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Systematic identification and functional analysis of potato (*Solanum tuberosum* L.) bZIP transcription factors and overexpression of potato bZIP transcription factor *StbZIP-65* enhances salt tolerance

Peng Zhao [a], Minghui Ye [a], Ruoqiu Wang [b], Dongdong Wang [a,*], Qin Chen [c,*]

[a] State Key Laboratory of Crop Stress Biology for Arid Areas, College of Agronomy, Northwest A&F University, Yangling, Shaanxi 712100, China
[b] Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, School of Life Sciences, Fudan University, Shanghai 200438, China
[c] College of Food Science and Engineering, Northwest A&F University, Yangling, Shaanxi 712100, China

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ABSTRACT

Basic leucine zipper (bZIP) transcription factors play important roles in numerous growth and developmental processes. Potato (*Solanum tuberosum* L.) is a worldwide important vegetable crop; nevertheless, no systematic identification or functional analysis of the potato *bZIP* gene family has been reported. In this research, 65 potato *bZIPs* distributed on 12 potato chromosomes were identified. According to the topology of Arabidopsis and potato bZIP phylogenetic tree, the bZIPs were classified into thirteen groups, designated as A-K, M, and S, with no potato bZIPs included in groups J and M. The bZIPs from the same group shared a conserved exon-intron structure, intron phase, and motif composition. Eighteen potato *bZIPs* were involved in segmental duplications, and the duplicated gene pairs were under purifying selection. No tandemly duplicated potato *bZIP* was found. Each potato *bZIP* promoter contained at least one kind of stress-responsive or stress-related hormone-responsive element. RNA-seq and qRT-PCR analyses revealed different expression patterns of potato *bZIPs* under abiotic stresses. The overexpression of *StbZIP-65* in Arabidopsis enhanced salt tolerance. The StbZIP-65 protein localized in the nucleus. β-Glucuronidase staining showed that promoter activity of *StbZIP-65* was induced by exogenous methyl jasmonate. These results may aid in further functional studies of potato bZIP transcription factors.

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1. Introduction

Transcription factors have vital functions during organism growth and development. Based on various eukaryotic genome sequences, transcription factors contribute 8% of the protein-encoding capacity [1]. In the model plant *Arabidopsis thaliana*, ~100 transcription factors have been defined [1]. The basic leucine zipper (bZIP) possesses numerous functions, for instance, light signaling, stress signaling, pathogen defense, seed maturation, and flower development [2]. The bZIP transcription factors are characterized by two regions, a basic DNA-binding region and a leucine-zipper region. The basic region usually contains ~16 amino acid residues, including a nuclear localization signal and a barely variant N-X_7-R/K motif (in which X represents any amino acid and the subscript indicates the quantity of amino acid). The leucine-zipper region comprises several heptad repeats of hydrophobic amino acids, such as leucine, isoleucine, valine, phenylalanine, and methionine. The first heptad repeat is located exactly 9 amino acids towards the R/K residue of the N-X_7-R/K motif. Anyhow, a typical consensus sequence of bZIP domain is N-X_7-R/K-X_9-L-X_6-L-X_6-L [2].

The basic region functions in DNA binding. To bind DNA sequence, two subunits adhere through van der Waals forces between the leucine residues or other hydrophobic residues, forming a coiled-coil structure, which is the so-called zipper [2]. The leucine-zipper region is responsible for forming homo- or heterodimers [3]. The flanking nucleotides of target DNA influence the binding specificity. In general, plant bZIPs bind to sequences with A-box (TACGTA), C-box (GACGTC), and G-box (CACGTG), which possess the ACGT core [4]. Interestingly, these binding sites are palindromic sequences. However, non-palindromic binding sites also exist. For instance, RSG specifically binds to the *rbe* sequence (TCC-AGCTTGA) [5].

To date, numerous functional data have been collected on plant bZIPs. The functional data shows that bZIPs participate in various processes and pathways during plant growth, development, reproduction and pathogen defense. Under energy-limiting conditions, S1-bZIPs promote survival by coordinating the expression of branched-chain amino acid catabolism relating genes to activate a mitochondrial respiratory pathway [6]. A low ratio of "red light:far red light" perception in the shoot reduces the lateral root emergence, which is modified by

* Corresponding authors.
E-mail addresses: dongdong-1025@hotmail.com (D. Wang), chenpeter2289@nwafu.edu.cn (Q. Chen).

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phytochrome-dependent accumulation of ELONGATED HYPOCOTYL5 in the lateral root primordia [7].

Both *in vitro* and *in vivo* analyses have shown that T-DNA-insertion mutants of *AtbZIP34* reduce pollen germination efficiencies and pollen tube growth rates, which indicates the function of AtbZIP34 in male reproductive development [8]. The bZIP transcription factor NPR1, which is regulated by posttranslational modifications, including sumoylation and phosphorylation, to keep it stable and quiescent, is a major regulator of basal and systemic acquired resistance in plants [9]. Additionally, plant bZIPs function in various abiotic stress responses. As a stress sensor, AtbZIP17 enhances salt tolerance in Arabidopsis when expressed by the stress-inducible promoter RD29A [10]. The overexpression of *bZIP16* in rice (*Oryza sativa*) significantly improves drought resistance at seedling and tillering stages [11]. Increased sensitivities to cold and drought occur in *OsbZIP52*-overexpressing rice plants, which indicates its potential as a negative regulator [12]. Constitutively expressing the tomato (*Solanum lycopersicum*) SlAREB in both Arabidopsis and tomato increases drought and salt tolerance [13].

As the most important non-grain food crop, the potato (*Solanum tuberosum* L.) is vital to worldwide food security [14]. Owing to its important roles in numerous physiological pathways, several studies on the function of bZIP have been performed in potato. Compared to the non-transgenic potato, the overexpression of a pepper bZIP-like transcription factor in potato improves drought tolerance and yield [15]. StbZIP61 regulates the dynamic biosynthesis of salicylic acid to contribute to immunity to *Phytophthora infestans* infection in potato [16]. StABF1 is phosphorylated during abscisic acid treatments and salt stress and is induced to express under ABA, drought, salt, and cold stresses or during tuberization [17]. With the improvement of sequencing technology, more plant genomes are being sequenced. Therefore, the *bZIP* gene family has been systematically identified in numerous plants, for instance, Arabidopsis [18], rice [19], maize [20], wheat [21], and tomato [22]. However, no systematic identification and analyses of potato bZIP transcription factors has been reported. Here, we focused on identification of potato bZIP family members and analyzed gene structures, motif distributions, phylogenetic relationships, *cis*-acting element composition and expression patterns of the identified potato bZIP transcription factors.

2. Materials and methods

2.1. Identification of potato bZIP family members

The potato representative protein sequences were downloaded from the Spud DB (http://solanaceae.plantbiology.msu.edu/index.shtml). We downloaded the Hidden Markov Model profiles of bZIP domains (PF00170 and PF07716) from Pfam [23], and searched in potato representative protein sequences using HMMER software [24]. The output putative bZIP protein sequences were checked by the Conserved Domain Database [25] and SMART [26] to confirm the presence of bZIP domains. Only protein sequences containing a complete bZIP domain were preserved. The preserved sequences were named based on their locations on pseudomolecules.

2.2. Sequence features and structural characterization

Every potato bZIP protein sequence was uploaded to ExPASy [27] to compute the amino acid quantity, molecular weight, and theoretical isoelectric point. The conserved motifs in bZIP proteins were analyzed using the MEME SUITE [28] in following parameters: any number of repetitions and optimum motif width from 10 to 100 amino acid residues. Motifs with e-values <1E-20 were retained for further analysis. The genomic and coding sequences of potato *bZIP* genes were submitted to Gene Structure Display Server [29] to show the exon–intron structures.

2.3. Chromosomal locations and gene duplications of potato bZIPs

The chromosomal location of each potato *bZIP* was retrieved from the Spud DB. Then, the physical map was generated using MapChart [30]. Two genes located within a <100-kb region and separated by five or fewer genes were considered as tandemly duplicated genes [31]. Segmentally duplicated *bZIP* genes were confirmed by Plant Genome Duplication Database [32], and the Ka and Ks values of segmentally duplicated genes were accessed in the same database. The segmental duplication diagram was generated using TBtools (https://github.com/CJ-Chen/TBtools).

2.4. Phylogenetic analysis and cis-acting element predictions

Protein sequences of bZIPs from Arabidopsis [18] and potato were used for a phylogenetic analysis. The protein sequences were aligned by MAFFT [33]. The unrooted phylogenetic trees were generated using MEGA X [34] with neighbor-joining method. The bootstrap test was performed 1000 times. To predict the *cis*-acting element composition, the 2-kb upstream sequence of each potato *bZIP* from the initiation codon ATG was submitted to PlantCARE [35]. The results were visualized using TBtools.

2.5. RNA-seq analysis of potato bZIP genes under abiotic stresses

The RNA-seq data of potato *bZIP* genes under control, heat, salt, drought, and wounding treatments were acquired from Spud DB. The RNA-seq data are presented as fragments per kilobase of exon model per million mapped fragments (FPKM) values. The *bZIPs* with FPKM values <2 under all of the treatments were considered to be non-expressed and were excluded from the analysis. The FPKM value of each *bZIP* gene under a stress treatment was divided by the FPKM value of the corresponding control. Then, the resulting data were normalized and clustered using MeV [36] to generate a heatmap.

2.6. Plant materials cultivation and treatments

The doubled monoploid *S. tuberosum* DM1–3 were cultured on solid Murashige and Skoog (MS) medium at 22 ± 1 °C. The photoperiod is 16-h light/8-h dark. Four-week old plantlets were transferred into cuvettes filled with 1/2 liquid MS medium. The plantlets grew in a growth chamber under the same temperature and photoperiod for a week. Then, heat, NaCl, and mannitol treatments were applied to the potato plantlets as described above [31]. Briefly, heat stress was carried out at 35 °C, and concentrations of NaCl and mannitol were 0.15 M and 0.26 M, respectively. After being stressed for 0, 3, and 24 h, the potato plantlets were sampled, and stored at −80 °C.

After surface sterilization, seeds of wild type (Col-0) and the homozygous T2 generation of overexpressing transgenic Arabidopsis were sown on 1/2 MS medium. The medium was kept at 4 °C for 2 d before transferred into a growth chamber. Two days after the seeds germinated, the seedlings were transplanted onto 1/2 MS medium (1.5% sucrose) with 125 mM NaCl. After another 7 d, primary root lengths were measured, and images were taken. Three replicates were conducted, and each replicate contained at least 15 seedlings.

2.7. Total RNA extraction and expression analysis of potato bZIPs

Total RNA was extracted by RNAsimple Total RNA Kit (BioTeke, Beijing, China). cDNA was synthesized by a 5× All-In-One RT MasterMix with an AccuRT Genomic DNA Removal Kit (Applied Biological Materials, Vancouver, Canada). All operation procedures followed the manufactures' protocols. The acquired cDNA was diluted 10-fold.

Quantitative real-time PCR (qRT-PCR) was carried out on a Bio-Rad real time PCR system. The reaction mixture preparation and procedure followed the same with we reported before [37]. Both biological and

technical replicates were repeated 3 times. We used potato *ef1α* as the internal reference gene. All the primers (Table S1) were designed using Primer-Premier 5 (Premier Biosoft Interpairs, Palo Alto, CA, USA), and were specificity checked by Primer-BLAST. The relative expression levels of potato *bZIPs* were calculated in $2^{-\Delta\Delta Ct}$ method.

2.8. Plasmid construction and genetic transformation

The full-length coding sequence of *StbZIP-65* was PCR amplified from the cDNA of DM1–3 using primers StbZIP-65-F and StbZIP-65-R with *Sal*I and *Sac*I restriction sites at the 5′ and 3′ ends of the amplified fragment, respectively. The amplified fragment was digested with *Sal*I/*Sac*I and inserted into the same sites of the pCambia1300 vector. The inserted sequence was driven by a cauliflower mosaic virus (CaMV) 35S promoter.

The 2154-bp upstream sequence from the ATG of *StbZIP-65* was PCR amplified from the genomic DNA of DM1–3 using primers StbZIP-65_pro-F and StbZIP65_pro-R. The *Pst*I restriction site plus a 14-bp upstream sequence and the *BamH*I restriction sites plus a 14-bp downstream sequence from the corresponding restriction sites of pCambia2300 were added to 5′ and 3′ ends of the amplified fragment, respectively. The pCambia2300 vector had been modified previously to contain the β-glucuronidase (*GUS*) gene. The amplified fragment was purified and inserted into the same site of the pCambia2300 vector using a seamless cloning kit (Biomed, Beijing, China).

The two resulting constructs, 35S::StbZIP-65 and StbZIP-65_promoter::GUS, were independently transferred into *Agrobacterium tumefaciens* strain GV3101. Wild type Arabidopsis (Col-0) was infected independently with *A. tumefaciens* contained the resulting constructs in floral-dipping method [38]. Homozygous T3 generation seedlings were used in the following analyses. The primer sequences could be found in Table S1.

2.9. GUS staining

Seeds of T2 generation homozygous transgenic Arabidopsis containing the *GUS* reporter gene were surface sterilized and sown on 1/2 MS. After vernalization for 2 days at 4 °C, the seeds grew under normal conditions in a growth chamber. Ten-day-old Arabidopsis was transferred onto 1/2 MS containing 50 μM methyl jasmonate (MeJA) or 150 mM NaCl. Afterwards, whole seedlings were sampled at 0 h and 16 h during the MeJA treatment and 0, 3, and 24 h during the salt treatment. The samples were incubated in the GUS staining buffer at 37 °C in darkness for 16 h. Then, the samples were decolored in 70% ethanol. The decolored samples were photographed using a stereomicroscope (Olympus, Japan). The GUS staining buffer contained 50 mM sodium phosphate buffer (pH 7.0), 10 mM $Na_2EDTA{\cdot}2H_2O$, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]\cdot 3H_2O$, and 0.1% Triton-100.

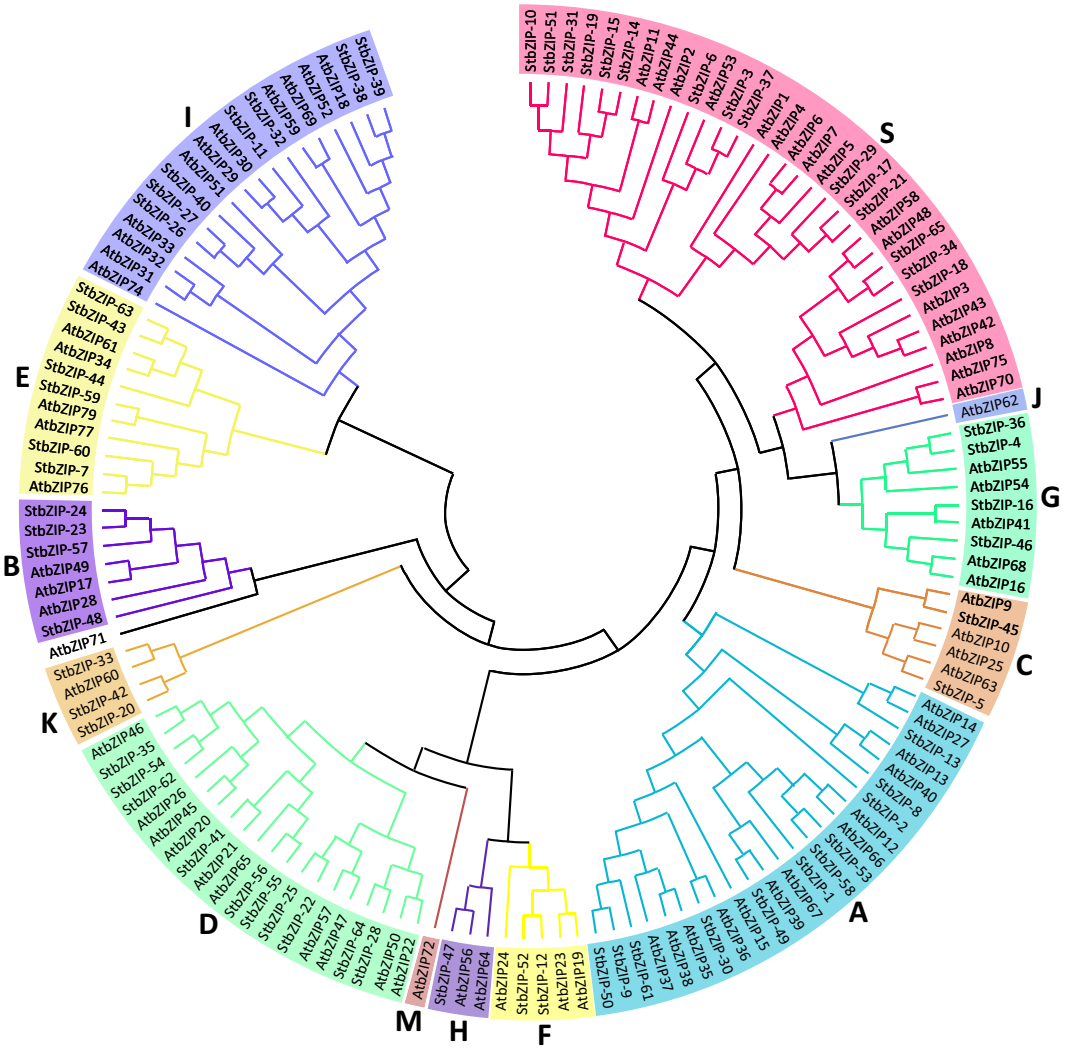


Fig. 1. The unrooted phylogenetic tree of Arabidopsis and potato bZIP proteins.

2.10. Subcellular localization of the StbZIP-65 protein

The control vector is a previously modified pCambia1300::35S::GFP vector. The coding sequences of *StbZIP-65* without the "TGA" stop codon and *GFP* gene were amplified from cDNA of DM1–3 and the plasmid, respectively. The *Xba*I restriction site plus a 15-bp upstream sequence from the same restriction site of pCambia1300 and a 20-bp coding sequence of GFP beginning from the "ATG" start codon were added to the 5'and 3′ ends of the amplified fragment, respectively. The last 21 bp of the coding sequence of *StbZIP-65* without the TGA stop codon and the *Sac*I restriction site plus a 15-bp downstream sequence of the same restriction site of pCambia1300 were added to the 5′ and 3′ ends of the amplified fragment, respectively. The two fragments were purified separately and were inserted into the same sites of the modified pCambia1300 containing the CaMV 35S promoter using a seamless cloning kit. The two constructs, pCambia1300::35S::GFP and pCambia1300::35S::StbZIP-65::GFP, were independently transferred into *A. tumefaciens* strain GV3101. According to the reported protocol [39], *A. tumefaciens* were infiltrated into tobacco (*Nicotiana benthamiana* L.) leaves. After incubation in a growth chamber for 48 h, the injected leaves were stained with 10 μg/ml DAPI solution. Then, a laser scanning confocal microscope (Olympus, Japan) were used to observe the DAPI and GFP fluorescence signals.

3. Results

3.1. Identification and characterization of potato bZIPs

We used the Hidden Markov Models of bZIP (PF00170 and PF07716) on Pfam as query to search the potato representative protein dataset downloaded from Spud DB. After removing the redundant sequences and the sequences containing an incomplete or no typical bZIP domain, 65 potato bZIP transcription factors were identified. The corresponding potato *bZIP* genes were designated as *StbZIP-1–65* based on their positions on pseudomolecules. In addition, two *bZIP* genes, *StABF1* (GenBank: HM988989) [17] and *StbZIP61* (XP_006348282) [16], have been reported previously in potato. However, using sequence alignments, neither of these *bZIP* genes was consistent with any gene in

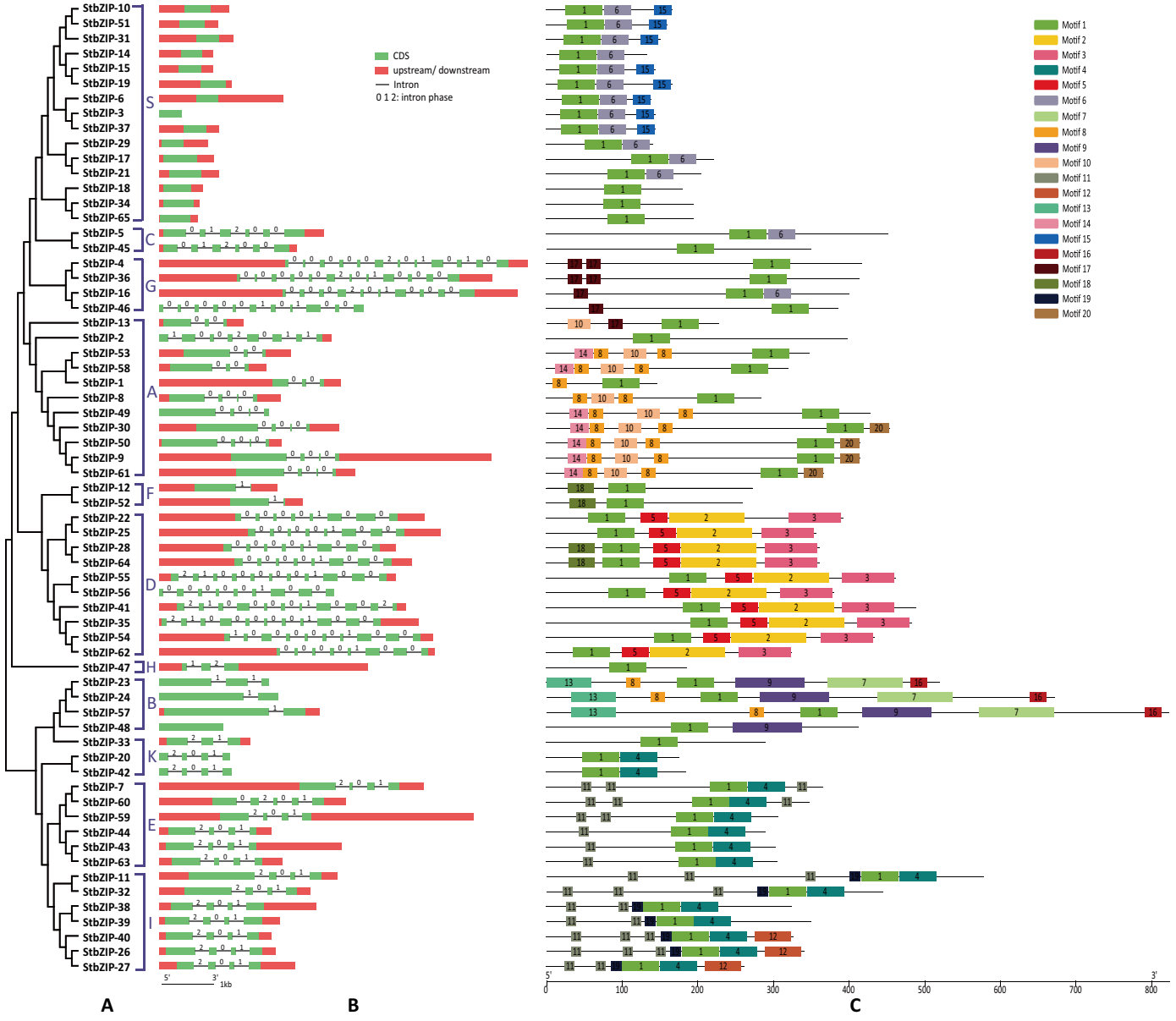


Fig. 2. Phylogenetic relationships, gene structures and conserved motifs of StbZIP transcription factors. (A) Phylogenetic tree of 65 StbZIPs. (B) Exon-intron organization of *StbZIP*s. (C) Conserved motif distributions of StbZIP transcription factors.

Fig. 3. Seqlogo of conserved motif 1 in potato bZIP proteins. The highly conserved amino acid sites in the typical bZIP domain feature are indicated by asterisks.

Spud DB (Fig. S1 and Fig. S2). Because the two *bZIP* genes were cloned from potato cultivars Spunta and Atlantic, and not DM1–3, which was used in our research, the results are reasonable. Of the 65 *bZIP* genes, 64 *bZIP* genes were located on 12 potato chromosomes, while one *bZIP* gene (*StbZIP-65*) could not be assigned to any potato chromosome. The isoelectric point range of StbZIPs range was 5.02–9.71, and their molecular weights varied from 15.4 to 73.4 kDa (Table S2).

3.2. Phylogenetic analysis of potato bZIPs

To explore the evolution of bZIP transcription factors, a phylogenetic tree were generated including 77 Arabidopsis bZIPs [18] and 65 potato bZIPs. The bZIP domain of AtbZIP78 (AT4G06598) was identified as being incomplete according to the Conserved Domain Database; therefore, it was not included in the phylogenetic tree. Based on the topology of phylogenetic tree and classification of Arabidopsis bZIPs [18], thirteen groups (designated as A-K, M, and S) were classified (Fig. 1). Groups J and M contained no potato bZIPs. The largest cluster, Group S, containing 17 Arabidopsis and 15 potato bZIPs. In general, the number of potato bZIPs was less than that of Arabidopsis bZIPs in most groups (Group A, C, F–J, M, and S). After excluding AtbZIP78, which was originally classified into group E, the numbers of potato and Arabidopsis bZIPs in groups E and D were equal. However, both groups B and K contained more potato than Arabidopsis bZIPs. According to a previous report, the bZIPs of groups B (AtbZIP17 and AtbZIP28) and K (AtbZIP60) act as significant regulators in responding to endoplasmic reticulum (ER)-stress [40]. Misfolded proteins accumulate in the ER under adverse environmental conditions and activate the ER-stress response. Then, the ER-stress response activates a cell survival response, autophagy, during a mild stress or leads to cell death during a severe stress. Thus, increasing the numbers of members of bZIP groups B and K in potato may facilitate the tolerance or resistance to severe environmental conditions.

3.3. Gene structure and conserved motif analyses

To further research the phylogenetic relationships among potato bZIP transcription factors, an unrooted phylogenetic tree only including potato bZIPs was generated (Fig. 2A), and the exon-intron structure

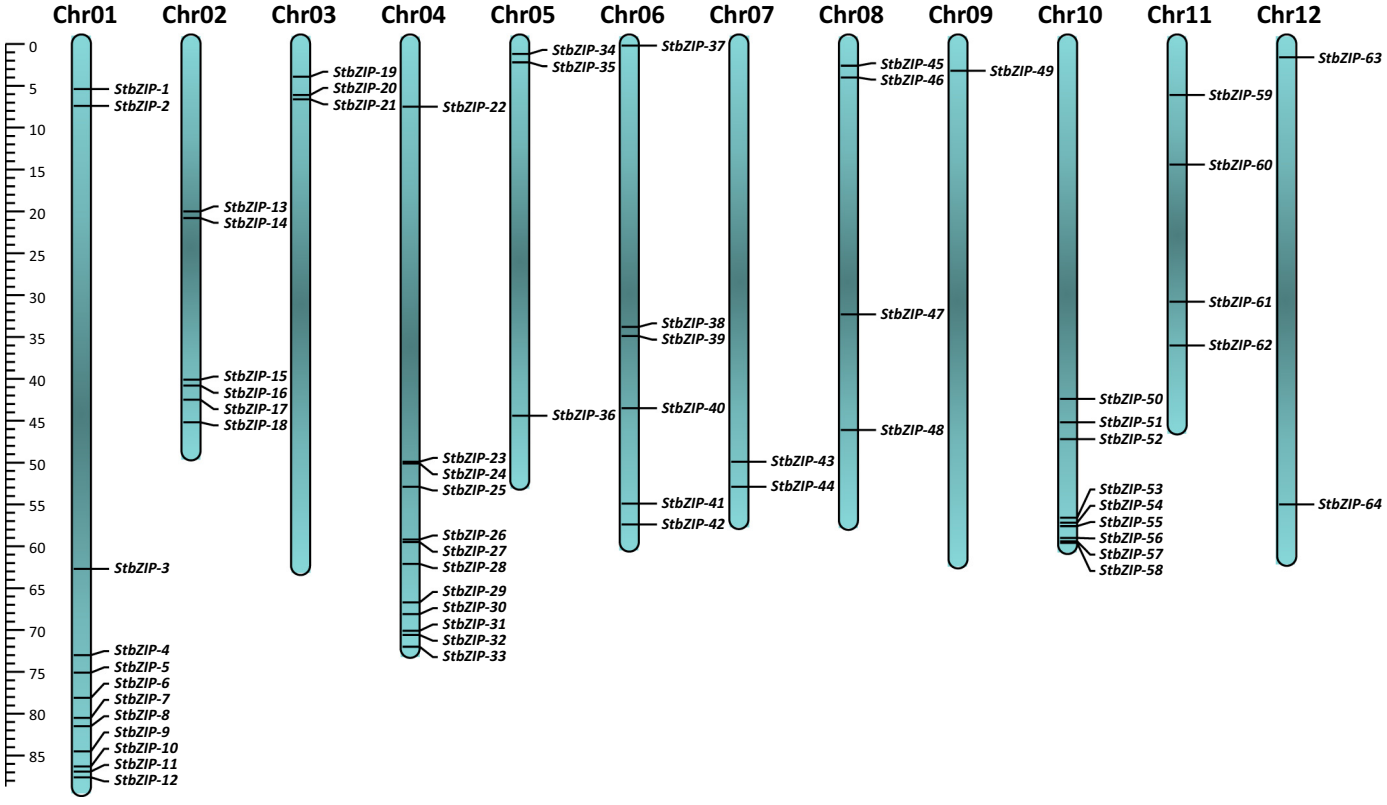


Fig. 4. Locations of potato *bZIP* genes on 12 chromosomes. The chromosome lengths and gene locations could be inferred from the scale on the left.

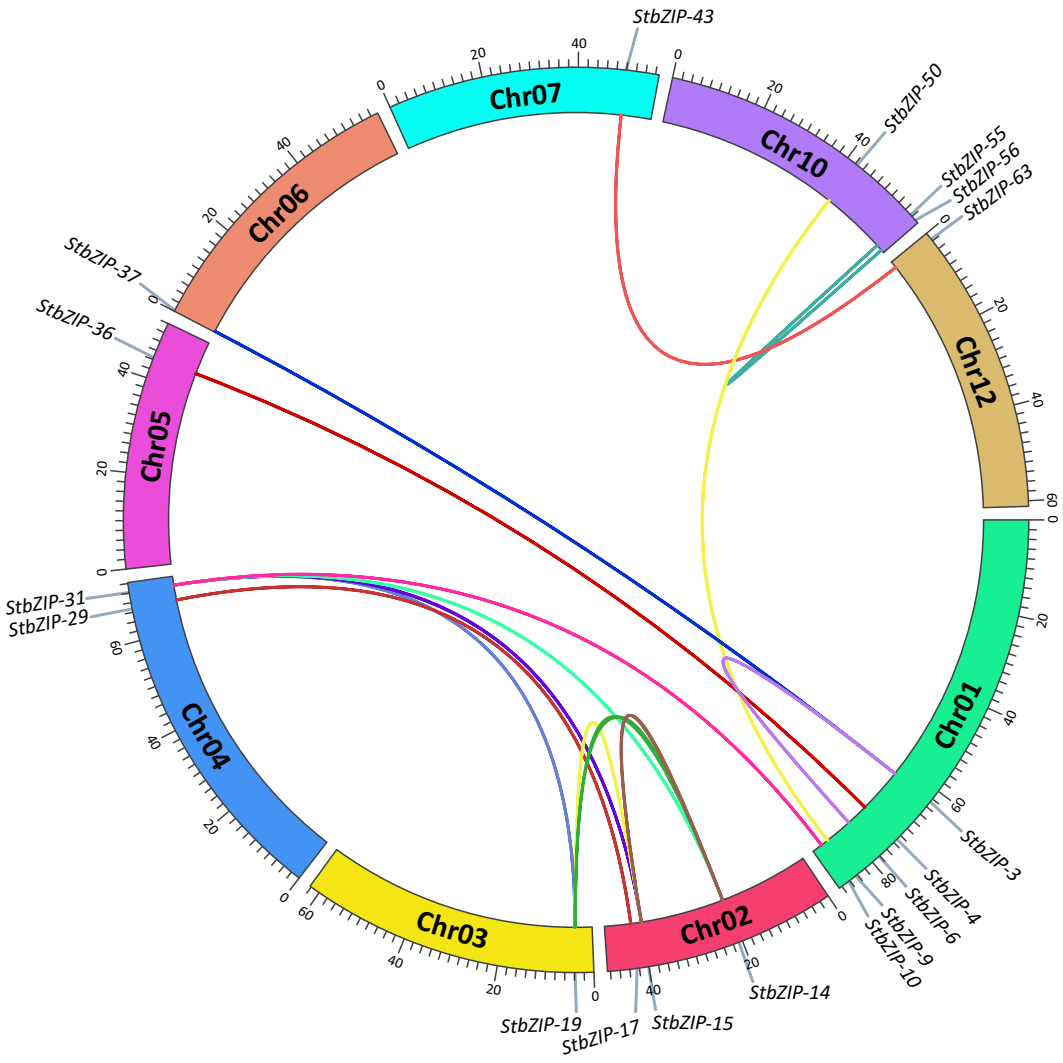


Fig. 5. Segmentally duplicated potato *bZIP* genes. Duplicated genes were linked by lines in the same color.

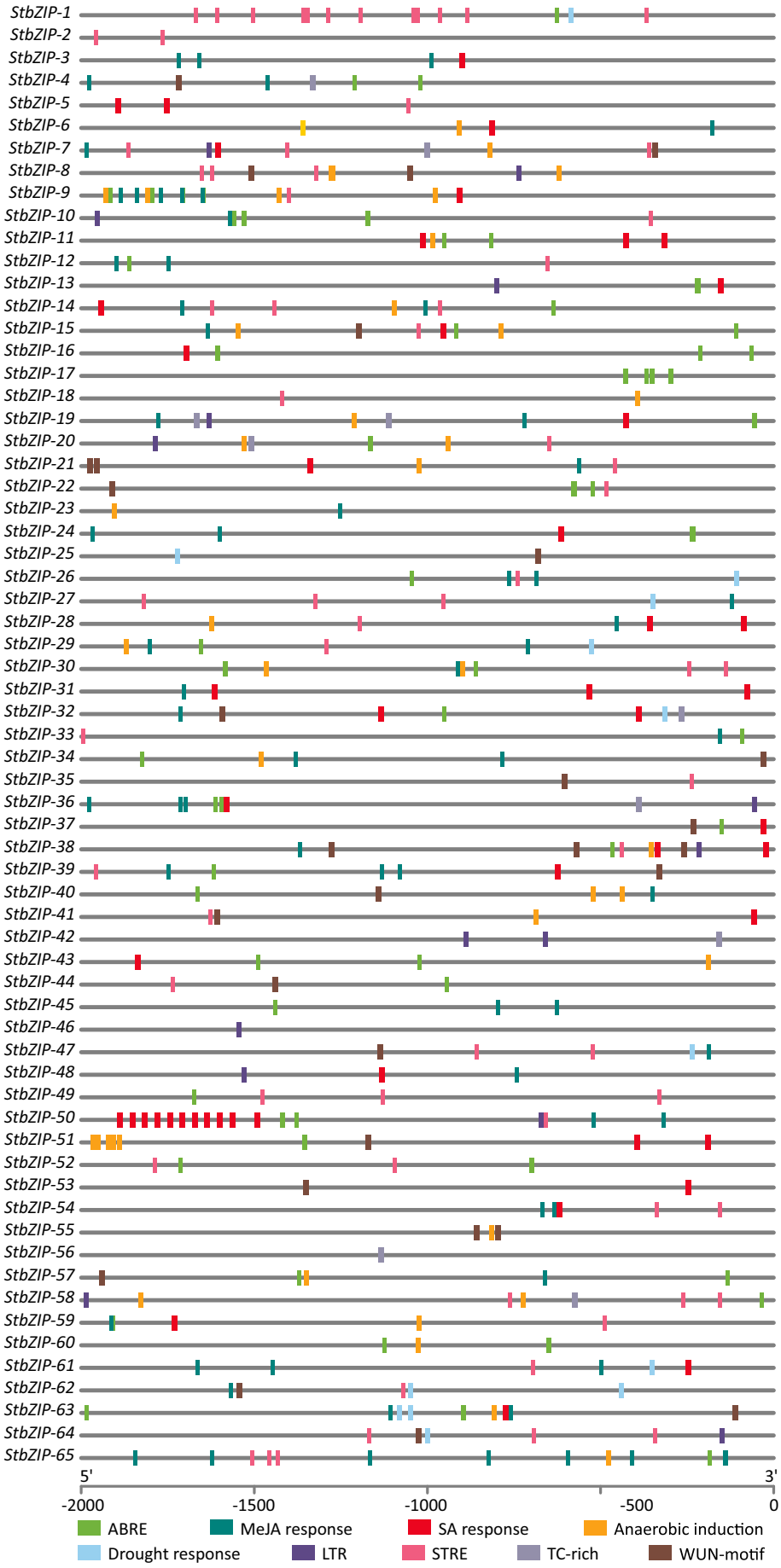
(Fig. 2B) of potato *bZIP* genes and distributions of conserved domains (Fig. 2C) in potato bZIP proteins were analyzed. The phylogenetic tree of potato bZIPs showed a highly similar topology with the phylogenetic tree that included both Arabidopsis and potato bZIPs. The potato bZIPs were classified and named as before. Most potato *bZIP* genes (75.4%) possessed at least one intron. All the members of group S were intronless. Groups D and G *bZIPs* comprised multiple introns, varying from 7 to 11. Although the exon-intron structures of *bZIPs* from different groups varied greatly, *bZIPs* from the same group generally possessed a conserved exon-intron structure. In those *bZIPs* with introns, the intron phase was also analyzed. The intron phases of *bZIPs* in the same group were uniform in general, while they differed greatly among *bZIPs* from different groups, showing a similar pattern similar to the exon–intron structure.

In total, 20 conserved motifs (Table S3) were identified in 65 potato bZIP proteins. Motif 1 existed in every potato bZIP, while the other motifs existed in several potato bZIPs. Thus, we inferred that the bZIP domain is highly conserved, while the other regions of bZIP transcription factors are variable. The motif 1 sequence alignment in Pfam indicated that it was the typical bZIP domain. The seqlogo of motif 1 revealed its amino acid composition and the conserved amino acids (Fig. 3). Potato bZIPs from the same group shared a similar composition and conserved domain distribution, but these differed vastly among the different groups. The analogous composition and distribution patterns of exon–intron structure, intron phase, and conserved domain supported phylogenetic relationship and classification of the potato bZIP transcription factors.

3.4. Chromosomal positions and duplications of potato bZIP genes

The physical map showed that potato *bZIP* genes were distributed among the 12 potato chromosomes (Fig. 4). Both chromosomes 1 and 4 contained 12 *bZIP* genes, while chromosome 9 only contained 1 *bZIP* gene. Gene duplication, such as tandem and segmental duplication, is important to large gene family evolution [41]. Based on the criteria, no tandem duplication events were found among the potato *bZIP* genes. However, 18 potato *bZIP* genes were involved in segmental duplication events (Fig. 5). The segmentally duplicated *bZIP* genes accounted for 27.7% of the total potato *bZIP* genes. Furthermore, of the 18 segmentally duplicated genes, 10 *bZIP* genes belonged to the largest potato *bZIP*

Fig. 6. *cis*-Acting element analysis of potato *bZIP* gene promoters. The relative position of each *cis*-acting element to the translation start site coud be figured out from the scale. ABRE: abscisic acid response; MeJA response: methyl jasmonate response; SA response: salicylic acid response; Anaerobic induction: *cis*-acting element essential for the anaerobic induction; Drought response: dehydration-responsive elements; LTR: low-temperature response; STRE: stress response; TC-rich: defense and stress responsive *cis*-acting element; WUN-motif: wounding response.


StbZIP-1
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StbZIP-3
StbZIP-4
StbZIP-5
StbZIP-6
StbZIP-7
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StbZIP-58
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StbZIP-62
StbZIP-63
StbZIP-64
StbZIP-65
5'
3'
-2000
-1500
-1000
-500
0
ABRE
MeJA response
SA response
Anaerobic induction
Drought response
LTR
STRE
TC-rich
WUN-motif

group, group S. The results suggested that segmental duplication played an important role in potato *bZIP* family expansion. Moreover, Ka/Ks ratios of the duplicated *bZIP* paralogs were <1, except for the duplicated paralogs of *StbZIP-6* and *StbZIP-3*, for which Ka/Ks values could not be estimated owing to a pipeline failure (Table S4). The results revealed that the duplicated potato *bZIPs* went through purifying selection.

3.5. cis-Acting element analysis of potato bZIP gene promoters

In plant responses and adaptions to environmental stresses, both physiological and molecular processes play important roles. The expressions of numerous plant genes are altered under abiotic stresses [42]. As important molecular switches, *cis*-acting elements contribute to transcriptional regulation of dynamic gene networks that respond to different abiotic stresses [43]. Thus, to explore potato *bZIP* gene function under abiotic stress, several *cis*-acting elements involved in stress and hormone response were identified in 65 potato *bZIP* gene promoters. The *cis*-acting elements are involved in anaerobic induction and responses to drought, low temperature, wound (Wun-motif), defense and stress (TC-rich), stress, ABA, MeJA, and salicylic acid. As showed in Fig. 6, at least one *cis*-acting element was identified in every potato *bZIP* gene promoter. In addition, in some *bZIP* gene promoters, there was more than one of the same *cis*-acting element. According to a previous report, the expression of an Arabidopsis dehydration-responsive gene, *rd29B*, requires two ABA-responsive *cis*-acting elements [44]. Thus, the potato *bZIP* genes with more than one of the same *cis*-acting element may be regulated in the same manner.

3.6. Expression of potato bZIPs under different abiotic stresses

To reveal potato *bZIPs* expression patterns under abiotic stresses, the corresponding RNA-seq data after heat, salt, drought, and wounding stresses (Table S5) were accessed in the Spud DB. Using the criteria described in the methods, nine potato *bZIP* genes were eliminated from the analysis owing to their low FPKM values. These nine *bZIP* genes may not respond to abiotic stress. The other potato *bZIP* genes showed several different expression patterns (Fig. 7). Some genes, such as *StbZIP-9*, *−47*, and *− 61*, were slightly upregulated under the four stress conditions. Most potato *bZIPs* showed conflicting expression patterns under abiotic stresses. For instance, *StbZIP-52* was insensitive to wounding stress. However, it was upregulated under drought and salt stresses, but downregulated under heat stress. In addition, some genes showed no obvious expression changes in responding to abiotic stresses, such as *StbZIP-57*. The various expression patterns reflected the different roles of potato *bZIP* genes in abiotic stress-response pathways.

3.7. The qRT-PCR analysis of potato bZIP genes

To further confirm the expression patterns of potato *bZIPs* under abiotic stress, several stress sensitive *bZIPs* were analyzed using qRT-PCR. The relative expression levels are represented by fold change in Fig. 8. The results were similar to RNA-seq results displayed in the heatmap (Fig. 7). Additionally, the potato *bZIP* genes showed different short- and long-term stress-response expression patterns. For example, after heat stressed for 3 h, the expression levels of *StbZIP-10* and *StbZIP-41* increased. As the stress continued, the expression of *StbZIP-10* increased but that of *StbZIP-41* decreased. In addition, *StbZIP-65* was insensitive to short-term salt stress but was up-regulated during a long-term salt stress. The results revealed the different response mechanisms of potato *bZIPs* under abiotic stress.

3.8. Overexpression of StbZIP-65 in Arabidopsis

To investigate the function of StbZIP-65, the *StbZIP-65* driven by a CaMV 35S promoter was transferred into Arabidopsis (Col-0). Five

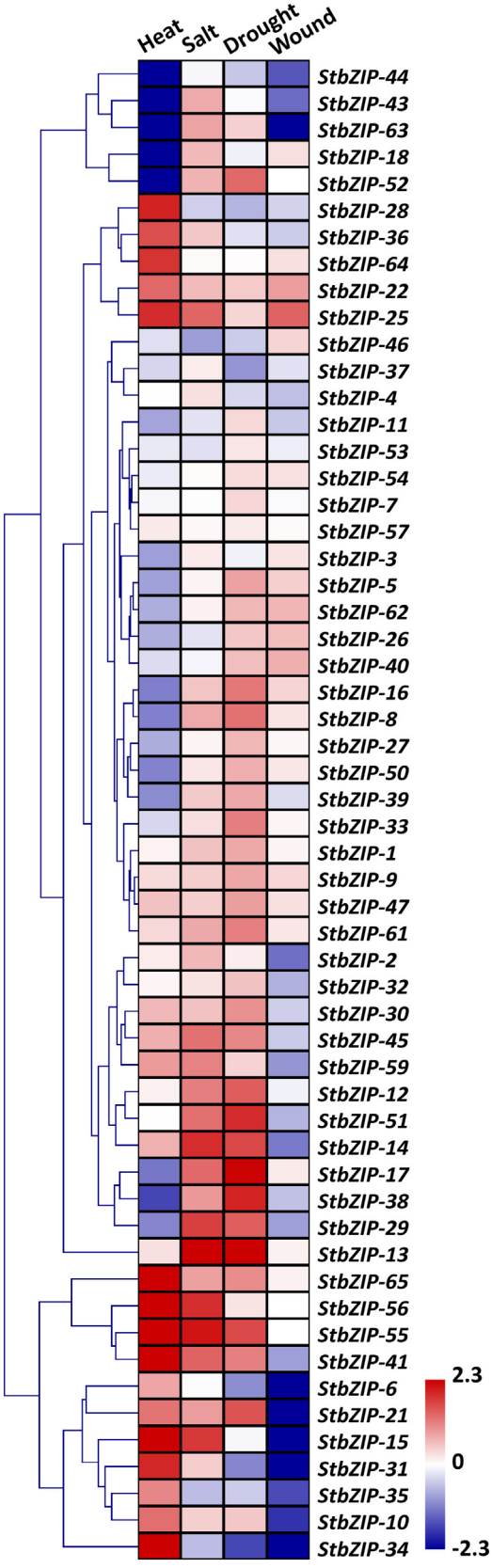

Fig. 7. Heatmap of potato *bZIP* genes under abiotic stresses. The FPKM values of each *bZIP* gene were normalized and clustered using MeV.

independent stable homozygous lines were acquired and were confirmed using semi-qRT-PCR (Fig. 9A). OE-2 and OE-4 lines were selected for functional identification. For the salt-tolerance analysis, 2-d-old

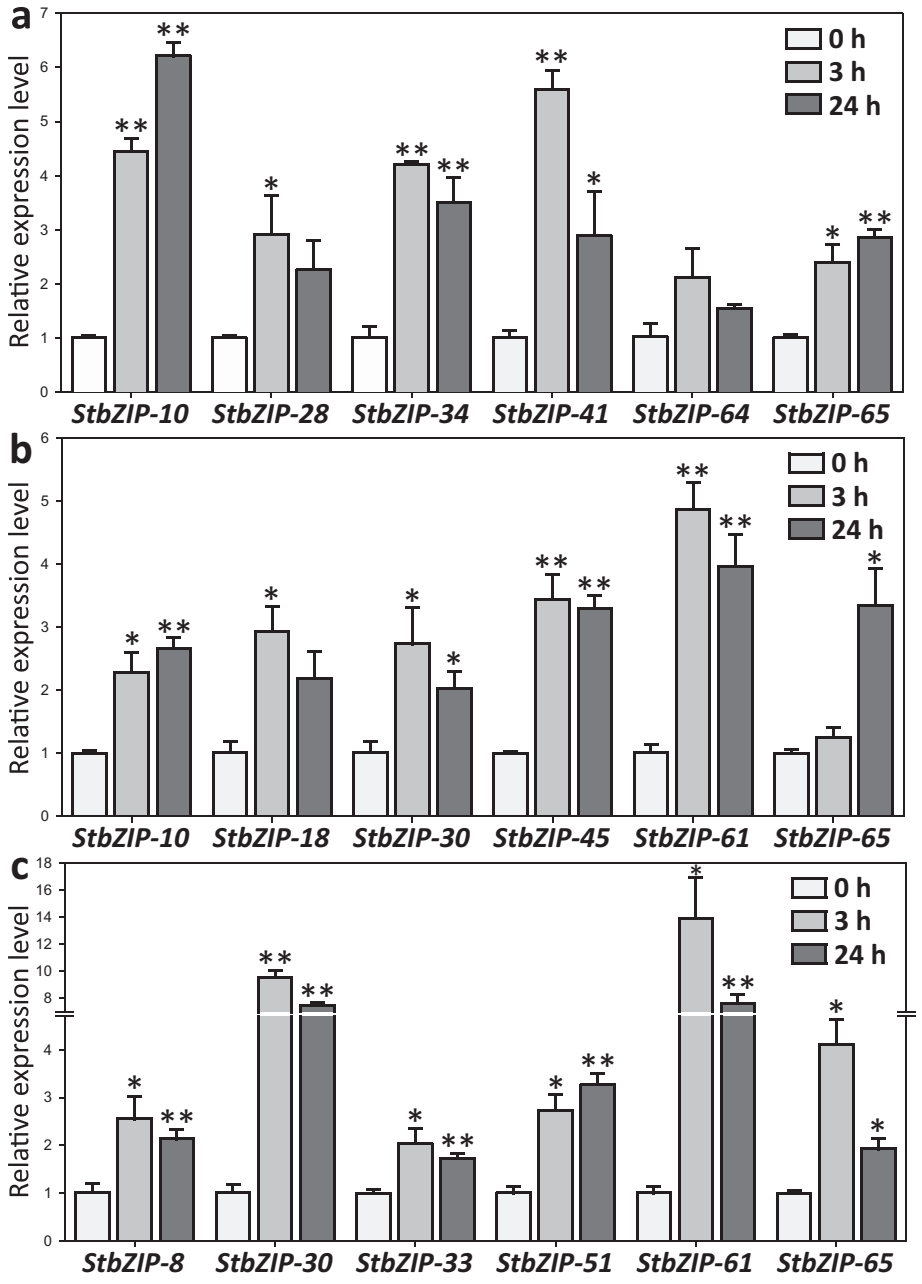

Fig. 8. Relative expression level of potato *bZIPs* under heat, salt, and drought stress. (a) heat stress; (b) salt stress; (c) drought stress. Each treatment contains 3 biological replicates. The results were shown as means ± standard deviations. The statistical significance of differences in target genes relative expression was analyzed between treated groups (3 h, 24 h) and control group (0 h) in Student's *t*-test method. *: $P < 0.05$; **: $P < 0.01$.

Arabidopsis were transferred onto 1/2 MS (1.5% sucrose) containing 125 mM NaCl. Wild type and transgenic Arabidopsis showed no growth difference under normal conditions. However, when exposed to salt stress, transgenic Arabidopsis showed longer primary root length than wild type (Fig. 9B-C). Transgenic Arabidopsis grew stronger than wild type (Fig. 9D-E). The results revealed that overexpression of *StbZIP-65* improved salt tolerance in Arabidopsis.

3.9. Subcellular localization and promoter activity of StbZIP-65

The StbZIP-65::GFP fusion protein was observed in tobacco epidermal cells to reveal the subcellular localization of StbZIP-65. The nuclei were visualized using DAPI staining. The GFP signals were observed in nuclei exclusively and coincided with the DAPI signals (Fig. 10). The results indicated that StbZIP-65 localized in the nucleus.

A *cis*-acting element analysis showed that multiple MeJA-response elements exist in the *StbZIP-65* promoter sequence, indicating that the expression of *StbZIP-65* may be regulated by MeJA signals. To verify the promoter activity of *StbZIP-65*, a 2154-bp upstream sequence from the ATG of *StbZIP-65* was cloned into pCambia2300 to drive the previously introduced *GUS* gene. The construct was transformed into Arabidopsis (Col-0), and T3 generation homozygous lines were acquired. Wild type and T3 transgenic Arabidopsis were germinated on 1/2 MS medium. After germinating for 10 d, the seedlings were transferred onto 1/2 MS medium containing 50 μM MeJA or 150 mM NaCl. The GUS staining results are shown in Fig. 11. GUS staining was not observed in wild type Arabidopsis (Fig. 11A). Under normal growth, the *StbZIP-65* promoter activity was low, and the staining only occurred in leaves (Fig. 11C). When treated with 50 μM MeJA for 16 h, the *StbZIP-65* promoter activity increased, and the staining could be observed in

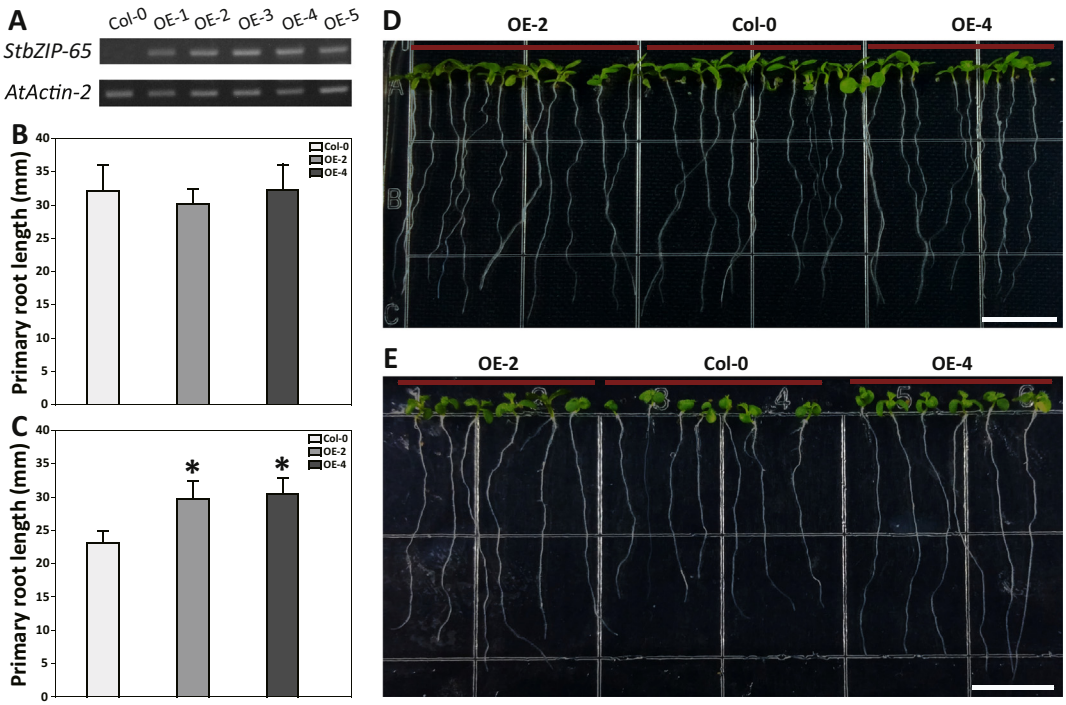

Fig. 9. Overexpression of *StbZIP-65* in Arabidopsis enhances tolerance and primary root length. (A) Identification of wild type and transgenic Arabidopsis by semi-quantitative RT-PCR. (B and D) Primary root length of wild type and overexpression Arabidopsis after germinated for 9 days on 1/2 MS (1.5% sucrose). Bar = 10 mm. (C and E) Primary root length of wild type and overexpression Arabidopsis under 125 mM NaCl treatment. *: $P < .05$, Student's *t*-test. Bar = 10 mm.

both leaves and roots (Fig. 11B). Compared with transgenic Arabidopsis under control treatment, the GUS staining became darker in leaves and roots after treating with 150 mM NaCl (Fig. 11D-E). Furthermore, after exposure to salt stress, the GUS staining was darker after 24 h than after 3 h, which was consistent with the expression pattern of *StbZIP-65* identified by qRT-PCR.

4. Discussion

In current study, 65 potato *bZIP* genes were identified. The potato genome is ~844 Mb [14], while the Arabidopsis genome size is only ~125 Mb [45]. Although the potato genome is much larger than Arabidopsis genome, there are less potato *bZIP* genes than Arabidopsis *bZIP* genes (78 genes) [18]. Similar results were also found in tomato (69 genes) [22], cucumber (64 genes) [46], grapevine (55 genes) [47], and castor bean (49 genes) [48]. Previous studies found that monocots usually possess more *bZIP* genes than dicots, and it was inferred that they evolved after monocots diverged from dicots [22,48,49]. In the current study, according to a phylogenetic analysis between Arabidopsis and potato, the potato bZIP family lacked J and M groups (Fig. 1). In addition, Arabidopsis bZIP J and M groups each only contained one member. Based on reported research, several Arabidopsis bZIP transcription factors function in a redundant manner, such as bZIP19 and bZIP23 [50] of group F, and ABF1, ABF2, ABF3, and ABF4 [51] of group A.

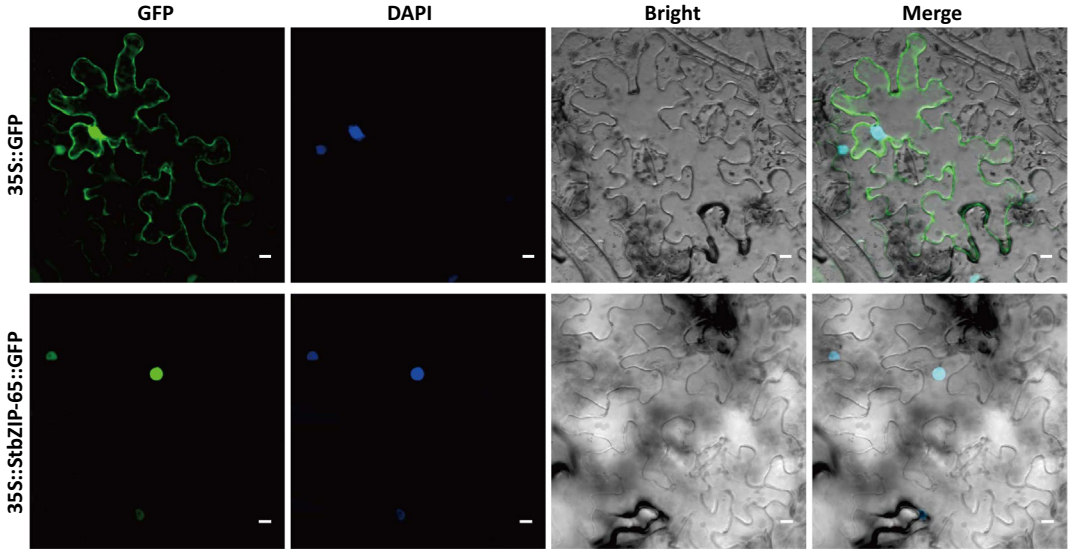

Fig. 10. Subcellular location of StbZIP-65 protein. Transient expression of GFP and StbZIP-65::GFP fusion protein was observed in tobacco epidermal cells at 48 h after *A. tumefaciens* infiltration. Bar = 10 μm.

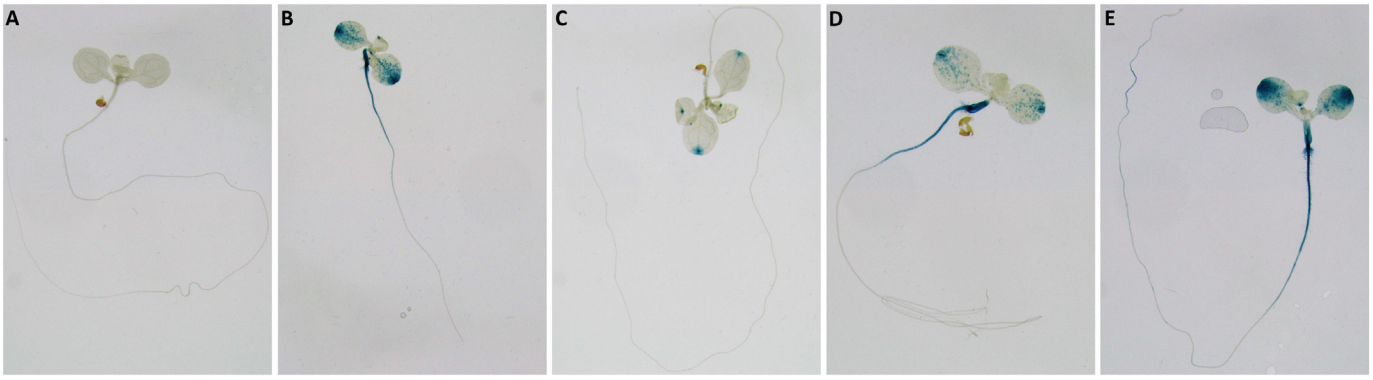

Fig. 11. Promoter activity of *StbZIP-65* gene. Detection of *StbZIP-65* promoter activity by GUS staining. A: GUS staining of wild type Arabidopsis; B: Transgenic Arabidopsis under 50 μM MeJA treatment for 16 h; C: Transgenic Arabidopsis under normal condition; D: Transgenic Arabidopsis under 150 mM NaCl treatment for 3 h; E: Transgenic Arabidopsis under 150 mM NaCl treatment for 24 h.

There were less members of the A and F groups in potato (11 and 2, respectively) than in Arabidopsis (13 and 3, respectively). Thus, potato may have lost several functionally redundant *bZIP* genes during evolution, resulting in less potato *bZIP* genes.

Within the same group, potato *bZIP* genes are highly conserved. The same group members possessed the same gene structure and the numbers of exons and introns (Fig. 2B). In those genes with introns, a similar intron phase composition generally occurred in the same group. The bZIP proteins from the same group had basically the same motifs arranged in same order (Fig. 2C). In addition, duplicated *bZIP* genes belonged to the same group, such as *StbZIP-4* and *StbZIP-36* in group G. The Ka/Ks values of duplicated potato *bZIP* genes were far less than one (Table S4), indicating a low Ka level between the two duplicated genes. The result also reflected that duplicated genes from the same group were highly conserved. However, potato bZIP transcription factors from different groups varied in gene structure and conserved motif composition. Conserved motif analysis suggested that potato bZIPs shared only one common motif, motif 1, which represented the typical bZIP domain. Thus, besides the characteristic bZIP domain, the other regions of the bZIP proteins were relatively variable. Similar results were also reported for other transcription factors, such as MYB [52]. The variable regions outside of the characteristic motif result in varied functions among different members from the same transcription factor family.

Tandem duplication and segmental duplication make a great contribution to the expansion of a gene family [41,53]. The gene duplication analysis of potato *bZIP* genes showed that no tandem duplications were found in the potato *bZIP* gene family, and 18 segmentally duplicated potato *bZIP* genes were identified. The segmentally duplicated genes accounted for 27.7% of potato *bZIPs*, which showed the vital role of segmental duplication to potato *bZIP* family expansion. Similar results were also acquired in other plant *bZIP* gene families, such as *Ipomoea trifida* [54], rice [19], grapevine [47], and *Brachypodium distachyon* [55]. Furthermore, many Arabidopsis proteins, such as MYB transcription factors, GTP binding proteins, glycosyl transferase, membrane transport protein, and proteasome 20S subunits, showed low tandem and high segmental duplication levels, but the reason is not known [41].

Gene expression is regulated by *cis*-acting elements. The *cis*-acting element analysis revealed that multiple different *cis*-acting elements responded to different abiotic stresses and hormone signals. The results suggested potential functions of potato bZIP transcription factors under abiotic stress conditions. The heatmap (Fig. 7) displayed the relative expression level changes of potato *bZIPs* under four kinds of abiotic stresses. Most potato *bZIP* genes responded to one or more kind of abiotic stress. Furthermore, the expression responses of potato *bZIPs* under abiotic stress conditions were confirmed by qRT-PCR. In total, 13 *bZIP* genes sensitive to abiotic stresses were chosen for the analysis. The qRT-PCR results revealed that the analyzed potato *bZIPs* expression patterns were consistent with the expression patterns presented in the heatmap, except for *StbZIP-64*. According to the qRT-PCR results, the expression level of *StbZIP-64* was not significantly upregulated under heat-stress conditions. This may result from different heat stress treatments. In the paper, the heat stress was imposed on potato seedlings under 16-h light/8-h dark. In accordance with the Spud DB instructions, the heat stress for RNA-seq was carried out under constant darkness. Five light-responsive elements, including LAMP-element, G-box, I-box, Box-4, and ATC-motif, were also identified in the promoter sequence of *StbZIP-64*. Thus, the expression of *StbZIP-64* in response to abiotic stress may be correlated with the photoperiod rhythm.

The ectopic expression of *StbZIP-65* in Arabidopsis preliminarily indicated the involvement of *StbZIP-65* in salt tolerance. Many studies have revealed the functions of *bZIPs* in abiotic stress tolerance. The stress-inducible expression of the activated AtbZIP17 under salt stress increased salt tolerance in Arabidopsis [10]. The overexpression of *SlAREB1* in tomato enhanced salt- and drought-stress tolerances. *SlAREB1* also regulates abiotic and biotic stress-related genes [56]. Silencing *ABF2* in cotton (*Gossypium hirsutum*) reduced tolerance to polyethylene glycol, osmotic, and salt stresses, while the overexpression of *GhABF2* in Arabidopsis and cotton increased tolerance levels to drought- and salt-stress [57]. Expression of *AtbZIP28* increased in Arabidopsis under heat-stress conditions. The null mutant of *AtbZIP28* was significantly heat-sensitive [58]. The *cis*-acting element and expression pattern analyses of potato *bZIP* genes indicated the possible involvement of potato *bZIP* genes in multiple abiotic stresses. Even though *StbZIP-65* was identified to be involved in salt-stress responses in this study, its function in potato and regulatory mechanism need to be studied further.

5. Conclusions

In summary, potato bZIP transcription factors were identified on genome-wide level. The gene structures, phylogenetic relationships, chromosomal locations, duplication events, stress and hormone-related *cis*-acting elements, and expression patterns under abiotic stresses of potato bZIP transcription factor were further analyzed by bioinformatics and qRT-PCR. Segmental duplication made a contribution to the expansion of potato bZIP transcription factors. Most potato bZIP transcription factors may participate in various abiotic stress responses. Overexpression of potato bZIP transcription factor *StbZIP-65* in Arabidopsis enhanced salt tolerance. However, the function of *StbZIP-65* in potato needs to be proved experimentally in future works.

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Author contribution statement

Peng Zhao collected the public dataset, did the bioinformatics analysis and biological experiments, and also drafted the manuscript. Minghui Ye participated in part of the bioinformatics analysis work. Ruoqiu Wang contributed to Arabidopsis transgene. Qin Chen and Dongdong Wang supervised and revised the manuscript. All of the authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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