

Article

Systematic Analysis of the bZIP Family in Tobacco and Functional Characterization of NtbZIP62 Involvement in Salt Stress

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Abstract: The basic leucine zipper (bZIP) transcription factors play important regulatory roles, influencing plant growth and responses to environmental stresses. In the present study, 132 bZIP genes identified in the tobacco genome were classified into 11 groups with *Arabidopsis* and tomato bZIP members, based on the results of a phylogenetic analysis. An examination of gene structures and conserved motifs revealed relatively conserved exon/intron structures and motif organization within each group. The results of an investigation of whole-genome duplication events indicated that segmental duplications were crucial for the expansion of the bZIP gene family in tobacco. Expression profiles confirmed that the NtbZIP genes are differentially expressed in various tissues, and several genes are responsive to diverse stresses. Notably, NtbZIP62, which was identified as an AtbZIP37/ABF3 homolog, was highly expressed in response to salinity. Subcellular localization analyses proved that NtbZIP62 is a nuclear protein. Furthermore, the overexpression of NtbZIP62 in tobacco significantly enhanced the salt stress tolerance of the transgenic plants. The results of this study may be relevant for future functional analyses of the bZIP genes in tobacco.

Keywords: tobacco; bZIP; abiotic stress; biotic stress; gene expression; gene family



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1. Introduction

Transcription factors (TFs) are important regulators of plant growth and development that bind to specific regions of target gene promoters to activate or suppress expression [1,2]. Including the bZIP TF family, more than 60 TF families have been identified in the plant kingdom [3]. The bZIP TFs usually contain a highly conserved bZIP domain comprising 40–80 amino acids [4]; this domain includes a highly conserved DNA recognition domain, a less conserved leucine zipper motif, which features on the contiguous α -helix [5,6]. Plant bZIP proteins generally bind to the ACGT core of the target gene promoter sequence and to the A-box (TACGTA), C-box (GACGTC), and G-box (CACGTG), in order of affinity [7].

Previous studies revealed that bZIP TFs participate in many essential biological processes in plants [8]. In *Arabidopsis*, 78 bZIP members have been identified and were divided into 13 groups. The contributions of a number of bZIP TFs to various developmental processes have been characterized [9]. For example, among the Group A members, AtbZIP39/ABI5 affects floral transitions by regulating the transcription of the gene encoding flowering site C (FLC), which suppresses the gene encoding the florigen flowering locus T (FT) [10]. Interestingly, function of FT is dependent on AtbZIP14/FD and AtbZIP27/FDP, which also belong to Group A [11]. The AtbZIP39 TF also influences seed maturation

and germination [12]. Moreover, AtbZIP53, in Group S, induces the expression of genes related to seed maturation [13], whereas AtbZIP9 (Group C) is reportedly involved in root vascular development [14]. Additionally, the gene encoding *Atb ZIP46/PAN* (Group D) is highly expressed in flowers, wherein it controls the number of floral organs [15], whereas AtbZIP47/TGA1 and AtbZIP57/TGA4 (from the same group) affect root hair patterning [16]. In Group G, AtbZIP41/GBF1 contributes to the regulation of lateral root development and natural senescence [17]. Furthermore, AtbZIP29 (Group I) functions in leaf and root development [18], and AtbZIP11/GBF6 (Group S) participates in primary root growth [19].

Several bZIP TFs modulate metabolism in *Arabidopsis*. Group H includes Atb ZIP56/HY5 and its homolog AtbZIP64/HYH, which have partly overlapping functions associated with anthocyanin biosynthesis [20]. In contrast to AtbZIP56, both Atb ZIP64 and AtbZIP41 (Group G) function synergistically to promote anthocyanin accumulation [21]. In Group S, AtbZIP11/GBF6 is believed to be involved in trehalose biosynthesis [22].

In *Arabidopsis*, many bZIP TFs were found to be involved in responses to multiple biotic and abiotic stresses [23]. For example, AtbZIP36/ABF2 and AtbZIP37/ABF3 (Group A) mediate responses to various abiotic stresses [24,25]. Among the Group B members, the expression of *AtbZIP17* is significantly induced by salt treatments, and *Atb ZIP17* overexpressing transgenic plants reportedly exhibit enhanced tolerance to salt stress [26]. In addition, *AtbZIP24* expression is induced by salt stress, and the encoded TF has been proposed to act as a negative regulator of salt-stress responses [27]. AtbZIP1 (Group S) controls the metabolic reprogramming in roots induced by salt stress; the salt resistance of transgenic *Arabidopsis* plants increases significantly following the overexpression of *AtbZIP1* [28]. Regarding defense responses, AtbZIP47/TGA1 and Atb ZIP57/TGA4 (Group D) directly activate the expression of CBP60g and SARD1, both of which are found to be involved in the biosynthesis of salicylic acid (SA), which helps to defend against pathogens [29]. Similarly, AtbZIP10 (Group C) and AtbZIP41 (Group G) help to protect plants from pathogens [30,31].

Tobacco is one of the most important crops, cultivated in more than 120 countries, but the yield of tobacco is under constant threat from various stresses. Furthermore, tobacco is a typical model plant in gene function research. However, there is little information about the bZIP family members in tobacco. The sequencing of the tobacco genome has set the foundation for the identification and analysis of the bZIP gene family in tobacco. In the current study, a comprehensive analysis was carried out, and the results suggest that tobacco's bZIP gene family members play multiple roles in various biological processes. These results could provide insight for further studies on their biological functions.

2. Materials and Methods

2.1. Identification and Phylogenetic Analysis of Tobacco bZIP Proteins

The genomic data for tobacco and tomato were obtained from the SGN database (<https://solgenomics.net/>), while the genomic data for *Arabidopsis* were downloaded from TAIR database (<http://www.arabidopsis.org/>). The potential bZIP proteins in tobacco were detected by a local BLASTP search with the previously reported full-length sequences of *Arabidopsis* bZIP proteins (E-value < 0.01). Then, the obtained sequences were queried against the SMART [32] and Pfam [33] databases to determine the number of bZIP domains. The remaining genes were renamed based on their physical locations on the chromosome/scaffold. The molecular weights and isoelectric points of the newly identified bZIP proteins were predicted using the ProtParam online tool [34].

The full-length sequences and DNA-binding domains of the NtbZIP proteins and previously reported *Arabidopsis* and tomato bZIP proteins were aligned using Clustal X 2.0 and visualized using R [35]. Based on the alignments, MEGA was used to build a neighbor-joining (NJ) tree with default parameters [36].

2.2. Conserved Motifs and Exon-Intron Structure Analysis of Tobacco bZIP Members

The online MEME tool was adopted to analyze conserved motifs by using the full-length protein sequences of the newly identified tobacco bZIP members [37]. Subsequently, the GSDS online tool was adopted to visualize the exon-intron structures of the tobacco bZIP genes by submitting their genomic and coding sequences [38].

2.3. Chromosomal Localization and Promoter Analysis of Tobacco bZIP Genes

Chromosomal location information for the *NtbZIP* genes was downloaded from the SGN database and visualized using the online MG2C tool [39].

The sequences 2000bp upstream of the *NtbZIP* genes, presumed to be the promoter regions, were extracted from the genome database using TBtools software [40]. The obtained sequences were subjected to PlantCARE platform analysis to further search for the putative cis elements in the promoter regions [41].

2.4. Analysis of Duplication Events in Tobacco bZIP Genes

Tandem duplication events were identified as previously described [42]. Segmental duplications were identified using MCScanX according to previous reports and visualized in Circos [43].

The synteny relationships of the orthologous genes obtained from tobacco and five other plant species (*Arabidopsis*, tomato, grape, rice, and maize) were analyzed using the Synteny Plotter [44]. The non-synonymous (ka) and synonymous substitutions (ks) rates were calculated using DnaSP 5.0 [45].

2.5. Expression Profiling of Tobacco bZIP Genes

To determine the expression of the *NtbZIP* genes in different tissues, RNA-seq data for the *NtbZIP* genes were downloaded from the GEO database (accession number: GSE95717) [46]. The expression data for the root, whole shoot, and shoot apex were selected and were normalized and illustrated using R.

2.6. Tobacco Plant Preparation and Treatments

The tobacco plants used in this study (*Nicotiana tabacum* L. Cv. K326) were cultivated in a growth chamber for eight weeks under conditions described in previous reports at 25 °C [47]. For abiotic stress treatments, the seedlings were treated with 50 μM Abscisic Acid (ABA) or 150 mM NaCl, and then harvested at 0, 1, 3, or 6 h after treatment. Different tissues, including the shoots, shoot apices, roots, and leaves were used to explore tissue-specific expression patterns. In addition, flower tissue was collected at the full-bloom stage.

2.7. RNA Extraction and RT-qPCR Analysis

Total RNA was extracted from the test samples using the method of Cao et al. [43]. The transcription of the tobacco ribosomal protein gene *L25* (GenBank No. L18908) was used as the internal control. In the present study, three biological replicates were performed, and relative expression of the *bZIP* genes was evaluated by the $2^{-\Delta\Delta C_t}$ method [48]. The gene-specific primers used in the current study were designed in Primer Premier 5.0 according to the cDNA sequences; details of the primers are provided in Supplementary Table S1.

2.8. Analysis of *NtbZIP62*'s Subcellular Localization

The coding sequence of *NtbZIP62* excluding the stop codon was amplified by using cloning primers. Subsequently, infusion recombinase was used to insert the coding sequences into the pCAMBIA1300-GFP vector, to construct a *NtbZIP62*-GFP fusion gene driven by the CaMV-35S promoter. After transient expression in the leaves of *N. benthamiana* for 3 days, the GFP fluorescence signal was detected by using a confocal microscope with a 488 nm exciting light, and the nuclear localization signal was confirmed by DAPI (4,6-diamidino-2-phenylindole) staining, as previously reported [49,50].

2.9. *NtbZIP62* Overexpression Analysis

The coding sequence of *NtbZIP62* was amplified using cloning primers and then inserted into the expression vector (pCAMBIA1300). The empty vector and *NtbZIP62* overexpression vector were transformed into tobacco wild type plants (K326) by an *Agrobacterium*-mediated method [51]. T0-generation seeds were screened in half-strength MS medium with 20 mg/L of hygromycin to identify *NtbZIP62*-overexpressing plants. One-week-old T1 transgenic and WT tobacco plants were transferred to MS plates with or without 150 mM NaCl, and three weeks later, changes in root length were recorded. All the plants were grown under continuous light at 25 °C, and then, changes in root length were observed. Significant differences were determined in GraphPad Prism 5 with Student's *t*-tests.

3. Results

3.1. Identification and Filtration of *bZIP* Genes in Tobacco

In this study, the *Arabidopsis* *bZIP* proteins were used to identify potential *bZIP* genes in tobacco. A total of 132 *bZIP* genes were thereby identified after manually removing repeats and incomplete sequences. For the convenience of further research, we renamed the newly identified *bZIP* genes from *NtbZIP1* to *NtbZIP132* based on their physical locations on each chromosome/scaffold.

The biochemical characteristics of the *NtbZIP* genes were analyzed, including coding sequence length, protein molecular weight (MW), and isoelectric point (pI). As shown in Supplementary Table S2, the coding sequence lengths ranged from 339bp to 2922bp, while the protein molecular weights ranged from 12.92 to 104.65 kDa. The isoelectric points values of the different *NtbZIP* proteins varied greatly, with a range of 4.70–10.79.

3.2. Multiple Sequence Alignment and Phylogenetic Analysis of the *NtbZIP* Members

The DNA-binding basic regions of *bZIP* transcription factors are highly conserved. In this study, the DNA-binding basic regions of the tobacco *bZIP* members were aligned using Clustal X 2.0 and then visualized with R. These results reveal that the basic region sequences of *NtbZIP* members harbor conserved amino acid residues (Supplementary Figure S1A). In addition, the characteristics of the basic region sequences were found to be conserved between *Arabidopsis* and tobacco (Supplementary Figure S1A,B).

To analyze the phylogenetic relationships among the *bZIP* family members, MEGA X was used to construct a Neighbor-Joining (NJ) tree based on the sequence alignments of 132 tobacco *bZIP* members and their homologs in *Arabidopsis* and tomato. The 132 tobacco *bZIP* members could be clustered into 11 groups according to the classification results for *Arabidopsis* (Figure 1). Notably, most of the groups contained two or more potato *bZIP* members, indicating that the differentiation of tobacco and *Arabidopsis* was later than that of the *bZIP* gene family. Among the 11 groups, Groups D and K contained the largest (30) and smallest (2) numbers of genes, respectively. Interestingly, the tobacco *bZIP* member was not detected in Group J or M.

3.3. Analysis of Gene Structure and Conserved Motifs

The determination of gene structure is an effective way to analyze the evolution of genes in a species. The gene structures of the 132 newly identified tobacco *bZIP* genes were detected using the online GSDS tool (Figure 2). The results showed that the *NtbZIP* genes clustered into the same group generally have similar gene structures. Most of the coding sequences of the *NtbZIP* genes were interrupted by introns, and the number of exons varied from 1 to 13. Among the 132 *NtbZIP* genes, there are 27 intron-less genes, and eight genes (*NtbZIP17*, *NtbZIP39*, *NtbZIP42*, *NtbZIP45*, *NtbZIP55*, *NtbZIP98*, *NtbZIP100*, and *NtbZIP131*) that only contain one intron and mostly belong to Groups S and B. By contrast, more than 10 introns were found in 11 *NtbZIP* genes, which were clustered into Groups D and G.

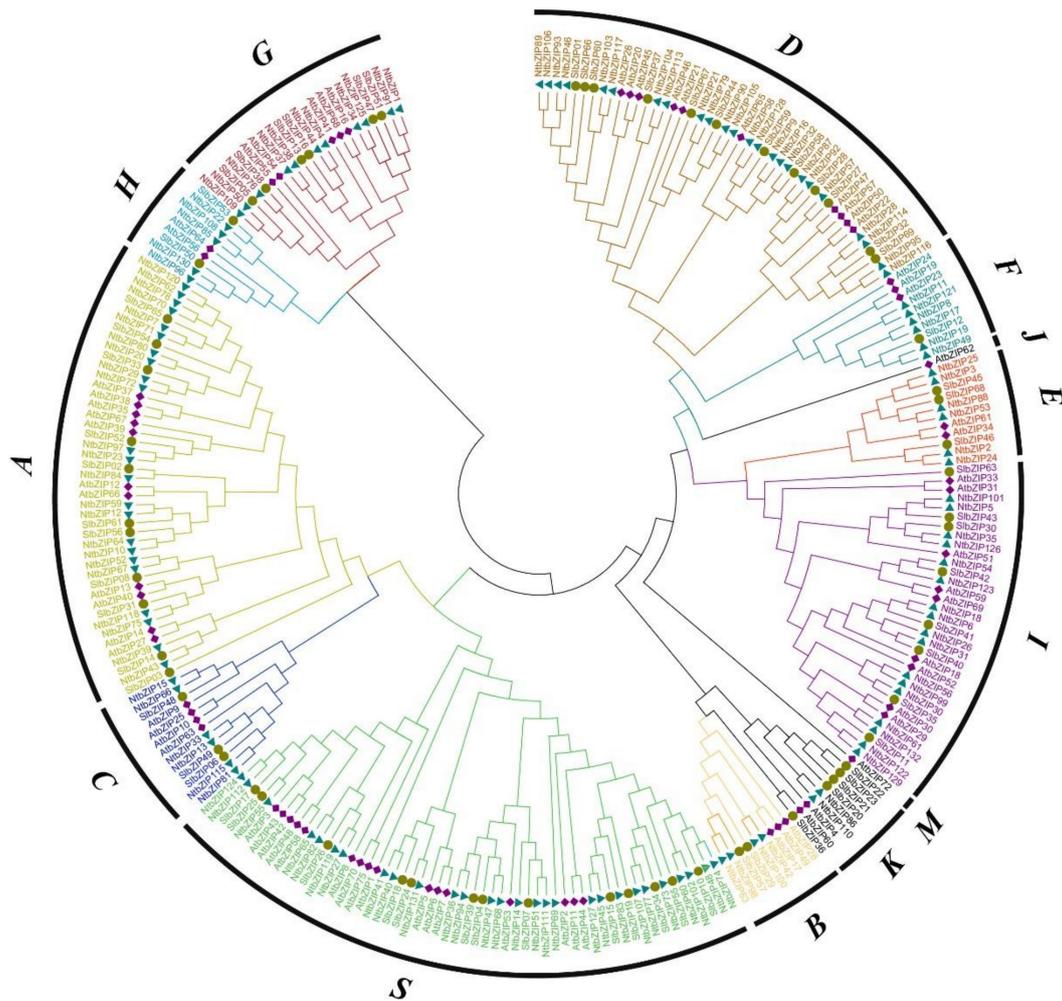


Figure 1. Phylogenetic analysis dendrogram of bZIP members in tobacco. The phylogenetic tree was constructed by bZIP proteins of tobacco, tomato and *Arabidopsis* with the Neighbor-Joining method.

The full-length sequences of the 132 *NtbZIP* genes were processed with the online MEME tool to detect conserved motifs. A total of 20 distinct motifs were identified and were named as Motif 1 to Motif 20 (Supplementary Figure S2). *NtbZIP* proteins in the same group usually have similar types and orders of motifs (Supplementary Figure S3). Moreover, some conserved motifs might exist only in specific groups. For example, Motifs 4 and 15 were found solely in Group D, while Motif 19 was only present in Group I with high specificity. The similarities in characteristic motifs in each group may reflect functional similarities and should be conducive to determining specific functions for each *bZIP* gene.

3.4. Syntenic Analysis of *NtbZIP* Genes

Syntenic analysis in species such as tobacco can help to elucidate genetic evolution. In the present study, we analyzed the syntenic relationship between the *NtbZIP* genes in tobacco and *bZIP* genes in five other species, including three dicotyledonous species (*Ara bidopsis*, tomato and grape) and two monocotyledonous species (maize and rice) (Figure 2). Collinear pairings between 73 of the *NtbZIP* genes with *bZIP* genes in tomatoes were identified, followed by 49 *NtbZIP* genes pairing with grape, 38 *NtbZIP* genes with *Arabidopsis*, and 11 and 7 *NtbZIP* genes with *bZIP* genes from maize and rice, respectively (Figure 2A). Notably, three *bZIP* genes were predicted to form collinear pairs with *bZIP* genes of all the other five species, which indicates that these genes may have existed before the divergence of these species (Figure 2B). Furthermore, 16 collinear *bZIP* gene pairings between tobacco and tomato/grape/*Arabidopsis* species were determined, but those collinear gene pairings were not detected in maize and rice, implying that these collinearities may

have arisen after the divergence of dicot and monocot species. Interestingly, eight *bZIP* genes in Arabidopsis were predicted to have collinear relationships with two or more tobacco genes, indicating that these *NtbZIP* genes may have arisen from duplication events. The details of the tobacco *bZIP* syntenic pairs can be found in Supplementary Table S3.

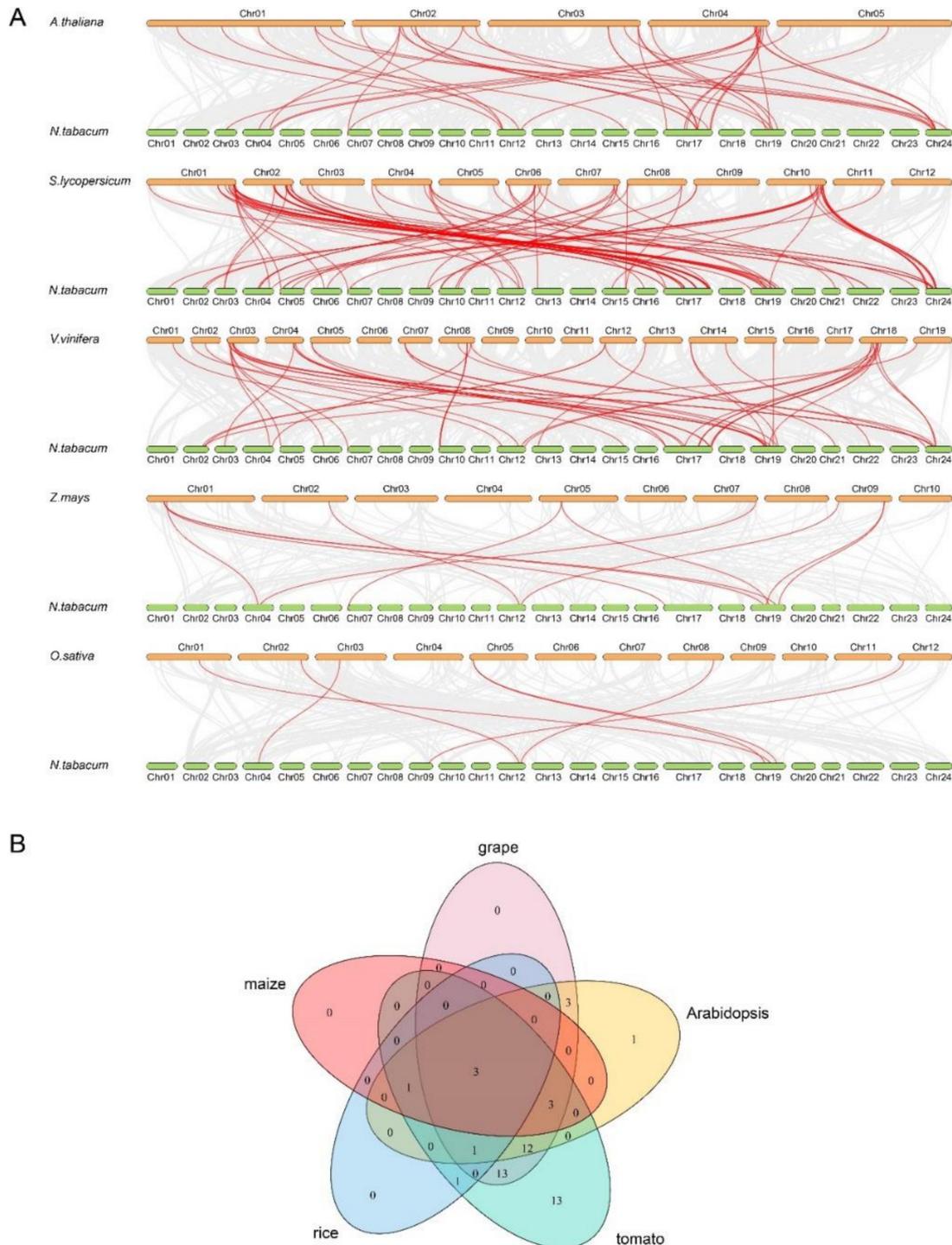


Figure 2. Syntenic relationship analysis between *bZIP* genes in tobacco and *bZIP* genes in five other species. (A) The collinear pairs between tobacco and five other species are represented by the gray line, while the collinear *bZIP* gene pairs are highlighted by the red color. (B) The *bZIP* genes formed the syntenic pairs between tobacco and all the other five selected species, visualized by R.

3.5. Chromosomal Distribution and Duplication Events

In this study, the chromosomal location information of 132 *NtbZIP* genes was obtained from the SGN database and visualized using the MG2C tool (Figure 3). Among the 132 genes, 65 were mapped to 20 chromosomes, and the remaining genes were located on scaffolds. Chromosome 17 harbored the most abundant *bZIP* genes of tobacco, while only one *bZIP* gene was located on chromosomes 1, 3, 7, 11, 13, 16, and 21. In the present study, the tobacco genome was carefully screened for tandem events; none were found to occur between the *bZIP* genes.

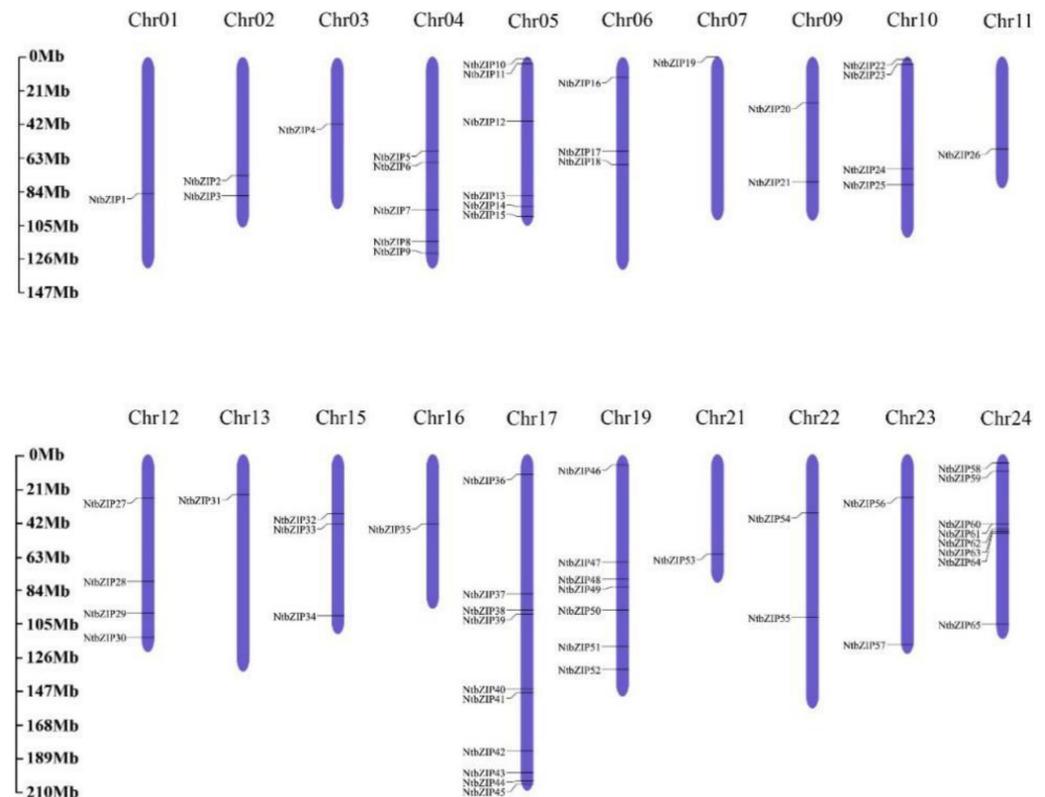


Figure 3. Distribution of 132 *bZIP* genes on the 20 chromosomes of tobacco.

Segmental duplication events for the *NtbZIP* genes were detected using the MCScanX tool. A total of 15 tobacco *bZIP* segmental duplication pairs were identified (Figure 4). Notably, 18.2% of the identified tobacco *bZIP* genes may have arisen through segmental duplication events, which have played important roles in the expansion of tobacco *bZIP* gene family. K_a/K_s is the ratio between non-synonymous and synonymous substitutions, which can be used to estimate purifying selection, neutral mutations, and beneficial mutations. The K_a/K_s ratios of 15 tobacco *bZIP* duplication gene pairs were calculated; the K_a/K_s ratios of the tested pairs were less than 1, suggesting that these *bZIP* genes may have undergone purifying selective pressure. The details of the segmental duplication genes are provided in Supplementary Table S4.

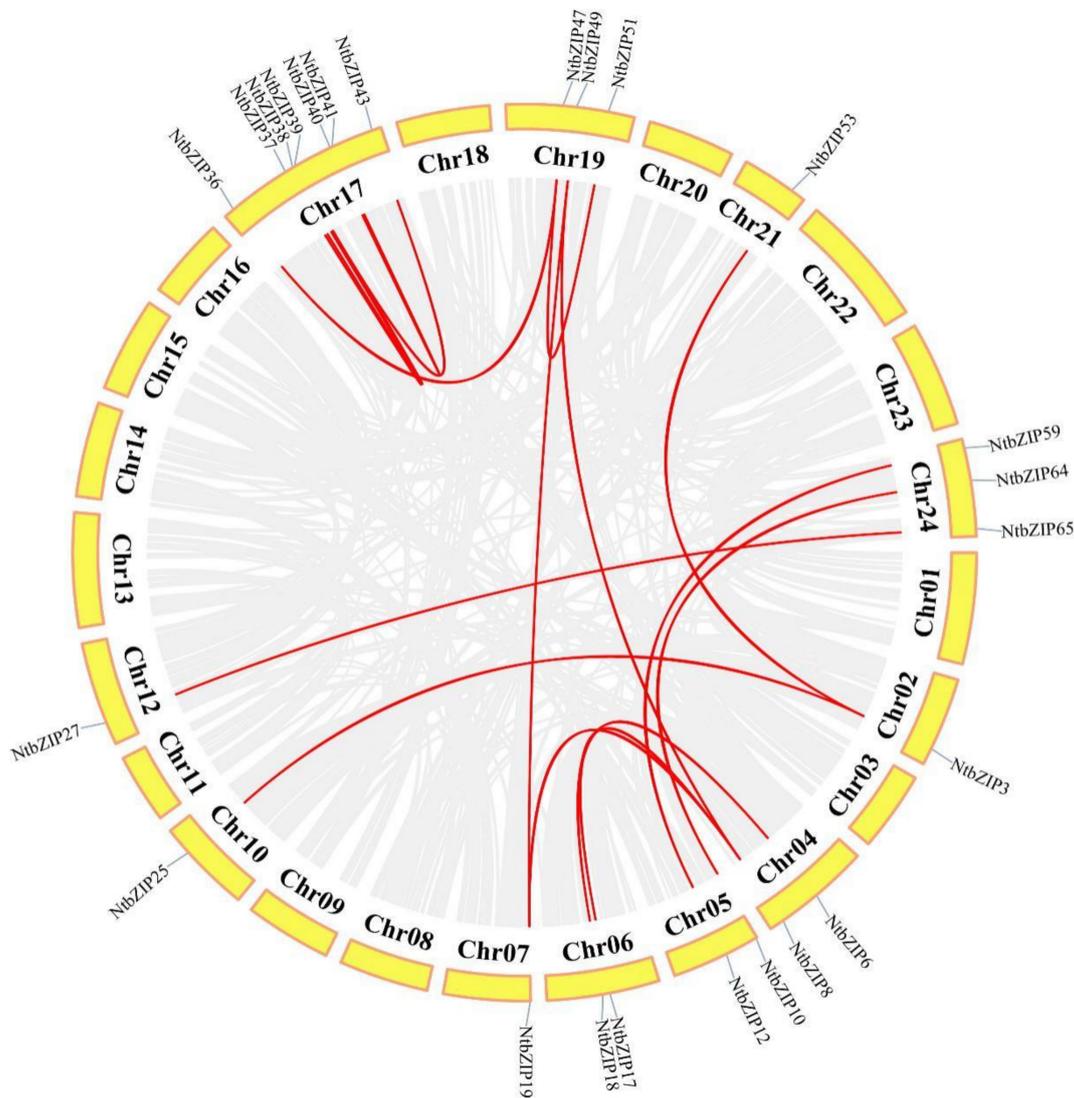


Figure 4. Segmental duplication analysis of the *NtbZIP* genes. The putative segmental duplication pairs in the tobacco genome are represented by the gray line, while the *NtbZIP* segmental duplication pairs are linked by the red line.

3.6. Analysis of *NtbZIP* Gene Promoters

To further explore the potential functions and regulation of the *NtbZIP* genes, *cis*-elements in the promoter regions of the 132 *NtbZIP* genes were analyzed (Figure 5). Many *cis*-elements related to stress responses were detected in the promoter regions of the genes, including W-box, MBS, TC-rich repeats, WUN-motif, LTR, and ARE, indicating that these genes might be involved in various stress responses. A number of hormone-response elements were also identified in promoter regions of *NtbZIP* genes, including ABRE, TCA-element, ERE, AuxRR-core, and the CGTCA-motif. In particular, the observation of the ABRE element in the promoter regions of more than 90 *NtbZIP* genes, suggests that those genes might be involved in ABA-mediated stress responses. Interestingly, only 13 *NtbZIP* promoter regions contained AuxRR-core: the respective genes might participate in auxin mediated plant development.

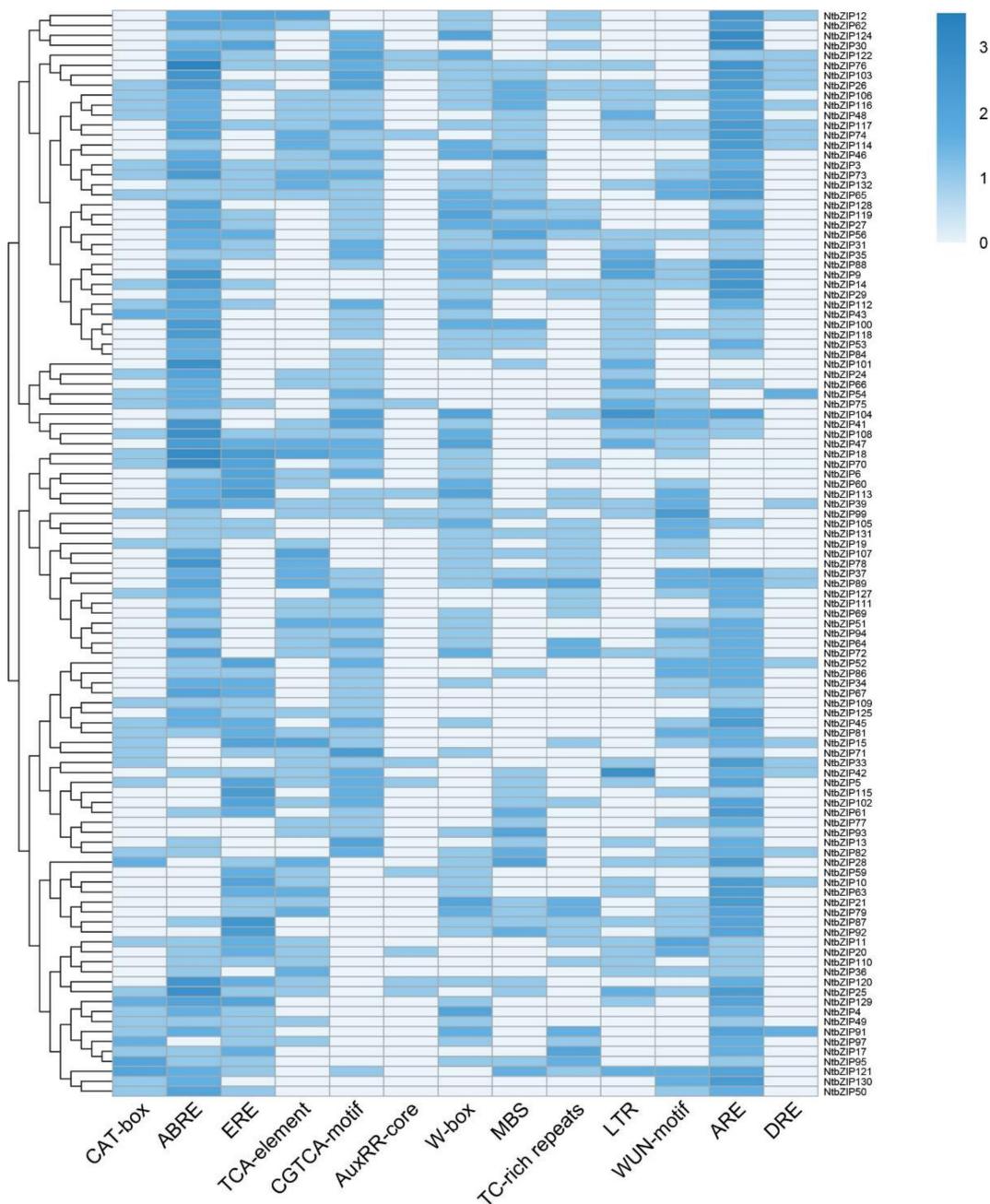


Figure 5. Statistics diagram of *cis*-elements in the promoter region of *NtbZIP* genes.

3.7. Gene Expression Profiles of *NtbZIP*s According to Transcription Data

To preliminarily elucidate the roles of *NtbZIP* genes in tobacco growth and development, the RNA-seq data for *bZIP* genes in three different tissues were downloaded from the NCBI database and analyzed (Figure 6). Among the 132 *NtbZIP* genes, 129 were detected to be expressed in at least one of the three tested tissues, and eight were highly expressed in all three tissues. In addition, many *NtbZIP* genes were expressed in a tissue-specific manner. For example, the expression of *NtbZIP45* and *NtbZIP127* in Group S was detected in the roots only. In Group G, *NtbZIP1*, *NtbZIP34*, and *NtbZIP125* were detected exclusively in the roots. Similarly, *NtbZIP2*, *NtbZIP3*, *NtbZIP25*, and *NtbZIP102* were detected exclusively in the shoot apex. High-level expression of *NtbZIP57*, *NtbZIP87*, *NtbZIP103*, *NtbZIP116*, and *NtbZIP117* was detected in roots and shoots.

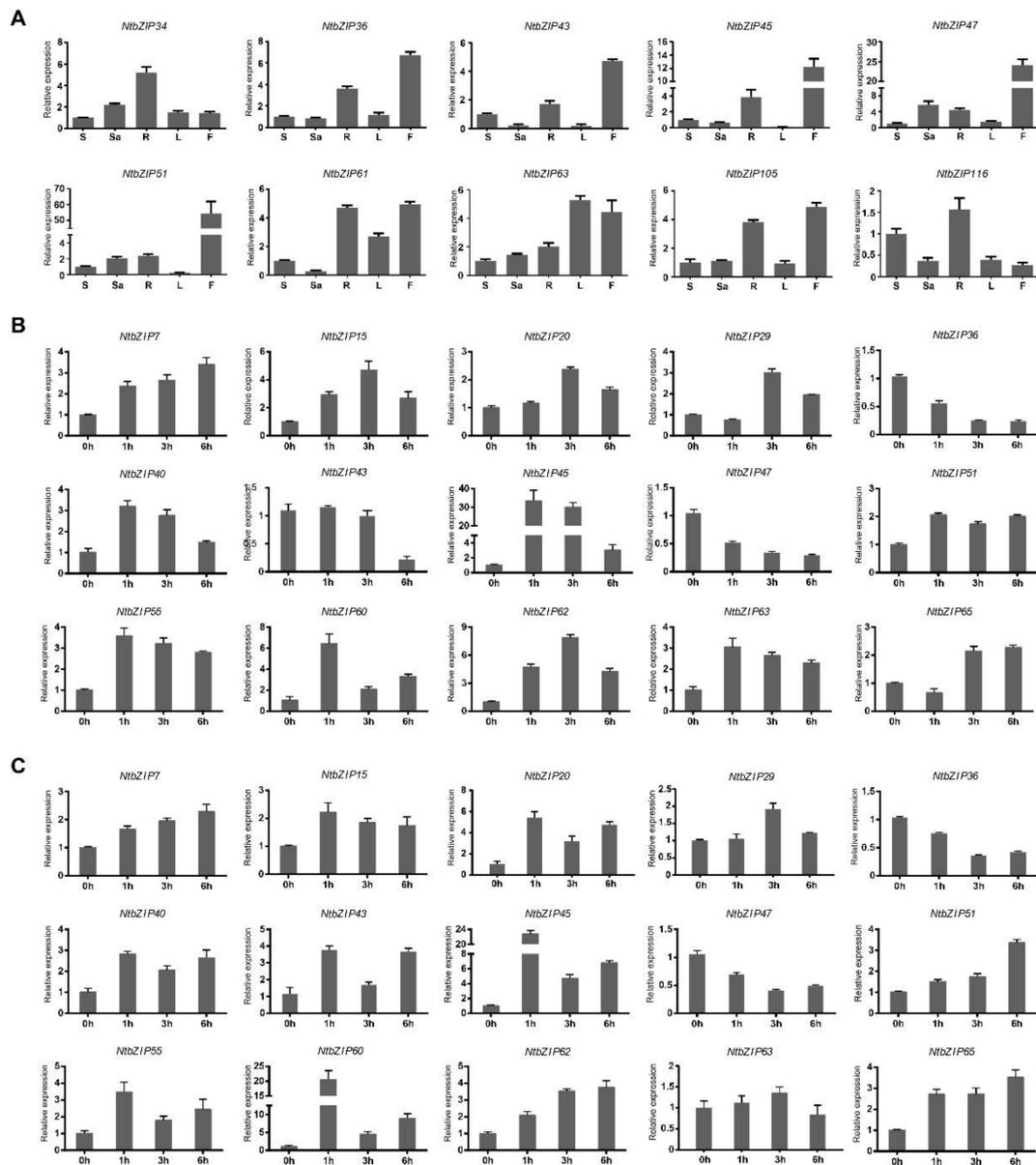


Figure 7. qRT-PCR analysis of representative *NtbZIP* genes from different groups. (A) To verify the tissue specificity expression of the representative *NtbZIP* genes, the expression level of each *NtbZIP* gene was calculated relative to the shoot. (B) The expression level of representative *NtbZIP* genes under salt stress treatments. (C) expression level of representative *NtbZIP* genes under Abscisic Acid (ABA) stress treatments.

3.9. Subcellular Localization Analysis

To further explore the potential functions of the *NtbZIP* genes, the stress-responsive gene *NtbZIP62* was selected for subcellular localization analysis (Figure 8). The gene's coding sequences were inserted into the pCAMBIA1300-GFP vector to analyze its subcellular localization, to provide a basis for revealing its potential function. The subcellular localization of *NtbZIP62* was detected by transient expression in *N.benthamiana* leaves. In the control, the green fluorescence signal was spread throughout the whole cell, while the signal of the GFP protein fused to *NtbZIP62* was only observed in the nucleus and overlapped with the signal of the nuclear-specific dye DAPI (Figure 8). The results indicated that *NtbZIP62* is a nuclear localization protein, which is consistent with its transcription factor characteristics.

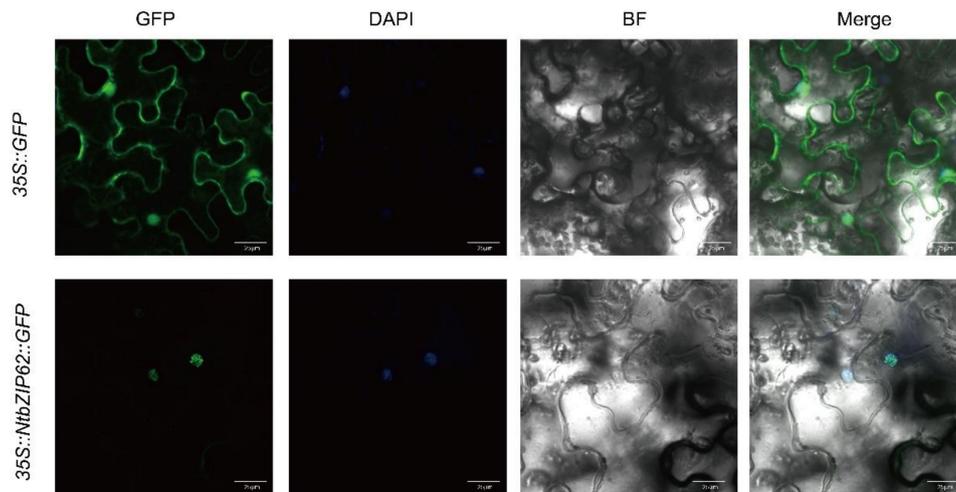


Figure 8. Subcellular localization of *NtbZIP62*. The coding sequence of *NtbZIP62* was inserted into pCAMBIA1300-GFP vector and transiently expressed in *N.benthamiana* leaves. The location of the nucleus was determined by 4,6-diamidino-2-phenylindole (DAPI) staining.

3.10. Overexpression of *NtbZIP62* Gene Enhanced Salt Tolerance in Tobacco

To analyze the function of the *NtbZIP62* gene in tobacco, the salt tolerance of wild-type and *NtbZIP62*-overexpressing tobacco were assessed via root elongation assay (Supplementary Figure S4, Figure 9). The WT and *NtbZIP62*-overexpressing tobacco plants showed no significant differences in root length under normal conditions. However, after three weeks of growing on 150 mM NaCl plates, the transgenic plants over-expressing *NtbZIP62* displayed longer root lengths than wild-type. Therefore, *NtbZIP62* was shown to confer enhanced salt-stress tolerance when overexpressed in tobacco.

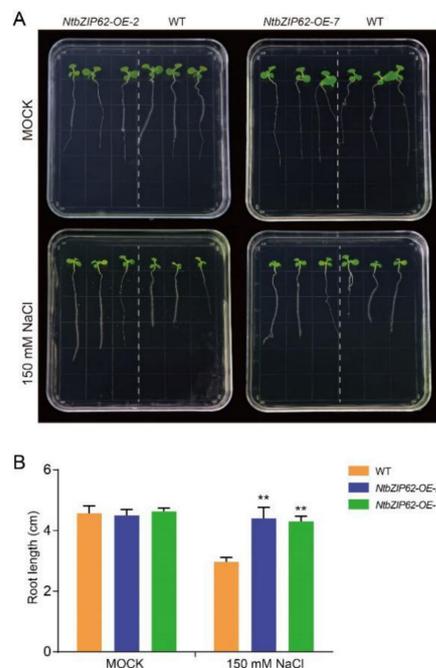


Figure 9. Effect of NaCl treatment on root growth of *NtbZIP62* transgenic lines. (A) Root growth of wild type and *NtbZIP62* overexpression lines under 0 or 150 mM NaCl treatment. (B) The quantification of primary root length on medium and the data were retrieved from more than 15 plants per genotype with three biological replicates. WT, wild type. Values represent means \pm SD. ** $p < 0.01$ (t -tests).

4. Discussion

Previous research proved that bZIP TFs have critical functions related to a plant's development and responses to diverse stresses [3]. The *bZIP* gene family has been identified in a number of plant species, including *Arabidopsis* [9], rice [52] and tomato [53]. However, there is relatively little available information regarding the functions of tobacco *bZIP* genes. In this study, a local BLASTP search was performed to identify the *bZIP* genes in the tobacco genome. Furthermore, the newly identified tobacco bZIP members were analyzed to determine their phylogenetic relationships, gene structures, motif organization, chromosomal distributions, duplication events, and expression profiles. In addition, *bZIP* genes homologous between *Arabidopsis* and tobacco were also studied to predict their potential functions.

A total of 132 *NtbZIP* genes were identified in tobacco genome by BLASTP searches and could be divided into 11 groups (Figure 1) [9,53]. Interestingly, the tobacco bZIP members were not detected in Groups J or M, implying that these *NtbZIP* members might have been lost during evolution (Figure 3). The results of the examination of gene structures and the conserved motifs in the *NtbZIP* genes were consistent with the findings of the phylogenetic analysis. Additionally, members in the same group were similar regarding gene structures and the numbers of exons and introns. The number of introns varied considerably among groups, from 0 to 13. The *NtbZIP* genes in Groups D and G have over six introns, similar to the *bZIP* genes in tomato [53] and potato [54].

The 132 *bZIP* genes identified in tobacco are significantly more than the number of *bZIP* genes in *Arabidopsis* (78) [9], potato (65) [54], cucumber (64) [55], and tomato (69) [53]. The expansion of the tobacco *bZIP* gene family may be attributable to whole-genome duplication events. Previous studies suggested that segmental duplications were more important than tandem duplications for the expansion of the *bZIP* gene family [56]. A total of 15 segmentally duplicated *NtbZIP* gene pairs were found in the tobacco *bZIP* gene family (Figure 4). The K_a/K_s ratios of all these duplicated pairs were lower than 1 (Supplementary Table S4), suggesting that these *bZIP* genes may have undergone purifying selective pressure. Notably, *NtbZIP47* and *NtbZIP51* arose from a duplicated gene pair, and both of these genes were found to exhibit similar expression patterns (Figure 7A). However, different *cis*-elements were predicted in the promoter regions of several duplicated tobacco *bZIP* gene pairs, such as *NtbZIP3/NtbZIP25*, which indicates diversity in their functions (Figure 5).

In *Arabidopsis*, several bZIP members function as regulators of plant development. Specifically, *AtbZIP29* (Group I) facilitates root and leaf development [18], whereas its tobacco homolog, *NtbZIP61*, is highly expressed in the roots and flowers, implying that *NtbZIP61* may modulate floral development (Figure 7A). In Group D, *AtbZIP46* is highly expressed in the floral meristem. A mutation in *AtbZIP46* in *Arabidopsis* increases the number of petals from four to five [15]. Its homolog in tobacco, *NtbZIP105*, is clustered with *AtbZIP46* (Figure 1), and is also highly expressed in flowers, suggesting that *NtbZIP105* may also affect floral development. Interestingly, *NtbZIP116* clusters together with *NtbZIP105*, which was highly expressed in roots, indicating possible functional divergence between these *bZIP* genes (Figure 7A). In plants, floral development influences seed formation, with implications for seed vitality. In Group S, *AtbZIP53* is reportedly involved in seed maturation [13]. The tobacco homologs, *NtbZIP34*, *NtbZIP47*, and *NtbZIP51*, are highly expressed in flowers (Figure 7A). *AtbZIP41* (Group G) helps to regulate root development [17], while its tobacco homolog, *NtbZIP34*, is highly expressed in the roots, implying that *AtbZIP41* and *NtbZIP34* may be functionally similar (Figure 7A).

Previous studies indicated that a number of *bZIP* genes encode important TFs mediating responses to abiotic and biotic stresses. In Group B, *AtbZIP17* expression increases the salt tolerance of *Arabidopsis* plants [26], and the expression of its tobacco homolog, *NtbZIP63*, is upregulated by salt treatments (Figure 7C). In Group S, *AtbZIP1*'s expression can be induced by several abiotic stresses, including salt, cold, and drought [28]. The overexpression of *AtbZIP1* in wild-type *Arabidopsis* improves tolerance to various stresses.

Similarly, the upregulation of *NtbZIP40*, which is closely related to *AtbZIP1*, enhances salt tolerance, suggesting that *NtbZIP40* may contribute to the salt-stress response of tobacco (Figure 7C).

In *Arabidopsis*, *AtbZIP36* and *AtbZIP37*, which belong to Group A, are reportedly involved in the signaling of the ABA synthesis pathway and response to abiotic stresses [24]. In plant tissues, *bZIP* gene expression can be induced by ABA via *cis*-elements in gene promoters, including the ABA response element (ABRE). The abiotic-stress-related elements are responsible for increasing gene expression following an exposure to cold, drought, or high salinity. Promoter analyses revealed that the *NtbZIP62* promoter region contains many ABREs (Figure 5). Additionally, *NtbZIP62* and *AtbZIP37* form a syntenic gene pair (Supplementary Table S3). Our qRT-PCR results indicate that *NtbZIP62* expression is significantly induced by ABA and salt treatments (Figure 7B,C), suggesting that *NtbZIP62* is involved in responses to environmental stresses. Furthermore, overexpression analyses further demonstrated that *NtbZIP62* can confer salt tolerance on transgenic tobacco plants (Figure 9). The *NtbZIP62*-GFP fusion protein was located in the nucleus, indicating that *NtbZIP62* acts as a transcription factor to regulate gene expression in response to stresses (Figure 8).

5. Conclusions

The systematic analysis of the tobacco genome in this study identified 132 *NtbZIP* genes, and a comprehensive analysis revealed that the tobacco *bZIP* gene family may play multiple roles in various biological processes. The *bZIP* members homologous between *Arabidopsis* and tobacco were found to be likely to play conserved roles in regulating plant development and stress responses. Notably, *NtbZIP62* was found to be induced by salt and ABA treatments. Furthermore, the overexpression of *NtbZIP62* in tobacco significantly enhanced the salt-stress tolerance of the transgenic plants. These results could provide insight for further studies on the biological functions of *NtbZIP* genes.

Supplementary Materials: Supplementary Materials can be found at <https://www.mdpi.com/2073-4395/11/1/148/s1>. Supplementary Table S1. The detailed information of qRT-PCR primers used in this study; Supplementary Table S2. The detailed information of identified *NtbZIP* members; Supplementary Table S3. Synteny blocks of *bZIP* genes between tobacco and other five plant species; Supplementary Table S4. The detailed information of segmental duplication gene pairs within tobacco genome; Supplementary Figure S1. Sequence logos of the basic region of *bZIP* members from tobacco (A) and *Arabidopsis* (B); Supplementary Figure S2. Detailed sequence information for each motif in *NtbZIP* proteins; Supplementary Figure S3. Conserved motifs and exon-intron structure organizations of *NtbZIP* members; Supplementary Figure S4. The expression level of *NtbZIP62* gene in wild type and two overexpression lines, the expression level of each genotype was calculated relative to the wild type.

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