Full Length Research Paper

Cloning and characterization of a putative transcription factor induced by abiotic stress in *Zea mays*

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bZIP transcription factors have been reported to play important roles in plant responses to abiotic stresses. Here, we reported the cloning and characterization of a putative bZIP transcription factor (*ZmbZIP17*) from maize inbred line Han21, which is up-regulated by drought treatment. The open reading frame sequence of *ZmbZIP17* was obtained by using 5'RACE and RT-PCR. Sequence analysis showed that *ZmbZIP17* encodes a polypeptide of 563 amino acids with predicted molecular mass of 59.8 kDa and pI of 5.6. Southern blot analysis showed that *ZmbZIP17* exists as a single copy gene in maize genome. Subcellular localization of ZmbZIP17 was identified in nucleus. The results of real-time PCR analysis indicated that *ZmbZIP17* was up-regulated by drought, heat, ABA and NaCl stress immediately, which suggested that *ZmbZIP17* is an early stage responsive gene to various abiotic stresses. The result also showed that *ZmbZIP17* expressed much higher in leaves than in other organs in maize seedlings.

Key words: Maize, *ZmbZIP17*, abiotic stress, subcellular localization.

INTRODUCTION

Environmental condition is the major stress that adversely affects plant growth and crop yield (Bartels and Nelson, 1994). Plants adapts to various environmental stresses such as drought, cold, heat and high salt through a lot of physiological and metabolic responses (Bray, 1993; Bohnert et al., 1995). Under these stress conditions, the plant hormone abscisic acid (ABA) level increases in vegetative tissues, triggering adaptive responses that are essential for their survival and productivity (Leung and Giraudat, 1998). Under water deficit stress, for example, ABA induces stomatal closure, minimizing water loss through transpiration. The ABA-controlled process is vital for plant survival.

At present, certain cellular and molecular responses of plant to abiotic stress have been investigated mainly using *Arabidopsis thaliana* as a model plant (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu, et al., 1997, Zhu, 2002). Many genes are involved in water stress response in plants, such as the enzymes required for osmolyte biosynthesis, chaperones, LEA proteins, mRNA binding proteins, water channel proteins, sugar and proline transporters, detoxification enzymes and various proteases (Ingram and Bartels, 1996; Seki et al., 2002). These proteins are probably regarded as functional proteins which directly respond to stress. Another group of genes called signal transduction gene also play essential roles in stress response. This group includes protein kinases, transcription factors and enzymes in phospholipid metabolism (Shinozaki and Yamaguchi-Shinozaki, 1997); they respond to stress by regulating the expression of other genes.

bZIP transcription factors are one of the most abundant transcription factor classes in eukaryotic class and they have been implicated in a diverse range of processes including commitment to cell survival versus apoptosis, the anti-inflammatory response and in the mammalian circadian oscillatory mechanism and so on (Cowell, 2002). During animal embryogenesis, bZIP factors are necessary for the proper development of organs and tissues such as the liver, bone, heart and fat (Wang et al., 1992; Darlington et al., 1998; Eferl et al., 1999). C/EBP family of bZIP transcription factors control the proliferation and

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differentiation of a variety of tissues (Grimm and Rosen, 2003). In yeast, bZIP factors are necessary for sexual differentiation and entry into stationary phase (Takeda et al., 1995). In plants, the functions of some bZIP transcription factors have been identified, especially in *A. thaliana*. The genome of *A. thaliana* encodes about 75 bZIP transcription factors. Genetic and molecular studies of some *A. thaliana* bZIP factors showed that they regulate diverse biological processes such as pathogen defense, light and stress signaling, seed maturation and flower development (Oyama et al., 1997; Chuang et al., 1999; Kim and Delaney, 2002; Kim et al., 2004). According to protein structural features, *Arabidopsis* bZIP transcription factors were divided into ten subgroups (Jakoby et al., 2002). Previous studies of bZIP transcription factors indicated that transcription factors belonging to the same group tend to exhibit similar functions. Among these ten subgroups, subgroupA transcription factors were usually considered as regulator in stress or ABA signaling pathway, *AtbZIP39*, *AtbZIP36*, *AtbZIP38*, *AtbZIP66*, *AtbZIP40*, *AtbZIP35* and *bZIP37* are all related to this pathway but other subgroups also participate in abiotic stress, for example *AtbZIP24*, regulates complex transcriptional networks involved in abiotic stress resistance (Yang et al., 2009).

To study the molecular mechanism of maize drought stress responses, we constructed a subtracted cDNA libraries from PEG-treated maize seedlings and analyzed the gene expression patterns by using cDNA microarray. Finally, hundreds of unique ESTs were isolated and validated as differentially expressed sequences in response to dehydration stress (Zheng et al., 2004). Among them, one EST was found to encode a putative transcription factor (*ZmbZIP17*). Here we report the cloning of *ZmbZIP17* gene from maize inbred line Han21 and the analysis of its expression pattern under various treatments. The subcellular localization and possible function of *ZmbZIP17* gene under drought stress are also discussed.

MATERIALS AND METHODS

Isolation of *ZmbZIP17* full length cDNA

One EST (CK986343) sequence was chosen from PEG-induced subtracted cDNA library (Zheng et al., 2004) and was used as a template for designing PCR primers. First, 5'RACE was performed using the SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech) by the manufacturer's instructions. After RT with primers supplied by the kit, the adaptor-ligated first strand cDNA was synthesized as PCR amplification template. PCR amplification reactions were set up using *Ex Taq* DNA polymerase (TaKaRa) and gene-specific primers: GSP1 (5'-AGGACGGCCGCGAGGTAG-3') and NGSP1 (5'-GCTCGGCGTCTGGGGAAATCTGCT-3'); GSP2 (5'-ATGCGGCCGACGGGGAAC-3') and NGSP2 (5'-CTGCAGGAATCAAACACCCACAAAG-3') for 5' RACE. The complete coding region of the cDNA was amplified by PCR approach using the specific primer pair, 5'-TCCATGGCGGAACCGGCC-3' (forward) and 5'-GGTGACCCTAAAGGTGAGGGCTATGG-3' (reverse). The

condition for amplification was at 94°C for 3 min followed by 30 cycles at 94°C for 30 s, at 62°C for 30 s and at 72°C for 1.5 min, plus a final extension at 72°C for 10 min. Three independent PCR products were purified, cloned into pGEM T-easy vector (Promega) and sequenced.

Southern blot analyses

For Southern blot analysis, genomic DNA (30 μg) from the seedlings of the maize inbred line Han21 was digested with *Bgl*II, *Kpn*I, *Eco*RV, *Eco*RI, *Hind*III, *Xba*I, separated by electrophoresis on a 0.8% agarose gel, transferred to a nylon membrane (Amersham) and hybridized for 16 h at 65°C with α-^{32}CTP-labeled partial coding region of the *ZmbZIP17* cDNA as a probe. The membrane was washed with 2 × SSC, 0.1% SDS; 1 × SSC, 0.1% SDS and 0.5 × SSC, 0.1% SDS for 15 min at 65°C, respectively. A 510 bp cDNA fragment was used as the probe and amplified by PCR using the primers: 5'- TGAAGTTTTCCAGTTCGATGTGT-3' and 5'-GGAAAT CAGCATCCGCGG-3'.

Maize material and stress treatments

Maize inbred line Han21 was used in this study. Seeds were directly sown in vermiculite saturated with water in pots. The seeds were allowed to grow for 9 days under a 16 h light/8 h dark cycle at 26°C. For NaCl and ABA treatments, the roots of seedlings were submerged into a water solution of 250 mM NaCl or 100 μm ABA for 1, 3, 6, 12 and 24 h, respectively. For drought treatment, the young seedlings were pulled out and dehydrated in the air for periods of 0.5, 1, 3, 6, 12 h. For heat treatment, the young seedlings were put into 42°C conditions for periods of 1, 3, 6 and 12 h. After these treatments, seedlings were collected. Organs: root, stem, leaf, spike, ear, silk, husk and coleoptile were collected at specific development stage.

Quantitative real-time PCR analysis

RNA was isolated from maize samples using the TRIzol reagent (Invitrogen) based on the standard protocol. The quality and quantity of every RNA sample was assessed by agarose gel electophoresis. The cDNA was synthesized from the total RNA using the M-MuLV reverse transcriptase (New England BioLabs) according to the manufacturer's instructions. The real-time PCR was conducted with SYBR Premix *Ex Taq™* (Takara) and carried out using a Biorad system. The primers for real-time PCR: 5'-TTCGCACGGTCAAG AACAGA-3' and 5'–TGAGGGCTATGGCTTTTGAAC-3'. The reaction procedures were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 5 s, 60°C for 15 s and 72°C for 15 s. Tubulin (accession number AY103544) was used as the internal control. Relative fold expression changes were calculated using the relative 2$^{-\ t}$ method (Livak and Schmittgrn, 2001).

Subcellular localization assay

The full-length cDNA of *ZmbZIP17* was amplified with primers: 5'-CCACTAGTATGGCGGAACCGG-3' and 5'-CGGTACCAAAGGTGA GGGCTATG-3', the DNA fragment obtained by PCR was digested with *Spe*I and *Kpn*I, fused to the pMDC85-derived GFP-expression vector, then transiently expressed in onion epidermal cells (Scott et al., 1999) using a biolistic PDS-1000/HeTM particle gun (Bio-Rad). After incubation for 18 - 24 h, fluorescence emission of GFP was observed under a confocal microscope a Nikon Eclipse TE2000-E inverted fluorescent microscope at an excitation wavelength of 488 nm.

RESULTS

Cloning and sequence analysis of *ZmbZIP17* cDNA

Our laboratory constructed a maize cDNA library. From this subtracted maize cDNA library, one EST was isolated as a differentially expressed sequence in response to osmotic stress. According to the blast results, this EST was aligned to one regions of an *Arabidopsis* gene, *AtbZIP17*, which encodes a putative transcription factor. This EST sequence was a partial cDNA sequence which had the complete 3' end sequence but didn't have the 5' end sequence via sequence analysis. 5' RACE was carried out to obtain the 5' end sequence. Finally, the open reading frame of this gene was amplified by RT-PCR and cloned. Three positive colonies were picked up and sequenced to avoid PCR-generated errors. Two of the sequences were identical, so these two clones were considered correct. Since the cDNA sequence was a member of the bZIP family and had high similarity with *AtbZIP17*, so we named the cDNA as *ZmbZIP17*. *Zmb ZIP17* has an open reading frame of 1692 nucleotides, encoding a protein containing 563 amino acids with predicted molecular mass of 59.8 kDa and pI of 5.6. *ZmbZIP17* cDNA sequence from Han21 has 99% identity with the newly published full-length cDNA sequenced from B73 (ACCESSION number: NM_001158672) and the encoded proteins have 99% identity. The difference was mainly caused by SNP existence between different varieties. The nucleotide difference causes 6 amino acids difference (Figure 1). Putative conserved domain detection tools at EBI sites (http://www.ebi.ac.uk/Inter-Pro Scan/) suggested the existence of a bZIP domain (amino acids 117–177) (Figure 1A) in the *ZmbZIP17* sequence, which suggested that *ZmbZIP17* belongs to the bZIP transcription factor family.

Multiple-sequence alignment demonstrated that *ZmbZI P17* had low similarity with bZIPs genes from other plants: 68% with OsbZIP50, 51% with AtbZIP17 and Gmb ZIP37. Lower sequence similarity with dicot bZIP transcription factors indicated that bZIP genes weren't very conservative among different species. To analyze the closeness of *ZmbZIP17* with related bZIPs, a phylogenetic tree was constructed based on their amino acid sequences (Figure 1B), which included one rice gene, three *Arabidospis* genes and two soybean genes. Based on this phylogram, *ZmbZIP17* was more similar to OsbZIP50 than other bZIPs genes.

Copy number analysis of *ZmbZIP17*

The copy number of *ZmbZIP17* in maize genome was determined by Southern blot analysis. We used six restriction enzymes to digest the genomic DNA: *Bgl*II, *Kpn*I, *EcoR*V, *EcoR*I, *Hind*III, *Xba*I. To avoid probe hybridization with other similar members of *ZmbZIP* family, a 510bp PCR fragment near 3' UTR of *ZmbZIP17* was selected as the probe and the restriction sites of these six enzymes were not found in the probe sequence. Southern blot analysis showed only one clear band in each lane (Figure 2), so we concluded that *ZmbZIP17* exist as a one copy gene in the maize genome.

Subcellular localization of *ZmbZIP17*

To monitor the subcellular localization of *ZmbZIP17*, a ZmbZIP17-GFP fusion construct, driven by the CaMV 35S promoter and GFP gene driven by CaMV 35S promoter as a control were transiently expressed in onion epidermal cells. By confocal microscopy, the GFP was observed only in the nucleus in the 35S-ZmbZIP17-GFP bombarded cells (Figure 3B). However, the GFP could be observed everywhere in the 35S–GFP bombarded cells (Figure 3D).

Expression of *ZmbZIP17* under various stresses

To analyze the expression pattern of *ZmbZIP17*, real-time quantitative PCR analysis was carried out under various stress treatments: drought, heat, ABA and NaCl. The results showed that *ZmbZIP17* expression was up-regulated by drought stress and its expression level was continuously increased with water loss (Figure 4A). Under heat shock treatment, *ZmbZIP17* expression was transiently up-regulated at 1 h treatment and down-regulated later (Figure 4B). Under NaCl treatment, *ZmbZIP17* was highly up-regulated at 1 and 3 h. After 3 hours, *ZmbZIP17* expression was maintained at a stable level (Figure 4C). For ABA treatment, *ZmbZIP17* was up-regulated two folds with different treat time (Figure 4D). These results indicated that *ZmbZIP17* was up-regulated rapidly by all these four treatments.

Expression of *ZmbZIP17* in various organs

The expression levels of *ZmbZIP17* in different organs: root, stem, leaf, spike, ear, silk, husk and coleoptile were also determined by real-time PCR analysis. The data showed that the transcript level of *ZmbZIP17* in maize leaves was noticeably higher than other organs (Figure 5). The result might indicate that *ZmbZIP17* mainly functions in leaves.

DISCUSSION

The open reading frame of *ZmbZIP17* has been successfully cloned and characterized. Sequence analysis showed that *ZmbZIP17* contains 1692 nucleotides and encodes a putative transcription factor containing a con-

A

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Han21      1 MAEPALLAPDPFADLPFPEFQAPIDGDSFALEDFDLEDLDLDVDFDLDLF     50
             |||||||||||||||||||||||||||||||||||||||||||||||||
B73        1 MAEPALLAPDPFADLPFPEFQAPIDGDSFALEDFDLEDLDLDVDFDLDLF     50

Han21     51 ASDGQPSQPPPLATSSSSAGSPVGGSSSSGAVGDGGGLRNEESSESSSRS    100
             |||||||||||||||||||||||||||||||||||||||||||||||||
B73       51 ASDGQPSQPPPLATSSSSAGSPVGGSSSSGAVGDGGGLRNEESSESSSRS    100

Han21    101 ASGTDGSGKGKGEEDEAKRRARRVRNRESAHLSRQRKKQYVEELEGKVKT    150
             |||||||||||||||||||||||||:|||||||||||||||||||||||.
B73      101 ASGTDGSGKGKGEEDEAKRRARQVRNRESAHLSRQRKKQYVEELEGKVKA    150

Han21    151 MQATIADLSARISCVTAENAALKQQLGGAAGAAPPPLPMYPAMYSLPMPW    200
             |||||||||||||||||||||||||||||||||||||:||||.||||||||
B73      151 MQATIADLSARISCVTAENAALKQQLGGAAGAAPPPMPMYPTMYSLPMPW    200

Han21    201 MHPSYPMRGSQVPLVPIPRLKPPQPASAVAEPPAKKARKTKKVASVSLLG    250
             ||||||||||||||||||||||||||.||||||||||||||||||||||
B73      201 MHPSYPMRGSQVPLVPIPRLKPPQPAPAVAEPPAKKARKTKKVASVSLLG    250

Han21    251 LICVAMLCGCLIPAVNRMYDSVDAGEGAAYGPSHRGRVLAVEGPHDNVSD    300
             |||||||||||||||||||||||||||||||||||||||||||||||||
B73      251 LICVAMLCGCLIPAVNRMYDSVDAGEGAAYGPSHRGRVLAVEGPHDNVSD    300

Han21    301 GVDPKPPQRASETLPALLYLPKNGKHVKINGNLVIKSIVASEKASLQMSG    350
             |||||||||||||||||||||||||||||||||||||||||||||||||
B73      301 GVDPKPPQRASETLPALLYLPKNGKHVKINGNLVIKSIVASEKASLQMSG    350

Han21    351 NDGKRPQNQEEETRLAIPGYVTPLKAGEVMESTEGMMNNELLALAPADGS    400
             |||||||||||||||||||||||||||||||||||||||||||||||||
B73      351 NDGKRPQNQEEETRLAIPGYVTPLKAGEVMESTEGMMNNELLALAPADGS    400

Han21    401 MYREEDGLLPQWFSEAMSGPLLSSGMCTEVFQFDVSPSSAHPNGIIPVYS    450
             |||||||||||||||||||||||||||||||||||||||||||||||||
B73      401 MYREEDGLLPQWFSEAMSGPLLSSGMCTEVFQFDVSPSSAHPNGIIPVYS    450

Han21    451 NAMSNSSQNFTQDLPPARVRTVKNRRISYSEAIPLRGSTSNDTEHLKSFG    500
             ||||||||||||||||||||||||||||||||||:|||||||||||||||
B73      451 NAMSNSSQNFTQDLPPARVRTVKNRRISYSEAIPVRGSTSNDTEHLKSFG    500

Han21    501 STKPVSSVVVSVLADPREAGDGDGEGRISSKSLSRILVVVLIDSVKYVTY    550
             |||||||||||||||||||||||||||||||||||||||||||||||||
B73      501 STKPVSSVVVSVLADPREAGDGDGEGRISSKSLSRILVVVLIDSVKYVTY    550

Han21    551 SCVLPFKSHSPHL        563
             |||||||||||||
B73      551 SCVLPFKSHSPHL        563
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B

Figure 1. Alignment of the deduced amino acid sequence of ZmbZIP17 from Han21 and B73, conserve domain analysis and phylogenetic tree of bZIP family member proteins. A. Alignment of amino acid (aa) sequence was conducted by using the CLUSTALW (1.83) programs at EBI server. B. Phylogenetic tree of bZIP family member proteins. The phylogram was constructed by using the DNASTAR software. One rice proteins (OsbZIP50), three *Arabidospis* proteins (AtbZIP17, AtbZIP28 and AtbZIP49) and two soybean proteins (GmbZIP37 and GmbZIP38) were compared with ZmbZIP17.

served domain of bZIP but the whole amino acid sequences of different species have big variations, especially between monocot and dicot. Our data revealed that *ZmbZIP17* belongs to the bZIP transcription factor family
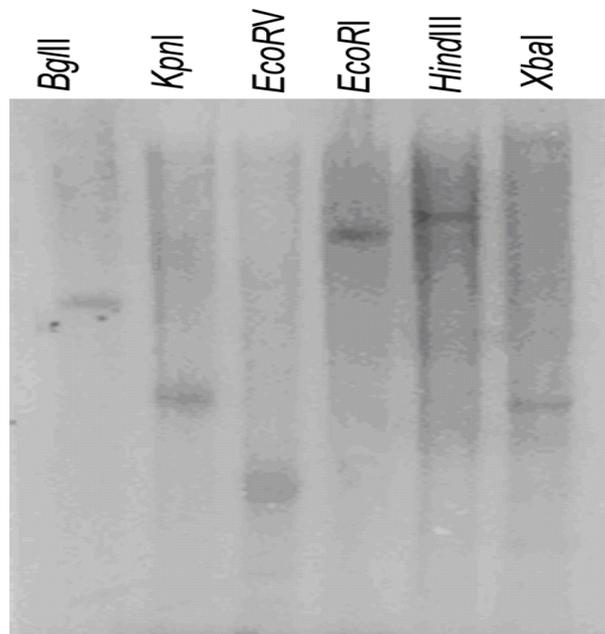
Figure 2. Southern blot analyses of *ZmbZIP17* to determine the copy number in the maize genome. Genomic DNA (30 μg) was completely digested with *Bgl*II, *Kpn*I, *EcoR*V, *EcoR*I, *Hind*III, *Xba*I, separated by electrophoresed on 0.8% (w/v) agarose gel and transferred to a nylon membrane. A 510 bp cDNA fragment located in the 3' region of the *ZmbZIP17* open reading frame was labeled with α-32p-dCTP as probe.
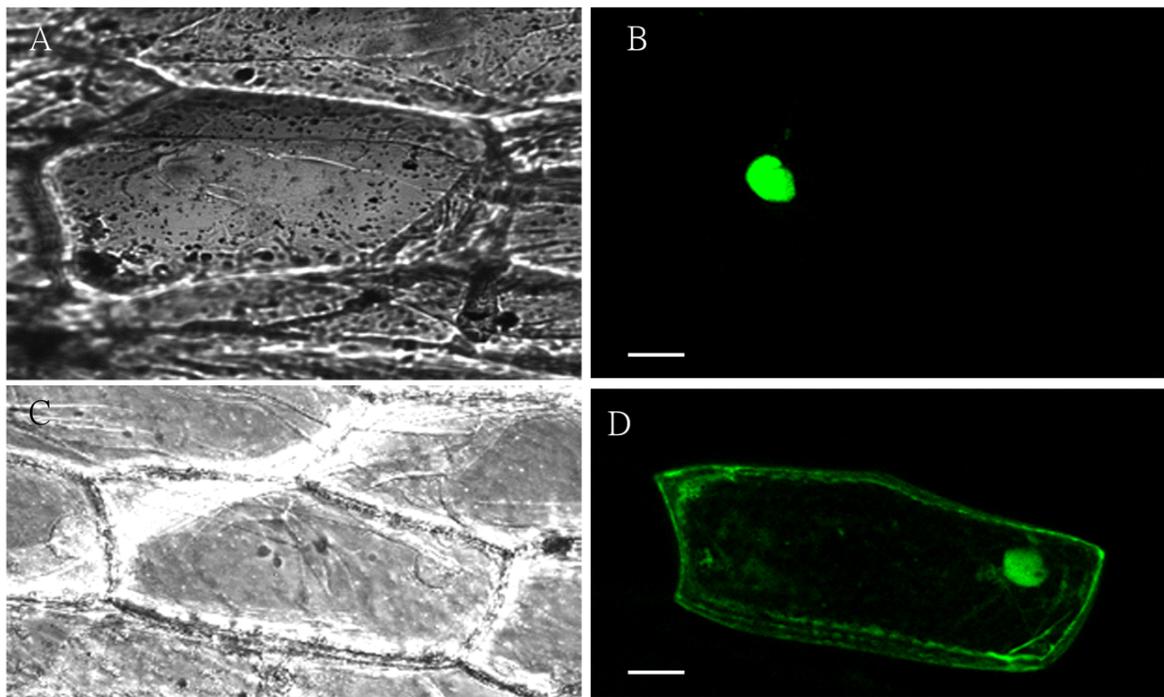
Figure 3. Confocal imaging of onion epidermal cells transiently expressing ZmbZIP17-GFP fusion protein. The onion epidermal transformed with empty vector (control), bars = 10 μm. A. 35S-ZmbZIP17-GFP observed under bright-field. B. 35S-ZmbZIP17-GFP observed under blue fluorescence. C. 35S-GFP observed under bright-field. D. 35S-GFP observed under blue fluorescence.
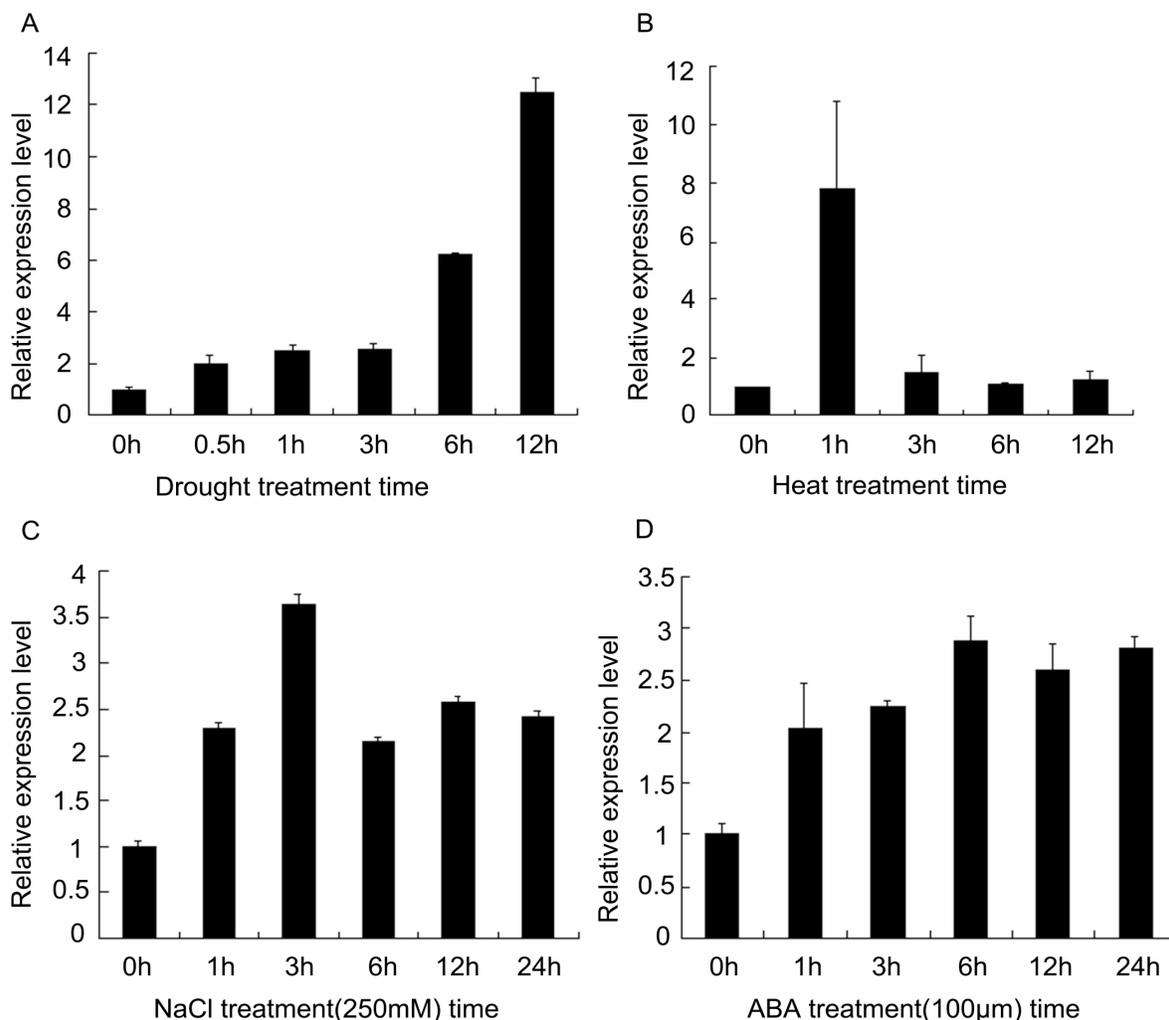
Figure 4. Expression of *ZmbZIP17* under various treatments in maize inbred line Han21: A. Drought treatment, B. heat treatment, C. NaCl treatment(250 mM), and D. ABA treatment(100 μm). The maize α-tubulin gene was used as the internal control for normalization of the template cDNA. Each PCR was repeated at least three times and the error bars represent the SD. The transcript level at time 0 h (untreated control) was used as the calibrator whose *ZmbZIP17* mRNA level was given as 1.

as a single copy gene existing in the maize genome. Real-time PCR analysis showed that the expression of *ZmbZIP17* was up-regulated by drought stress continuously within the treatment period. In addition, *Zmb ZIP17* transcription was also induced by heat, ABA and high salt stresses immediately, these results demonstrated *ZmbZIP17* has different expression patterns under different treatments. Expression in different organs showed that its transcription level is very low in other organs but high in leaves. As conclusion, *ZmbZIP17* is an abiotic stress response factor mainly functioning in leaves.

Subcellular localization analysis indicated that *ZmbZIP 17* is localized to nucleus, this result is consistent with former data showing that transcription factors are localized in nucleus and bind to specific DNA sequences to regulate the expression of downstream genes

(Bensmihen et al., 2005). However this result is different from its similarity *Arabidopsis* gene *AtbZIP17* which is localized to ER (Liu et al., 2007). According to protein structure analysis, AtbZIP17 has a N-terminal bZIP domain, a transmembrane segment and a canonical [RXXL or RXLX] S1P cleavage site on the C-terminal side of the transmembrane segment, AtbZIP17 is cleaved and the N-terminal bZIP component is translocated to the nucleus, where it activates the expression of salt stress response genes (Liu et al., 2007). However, protein of *ZmbZIP17* have no canonical [RXXL or RXLX] S1P cleavage site on the C-terminal side of the transmembrane segment, and so *ZmbZIP17* could not be cleaved before translocation to the nucleus. Maybe that is the reason why *ZmbZIP17* is directly localized to nucleus as a common transcription factor. By now, no study indicated *AtbZIP17* overexpression in *Arabidopsis* can bring any
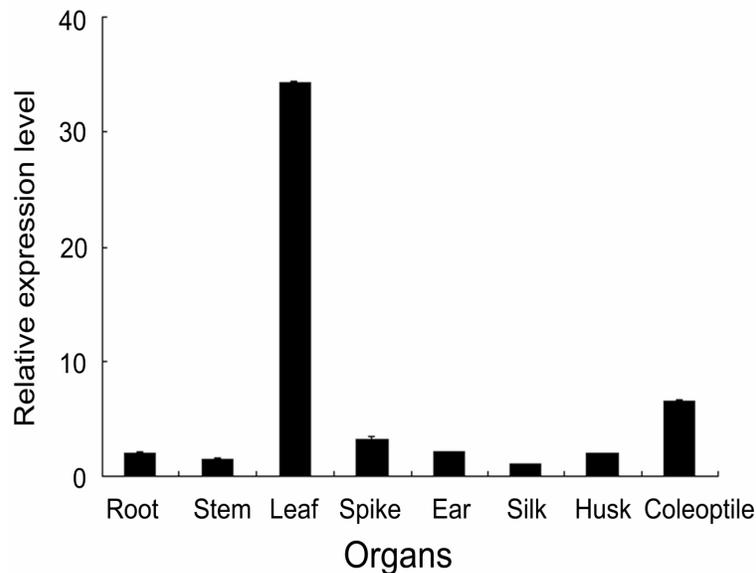
Figure 5. Expression of *ZmbZIP17* in various organs. The maize α-tubulin gene was used as the internal control for normalization of the template cDNA, each PCR was repeated at least three times and the error bars represent the SD. The transcript level in root was used as the calibrator whose *ZmbZIP17* mRNA level was given as 1.

obvious phenotype. We also constructed a *ZmbZIP17* over-expression vector and transferred this gene into *Arabidopsis*. However, no obvious phenotype was observed on 8 independent transgenic lines (data not shown). Two reasons may lead to this result: one reason is that *ZmbZIP17* overexpression in *Arabidopsis* is hard to regulate the expression of *Arabidopsis* downstream genes, and another reason is that *ZmbZIP17* over-expression in *Arabidopsis* caused weak phenotype, so no visible phenotype was observed.

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