Bioinformatic Analyses of Subgroup-A Members of the Wheat bZIP Transcription Factor Family and Functional Identification of TabZIP174 Involved in Drought Stress Response

Xueyin Li¹, ², Biane Feng², ³, Fengjie Zhang², ³, Yimiao Tang², Liping Zhang², Lingjian Ma¹*, Changping Zhao²* and Shiqing Gao²*

¹ College of Agronomy, Northwest A & F University, Yangling, China, ² Beijing Municipal Key Laboratory of Molecular Genetics of Hybrid Wheat, Beijing Engineering Research Center for Hybrid Wheat, Beijing Academy of Agriculture and Forestry Sciences, Beijing, China, ³ College of Agriculture, Shanxi Agricultural University, Taigu, China

Extensive studies in Arabidopsis and rice have demonstrated that Subgroup-A members of the bZIP transcription factor family play important roles in plant responses to multiple abiotic stresses. Although common wheat (Triticum aestivum) is one of the most widely cultivated and consumed food crops in the world, there are limited investigations into Subgroup A of the bZIP family in wheat. In this study, we performed bioinformatic analyses of the 41 Subgroup-A members of the wheat bZIP family. Phylogenetic and conserved motif analyses showed that most of the Subgroup-A bZIP proteins involved in abiotic stress responses of wheat, Arabidopsis, and rice clustered in Clade A1 of the phylogenetic tree, and shared a majority of conserved motifs, suggesting the potential importance of Clade-A1 members in abiotic stress responses. Gene structure analysis showed that TabZIP genes with close phylogenetic relationships tended to possess similar exon–intron compositions, and the positions of introns in the hinge regions of the bZIP domains were highly conserved, whereas introns in the leucine zipper regions were at variable positions. Additionally, eleven groups of homologs and two groups of tandem paralogs were also identified in Subgroup A of the wheat bZIP family. Expression profiling analysis indicated that most Subgroup-A TabZIP genes were responsive to abscisic acid and various abiotic stress treatments. TabZIP27, TabZIP74, TabZIP138, and TabZIP174 proteins were localized in the nucleus of wheat protoplasts, whereas TabZIP9-GFP fusion protein was simultaneously present in the nucleus, cytoplasm, and cell membrane. Transgenic Arabidopsis overexpressing TabZIP174 displayed increased seed germination rates and primary root lengths under drought treatments. Overexpression of TabZIP174 in transgenic Arabidopsis conferred enhanced drought tolerance, and transgenic plants exhibited lower water loss rates, higher survival rates, higher proline, soluble sugar, and leaf chlorophyll contents, as well as more stable osmotic potential under drought conditions. Additionally, overexpression of TabZIP174 increased the...
expression of stress-responsive genes (RD29A, RD29B, RAB18, DREB2A, COR15A, and COR47). The improved drought resistance might be attributed to the increased osmotic adjustment capacity. Our results indicate that TabZIP174 may participate in regulating plant response to drought stress and holds great potential for genetic improvement of abiotic stress tolerance in crops.

Keywords: wheat, bZIP, Subgroup A, expression profile, subcellular localization, TabZIP174, transgenic Arabidopsis, drought tolerance

INTRODUCTION

Plants often undergo adverse environmental stresses, such as drought, high salinity, and low temperature, which hinder plant growth and development, and decrease grain yield. Plants have evolved diverse regulatory mechanisms at the biochemical, cellular, physiological, and morphological levels to cope with these unfavorable environmental conditions (Zhu, 2002; Hiyama and Shinozaki, 2010; Krasensky and Jonak, 2012; Zhang et al., 2015).

During the response and adaptation to various abiotic stresses, transcription factors act as triggers of gene expression and play important regulatory roles (Xiang et al., 2008). The basic leucine zipper (bZIP) proteins make up one of the largest and most diverse transcription factor families in plants, and regulate various biological processes, including seed maturation and germination, photomorphogenesis, floral induction and development, and pathogen defense (Jakoby et al., 2002; Nijhawan et al., 2008). The bZIP proteins possess an eponymous bZIP domain, which is comprised of a basic region and a leucine zipper (Hurst, 1994). The basic region is highly conserved and is composed of approximately 16 amino acid (aa) residues, including an invariant N-X7-Y/R/K motif. The basic region takes charge of sequence-specific DNA binding. By comparison, the leucine zipper is less conserved and comprises heptad repeats of leucine (Leu) or other bulky hydrophobic amino acid (such as Ile, Val, Phe, or Met) residues positioned accurately nine amino acid residues toward the C-terminus to create an amphipathic helix that confers homo- or hetero-dimerization specificity (Jakoby et al., 2002; Nijhawan et al., 2008; Li X. et al., 2015).

Members of the bZIP transcription factor family were comprehensively identified or predicted in some plant genomes, such as Arabidopsis, rice, soybean, sorghum, maize, cucumber, grapevine, castor bean, Brachypodium distachyon, tomato, wheat, Triticum urartu, Aegilops tauschii, barley, and pearl barley (Jakoby et al., 2002; Liao et al., 2008; Nijhawan et al., 2008; Wang et al., 2011; Wei et al., 2012; Baloglu et al., 2014; Jin et al., 2014; Liu et al., 2014; Li D. et al., 2015; Li X. et al., 2015; Liu and Chu, 2015; Zhao et al., 2016). bZIP family members were classified into 14 subgroups (Jakoby et al., 2002; Li X. et al., 2015). Extensive studies in Arabidopsis and rice demonstrated that Subgroup-A proteins of the bZIP family participated in mediating abscisic acid (ABA) signaling and/or regulating abiotic stress responses in plants, and held great potential in enhancing the resistance of transgenic plants to multiple abiotic stresses. In Arabidopsis, Subgroup-A members of the bZIP family were designated as ABFs (ABA-responsive element binding factors), AREBs (ABA response element binding factors), or DPBFs (D3 promoter binding factors) in different studies (Kim S. Y. et al., 1997; Choi et al., 2000; Uno et al., 2000; Kim et al., 2002). However, several of these independently identified loci were identical. Most AbtZIPs in Subgroup A were characterized in previous studies, including AtbZIP12/DPBF4/EEL, AtbZIP35/ABF1, AtbZIP36/ABF2/AREB1, AtbZIP37/ABF3/DPBF5, AtbZIP38/ABF4/AREB2, AtbZIP39/ABF5/DPBF1, AtbZIP40/GBF4, AtbZIP46/AREB3/DPBF3, and AtbZIP67/DPBF2 (Kim J. et al., 1997; Choi et al., 2000; Bensmihen et al., 2002; Jakoby et al., 2002; Kim et al., 2002). The AREBs/ABFs were important transcription factors involved in response to ABA and various abiotic stresses, including drought and salinity (Kobayashi et al., 2008). Expression of ABF genes was induced by ABA and various abiotic stress treatments, and as master transcription factors, ABF2, ABF3, and ABF4 cooperatively mediated drought-responsive ABA signaling (Choi et al., 2000; Yoshida et al., 2010). ABF2 overexpression affected multiple stress tolerance and ABA sensitivity of transgenic Arabidopsis (Kim et al., 2004). Overexpression of ABF3 or ABF4 reduced transpiration, enhanced drought tolerance and resulted in ABA hypersensitivity in transgenic Arabidopsis. However, the abf3 and abf4 mutants were defective in dehydration, salt, and ABA responses (Kang et al., 2002; Kim et al., 2004). In rice (Oryza sativa), overexpression of OsbZIP23 (LOC_Os02g52780) significantly increased ABA sensitivity and resistance to drought and high-salinity stresses. The mutation of OsbZIP23 led to decreased ABA sensitivity and decreased tolerance to drought and salinity stresses; however, this phenotype could be restored after transforming OsbZIP23 back into the mutant (Nijhawan et al., 2008; Xiang et al., 2008). OsbZIP46 (OsABF2, LOC_Os06g10880) and OsbZIP72 (LOC_Os09g28310) proteins participated in the positive regulation of ABA response and drought resistance of rice (Nijhawan et al., 2008; Lu et al., 2009; Hossain et al., 2010a; Tang et al., 2012). Mutation of OsABF1 (OsbZIP12, LOC_Os10g64730) led to increased sensitivity to salinity and drought stress treatments, and suppressed the expression of ABA- and stress-responsive genes (Nijhawan et al., 2008; Hossain et al., 2010b). Additionally, a recent study revealed...
that OsABF1 might act as a drought-induced suppressor of floral transition (Zhang et al., 2016). OsABF5 (OsbZIP10, LOC_Os01g64000) was closely related to stress responses of rice (Nijhawan et al., 2008; Zou et al., 2008).

Bread wheat (*Triticum aestivum*; 2n = 6x = 42; AABBDD) is one of the most widely cultivated and consumed food crops in the world (Jia et al., 2013; Ling et al., 2013). Great efforts have been made worldwide to sequence and annotate the complex hexaploid wheat genome (Brenchley et al., 2012; Jia et al., 2013; Ling et al., 2013; Luo et al., 2013; Choulet et al., 2014; Marcussen et al., 2014; Pfeifer et al., 2014), and recently, a draft sequence of the 17 Gb hexaploid wheat genome has been generated by sequencing isolated chromosome arms (IWGSC, 2014). However, compared with *Arabidopsis* and rice, there were fewer investigations into Subgroup A of the bZIP family in wheat. The wheat WAB15 gene was orthologous to barley HvAB15, and overexpression of WAB15 in transgenic tobacco plants greatly improved the tolerance to osmotic and freezing stresses, and resulted in a hypersensitivity to ABA at the seedling stage (Kobayashi et al., 2008). A novel ABI-like bZIP transcription factor gene, TaAABL1, was cloned from wheat in our previous study (Xu et al., 2014). The gene TabZIP60 shared high similarity in protein sequence with TaAABL1. The expressions of both TaAABL1 and TabZIP60 were strongly induced by drought, salt, low temperature, and exogenous ABA treatments. Transgenic plants overexpressing TaAABL1 or TabZIP60 exhibited improved tolerance to multiple abiotic stresses and increased ABA sensitivity (Xu et al., 2014; Zhang et al., 2015). Furthermore, TaAABL1 overexpression hastened stomatal closure of transgenic plants under abiotic stress conditions (Xu et al., 2014). Numerous studies in *Arabidopsis* and rice showed that Subgroup-A bZIP genes played vital roles in regulating abiotic stress responses of plants. However, more wheat bZIP genes involved in abiotic stress responses remain to be identified from Subgroup A.

Members of the bZIP family in wheat, including most of Subgroup-A members, were identified in our previous study (Li X. et al., 2015). In the present study, we first added a few more bZIP members to Subgroup A. And we report the bioinformatic analyses of Subgroup-A members of the wheat bZIP family. Phylogenetic and conserved motif analyses were performed to reveal the similarities among Subgroup-A bZIP proteins from wheat, *Arabidopsis*, and rice, which suggests the potential importance of Clade-A1 bZIP proteins in abiotic stress response. Gene structures were analyzed to obtain a deeper insight into the exon–intron compositions of Subgroup-A TabZIP genes, as well as intron positions within the bZIP domains. Additionally, we also identified homologous TabZIP genes within Subgroup A of the wheat bZIP family. Gene expression analysis was performed to characterize the expression profiles of Subgroup-A TabZIP genes in response to ABA and multiple abiotic stresses. Subsequently, the subcellular localizations of several TabZIP proteins were confirmed. Functional analysis of the gene TaTabZIP174 was carried out by investigating the drought stress tolerance of transgenic *Arabidopsis* plants overexpressing TaTabZIP174, and meanwhile, their physiological traits were monitored. The expression of several stress-responsive genes was also detected by quantitative real-time PCR (qRT-PCR). Our results indicated that TabZIP174 might participate in regulating the response to drought stress. Finally, we proposed the putative mechanism by which TabZIP174 enhanced the drought tolerance of transgenic *Arabidopsis* plants.

**MATERIALS AND METHODS**

**Database Search for Full-Length Coding Sequences**

In our previous study, 187 bZIP transcription factor family members were identified from the wheat genome (Li X. et al., 2015). Among these, 35 novel TabZIP proteins belonged to Subgroup A of the wheat bZIP family. To obtain the full-length coding sequences (CDSs) of these Subgroup-A TabZIP genes, their CDSs were used as queries to perform similarity search in the Triticeae full-length CDS database (TriFLDB; http://trifldb.psc.riken.jp/v3/index.pl; Mochida et al., 2009).

**Phylogenetic and Conserved Protein Motif Analyses**

Multiple protein sequence alignment of the Subgroup-A members of the bZIP family in wheat, *Arabidopsis* and rice, including 41 TabZIP, 13 AtbZIP, and 17 OsbZIP proteins, respectively, was performed using the ClustalX program (Version 2.1) with the default settings (Larkin et al., 2007). An unrooted phylogenetic tree was constructed with the neighbor-joining method using MEGA5.0 software (Tamura et al., 2011).

Protein sequences of TabZIPs, AtbZIPs, and OsbZIPs were searched for common conserved motifs using the online tool MEME (Version 4.10.2, http://meme-suite.org/tools/meme; Bailey et al., 2009), and the number of motifs was specified with all other parameters set to default.

**Gene Structure Analysis**

GSDS V2.0 (http://gsds.cbi.pku.edu.cn; Hu et al., 2015) was used to align the CDSs of the Subgroup-A TabZIP genes with their corresponding genomic sequences to analyze their exon–intron structures.

To further analyze the intron-position patterns within the bZIP domains, protein sequences of the bZIP domains of the Subgroup-A TabZIP proteins were extracted using ScanProsite (http://prosite.expasy.org/; de Castro et al., 2006).

**Identification of Homologous Genes**

To identify homologous genes within Subgroup A of the wheat bZIP family, pairwise protein sequence alignments were performed using the BLASTP 2.3.0+ program (Altschul et al., 2005). A pair of homologous genes was defined based on the following criteria: (1) the FASTA-aligned region between their protein sequences covered ≥70% of the longer sequence and (2) the identity of the aligned region was ≥75% (Gu et al., 2002).

**Plant Materials and Abiotic Stress Treatments**

The wheat (*T. aestivum* L.) genotype “Luohan 9769” was used in this study. After sterilization with 75% ethanol and washing with sterilized water, wheat seeds were germinated and cultivated...
with double-distilled water in a growth chamber (25°C, 500 μmol/m²/s, 12/12 h light/dark photoperiod). Twelve-day-old wheat seedlings were treated with 15% (w/v) polyethylene glycol-6000 (PEG-6000), 250 mM NaCl, 200 μM ABA, or low temperature (4°C). The treated plants were stressed in the PEG or NaCl solutions for 24 h, cultivated under low temperature conditions for 24 h or sprayed once with the ABA solution. Three entire wheat seedlings were harvested as a pooled sample at 0, 1, 3, 6, 12, and 24 h after treatment, respectively. To investigate the tissue-specific expression pattern of the gene TabZIP174 in wheat, roots, and leaves of 12-day-old seedlings, as well as roots, stems, leaves, and anthers at the blooming stage, were harvested. At harvest, plant materials were frozen immediately in liquid nitrogen and stored at −80°C.

**RNA Extraction and qRT-PCR**

Total RNA extraction and first-strand cDNA synthesis were performed as described previously (Li X. et al., 2015). The qRT-PCR analysis was performed using an Eco Real-Time PCR system (Illumina, San Diego, CA, USA) with TaKaRa SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China). The wheat Actin (Gene ID: 542814) and Arabidopsis Actin2 (AT3G18780) were used as the internal controls for the expression analysis of TabZIP genes in wheat and stress-responsive genes in Arabidopsis, respectively. The gene-specific primers were designed using Primer Premier 5.0 (Singh et al., 1998). Each reaction was repeated in triplicate in a reaction volume of 10 μl as described previously (Li X. et al., 2015). The expression analysis was conducted using the same PCR procedure or with a slightly adjusted annealing temperature. The relative gene expression levels were calculated according to the 2−ΔΔCT method (Livak and Schmittgen, 2001).

**Subcellular Localization in Wheat Protoplasts**

Nuclear localization signal (NLS) prediction for the Subgroup-A TabZIP proteins was performed with the online tool NucPred (http://www.sbc.su.se/~macallr/nucpred/; Brameier et al., 2007). To further examine the subcellular localization of the Subgroup-A TabZIP proteins, green fluorescent protein (GFP) expression vectors (CaMV35S-GFP-NOS) were constructed. The coding regions of TabZIP9, TabZIP27, TabZIP74, TabZIP138, and TabZIP174 were amplified by PCR with gene-specific primers and independently fused to the N-terminus of GFP in the expression vector. Wheat protoplasts were isolated from the mesophyll tissue of 2-week-old wheat seedlings, and then transformed using the PEG transfection method separately with the plasmid DNA of 35S::TabZIP9-GFP, 35S::TabZIP27-GFP, 35S::TabZIP74-GFP, 35S::TabZIP138-GFP, 35S::TabZIP174-GFP, and 35S::GFP control as described previously (Shan et al., 2014). After PEG transfection, wheat protoplasts were incubated in W5 solution (2 mM MES, 154 mM NaCl, 125 mM CaCl2, and 5 mM KCl, pH = 5.7) in a dark chamber at 23°C for 18 h, and GFP fluorescence was monitored under a laser-scanning confocal microscope (A1, Nikon Corporation, Tokyo, Japan).

**Generation of Transgenic Arabidopsis**

The full-length opening reading frame of TabZIP174 was amplified from wheat cDNA with gene-specific primers (forward: 5′-ATGGAGATGCGGGAGGGA-3′; reverse: 5′-CTACACACGACCCGTAGAGTT-3′), and cloned into the pBI121 vector driven by cauliflower mosaic virus (CaMV) 35S promoter to construct the recombinant vector (35S::TabZIP174). The recombinant vector (35S::TabZIP174) was introduced into Agrobacterium tumefaciens and transformed into Arabidopsis (ecotype Columbia-4) using the floral dip method (Zhang et al., 2006). Positive transgenic lines were screened on Murashige and Skoog (MS) medium containing 50 μg/ml kanamycin, and then confirmed by reverse transcription PCR. Independent T3-generation transgenic Arabidopsis lines with relatively higher TabZIP174 transcript levels were chosen for further analyses.

**Germination and Primary Root Growth Assays**

Homozygous T3 seeds of TabZIP174 transgenic lines were used for germination and primary root growth assays. Homozygous T3 transgenic and wild type (WT) seeds were surface-sterilized, kept at 4°C in the dark for 3 days and then sown on ½ MS medium solidified with 1.0% (w/v) agar. For the germination assay, the seeds of transgenic lines and WT were placed on ½ MS medium containing no or 5% (w/v) PEG-6000. The germination percentages were calculated daily for 7 days. For the primary root growth assay, 3-day-old seedlings grown on ½ MS medium were transferred to ½ MS medium supplemented with or without 5% PEG-6000, and grown vertically for 4 days prior to measuring primary root lengths.

**Drought Tolerance Assay**

Homozygous T3-generation TabZIP174 transgenic lines were used in the drought tolerance assay. Both transgenic and WT seeds were kept at 4°C in the dark for 3 days and then germinated in a soil mixture (1:1 of vermiculite:humus) in a greenhouse (22°C, relative humidity 70%, and 12/12 h light/dark photoperiod). Two-week-old seedlings were transferred to identical rectangular pots filled with the soil mixture and were regularly watered for two weeks. Subsequently, transgenic and WT plants were cultivated without watering for 4 weeks, and were then rewatered. Survival rates were calculated at 4 days after rewatering. The drought tolerance experiment was carried out in triplicate.

**Physiological Characterization of Transgenic Arabidopsis**

To determine the water loss rate under water deficit conditions, the aboveground parts were excised from 4-week-old transgenic and WT plants, and weighted immediately (fresh weight, FW). The samples were then placed on the laboratory bench (22–24°C, relative humidity 40–45%) and weighted at the designated time-points. The samples were finally oven dried at 80°C for 24 h to a constant dry weight (DW) (Mao et al., 2012). The percentages of water loss were measured relative to the initial water contents. Subsequently, relative water contents (RWGs) before oven drying
were also calculated. Ten plants for each of transgenic lines and WT were used in this assay.

Arabidopsis leaves were harvested at designated time-points during drought treatment and used to measure the free proline content, total soluble sugar content, and osmotic potential. To maximize the sample uniformity at each time-point, leaves of the same size and location were detached from transgenic and WT plants. The free proline content was determined as described previously (Bates et al., 1973). Samples (~0.1 g) were homogenized in 3% sulfosalicylic acid and boiled for 10 min. After the reaction between proline and acid ninhydrin, the absorbance of sample solutions was measured at 520 nm.

The total soluble sugar content was measured using the anthrone colorimetric method (Yemm and Willis, 1954). Soluble sugars were extracted from homogenized samples (~0.1 g). After the reaction of soluble sugars with anthrone and concentrated sulfuric acid, the absorbance of sample solutions was measured at 620 nm. The proline and soluble sugar contents were determined using their respective standard curves and calculated based on fresh weights. Osmotic potential was measured with an automatic freezing-point osmometer (Multi-OSMETTE™, Model 2430E, Precision Systems Inc., Natick, MA, USA). Ten leaves per Arabidopsis line were harvested as a pooled sample and transferred to an injection syringe, and the liquid was then squeezed out of the leaves. The supernatant tissue sap was collected after centrifugation at 13,400 × g for 1 min at room temperature, and then filtered with a filtration column. The filtrate was loaded onto the osmometer to measure the osmotic concentration according to the manufacturer’s instructions. The osmotic concentration was measured three times for each sample. Subsequently, the osmotic potential was calculated from the osmotic concentration using the Van’t Hoff equation. All of these measurements were repeated three times.

The total chlorophyll content was determined with a portable chlorophyll meter (SPAD-502Plus, Konica Minolta Inc., Tokyo, Japan). Thirty leaves of similar size were selected from each Arabidopsis line and used for the in situ measurement of the chlorophyll content at each designated time-point during the drought treatment. To minimize the measurement deviations of different leaves, the chlorophyll meter was applied to the same position on the leaves.

RESULTS

Phylogenetic and Conserved Motif Analyses of Subgroup-A Members in the bZIP Family

In our previous study, 187 wheat bZIP transcription factor genes were identified from the wheat genome and named based on their chromosomal locations (Li X. et al., 2015). And 35 novel TabZIP, 13 AtbZIP, and 17 OsbZIP genes were identified as Subgroup-A members of the bZIP family based on the phylogenetic analysis (Li X. et al., 2015). To obtain the full-length CDSs of the 35 Subgroup-A TabZIP genes, the Triticeae full-length CDS database (TriFLDB) (Mochida et al., 2009) was used to perform similarity search with the CDSs of these 35 TabZIP genes as queries. The basic information on these 35 TabZIP genes is shown in Table 1. In addition to these 35 novel TabZIP genes, Subgroup A of the wheat bZIP family included TaABF1, TaAB15, WAB15 (WAB15-1, WAB15-2, and WAB15-3), TaAB1L, and TabZIP60 (TabZIP60-A, TabZIP60-B, and TabZIP60-D) (Johnson et al., 2002, 2008; Kobayashi et al., 2008; Rikiishi et al., 2010; Harris et al., 2013; Xu et al., 2014; Zhang et al., 2015). Therefore, 35 TabZIP genes were identified from the wheat genome.

Multiple protein sequence alignment of the Subgroup-A members of the bZIP family in wheat, Arabidopsis, and rice (including 41 TabZIP, 13 AtbZIP, and 17 OsbZIP proteins) was performed, and then, an unrooted phylogenetic tree was constructed (Figure 1). A majority of the 12 AtbZIPs in the phylogenetic tree had been characterized in previous studies (Menkens and Cashmore, 1994; Choi et al., 2000; Finkelstein and Lynch, 2000, 2002; Uno et al., 2000; Lopez-Molina et al., 2001; Bomsilhen et al., 2002, 2005; Carles et al., 2002; Kang et al., 2002; Kim et al., 2002, 2004; Lopez-Molina et al., 2002; Finkelstein et al., 2005; Yoshida et al., 2010; Mendes et al., 2013), and the protein sequence of each motif was highly conserved among these bZIP proteins. Therefore, protein sequence similarity of this subset of proteins, which shared most of the above-mentioned bZIP proteins, was analyzed (Supplemental Figure 1). Most (9/11) of the nine AtbZIPs played important roles in regulating abiotic stress resistance in Arabidopsis (Choi et al., 2000; Kang et al., 2002; Kim et al., 2004; Finkelstein et al., 2005). Additionally, 5 of the 17 OsbZIPs in the phylogenetic tree had important functions in enhancing tolerance of transgenic rice to multiple abiotic stresses (Xiang et al., 2008; Zou et al., 2008; Lu et al., 2009; Hossain et al., 2010a,b; Tang et al., 2012). Among the 41 TabZIPs of Subgroup A, only WAB15-1, TaAB1L, and TabZIP60 were involved in regulating plant responses to abiotic stress. These 12 TabZIPs were more likely associated with abiotic stress responses.

Common conserved motifs in the Subgroup-A proteins of the bZIP family in wheat, Arabidopsis and rice were also analyzed (Supplemental Figure 1). Most (9/11) of the Subgroup-A bZIP proteins related to abiotic stress responses (namely, AtbZIP36, AtbZIP37, AtbZIP38, OsbZIP23, OsbZIP46, WAB15-1, TaAB1L, and TabZIP60) shared a majority of conserved motifs (Supplemental Figure 1). And, notably, TaAB1L and TabZIP174 shared most of the conserved motifs with the above-mentioned bZIP proteins. Therefore, protein sequence similarity of this subset of proteins, which shared most of the conserved motifs, was further analyzed (Figure 2). These 12 Subgroup-A bZIP proteins had six conserved motifs in common, and the protein sequence of each motif was highly conserved among these bZIP proteins (Figure 2). Nine of the 12 bZIP proteins (AtbZIP36, AtbZIP37, AtbZIP38, OsbZIP23, OsbZIP46,
Table 1: Basic information for the Subgroup-A TabZIP genes.

| TabZIP  | Gene ID                  | CDS(nt) | PEP(aa) | bZIP domain | Chromosomal location | Precise position on the chromosome |
|---------|--------------------------|---------|---------|-------------|----------------------|-----------------------------------|
| TabZIP5 | Traes_1AL_1FFBF058.1     | 987     | 328     | 256–301     | 1AL                  | 220288133–220292420               |
| TabZIP9 | Traes_2AS_3A8C06B06.1    | 276     | 91      | 1–38        | 2AS                  | 15377631–15378760                 |
| TabZIP14| Traes_2AL_3D7807781.1    | 396     | 131     | 54–110      | 2AL                  | 221473224–221473242               |
| TabZIP20| Traes_3AL_58F294736.2    | 381     | 126     | 57–102      | 3AL                  | Scaffold                          |
|          |                          |         |         |             |                      | IWGSC_CSS_3AL_scaffold_3842941:1–824 |
| TabZIP23| Traes_3AL_FC523394.2     | 1176    | 391     | 305–350     | 3AL                  | 158927528–158928568               |
| TabZIP27| Traes_4AS_F9C171219.1    | 678     | 225     | 153–197     | 4AS                  | 86640961–86645921                 |
| TabZIP40| Traes_5AL_79E6A58E6.1    | 213     | 70      | 1–43        | 5AL                  | Scaffold                          |
|          |                          |         |         |             |                      | IWGSC_CSS_5AL_scaffold_2772028:6–1337 |
| TabZIP51| Traes_6AL_E8C2D2C02B.1/  | 1086    | 361     | 279–328     | 6AL                  | 193103591–193107395               |
|          | KJ865658 (GenBank)       |         |         |             |                      |                                   |
| TabZIP54| Traes_7AL_3ED7A9663.1    | 255     | 84      | 12–68       | 7AL                  | Scaffold                          |
|          |                          |         |         |             |                      | IWGSC_CSS_7AL_scaffold_4376338:4753–7569 |
| TabZIP65| Traes_1BL_DE2CF9613.1    | 981     | 326     | 254–299     | 1BL                  | Scaffold                          |
|          |                          |         |         |             |                      | IWGSC_CSS_1BL_scaffold_3907952:2851–6847 |
| TabZIP68| Traes_2BS_84FB90D98.1    | 276     | 91      | 1–38        | 2BS                  | Scaffold                          |
|          |                          |         |         |             |                      | IWGSC_CSS_2BS_scaffold_5141107:3–1150 |
| TabZIP73| Traes_2BL_70E67A02.1     | 528     | 176     | 104–149     | 2BL                  | 333582239–333582788               |
| TabZIP74| Traes_2BL_94E5996F7.1    | 405     | 134     | 57–113      | 2BL                  | 300904368–300905361               |
| TabZIP76| Traes_2BL_D0DF6F846.1    | 552     | 183     | 93–138      | 2BL                  | 30275846–302758882                |
| TabZIP77| Traes_3B_1F253F060.1     | 273     | 90      | 8–72        | 3B                   | 629627371–629627608               |
| TabZIP80| Traes_3B_3A822421E.1     | 1176    | 391     | 305–350     | 3B                   | –                                 |
| TabZIP83| Traes_3B_50DF7382A.1     | 1023    | 340     | 271–316     | 3B                   | –                                 |
| TabZIP84| Traes_3B_6B26C136.1      | 858     | 286     | 245–286     | 3B                   | –                                 |
| TabZIP86| Traes_3B_A796206A0.2     | 1164    | 387     | 305–350     | 3B                   | 8982474–8985806                   |
| TabZIP90| Traes_4BL_50AA15F3.1     | 683     | 220     | 148–192     | 4BL                  | 584035636–58410423                |
| TabZIP111| Traes_5BL_DE519903.3    | 1098    | 365     | 284–333     | 5BL                  | 144705569–144709291               |
| TabZIP114| Traes_5BL_FB4EDEA83.2   | 972     | 323     | 251–296     | 5BL                  | Scaffold                          |
|          |                          |         |         |             |                      | IWGSC_CSS_5BL_scaffold_10907354:7912–12081 |
| TabZIP127| Traes_1DL_D9BA83221.2    | 1107    | 388     | 254–299     | 1DL                  | 106298159–106302141               |
| TabZIP128| Traes_1DL_DA67871E9.1    | 762     | 253     | 171–235     | 1DL                  | 86554441–86556883                 |
| TabZIP133| Traes_2DL_1F0CDB1CE.1    | 396     | 131     | 54–110      | 2DL                  | 133012655–133013459               |
| TabZIP134| Traes_2DL_F0CDDB1CE.1    | 396     | 131     | 54–110      | 2DL                  | 133008772–133009276               |
| TabZIP135| Traes_2DL_5610B574.1     | 453     | 151     | 77–122      | 2DL                  | 149025389–149026423               |
| TabZIP138| Traes_3DL_20ED2EA4.1     | 1014    | 337     | 265–310     | 3DL                  | 85255062–85255465                 |
| TabZIP141| Traes_3DL_E11790E8.1     | 630     | 209     | 90–135      | 3DL                  | 90533952–90535138                 |
| TabZIP148| Traes_4DL_F38ED7FB6.1    | 672     | 223     | 151–195     | 4DL                  | 38815726–38820767                 |
| TabZIP158| Traes_5DL_73CE92096.2    | 972     | 323     | 251–296     | 5DL                  | Scaffold                          |
|          |                          |         |         |             |                      | IWGSC_CSS_5DL_scaffold_2974210:869–5254 |
| TabZIP162/WABI5-3| Traes_5DL_895AA6D35.1/  | 1069    | 352     | 272–321     | 5DL                  | 77875198–77878011                 |
|          | AB382280 (GenBank)       |         |         |             |                      |                                   |
| TabZIP171| Traes_6DL_F7015CE89.2    | 525     | 175     | 91–136      | 6DL                  | 157536269–157540353               |
| TabZIP174| Traes_7DS_G8A3C10A6.1    | 1023    | 340     | 258–303     | 7DS                  | Scaffold                          |
|          |                          |         |         |             |                      | IWGSC_CSS_7DS_scaffold_3962964:4–4775 |
| TabZIP182| Traes_7DL_EECCC4DBF.1    | 496     | 164     | 92–148      | 7DL                  | 234881263–234884437               |
OsBZIP72, WABI5-1, TaABL1, and TabZIP60) played important roles in plant responses to abiotic stress (Choi et al., 2000; Kang et al., 2002; Kim et al., 2004; Finkelstein et al., 2005; Kobayashi et al., 2008; Xiang et al., 2008; Lu et al., 2009; Hossain et al., 2010a; Tang et al., 2012; Xu et al., 2014; Zhang et al., 2015). The phylogenetic analysis and similarity in the conserved motifs indicated that TabZIP111 and TabZIP174 were possibly involved in abiotic stress responses.

### Gene Structure Analysis

To obtain a deeper insight into the structures of the 35 novel TabZIP genes in Subgroup A, we mapped their exon/intron organizations. Only two (5.7%) of these genes were intronless, and the others had 1–3 introns (Figure 3). Specifically, 10 TabZIP genes had three introns, 15 had two introns, and 8 had one intron.

Not surprisingly, neighboring TabZIP genes in the phylogenetic tree tended to share similar gene structures (Figure 3). TabZIP9 and TabZIP68 possessed identical exon/intron organizations, as did TabZIP23/TabZIP80, TabZIP114/TabZIP158, and TabZIP14/TabZIP133/TabZIP134. Except slight variations in the first exon lengths, TabZIP5 and TabZIP65, as well as TabZIP27, TabZIP90, and TabZIP148, had nearly identical exon/intron compositions. Similarly, TabZIP80 differed from TabZIP66 only by 12 nucleotides (nt) in the last exon lengths. Both TabZIP73 and TabZIP135 had only one exon, despite a difference in exon length. Differences in exon lengths between these homologous TabZIP genes (Table 2) could potentially lead to their functional divergences.

In addition, for 30 (85.7%) of these 35 Subgroup-A TabZIP genes, the first exon was longer than the subsequent exons in the CDS. Statistical analysis showed that exons of 72, 81, 30, and 93 nt in length appeared frequently within the CDSs of the 35 Subgroup-A TabZIP genes, with 23 (72 nt-exon), 13 (81 nt), 10 (30 nt), and 6 (93 nt) occurrences, respectively. Moreover, the exon lengths of the Subgroup-A TabZIP genes were all multiples of three without exception (Figure 3), demonstrating that all of their introns were in Phase 0 (P0).

The intron positions within the bZIP domains were also analyzed. Among the 33 intron-containing TabZIP genes, 32 had introns within the bZIP domain region. The 35 Subgroup-A TabZIP genes were divided into seven patterns (a–g) (Supplementary Figure 2), based on intron numbers and positions, which was consistent with previous studies in rice and maize (Nijhawan et al., 2008; Wei et al., 2012).

A majority (57.1%) of the Subgroup-A TabZIP genes were of Pattern a, and these TabZIP genes possessed only one intron, which was in P0 and located between the codons encoding the amino acids Gln and Ala (or Ser) in the hinge region (Supplementary Figures 2, 3). This was in accordance with previous studies (Nijhawan et al., 2008; Wei et al., 2012). Patterns b and d were similar in that both had two introns in P0, one within the hinge region and the other within the leucine zipper region. The only difference was that the intron within the leucine zipper region was inserted between Gln at Position +19 and Ala (or Lys) at Position +20 in Pattern b, but was inserted between Glu at Position +21 and Leu at Position +22 in Pattern d. Pattern e had three introns, one in the hinge region and two in the leucine zipper region. Two of them shared the same positions with the two introns of Pattern b, one intervening between the amino acids at Positions −5 and −6 in the hinge region and the other between the amino acids at Positions +19 and +20 in the leucine zipper region. Pattern f had only one intron within the leucine zipper region, and was only found in one TabZIP gene. Pattern g lacked any intron in the bZIP domain region and was found in three TabZIP genes.

Notably, if present, introns within the hinge region always occurred at the same position, between Gln (or Arg) at Position −6 and Ala (or Ser) at Position −5 (such as Patterns a, b, d, and e), which was in agreement with the findings of a previous study (Nijhawan et al., 2008). However, introns present in the leucine zipper region were at variable positions. In our case, no introns were within the basic region of the bZIP domain. To sum up, TabZIP genes with close phylogenetic relationships tended to share similar exon/intron organizations, and the positions of the introns within the hinge regions of the bZIP domains remained well conserved during the evolution of the Subgroup-A TabZIP genes.

### Identification of Homologous Genes

As homologous genes typically retain similar biological roles, identifying homologous genes is important for transferring functional information between genes (Remm et al., 2001). To identify homologous genes within Subgroup A of the wheat bZIP family, pairwise protein sequence alignments were performed using the BLASTP 2.3.0+ program (Altschul et al., 2005). A total

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**Table 1 | Continued**

| TabZIP | Gene ID | CDS(nt) | PEP(aa) | bZIP domain | Chromosomal location | Precise position on the chromosome |
|--------|---------|---------|---------|-------------|----------------------|-------------------------------------|
| TabZIP60-B | KJ806559 (GenBank) | 1069 | 352 | 270–319 | 6B | – |
| TabZIP60-D | KJ806560 (GenBank) | 1066 | 361 | 279–328 | 6D | – |
| WABI5-1 | AB362818 (GenBank) | 1059 | 355 | 274–323 | 5A (or 5B) | – |
| WABI5-2 | AB362819 (GenBank) | 1059 | 352 | 272–321 | 5B (or 5A) | – |
| TaABI5 | AB238934 (GenBank) | 1173 | 390 | 304–349 | – | – |
| TaABL1 | – | 1083 | 360 | 279–328 | – | – |

*The column entitled “bZIP domain” indicates the positions of the bZIP domains in the protein sequences.*
FIGURE 1 | Phylogenetic analysis of Subgroup-A members of bZIP transcription factor family in wheat, Arabidopsis, and rice. The phylogenetic analysis was performed using the protein sequences of Subgroup-A members (including 41 TabZIPs, 13 AtbZIPs, and 17 OsbZIPs) in the bZIP family. The unrooted phylogenetic tree was generated by the neighbor-joining method using MEGA5.0 software. All of these Subgroup-A members are further classified into six clades (A1–A6). Numbers above or below branches of the tree indicate bootstrap values. TabZIPs, AtbZIPs, and OsbZIPs are indicated by green, red, and blue circles, respectively, and if they had been characterized in previous studies, their names are colored with green, red, or blue, respectively. Those that had been reported to play important roles in abiotic stress responses are emphasized with asterisks beside their names.
of 13 groups of homologous bZIP genes were identified from Subgroup A and included two categories: homologs (11 groups) and paralogs (2 groups) (Table 2).

Modern wheat (T. aestivum, 2n = 6x = 42, AABBDD) derives from two hybridizations between three gramineous ancestors (Brenchley et al., 2012; Jia et al., 2013). The wheat genome is composed of A, B, and D sub-genomes. In wheat, homologous genes located at similar positions on three corresponding chromosomes from the A, B, and D sub-genomes are generally referred to as homologs. For instance, TabZIP14, TabZIP74, and TabZIP133 were three homologs located at similar positions on the long arms of chromosomes 2A, 2B, and 2D, respectively (Figure 4). They shared high similarities in protein sequences with the aligned region between each pair covering the whole sequences, and their identities were 92, 98, and 94%, respectively.

Tandem paralogs are homologous genes located at neighboring positions of the same chromosome. In this study, we identified two groups of tandem paralogs within Subgroup A of the wheat bZIP family, TabZIP133/TabZIP134 and TabZIP80/TabZIP84/TabZIP86 (Table 2). Paralogs TabZIP133 and TabZIP134 possessed identical protein sequences and were adjacent to each other in the long arm of chromosome 2D (Figure 4), indicating that this pair of paralogs probably derived from one tandem duplication event, which often led to the generation of paralogs. Paralogs TabZIP80, TabZIP84, and TabZIP86 were all located on chromosome 3B and shared high similarities in protein sequences. Specifically, the aligned region between TabZIP80 (391 aa) and TabZIP86 (387 aa) covered their entire sequences, and the identity within the aligned region was 89%. The aligned region between TabZIP80 and TabZIP84 (286 aa) covered the entire TabZIP84 sequence and 73% of the TabZIP80 sequence, with an identity of 90%. The aligned region between TabZIP84 and TabZIP86 covered the entire TabZIP84 sequence and 74% of the TabZIP86 sequence, with an identity of 87%. This suggested that this paralogous group (TabZIP80/TabZIP84/TabZIP86) might have been derived from two tandem duplication events. Further genomic sequence alignment among them showed that the unspliced transcript sequence of TabZIP84 (including 5′- and 3′-untranslated regions, two exons and one intron) was aligned with only a portion of the unspliced transcript sequence of TabZIP80 (or TabZIP86) (data not shown), indicating that the gene TabZIP84 derived from only a segment of TabZIP80 (or TabZIP86).

**Expression Profiles of Subgroup-A TabZIP Genes in Response to Various Abiotic Stresses**

Compelling evidence demonstrated that the expression of Subgroup-A bZIP genes in Arabidopsis and rice was induced by ABA and various abiotic stress treatments (Choi et al., 2000; Xiang et al., 2008; Hossain et al., 2010b; Tang et al., 2012). To further confirm whether the expression of Subgroup-A bZIP genes in wheat was induced by different abiotic stresses, quantitative real-time PCR (qRT-PCR) was performed to analyze the expression profiles of these TabZIP genes following PEG, NaCl, cold (4°C) orABA treatments. Overall, the expression of 28 of these 35 Subgroup-A TabZIP genes responded to all four treatments and exhibited complicated variation trends following the treatments (Figure 5).

Among the expression profiles of the 28 TabZIP genes under the four different treatments (8 × 4 = 112), 20 exhibited a variation trend in which the transcript level gradually ascended and reached a maximum, followed by a continuous decline. These included TabZIP5 (under PEG treatment), TabZIP9 (cold), TabZIP20 (cold), TabZIP40 (NaCl, cold or ABA), TabZIP51 (cold), TabZIP65 (cold), TabZIP76 (PEG), TabZIP77 (cold), TabZIP83 (cold), TabZIP114 (PEG or cold), TabZIP127 (ABA),
FIGURE 3 | Phylogenetic analysis (left) and exon–intron structures (right) of 35 novel Subgroup-A TabZIP genes. (Left) The phylogenetic analysis was performed using the protein sequences of the 35 novel Subgroup-A TabZIP genes. The unrooted phylogenetic tree was generated by the neighbor-joining method using MEGA5.0 software. Numbers above or below branches of the tree indicate bootstrap values. (Right) 5′- and 3′-untranslated regions (represented by blue boxes) and exons (represented by green boxes) are drawn to scale. Black lines connecting exons represent introns. Numbers above the exons of each gene structure indicate the size of the exons.

TabZIP133 (cold), TabZIP138 (cold), TabZIP158 (PEG or ABA), TabZIP171 (cold), and TabZIP174 (cold).

In addition, 10 other expression profiles had an inverse variation trend in which the transcripts decreased to the lowest level and then increased. These included TabZIP14 (NaCl or cold), TabZIP74 (PEG or ABA), TabZIP77 (ABA), TabZIP80 (cold), TabZIP133 (PEG or NaCl), and TabZIP174 (PEG or NaCl).

Under the NaCl treatment, the expression level of TabZIP86 continuously increased and reached its maximum at 24 h after treatment. In contrast, for TabZIP27 (PEG) and TabZIP74 (cold), the transcript levels declined continuously until reaching their lowest levels at 24 h after treatment. After the 24-h PEG treatment, the expression level of TabZIP27 was only one-seventh of that before treatment. The transcript level of TabZIP74 after the 24-h cold treatment dropped to one-tenth of that before treatment. Thus, the expression levels of TabZIP27 and TabZIP74 were down-regulated by PEG and cold treatments, respectively.

Notably, the expression of several TabZIP genes (such as TabZIP40, TabZIP76, and TabZIP111) responded rapidly to the ABA treatment because their expression levels increased rapidly immediately after a 1-h exposure to exogenous ABA. For instance, the transcript level of TabZIP76 was more than 10 times that before treatment, while the transcript levels of TabZIP40 and TabZIP111 were ~6.5 and 7 times those before treatment, respectively.

Interestingly, some homologs (or paralogs) exhibited similar expression profiles following certain treatments, such as TabZIP5/TabZIP65 (cold), TabZIP14/TabZIP133 (NaCl), TabZIP74/TabZIP133 (PEG), TabZIP27/TabZIP90 (PEG), TabZIP27/TabZIP90/TabZIP148 (cold), TabZIP27/TabZIP148 (NaCl or ABA), TabZIP83/TabZIP138 (cold), and TabZIP114/TabZIP158 (PEG). However, most homologous TabZIP genes were expressed differently under the same treatments (Figure 5). Indeed, TabZIP80 and TabZIP86 even displayed inverse variation trends of expression under...
TABLE 2 | Homologous genes within Subgroup A of the wheat bZIP family.

| Category         | TabZIP          | Chromosomal location |
|------------------|-----------------|----------------------|
| Homologs         | TabZIP5         | 1AL                  |
|                  | TabZIP65        | 1BL                  |
|                  | TabZIP127       | 1DL                  |
|                  | TabZIP9         | 2AS                  |
|                  | TabZIP68        | 2BS                  |
|                  | TabZIP14        | 2AL                  |
|                  | TabZIP74        | 2BL                  |
|                  | TabZIP133       | 2DL                  |
|                  | TabZIP73        | 2BL                  |
|                  | TabZIP35        | 2DL                  |
|                  | TabZIP82/TabABF1| 3AL                  |
|                  | TabZIP80        | 3B                   |
|                  | TabZIP83        | 3B                   |
|                  | TabZIP138       | 3DL                  |
|                  | TabZIP27        | 4AS                  |
|                  | TabZIP90        | 4BL                  |
|                  | TabZIP148       | 4DL                  |
|                  | WAB15-1         | 5A (or 5B)           |
|                  | WAB15-2         | 5B (or 5A)           |
|                  | TabZIP162/WAB15-3| 5DL                |
|                  | TabZIP114       | 5BL                  |
|                  | TabZIP158       | 5DL                  |
|                  | TabZIP51/TabZIP60-A| 6AL               |
|                  | TabZIP60-B      | 6B                   |
|                  | TabZIP60-D      | 6D                   |
|                  | TabZIP54        | 7AL                  |
| Paralogs (tandem)| TabZIP133       | 2DL                  |
|                  | TabZIP134       | 2DL                  |
|                  | TabZIP80        | 3B                   |
|                  | TabZIP84        | 3B                   |
|                  | TabZIP86        | 3B                   |

4 Homologous genes within the Subgroup A of the wheat bZIP family are divided into two categories (homologs and paralogs). Generally, homologs refer to those homologous genes located at similar positions on three corresponding chromosomes from the A, B, and D sub-genomes of wheat, respectively. Tandem paralogs refer to those homologous genes located at neighboring positions on the same chromosome.
5 TabZIP54 and TabZIP182 may be homologs.

Subcellular Localization of Subgroup-A TabZIP Proteins in Wheat Protoplasts

Our phylogenetic and conserved motif analyses suggested that TabZIP174 (belonging to Clade A1) were more likely involved in abiotic stress response. In addition to TabZIP174, several Subgroup-A TabZIP members (TabZIP9, TabZIP27, TabZIP74, and TabZIP138) were selected from other clades to confirm their subcellular localization. First, the NLSs were predicted using their protein sequences. A NLS, consisting of 4–8 amino acid (e.g., Pro, Lys, or Arg) residues, commonly exists within a transcription factor protein (Kalderon et al., 1984). The basic region within the bZIP domain generally includes a NLS followed by an invariant motif (N-X7-R/K) that is responsible for contacting the DNA (Jakoby et al., 2002). According to the NLS prediction result by NucPred (Brameier et al., 2007), TabZIP27 possessed a NLS sequence (RRKKR) positioned three amino acid residues ahead of the N-terminus of the N-X7-R/K motif, and TabZIP138 also had a NLS sequence (GRKKK) located away from the N-terminus of the bZIP domain.

To further examine the subcellular localizations of these five TabZIP proteins, wheat protoplasts were separated from mesophyll tissue and then transformed with 35S::TabZIP9-GFP, 35S::TabZIP27-GFP, 35S::TabZIP74-GFP, 35S::TabZIP138-GFP, and 35S::TabZIP174-GFP fusion vectors, respectively. The 35S::GFP vector served as the control. Subsequently, GFP expression was monitored by confocal microscopy at 18 h after PEG transformation.

Green fluorescence was detected in the nucleus of wheat protoplasts for TabZIP27-GFP, TabZIP74-GFP, TabZIP138-GFP, and TabZIP174-GFP fusion proteins (Figure 6), indicating that TabZIP27, TabZIP74, TabZIP138, and TabZIP174 proteins were exclusively localized in the nucleus. Nevertheless, green fluorescence of TabZIP9-GFP fusion protein was simultaneously present in the nucleus, cytoplasm, and cell membrane.

Germination Rate and Primary Root Growth of TabZIP174 Transgenic Arabidopsis

The foregoing phylogenetic and conserved motif analyses suggested that TabZIP174 potentially participated in regulating abiotic stress response. To further investigate the role of TabZIP174 in response to abiotic stress, 35S::TabZIP174 transgenic Arabidopsis lines were generated. To examine differences in the germination rates and primary root growth between TabZIP174 transgenic and WT Arabidopsis, seeds of three transgenic lines (OE-2, OE-4, and OE-5) and WT were germinated on MS medium.

Under normal conditions, there were no significant differences in germination rates and primary root lengths between the transgenic lines (OE-2, OE-4, and OE-5) and WT. However, 3 days after the PEG treatment, the germination rates of the transgenic lines (OE-2, OE-4, and OE-5) were higher than that of WT (Figure 7A), and the differences between OE-2 and WT and between OE-4 and WT both reached the significant level (t-test, P < 0.05). Compared with WT, the germination rates of the transgenic lines OE-2, OE-4, and OE-5 increased by ~14, 17, and 12%, respectively.

the NaCl (or cold) treatment. After the NaCl treatment, TabZIP86 transcripts accumulated gradually and reached a maximum at 24 h post-treatment, while, in contrast, TabZIP80 expression was down-regulated by NaCl, with its transcript level dropping to a relatively lower level after the 24-h treatment. This suggested that homologous TabZIP genes in Subgroup A had undergone expression pattern shifts.
In the presence of PEG, primary root elongation was significantly inhibited for both the transgenic lines and WT, however, the primary root lengths of lines OE-2, OE-4, and OE-5 were significantly greater than those of WT plants (t-test, \( P < 0.01 \)) (Figures 7B,C). This indicated that the inhibitory effect of PEG on primary root growth was more serious in WT than in the transgenic plants. Thus, our results suggested that TabZIP174 overexpression enhanced the tolerance of transgenic Arabidopsis to the imposed drought stress.

**TabZIP174-Overexpressing Transgenic Arabidopsis had Enhanced Drought Tolerance**

Under well-watered conditions, there were no evident morphological differences between transgenic and WT Arabidopsis throughout their life cycles.

Four TabZIP174 transgenic lines were selected for testing in soil to characterize their performances under drought stress. At the early stage of the drought treatment (e.g., 2 weeks before rewatering), TabZIP174 transgenic and WT plants grew normally, with no notable phenotypic differences between them (Figure 8A: the upper row).

Eighteen days after withholding water, the lower rosette leaves of WT plants showed slight wilting, whereas TabZIP174 plants still grew normally. After 4-week drought treatment (just before rewatering), most WT plants were severely wilted and a number of rosette leaves were yellow or dead. In contrast, although most TabZIP174 plants were wilted and many rosette leaves were severely curled, most leaves remained green and only some TabZIP174 plants displayed symptoms of severe water deficit (Figure 8A: the middle row).

Four days after rewatering, 38–60% of the TabZIP174 plants had survived, whereas only 18% of the WT plants had survived (Figure 8A: the lower row, Figure 8B). The survival rates of lines OE-2, OE-4, and OE-5 were 42, 60, and 49%, respectively, which were much higher (t-test, \( P < 0.01 \)) than that of WT (18%). Thus, the overexpression of TabZIP174 greatly improved drought resistance in transgenic Arabidopsis.

**Physiological Changes in TabZIP174 Transgenic Arabidopsis**

Water loss rate is an important index that has been used to evaluate the water status of plants under water deficit conditions (Mao et al., 2012). In the present study, the water loss rates of the detached rosettes for transgenic lines were lower than that of WT plants, and the final relative water contents of transgenic lines were significantly higher than that of WT (t-test, \( P < 0.01 \)) (Figure 9), indicating that TabZIP174 transgenic plants had stronger water retention capacity.

To explore whether TabZIP174 overexpression influenced proline accumulation, the free proline contents in the transgenic and WT plants were measured. After drought treatment, the proline contents of the transgenic lines were higher than those of the WT plants. And the difference between each transgenic line (OE-2, OE-4, or OE-5) and WT reached the significant level (t-test, \( P < 0.01 \)) whether 2 or 3 weeks after drought treatment (Figure 10A).

The contents of total soluble sugars were also measured in the transgenic and WT plants under drought stress to determine whether the enhanced drought tolerance of the transgenic plants was associated with soluble sugars. Under water deficit conditions, the soluble sugar contents obviously increased compared with those before drought treatment for both transgenic and WT plants, and notably, the soluble sugar contents of transgenic lines OE-2, OE-4, and OE-5 were significantly higher than that of WT 2 weeks after drought treatment (t-test, \( P < 0.01 \)) (Figure 10B). The results suggested that TabZIP174 probably participated in proline and carbohydrate metabolism.

To retain a relatively stable intracellular environment under abiotic stress conditions, many plants decrease their intracellular osmotic potential by accumulating organic osmolytes (such as...
| TabZIP | PEG | NaCl | Cold (4°C) | ABA |
|--------|-----|------|------------|-----|
| TabZIP5 | ![Graph](image1) | ![Graph](image2) | ![Graph](image3) | ![Graph](image4) |
| TabZIP9 | ![Graph](image5) | ![Graph](image6) | ![Graph](image7) | ![Graph](image8) |
| TabZIP14 | ![Graph](image9) | ![Graph](image10) | ![Graph](image11) | ![Graph](image12) |
| TabZIP20 | ![Graph](image13) | ![Graph](image14) | ![Graph](image15) | ![Graph](image16) |
| TabZIP27 | ![Graph](image17) | ![Graph](image18) | ![Graph](image19) | ![Graph](image20) |
| TabZIP40 | ![Graph](image21) | ![Graph](image22) | ![Graph](image23) | ![Graph](image24) |
| TabZIP51 | ![Graph](image25) | ![Graph](image26) | ![Graph](image27) | ![Graph](image28) |
| TabZIP54 | ![Graph](image29) | ![Graph](image30) | ![Graph](image31) | ![Graph](image32) |
| TabZIP65 | ![Graph](image33) | ![Graph](image34) | ![Graph](image35) | ![Graph](image36) |
| TabZIP73 | ![Graph](image37) | ![Graph](image38) | ![Graph](image39) | ![Graph](image40) |
| TabZIP74 | ![Graph](image41) | ![Graph](image42) | ![Graph](image43) | ![Graph](image44) |
| TabZIP76 | ![Graph](image45) | ![Graph](image46) | ![Graph](image47) | ![Graph](image48) |
| TabZIP77 | ![Graph](image49) | ![Graph](image50) | ![Graph](image51) | ![Graph](image52) |
| TabZIP80 | ![Graph](image53) | ![Graph](image54) | ![Graph](image55) | ![Graph](image56) |
| TabZIP83 | ![Graph](image57) | ![Graph](image58) | ![Graph](image59) | ![Graph](image60) |
| TabZIP86 | ![Graph](image61) | ![Graph](image62) | ![Graph](image63) | ![Graph](image64) |
| TabZIP90 | ![Graph](image65) | ![Graph](image66) | ![Graph](image67) | ![Graph](image68) |

**FIGURE 5** | Continued
FIGURE 5 | Expression profiles of Subgroup-A TabZIP genes in response to multiple abiotic stress and ABA treatments. The $2^{-\Delta\Delta C_{T}}$ method was used to calculate the relative expression levels of TabZIP genes. The expression of each TabZIP gene at 0 h after treatment is regarded as a reference, and other values represent the expression levels relative to the reference. Mean values and SDs were obtained from three replicates.

proline, mannitol, and glycine betaine) in cells (Zhang et al., 2011). Our result revealed that 2 weeks after drought treatment, the transgenic plants had lower osmotic potential than WT. In contrast, the osmotic potential was higher in the transgenic plants than in WT 3 weeks after drought treatment (Figure 10C). And whether 2 or 3 weeks after drought treatment, the difference between transgenic line OE-4 (or OE-5) and WT reached the significant level ($t$-test, $P < 0.05$). Compared with WT plants, TabZIP174 transgenic lines maintained a relatively stable osmotic potential under water deficit conditions.

Additionally, the leaf chlorophyll content was measured to further determine possible physiological differences between transgenic and WT plants. Two weeks after drought treatment, the rosette leaves of all of the transgenic and WT plants remained green (Figure 8A: the upper row); with no evident difference in the chlorophyll content (Figure 10D). Three weeks after drought treatment, however, transgenic lines OE-4 and OE-5 had significantly higher chlorophyll contents than WT ($t$-test, $P < 0.05$) (Figure 10D). The severe drought resulted in a more rapid decrease in the chlorophyll content of WT than transgenic plants. This suggested that the overexpression of TabZIP174 could directly or indirectly slow chlorophyll degradation in leaves of transgenic plants under severe drought conditions.

Enhanced Expression of Stress-Responsive Genes in TabZIP174 Transgenic Plants

Our results of phenotypic studies showed that TabZIP174 transgenic lines had improved resistance to drought stress.
FIGURE 6 | Subcellular localization of TabZIP-GFP fusion proteins in wheat mesophyll protoplasts. The 35S::TabZIP9-GFP, 35S::TabZIP27-GFP, 35S::TabZIP74-GFP, 35S::TabZIP138-GFP, and 35S::GFP control vectors were independently transformed into wheat mesophyll protoplasts by PEG transfection. Green fluorescent was monitored under a laser scanning confocal microscope.

FIGURE 7 | Comparison of germination rates and primary root lengths between TabZIP174 transgenic and wild type (WT) Arabidopsis. OE-2, OE-4 and OE-5 represent three TabZIP174 transgenic Arabidopsis lines. (A) Germination rates of TabZIP174 transgenic and WT seeds after the 5% (w/v) PEG treatment. Germination rates were determined daily for 7 days following 2-day stratification. In each of three independent repetitions, 169 (13 × 13) seeds per Arabidopsis line were used. Data represent the means ± SDs. (B) Primary root growth of TabZIP174 transgenic and WT plants on Murashige and Skoog (MS) medium with or without 5% PEG. Thirty plants per Arabidopsis line were used in each of three independent repetitions. Representative transgenic and WT plants were photographed after the 7-day growth. (C) Statistical analysis of primary root lengths. Primary root lengths were measured after the 7-day growth. Data represent the means ± SDs. Asterisks indicate statistically significant differences between transgenic and WT plants (Student’s t-test, **P < 0.01).
FIGURE 8 | Overexpression of TabZIP174 in transgenic Arabidopsis improved drought tolerance. Fifteen plants per Arabidopsis line were used in each of three independent repetitions. OE-2, OE-4, OE-5, and OE-8 represent four TabZIP174 transgenic Arabidopsis lines. (A) Phenotypes of TabZIP174 transgenic and wild type (WT) plants under water deficit conditions and after rewetting. (B) Survival rates of TabZIP174 transgenic and WT plants 4 days after rewetting. Data represent the means ± SDs. Asterisks indicate statistically significant differences between transgenic and WT plants (Student’s t-test, *P < 0.05, **P < 0.01).

FIGURE 9 | TabZIP174 transgenic plants have stronger water retention ability. OE-2, OE-4, OE-5, and OE-8 represent four TabZIP174 transgenic lines. Data represent the means ± SDs. (A) Comparison of water loss rates for detached rosettes between transgenic and wild type (WT) plants under dehydration conditions. (B) Comparison of relative water contents (RWCs) for detached rosettes between transgenic and WT plants after a 7-h dehydration treatment. Asterisks indicate statistically significant differences between transgenic and WT plants (Student’s t-test, **P < 0.01).

To further explore the underlying molecular mechanisms, we performed the expression analyses of nine stress-response-related genes in transgenic and WT plants under normal and water deficit conditions. Transcript levels of six genes (RD29A, RD29B, RAB18, DREB2A, COR15A, and COR47) were similar between transgenic lines and WT under normal conditions, whereas after PEG treatment, their transcript levels were significantly higher in transgenic lines than in WT (t-test, P < 0.05 or P < 0.01) (Figure 11). However, there were no significant differences in transcript levels of the other three genes (P5CS, ABI1, and ABI2) between transgenic lines and WT (data not shown).

DISCUSSION

In this study, we performed a series of bioinformatic analyses of the Subgroup-A members of the wheat bZIP transcription factor family for the first time. Among the Subgroup-A bZIP proteins, 15 bZIPs (7 TabZIPS, 3 AtbZIPS, and 5 OsbZIPS) played important roles in regulating abiotic stress responses of plants (Kang et al., 2002; Kim et al., 2004; Kobayashi et al., 2008; Xiang et al., 2008; Zou et al., 2008; Lu et al., 2009; Hossain et al., 2010b; Tang et al., 2012; Xu et al., 2014; Zhang et al., 2015). Most (13/15) of these 15 bZIPs clustered in Clade A1 of the phylogenetic tree (Figure 1). Thus, the study of Subgroup-A bZIP genes, especially those in Clade A1, should be emphasized because of their potential importance in responses to abiotic stress. In addition, the expression of most TabZIP genes in Subgroup A were induced by PEG, NaCl, cold, and exogenous ABA treatments, implying that they might play roles in response to ABA or various abiotic stresses, just as those bZIP genes in Arabidopsis and rice (Choi et al., 2000; Xiang et al., 2008; Hossain et al., 2010b; Tang et al., 2012). The complicated variation trends in the gene expression reflected the complexity of the possible regulatory mechanisms of
abiotic stress responses by transcription factors. The subcellular localization results confirmed that most TabZIP proteins acted as transcription factors and performed their functions in the nucleus (Figure 6). Our analyses proposed the importance of the wheat Subgroup-A bZIP genes in abiotic stress responses, and laid a foundation for further studies on these TabZIP genes, which would facilitate the excavation of valuable gene resources with great potential for genetic improvement of abiotic stress tolerance in crops.

A novel Subgroup-A bZIP gene, TabZIP174, was cloned from wheat in this study. TabZIP174 was predominantly expressed in leaf (Supplementary Figure 4) and encoded a putative protein of 340 aa, with a theoretical isoelectric point of 8.84 and a molecular weight of 36.78 kDa (ExPaSy, Compute pi/Mw tool, http://web.expasy.org/compute_pi/). TabZIP174 protein possessed a typical bZIP conserved domain and was exclusively localized in the nucleus (Figure 6), which was consistent with its putative function as a transcription factor. Sequence analysis showed that TabZIP174 had a highly similar protein sequence and shared the vast majority of conserved motifs with OsbZIP23 and OsbZIP46 (Figure 1, Supplementary Figure 1), suggesting that they might have similar roles in regulating abiotic stress responses (Xiang et al., 2008; Hossain et al., 2010a; Tang et al., 2012).

To investigate the role of TabZIP174 in abiotic stress response, TabZIP174 was transformed into Arabidopsis, and its overexpression was confirmed by reverse transcription PCR. Germination, primary root growth and drought tolerance assays demonstrated that the overexpression of TabZIP174 in transgenic Arabidopsis significantly enhanced drought tolerance (Figures 7, 8). Our results were consistent with previous studies on other Subgroup-A bZIP proteins. For instance, the overexpression of TaABL1 in transgenic plants conferred enhanced resistance to drought stress (Xu et al., 2014). In addition, our expression analysis demonstrated that the expression of TabZIP174 was rapidly responsive to both PEG and ABA treatments (Figure 5). Our findings suggested that TabZIP174 participated in regulating plant response to drought stress, likely through an ABA-dependent pathway, which was similar to the regulatory mechanisms of several other bZIP transcription factors in Subgroup A (Kang et al., 2002; Xiang et al., 2008).
Interestingly, TabZIP174 transgenic plants exhibited higher germination rates and longer primary roots than WT plants after the PEG treatment (Figures 7A–C), suggesting that the overexpression of TabZIP174 weakened the adverse effects of PEG on germination and primary root growth. However, there were no evident differences in both germination rates (data not shown) and primary root lengths (Figures 7B,C) between transgenic and WT plants under normal conditions. Although TabZIP174 was constitutively overexpressed in transgenic plants under the control of CaMV 35S promoter, TabZIP174 protein may not be activated under normal conditions. Besides, under normal conditions, the expression levels of six stress-responsive genes were similar between transgenic lines and WT (Figure 11), which indicated that the overexpression of TabZIP174 was not sufficient to activate transcription of downstream target genes. As reported previously, ABA-activated SnRK2 protein kinases regulated ABA-induced genes by phosphorylating AREBs, such as TRAB1 (Kobayashi et al., 2005). The potential phosphorylation sites of TabZIP174 included 5 Ser, 10 Thr, and 1 Tyr residues (NetPhos 2.0 Server, http://www.cbs.dtu.dk/services/NetPhos/). The activation of TabZIP174 might require its own phosphorylation by ABA-activated SnRK2 protein kinases (Kobayashi et al., 2005; Furihata et al., 2006; Fujita et al., 2013), given that the PEG treatment could...
cause an increase in the endogenous ABA level (Lata and Prasad, 2011; Fujita et al., 2013; Chiappetta et al., 2015).

Gene overexpression might cause the growth retardation of transgenic plants (Kasuga et al., 1999; Kim et al., 2004; Maruyama et al., 2004; Dai et al., 2007), restricting the applicability of target genes in transgenic breeding. The morphological features of TabZIP174 transgenic plants were closely monitored, and no obvious adverse effects were observed, indicating the potential of TabZIP174 in plant breeding.

Adverse environmental factors often cause physiological changes in plants. Meanwhile, physiological indices can be used to evaluate the abiotic stress resistance of crops (Mao et al., 2012). Osmotic adjustment is defined as the decrease in osmotic potential achieved by osmolyte accumulation in response to osmotic stress, and is regarded as a beneficial mechanism for drought adaptation in some crop species (Girma and Krieg, 1992; Subbarao et al., 2000; Chen and Jiang, 2010). Generally, a higher capacity in the osmotic adjustment indicates a stronger tolerance and adaptation to osmotic stress. Osmotic potential is an effective physiological index, which is used to directly reflect the osmotic adjustment capacity and to evaluate the osmotic stress resistance of plants (Mao et al., 2012). In this study, our result of osmotic potential measurement indicated that TabZIP174 overexpression enhanced the osmotic adjustment capacity of transgenic Arabidopsis (Figure 10C). Proline or soluble sugars accumulation is a common physiological response to water stress in plants. Proline and soluble sugars are both regarded as important osmolytes in osmotic adjustment and play vital roles in weakening the adverse effects of osmotic stress (Kameli and Lösel, 1993, 1995, 1996; Igarashi et al., 1997; Kumar et al., 2003; Bartels and Sunkar, 2005; Claussen, 2005; Verbruggen and Hermans, 2008; Zhang et al., 2011; Mao et al., 2012). In this study, our results suggested that TabZIP174 overexpression accelerated the accumulation of proline and soluble sugars (Figures 10A,B), and that their rapid accumulation in transgenic plants probably contributed to the enhanced tolerance to drought stress. However, the osmotic potential for four transgenic lines was not completely consistent with either their proline or soluble sugar contents, implying that proline and soluble sugars might only partially account for the osmotic potential changes. At the early stage of drought treatment (e.g., 2 weeks after withholding water), transgenic plants possessed relatively lower osmotic potential, which indicated higher water retention capability. Therefore, at the later stage of drought treatment (e.g., 2 weeks after withholding water), transgenic plants had higher osmotic potential because of higher water contents.

Previous studies showed that the overexpression of maize ZmbZIP72 significantly increased the transcript levels of RD29B, RAB18, and HISI-3 and conferred enhanced abiotic stress tolerance in transgenic Arabidopsis (Ying et al., 2012). TaABL1 activated the expression of two stress-responsive genes (RD29B and RAB18) and two guard cell ion channel genes (KAT1 and KAT2) (Xu et al., 2014). TabZIP60 functioned in response to multiple abiotic stresses via an ABA-dependent pathway and up-regulated the expression of RD29A, RD29B, MYB2, COR47, RD20, DREB2A, and ERD6 under salt stress conditions (Zhang et al., 2015). Higher expressions of six stress-responsive genes (RD29A, RD29B, RAB18, DREB2A, COR15A, and COR47) were detected in TabZIP174 transgenic lines than in WT under water deficit conditions (Figure 11). RD29A possesses an ABA-responsive element (ABRE) and a dehydration-responsive element (DRE) in its promoter region, and is induced by drought, cold, and ABA treatments (Shinozaki and Yamaguchi-Shinozaki, 1997). The significant increase in transcription levels of RD29A, RD29B, RAB18, and COR15A (encoding low-molecular-weight hydrophilic proteins) accelerates osmolyte accumulation in cells, leading to the decrease of osmotic potential, and reduction of water loss rates under drought stress conditions (Láng and Palva, 1992; Yamaguchi-Shinozaki and Shinozaki, 1993; Zhou et al., 2009; Mao et al., 2012).

In conclusion, both phenotypic and physiological evidence demonstrated that the overexpression of TabZIP174 conferred the enhanced drought tolerance in transgenic Arabidopsis. In our view, the improved drought resistance was likely due to the increased osmotic adjustment capacity. The increased osmotic adjustment capacity enabled plants to maintain relatively lower cellular osmotic potential at the early stage of water deficit, which facilitated water retention and decreased water loss, thus strengthening drought resistance.

AUTHOR CONTRIBUTIONS

XL conceived of the study, performed the bioinformatics analysis and the experimental work, and wrote the manuscript. SG and YT helped in conceiving of the study and revised the manuscript. BF, FZ, and LZ helped in experimental work and data analysis. LM and CZ contributed with valuable discussion. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Availability of Data and Material

The datasets supporting the conclusions of this article are included within the article and its Supplementary Material.
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Conflicts of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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