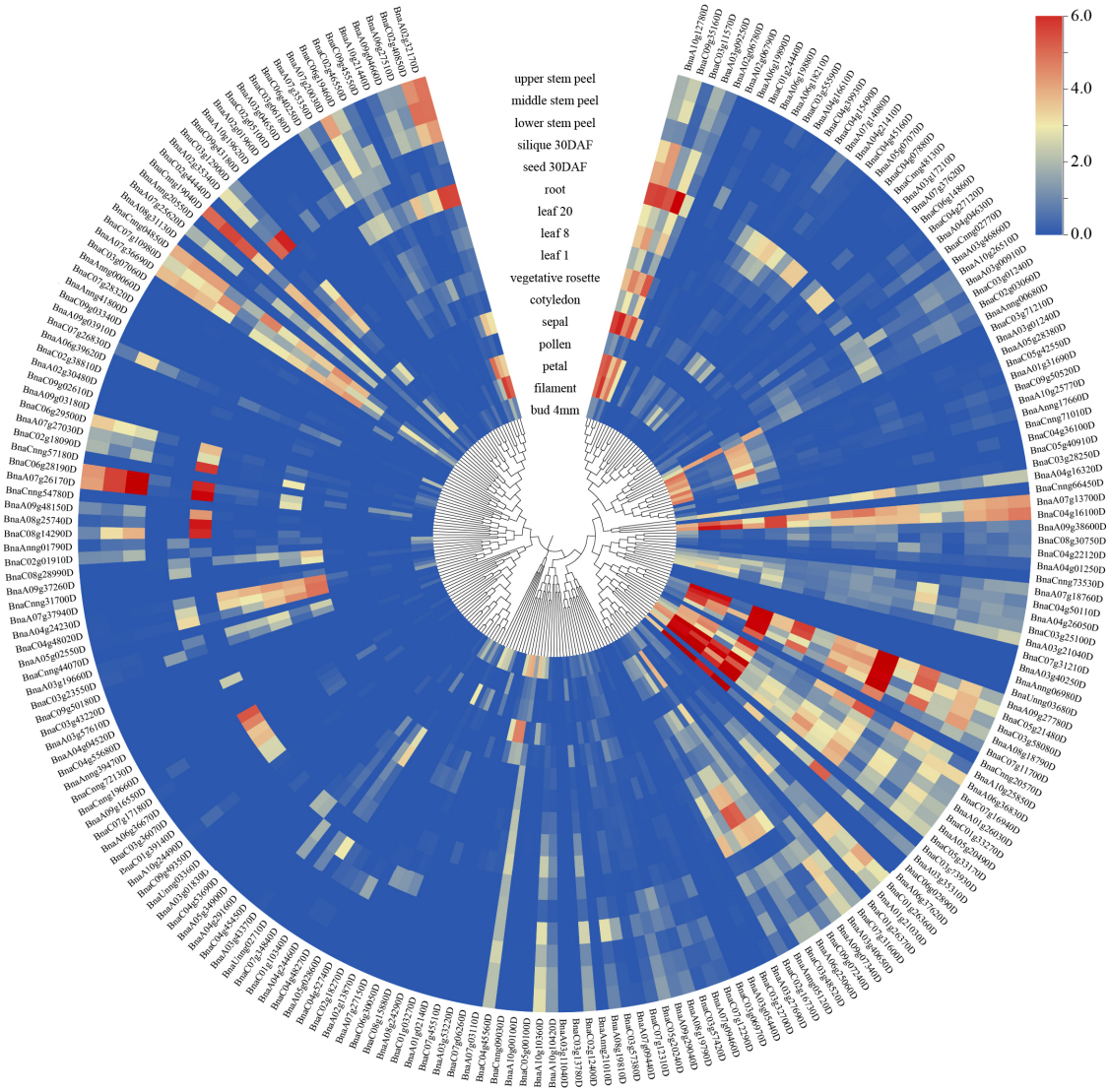


Figure 4. The expression profiles of *BnaQ-type C2H2-ZFP* genes in different tissues of *B. napus* at full-bloom stage. Note: The heatmap was plotted using the $\log_2$FPKM values. The color scale represents the relative expression levels, ranging from low (blue) to high (red).

3.7. Expression Analysis of BnaQ-Type C2H2-ZFPs Under Different Abiotic Stress

To further elucidate the response of *BnaQ-type C2H2-ZFPs* to abiotic stress, the expression profiles of "ZS11" were obtained from the BnIR database [32]. As shown in Figure 5 and Table S7, under salt, drought, freezing, cold, heat, and osmotic stress, 27 genes showed no differential expression (log2FC = 0) across all these six abiotic stresses, whereas 101 genes displayed upregulation ($\log_2$FC > 1) or downregulation ($\log_2$FC < 1) in the leaves under these abiotic stress. Under the six abiotic stress treatments, the expression levels of *BnaA04g16320D*, *BnaA03g43370D*, *BnaA04g21410D*, *BnaA04g24230D*, *BnaA05g02550D*, *BnaA09g37260D*, *BnaC04g22120D*, *BnaC04g45160D*, *BnaC04g48020D*,

BnaC07g26830D, *BnaC07g31600D*, *BnaC08g30750D*, and *BnaC06g40250D* in leaves were downregulated ($\log_2$FC < 1) after 1 h or 24 h treatment. These findings suggest that the expression of these genes was inhibited under these six abiotic stresses. In contrast, the genes *BnaA01g26030D*, *BnaA03g35310D*, *BnaA05g20490D*, *BnaA08g18790D*, *BnaA10g25850D*, *BnaC03g11570D*, *BnaC03g58080D*, and *BnaC05g33170D* were upregulated ($\log_2$FC > 1) under six abiotic stresses following 1 h or 24 h treatments, with the exception of osmotic stress. The expression levels of *BnaA03g09250D*, *BnaA10g12780D*, *BnaC09g35160D*, *BnaA02g06780D*, *BnaC03g11570D*, *BnaA08g18790D*, *BnaA03g35310D*, *BnaA05g20490D*, *BnaC05g21480D*, *BnaC05g33170D*, and *BnaA10g25850D* were significantly upregulated ($\log_2$FC > 3) under freezing and cold treatments. Notably, among these 11 genes, *BnaA03g09250D*, *BnaA10g12780D*, and *BnaC09g35160D* exhibited significantly high expression levels (FPKM > 100) under cold and freezing stresses. These findings suggest that *BnaA03g09250D*, *BnaA10g12780D*, and *BnaC09g35160D* may play crucial roles in the response to low-temperature stress in leaves of *B. napus*.

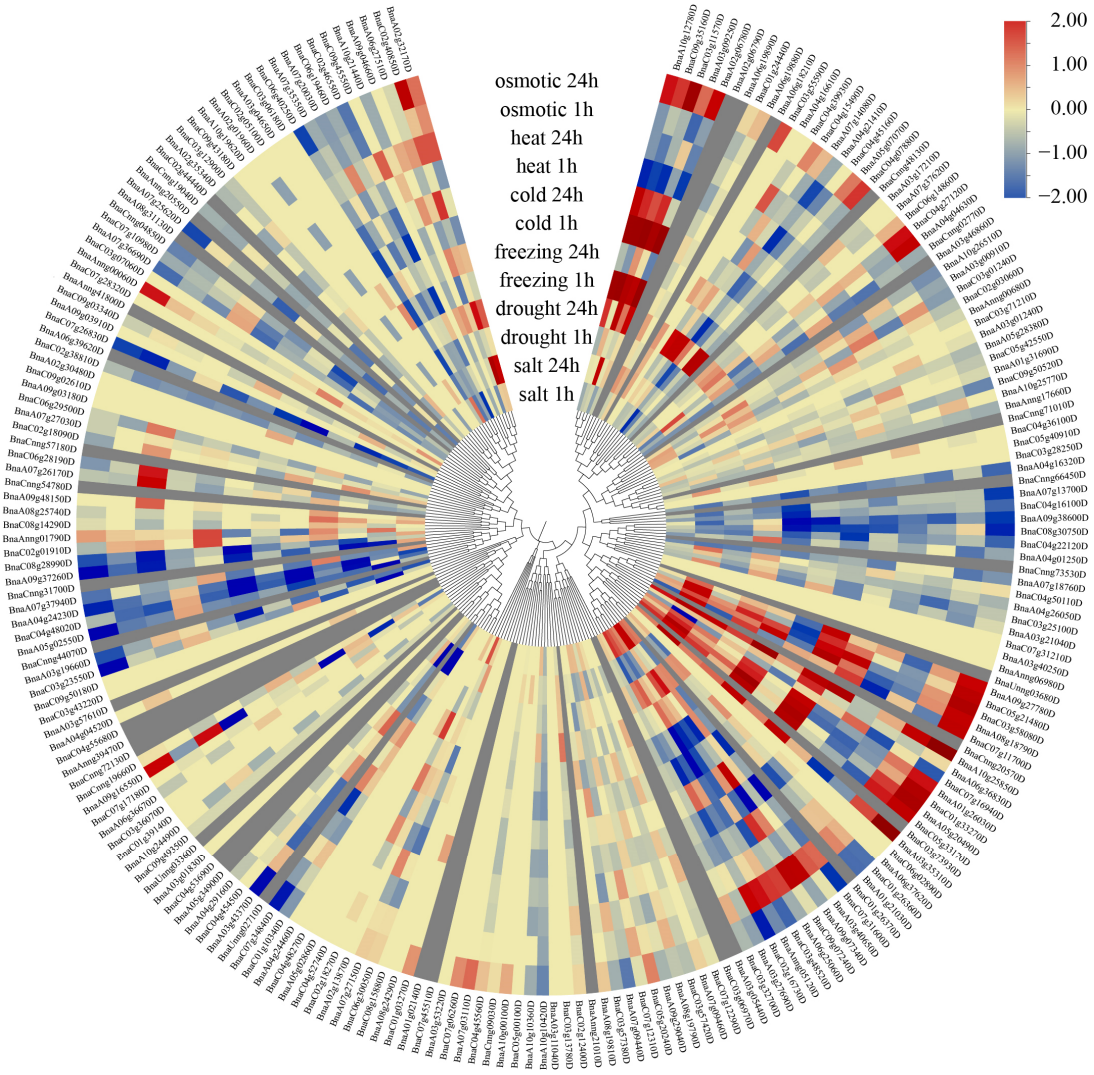


Figure 5. Heatmap showing the expression of *BnaQ-type C2H2-ZFP* genes in leaf tissue under abiotic stress. Note: The expression profiles of 216 *BnaQ-type C2H2-ZFP* genes in leaves under salt, drought, freezing, cold, heat, and osmotic stresses at 1 h and 24 h. The heatmap was plotted using $\log_2$FC value. The color scale represents the $\log_2$(FC + 1) values ranging from low (blue) to high (red).

Under the six abiotic stresses, 158 genes exhibited differential expression ($|\log_2\text{FC}| > 1$) and 58 genes showed no differential expression ($\log_2$FC = 1) in the roots of *B. napus*.

Among these genes, *BnaA03g09250D*, *BnaC03g11570D*, *BnaA05g20490D*, *BnaA10g25850D*, *BnaA01g21030D*, *BnaC03g01240D*, and *BnaA03g00910D* were upregulated under six abiotic stresses ($log_2FC > 1$). In contrast, *BnaA09g48150D*, *BnaC02g46550D*, *BnaA07g27030D*, *BnaC06g29500D*, *BnaA07g20030D*, *BnaC06g40250D*, *BnaC06g19460D*, and *BnaA07g35350D* were downregulated ($log_2FC < 1$) under all these stresses when treated for either 1 h or 24 h. The genes *BnaA03g09250D*, *BnaC03g11570D*, *BnaA02g06780D*, *BnaC09g35160D*, *BnaA05g20490D*, and *BnaC01g33270D* were significantly upregulated under salt, drought, freezing, and cold stresses. Notably, *BnaA03g09250D*, *BnaA10g12780D*, *BnaC09g35160D*, *BnaC03g11570D*, *BnaA08g18790D*, *BnaC05g21480D*, *BnaA10g25850D*, and *BnaA09g27780D* exhibited significantly high expression levels under cold and freezing stresses ($log_2FC > 3$ and FPKM > 100). Interestingly, these genes are also highly expressed in leaves. These findings provided an insight into the responses of *BnaQ-type C2H2-ZFPs* to abiotic stress, particularly salt, freezing, and cold treatments (Figure 6 and Table S8).

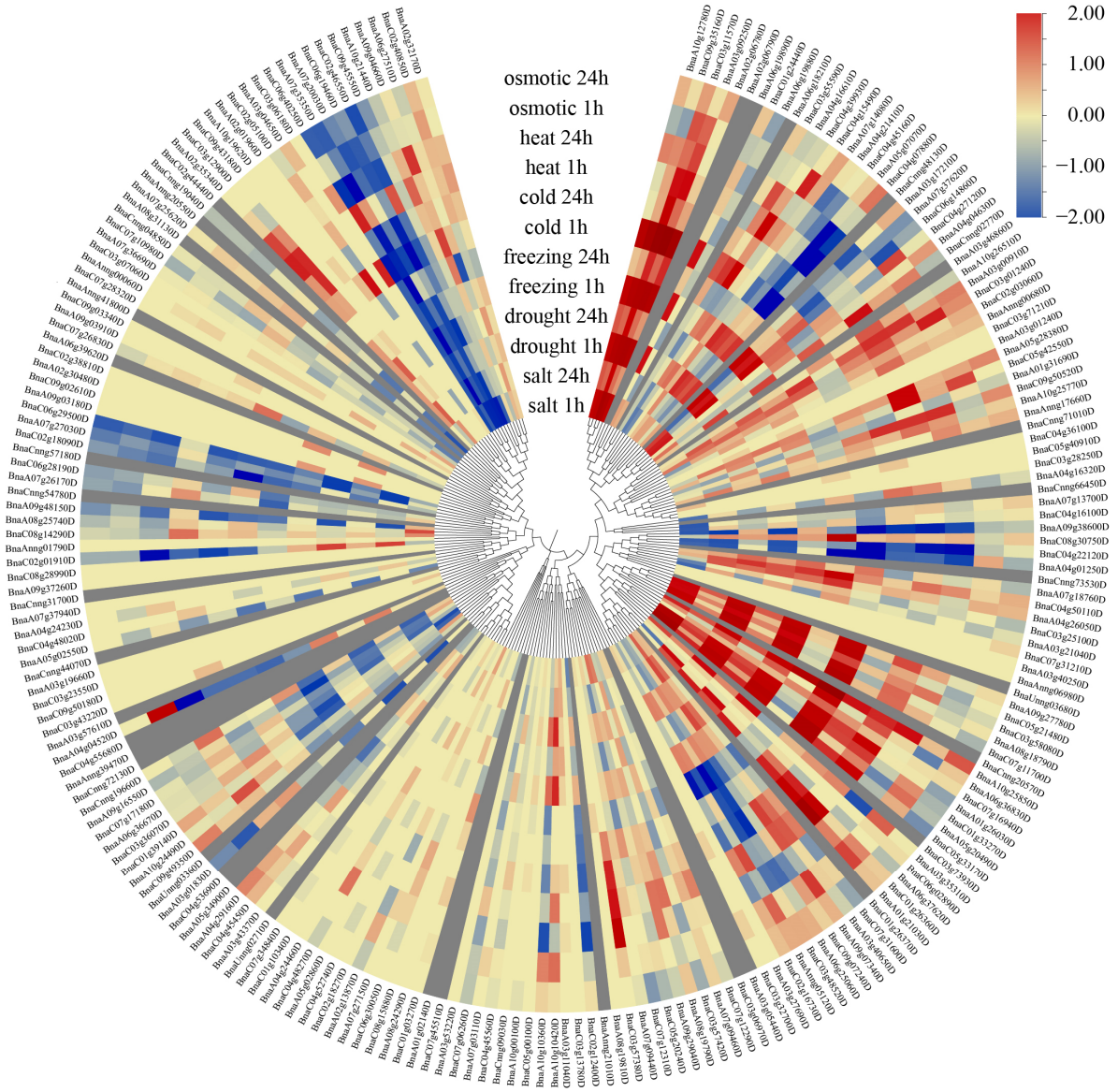


Figure 6. The expression patterns of *BnaQ-type C2H2-ZFP* genes in root tissue under abiotic stress. Note: The expression levels of 216 *BnaQ-type C2H2-ZFP* genes in roots under salt, drought, freezing, cold, heat, and osmotic stresses at 1 h and 24 h. The heatmap was plotted using the log_2FC value. The color scale represents the log_2FC value from low (blue) to high (red).

3.8. Expression Levels of BnaQ-Type C2H2-ZFPs in Different Winter-Type B. napus Varieties Under Freezing Stress

The expression patterns of *BnaA03g09250D*, *BnaA10g12780D*, *BnaC09g35160D*, *BnaC03g11570D*, *BnaA05g20490D*, *BnaC05g21480D*, *BnaA09g27780D*, *BnaA10g25850*, *BnaC03g58080D*, and *BnaA08g18790D* in leaf tissue under abiotic stress are shown in Figure 5. Among these ten genes, five exhibited high expression (FPKM > 50 and $\log_2$FC > 4) under freezing stress, seven exhibited high expression (FPKM > 50 and $\log_2$FC > 5) under cold stress, and three genes (*BnaA03g09250D*, *BnaA10g12780D*, and *BnaC09g35160D*) were highly expressed under both freezing and cold stress, with FPKM > 140 and $\log_2$FC > 4. Notably, in our previous study, *BnaA03g09250D*, *BnaA10g12780D*, *BnaC09g35160D*, *BnaC03g11570D*, *BnaC03g58080D*, and *BnaA08g18790D* were strongly inducted (FPKM > 160 and $\log_2$FC > 4) after 24 h of freezing treatment (−2 °C) in the strongly cold-tolerant line "2016TS(G)10" [3]. Based on these results, we further investigated the expression patterns of ten *BnaQ-type C2H2-ZFP* genes in winter-type *B. napus* with differing cold tolerance under freezing stress. qPCR analysis was conducted on the strongly cold-tolerant variety "Longyou 88" and the cold-sensitive variety "Tianyou 2288" after exposure to −4 °C for 0 h, 1 h, 24 h, and 48 h. As shown in Figure 7, all 10 genes exhibited continuous upregulation from 1 h to 24 h under freezing stress. From 24 h to 48 h, the expression levels of *BnaC03g11570D* and *BnaA05g20490D* in "Tianyou 2288" showed a significant decrease ($p < 0.05$), whereas *BnaA10g25850* exhibited a non-significant reduction ($p < 0.05$). Meanwhile, the expression levels of seven other genes continued to increase during this period. Notably, the upregulation of all 10 genes in "Longyou 88" after 1 h of freezing stress was significantly lower than in "Tianyou 2288", except for *BnaA10g25850*. However, after 48 h, the expression levels of six genes in "Longyou 88" were significantly higher than those in "Tianyou 2288". These results indicated that the expression of these 10 genes was induced by freezing treatment in winter-type *B. napus*, but the response speed and expression levels varied between different cold-tolerant varieties.

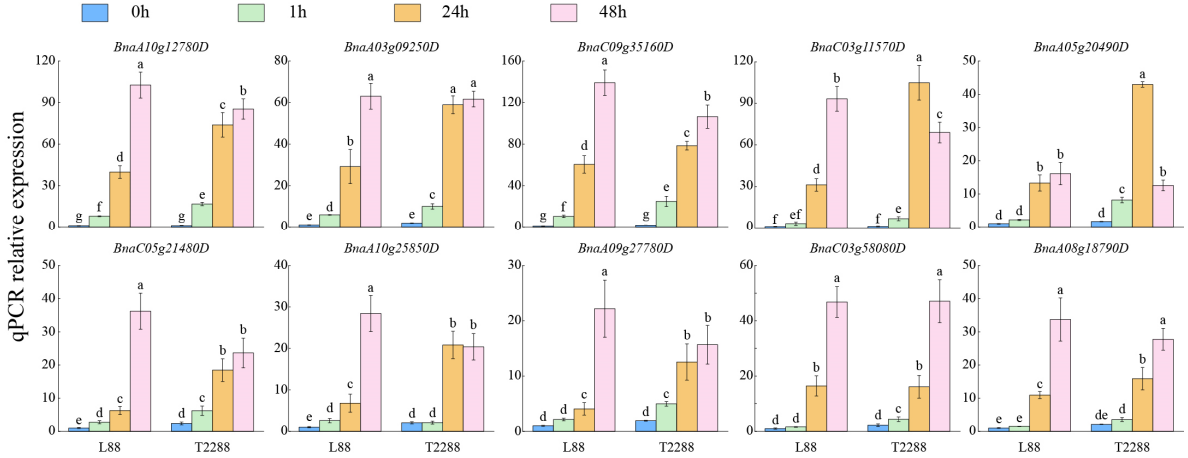


Figure 7. Expression of *BnaQ-type C2H2-ZFP* genes in *B. napus* under −4 °C for 0 h, 1 h, 24 h, and 48 h. Note: L88 and T2288 represent "longyou88" and "Tianyou2288". Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Data were analyzed by one-way ANOVA in SPSS. Data are presented as the mean ± standard error (SE) from three independent biological replicates. SEs are indicated by bars above the columns. The letters a–g denote significant differences between varieties and treatments at $p < 0.05$.

3.9. Phenotypic Analysis of BnaC09g35160D Overexpression in Transgenic Arabidopsis Under Freezing Stress

The *BnaC09g35160D* cDNA sequence was cloned from "Longyou88" (L88), showing 98.76% similarity with the reference genome sequence (version 5.0) (Figure S4). *Arabidopsis*

plants were then transformed, and positive transgenic lines were selected using Basta. Transgenic plants overexpressing *BnaC09g35160D*, driven by the CaMV35S promoter, were successfully obtained. To elucidate the function of *BnaC09g35160D* under freezing stress, four-week-old plants were exposed to −4 °C for 24 h. As shown in Figure 8, no significant differences were observed between the wild-type (WT) and transgenic plants under normal conditions or after 1 h of freezing treatment. After 12 h of freezing, WT plants began to exhibit wilting and curling, with older leaves showing more severe symptoms than younger leaves, and overall, WT plants displayed greater damage than the transgenic line. After 24 h of continuous freezing, the leaves of the transgenic plants turned dark green, with curling, drooping, and wilting observed mainly at the edges of older leaves. In contrast, most WT plants exhibited severe wilting or died. These results indicated that *BnaC09g35160D* overexpression enhances freezing tolerance in plants.

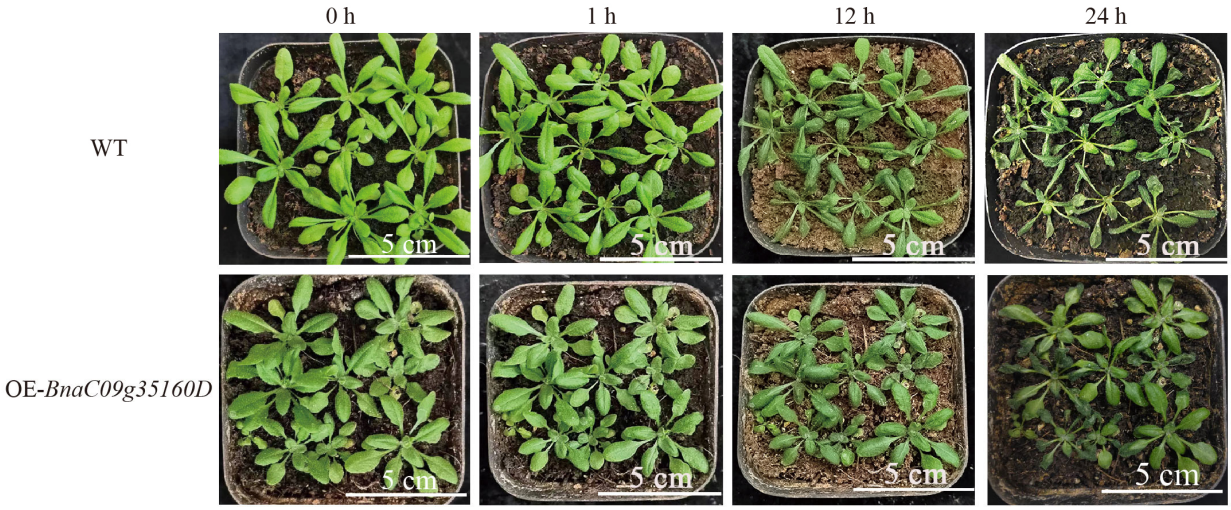


Figure 8. Phenotype of *BnaC09g35160D*-overexpressing transgenic *Arabidopsis* plants after freezing treatment.

4. Discussion

Q-type C2H2-ZFPs represent one of the largest transcription factor families in higher plants, and play crucial roles in growth, development, and stress response. In recent years, large-scale identification and analysis of gene families have become a central focus in functional genomics research. To date, numerous studies have been conducted in model plants, staple crops, fruit crops, and other plant species. As an important oilseed crop, *B. napus* has been extensively studied in the identification of gene families, including the AP2/ERF [38], Aux/IAA [39], R2R3-MYB [40], and WRKY [41] gene families. The genome resequencing in *B. napus* has provided valuable resources for the study of gene family functions [2]. However, despite extensive research on various gene families in *B. napus*, the identification of a Q-type C2H2-ZFP subfamily has yet to be reported. But these genes have been identified in many other species, including 52 members in *Vitis vinifera*, 54 in *Vitis riparia*, 55 in *Vitis amurensis*, 79 in *Solanum tuberosum*, 58 in *Medicago sativa*, 159 in *Triticum aestivum*, 37 in *Brassica oleracea*, and 110 in *Brassica rapa*, among others [42–47]. In this study, a total of 216 *Q-type C2H2-ZFPs* were identified in the *B. napus* genome, each containing at least one conserved "QALGGH" motif, characteristic of the Q-type C2H2-ZFP domain. The amino acid lengths of these members ranged from 135 to 948, with molecular weights varying between 14.98 kDa and 106.74 kDa, and pI varying between 4.42 and 10.03, indicating substantial physicochemical diversity among the members. All the genes were unevenly distributed across all 19 Chr., but showed nearly equal distribution between the An and Cn subgenomes. Segmental duplication was identified as the predominant

mechanism driving the expansion of the BnQ-type C2H2-ZFPs transcription factor family. Only three pairs (6/216) of members were identified as tandemly duplicated, located on ChrA02, ChrA06, and ChrC01. Gene duplication plays a crucial role in genome rearrangement, expansion, functional diversification, and the evolution of gene families. Previous studies on *B. napus* gene families, such as WOX [48], Aux/IAA [39], and WRKY [41], have demonstrated that segmental duplication is the primary mechanism driving the expansion of gene family expansion. Similarly, our findings reveal that 74.07% (160/216) of *Q-type C2H2-ZFP* genes originated from segmental duplication, whereas only 2.78% (6/216) arose from tandem duplication. These results suggest that segmental duplication is the primary mechanism underlying the expansion of the *Q-type C2H2-ZFP* gene family in *B. napus*.

The EAR motif has a highly conserved amino acid sequence, specifically "L/FDLNL/F(x)P" and "LxLxL". Many previously identified plant ZFPs contain EAR motifs in their C-terminal regions [44]. Transcription factors containing the EAR motif contribute to maintaining the normal physiological state of plants in diverse environments by negatively regulating the expression of genes involved in growth and development, as well as in responses to abiotic and biotic stresses [49]. In this study, motif 4 contains the highly conserved amino acid sequence "L/FDLNL/F(x)P", which represents one type of EAR motif, while the other type of EAR motif, characterized by the conserved sequence "LxLxL", corresponds to motif 7. More than half of the 216 BnaQ-type C2H2-ZFP members (111/216) contain the "L/FDLNL/F(x)P" motif, 100 members contain the "LxLxL" motif, and 18 members contain both motifs. Among these 18 members, 4 contain one "L/FDLNL/F(x)P" and two "LxLxL" motifs: BnaA07g36690D, BnaC07g10980D, BnaCnng04850D, and BnaA08g31130D. In the present study, these four proteins were clustered in subgroup C3-ii along with the ZFP7 from *Arabidopsis*. Previous studies have shown that overexpression of *EgrZFP7* in *Arabidopsis* increases both the number and length of lateral roots and also enhances the sensitivity of overexpressing lines to freezing stress. Except for these 18 proteins, all other members belonging to subgroups C3-iii, C2-iii, C1-i, and C1-ii contain either the "LxLxL" motif or the "L/FDLNL/F(x)P" motif. These findings suggest that *BnaQ-type C2H2-ZFP* genes are enriched in potential transcriptional repressors that may negatively regulate the expression of genes involved in development, as well as in abiotic and biotic stress responses in *B. napus*.

In terms of gene structure, over 70% of the *BnaQ-type C2H2-ZFP* genes (a total of 154 genes) consist of only one exon, while 37 genes contain two exons, and 25 genes contain three to eight exons. This distribution suggests that the *BnaQ-type C2H2-ZFP* gene family has remained relatively conserved during evolution. However, the observed structural variations indicate that divergence has occurred over long evolutionary timescales, likely due to mechanisms such as exon gain/loss or intron insertion/deletion [5]. Notably, genes within the same phylogenetic clade display highly similar exon–intron structures, thereby providing further evidence of functional conservation among closely related members. These findings indicate that although the *Q-type C2H2-ZFP* gene family in *B. napus* has undergone structural diversification, evolutionary constraints have preserved conservation within certain lineages, potentially reflecting selective pressures to maintain critical functional domains.

Gene expression patterns can reflect their functional roles [50]. Previous studies have demonstrated that *Q-type C2H2-ZFP* genes exhibited different expression patterns across various tissue, growth stage, and developmental processes in many species. In potato, *PG0005486* and *PG0030311*, which belong to the *Q-type C2H2-ZFP* gene family, were predominantly expressed in leaves and roots, displaying tissue-specific expression patterns. *PG0015531*, *PG0015532*, *PG0015533*, *PG0015534*, *PG0015557*, *PG0015558*, *PG0041467*, and *PG0011548* were specifically expressed in the stolon and *PG0018743* was specifically expressed in both immature and mature potato fruits, whereas *PG0023666* did not exhibit

tissue-specific expression across different tissues [43]. In sugar beet, *BvZFP3*, *BvZFP9*, *BvZFP16*, *BvZFP18*, *BvZFP19*, *BvZFP32*, and *BvZFP35* showed specific expression in roots, whereas *BvZFP2*, *BvZFP6*, *BvZFP7*, *BvZFP14*, *BvZFP17*, *BvZFP20*, *BvZFP27*, *BvZFP28*, *BvZFP30*, and *BvZFP34* were expressed in both leaves and roots [51]. *Csa1G132120* was specifically expressed in roots and hypocotyls and *Csa3G199020* exhibited the highest expression in flowers, whereas *Csa2G354820*, *Csa7G428820*, *Csa3G697940*, *Csa1G613510*, *Csa7G428830*, *Csa4G642460*, *Csa6G452080*, *Csa6G152950*, *Csa1G657150*, and *Csa4G290810* showed consistently high expression across 23 cucumber tissues [52]. Some genes were barely expressed or showed no detectable expression. In our study, *BnaA10g12780D*, *BnaC09g35160D*, *BnaA01g26030D*, *BnaC01g33270D*, *BnaC05g33170D*, *BnaA04g16320D*, *BnaA07g13700D*, and *BnaC04g16100D* were expressed across 16 tissues. Among them, *BnaA01g26030D* and *BnaC05g33170D*, which are orthologs of *Arabidopsis AZF2*, exhibited particularly high expression in the filament, petal, and sepal. *BnaA03g35310D*, *BnaA05g20490D*, and *BnaA09g27780D* were highly expressed in the filament, petal, and sepal as well, but did not exhibit tissue-specific expression. *BnaA03g09250D* was specifically expressed in roots, showing high expression level. Thirty-four members exhibited no expression across 16 tissues. These results demonstrated distinct expression patterns of *BnaQ-type C2H2-ZFP* genes in different tissues of *B. napus*, suggesting that these genes may play diverse roles during plant development.

Under abiotic stress, the expression patterns of the *Q-type C2H2-ZFP* gene family varied significantly. In foxtail millet, five genes showed no expression under salt stress in both salt-tolerant and salt-sensitive cultivars. Nine genes were upregulated, with one gene (*SiC2H2-23*) showing the most significant induction under salt stress, whereas *SiC2H2-30* was significantly repressed [53]. In cucumber, only *Csa3G697940* responded to cold, drought, NaCl, and ABA treatments, two genes responded to three treatments, and thirteen members responded to one or two treatments [52]. In the present study, 85 genes showed no expression in roots or leaves under salt, drought, freezing, cold, heat, and osmotic treatments. Eighteen genes responded to five stresses in roots, while one gene (*BnaA05g02550D*) was expressed in leaves under the same five stresses. Eleven genes exhibited responses to all treatments in both roots and leaves, with the exception of heat stress. Notably, the transcript of *BnaA09g27780D* was strongly upregulated ($\log_2$FC > 40) and showed high expression levels after 1 h of exposure to salt, drought, freezing, cold, and osmotic stresses in roots. Interestingly, *BnaA03g09250D*, *BnaC09g35160D*, and *BnaA10g12780D*, which are orthologs of *Arabidopsis ZAT12*, were strongly upregulated under freezing stress and exhibited high expression levels in both roots and leaves. In winter rapeseed variety "L88" and "T2288", the relative expression levels of these three genes were consistently upregulated under freezing stress. These results indicated that these three genes may play an important role in the response to freezing stress in *B. napus*. Previous studies have demonstrated that *ZAT10* and *ZAT12* positively regulate the expression of *COR* genes under freezing stress, thereby enhancing plant freezing tolerance [54,55]. In the *Arabidopsis* ecotype Col-0, cold conditions induce the expression of *HSFC1*, *ZAT12*, *ZAT10*, *ZF*, and *CZF* genes, which subsequently activate one or more *CBF* genes [54]. A similar rapid cold-induced expression of these genes in response to cold stress was observed in other *Arabidopsis* ecotypes, including those from Italy and Sweden [55]. Overexpression of *ZAT12* and *HSFA1* has been shown to improve cold tolerance in transgenic plants [15]. Similar results were observed in transgenic *BrZAT12* plants, where *BrZAT12*-transformed *Arabidopsis* exhibited higher survival rate, as well as increased POD, SOD, and CAT enzyme activities, compared to wild-type plants [56]. In our previous study, strong and progressive upregulation of 11 genes in *B. napus* were observed, including *BnaA08g18790D*, *BnaA09g27780D*, *BnaC03g58080D*, *BnaC05g21480D*, *BnaA03g09250D*, *BnaA10g12780D*, *BnaC03g11570D*, *BnaC09g35160D*, *BnaA10g25850D*, *BnaA05g20490D*, and

BnaC05g33170D. These genes cluster with *AtZAT10* and *AtZAT12* and exhibited sustained and significant upregulation during prolonged freezing stress [3]. The results of the present study are consistent with these findings and demonstrate that *ZAT12* may play a role in the plant response to freezing stress and is worthy of further investigation.

5. Conclusions

In this study, we performed a genome-wide analysis and identified a total of 216 *Q-type C2H2-ZFP* genes, each containing one to three copies of conserved "QALGGH" motifs. We analyzed physiochemical properties, chromosomal localization, gene structure, conserved motif compositions, gene duplication events, and genomic collinearity. Additionally, we examined the expression profiles of *BnaQ-type C2H2-ZFPs* in different tissues and under abiotic stress, identifying *BnaA03g09250D*, *BnaC09g35160D*, *BnaC03g11570D*, and *BnaA10g25850D* as potential candidates involved in the response to freezing stress. Overexpression of *BnaC09g35160D* provided preliminary evidence of its role in enhancing freezing tolerance in plants. Our findings contribute to the understanding of the roles of *BnaQ-type C2H2-ZFP* genes in response to abiotic stress, and these genes might be used as potential genetic resources for improving abiotic stress tolerance in *B. napus* breeding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy15092085/s1. Table S1. Characteristic features of 216 BnaQ-type C2H2-ZFPs. Table S2. The conserved motifs identified in 216 BnaQ-type C2H2-ZFPs by MEME. Table S3. The conserved motif distribution in each BnaQ-type C2H2-ZFP. Table S4. Tandemly and segmentally duplicated *Q-type C2H2-ZFP* genes. Table S5. Ka, Ks, and Ka/Ks values of *BnaQ-type C2H2-ZFP* genes with tandem and segmental duplications. Table S6. The FPKM values of *BnaQ-type C2H2-ZFP* genes in diverse tissues. Table S7. The FPKM values of *BnaQ-type C2H2-ZFP* genes in leaf tissue under abiotic stress. Table S8. The FPKM values of *BnaQ-type C2H2-ZFP* genes in root tissue under abiotic stress. Figure S1. Phylogenetic tree, conserved motifs, and gene structure analysis of the 216 *BnaQ-type C2H2-ZFP* genes in *B. napus*. Figure S2. Ka, Ks, and Ka/Ks values of *BnaQ-type C2H2-ZFP* genes with tandem and segmental duplications. Figure S3. Collinearity analysis of *BnaQ-type-C2H2-ZFP* genes in *B. napus* genome with in *B. rapa* and *B. oleracea*. Figure S4. Sequence alignment between the sequencing results and reference genes revealed a 98.76% similarity to the reference genome.

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Conflicts of Interest: The authors declare no conflicts of interest.

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