

Cloning and Expression Analysis of a Salt-Stress-Induced HD-Zip Transcription Factor *HB-12* from Sunflower (*Helianthus annuus* L.)

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Abstract *HB* transcription factor genes play a significant role in plant growth and development, including response to biotic and abiotic stresses. In this study, an HD-Zip transcription factor *HB-12* was cloned from sunflower using SMARTer RACE technology based on Unigene551_All known sequence. The full-length *HB-12* cDNA sequence is 821 bp, including 573 bp open reading frame and encoding 190 amino acids. The predicted protein molecular weight and isoelectric point are 22.55 kD and 5.58, respectively, with a homeobox domain (HD) and a homeobox-associated leucine zipper domain (HALZ). *HB-12* belongs to the sunflower HD-Zip I subfamily proteins. The GenBank sequence accession number is KU315052. *HB-12* protein does not exist in the transmembrane domain, and its subcellular localization predicted that it might be in the nucleus. Cluster analysis revealed that the sunflower *HB-12* is closely related to the *HB-12* of potato and tomato crops. Genomic DNA sequence corresponding to the full-length cDNA of *HB-12* was amplified using polymerase chain reaction (PCR). The full length of the coding region is 652 bp, and two exons are separated by one intron. The sequence has been submitted to GenBank (Accession No. KU315053). Real-time PCR analysis showed that *HB-12* expression was induced by salt, abscisic acid (ABA), and polyethylene glycol (PEG) and varied in different organs, such as roots, hypocotyls, and leaves. This study lays a foundation for research in molecular breeding of sunflower.

Keywords Sunflower (*Helianthus annuus* L.); *HB-12*; HD-Zip; Stress; Expression analysis

Understanding the molecular basis of plant response to abiotic stresses, such as drought and salinity, is crucial for improving crop resistance to these stresses. At present, thousands of stress response genes have been identified, some of which have been used as target genes to improve crop stress resistance through genetic engineering. Transcription factors are excellent target genes for studying the molecular mechanism of plant response to abiotic stresses because they regulate the expression of many downstream target genes individually or in combination. Among the transcription factors, *Homeobox (HB)* gene plays a vital role in plant growth, development, and response to abiotic stresses. This has been confirmed through various studies involving *HB*-overexpression and *HB*-mutant plants. Maize *knotted1* gene is the first *HB* gene to be identified in plants (Vollbrecht et al., 1991). Several *HB* homologous genes have since been discovered in many plants. The most important feature of *HB* transcription factors is that they all have a highly conserved domain- Homeodomain (HD) composed of 60 (or 61) amino acids. In plants, *HB* protein contains other conserved domains besides HD. According to the position and sequence difference of HD motif and the homology of its two sides and other conserved domains, plant *HB* genes are categorized in six families, including HD-Zip (Homeodomain-leucine-zipper), PHD-Finger (Homeodomain-finger), ZF-HD (Zinc finger-Homeodomain), KNOX (KNO TTED1-like homeobox), WOX (Wuschel-related homeobox), and BELL (Ariel et al., 2007). Among them, HD-Zip family proteins are unique to higher plants.

In addition to a highly conserved homeodomain (HD), HD-Zip protein is closely linked with a leucine zipper (LZ) domain (known as HALZ, generally represented by Zip) at the carboxyl end of HD. HD-Zip protein forms dimer through the LZ domain and then binds to a specific DNA sequence (Meijer et al., 1997; Frank et al., 1998; Johannesson et al., 2001). A total of 42 HD-Zip family genes have been found in *Arabidopsis thaliana* (Mattsson et al., 1992; Schena and Davis, 1992). Based on their domain conservation, HD-Zip family is divided into four

subfamilies: HD-Zip I, HD-Zip II, HD-Zip III, and HD-Zip IV (Ariel et al., 2007; Elhiti and Stasolla, 2009). HD-Zip transcription factors have 17 members that mainly participate in responses to abiotic stress, ABA, blue light, etiolation, and embryonic development (Ariel et al., 2007; Elhiti and Stasolla, 2009). For example, *ATHB-12* expression is induced by water and exogenous ABA in *Arabidopsis thaliana* (Son et al., 2010). Also, *ATHB-7* can be induced by drought, exogenous ABA, and salt stress (Olsson et al., 2004). HD-Zip II transcription factors have nine members that mainly facilitate various light responses and auxin signal transduction (Ariel et al., 2007; Elhiti and Stasolla, 2009). For example, *ATHB-2* controls hypocotyl development through far-infrared light regulation in *A. thaliana* (Morelli and Ruberti, 2002; Ciarelli et al., 2008). HD-Zip III transcription factors have five members that mainly control embryonic development, leaf polarity, lateral organ initiation, and meristem development (Ariel et al., 2007; Elhiti and Stasolla, 2009). For example, *PHB*, *PHV*, *REV*, and *ATHB15* regulate apical meristem differentiation, polarity of lateral organs, and embryonic development in *Arabidopsis thaliana* (Byrne, 2006). HD-Zip IV transcription factors consist of 16 members that regulate anthocyanin accumulation, epidermal cell differentiation, hairy body formation, and root development (Ariel et al., 2007; Elhiti and Stasolla, 2009). Specifically, *PDF2* and *ATML1* of *Arabidopsis thaliana* participate in epidermal cell differentiation and regulate epidermal-specific gene expression (Sahu and Shaw, 2009). Additionally, *ATHB-10* is mainly involved in the formation of the seed coat, epidermal hair, and root hair (Ariel et al., 2007; Elhiti and Stasolla, 2009). Besides *Arabidopsis thaliana*, HD-Zip proteinoid are found in *Oryza sativa* (Meijer et al., 1997; Itoh et al., 2008), *Glycine max* (Wang et al., 2005), and *Zea mays* (Vernoud et al., 2009). At present, HD-Zip I transcription factor *Hahb-4* (Dezar et al., 2005a) and HD-Zip II transcription factor *Hahb-10* (Rueda et al., 2005) have been identified and studied in *Helianthus annuus*. *Hahb-4* expression is induced by water, high salt, drought, ABA, mechanical injury, SA, JA, ethylene, and darkness (Dezar et al., 2005b; Manavella et al., 2006; 2008a), whereas *Hahb-10* expression is regulated by the quality and quantity of light. Studies have shown that *Hahb-10* expression can be induced by gibberellin treatment, which correlates with early flowering (Rueda et al., 2005; Dezar et al., 2011).

In this study, HD-Zip I transcription factor *HB-12* was cloned from salt-tolerant sunflower cultivar Neikuiza 4 (P50), based on the Unigene551_All obtained from the transcriptome of sunflower grown under salt stress (Sun et al., 2015a) and the sequencing results of expression profile (unpublished). Subsequently, *HB-12* sequence analysis, function prediction, and expression analysis were performed. This study lays a foundation for understanding the *HB-12* regulation mechanism in response to abiotic stress in sunflower. Our findings can be applied to develop abiotic stress-resistant crops through genetic engineering or breeding.

1 Results and Analysis

1.1 Full-length cDNA cloning and *HB-12* sequence analysis

Gel electrophoresis of the total RNA showed that the brightness of 28S band was twice that of 18S. Also, the ratio of *OD*₂₆₀ to *OD*₂₈₀ was 1.8, indicating that the extracted RNA had good integrity and high purity, and therefore met the requirements of reverse transcription (Figure 1A). SMARTer RACE 5' and 3' primers were designed based on the Unigene551_All known sequence obtained through high throughput screening. The 5' and 3' end sequences were amplified using PCR, and about 700 bp (Figure 1B) and 500 bp (Figure 1C) bands were obtained. The PCR products were cloned and sequenced, and then the sequences were spliced with known sequences. The obtained sequences were 5' and 3' terminal sequences of Unigene551_All. The full-length cDNA of the splicing sequence was 821 bp. The expected band of 700 bp was obtained by RT-PCR amplification using specific primers (Figure 2, Lane 2). The PCR product was cloned and sequenced. The sequence was consistent with the splicing sequence, which verified the correctness of the gene. The maximum open reading frame (ORF) was predicted to be 573 bp and encoding 190 amino acids. The 5' and 3' untranslated regions (5'Utr and 3'Utr) were 41 bp and 207 bp, respectively, including a poly (A) (Figure 3). The predicted protein molecular weight and isoelectric point were 22.55 kD and 5.58, respectively. Amino acid sequence alignment indicated that the gene had high similarity (36%~57%) with the HD-zip transcription factor *ATHB-12* reported in many species. Therefore, the gene was named *HB-12*, and the sequence was submitted to the GenBank under accession number KU315052. Online analysis (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) revealed that *HB-12* and other homologous proteins have conserved homeobox domain (HD) sequence and homeobox associated leucine zipper domain

(HALZ) sequence. The former is the region between the 29th and 79th amino acids, and the latter is the region between the 81st and 125th amino acids, conforming with the typical characteristics of HALZ domain: a leucine residue appears every seven amino acids and repeated 5~6 times (Figure 3; Figure 4). TMHMM results indicated that there is no transmembrane domain in HB-12 protein. Meanwhile, subcellular localization prediction analysis showed that HB-12 protein might be in the nucleus. Phylogenetic comparison of sunflower HB-12 amino acid sequences with those of *A. thaliana*, potato, tomato, and other 12 species revealed that sunflower *HB-12* gene has a closest genetic relationship with *ATHB-12* gene of potato and tomato, and furthest genetic relationship with *ATHB-12* gene of *Beta vulgaris* (Figure 5).

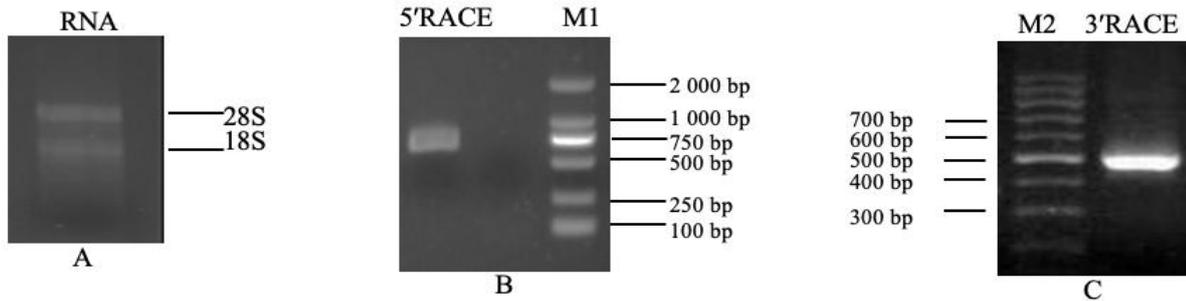


Figure 1 Electrophoretogram of total RNA and RACE amplification of *HB-12* in sunflower
 Note: M1: DL2000 Marker; M2: 100 bp Marker

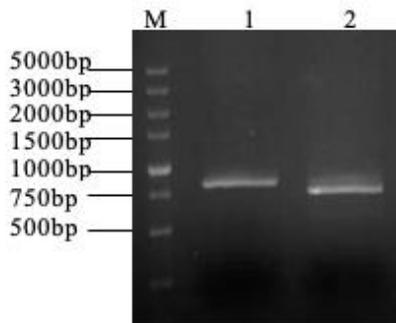


Figure 2 Electrophoresis of ORF cDNA and gDNA amplification
 Note: M: DL5000 Marker; 1: ORF gDNA; 2: ORF cDNA

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1      gactacaacccccctcttgggtcacatccttctaaaatcatac
42  ATGTTAGACATGGGATCAGACCCATATTCAGAATGCAGTTTCACTGCCTTCTTCAACAACAAGAACAACAAC
    M L D M G S D P Y S E C S F T A F F N N K N N N
114 AAAATACAAAGGCGGTTCAACCAACCAACAGATCGAATCACTCGAGACCATTTCAGACCGAATCCAAATC
    K I Q R R F T N Q Q I E S L E T I F Q T E S K L
186 GAACCTCGTAAGAAGTTGAGCTAGCTAAAGATCTTGGACTACAACCGGACAGGTAGCAATTTGGTTTCAA
    E P R K K L Q L A K D L G L Q P R Q V A I W F Q
258 AATAAGCGAGCTCGATGGCGATCAAAGCAATTAGAAAAAGACTACAACATACTAAAAGCTAGTTATGATTG
    N K R A R W R S K Q L E K D Y N I L K A S Y D S
330 CTTTCATCGGATACGACCTCCTCAGTAGAGAGCATCAGTCATTAACCCACCAGGTGAATAAGCTACGTGAA
    L S S R Y D L L S R E H Q S L T H Q V N K L R E
402 GGGATTGAGAAGAAAGCAAAAAGCTGTAGCACAGGGGAACTTCATACAATTCAATGGAAGAGAAGGTGTCT
    G I E K K A K S C S T G E T S Y N S M E E K V S
474 GATGCTGACAGCAACATGAAACAAGAATATATTGAAGATGAGAAAATGAATAATAATAATATGGGTTTTGTG
    D A D S N M K Q E Y I E D E K M N N N N M G F V
546 GAGAATTGCAAAAATGTGGAGTGTGGGATGATTGGAGTGTGATCAATGGTGGGACCTTTGGTCTTAA-614
    E N C K N V E C W D D L E C D Q W W D L W S *
615 attatattgagatttcttgatataataatcattttatgtacaataaggatgggtcatggatgataatatatta
687 tgatgatgattcttacgttaagccttgaggggatgtcataattacttacatatacatttagtaaatataaga
759 tcatgttggatattaaccaactaactttcttggatataaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
    
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Figure 3 The full-length nucleotide sequence of *HB-12* cDNA and its deduced amino acid sequence
 Note: The italic sequence represents the homeobox domain, whereas the underlined sequence indicates the homeobox associated leucine zipper domain

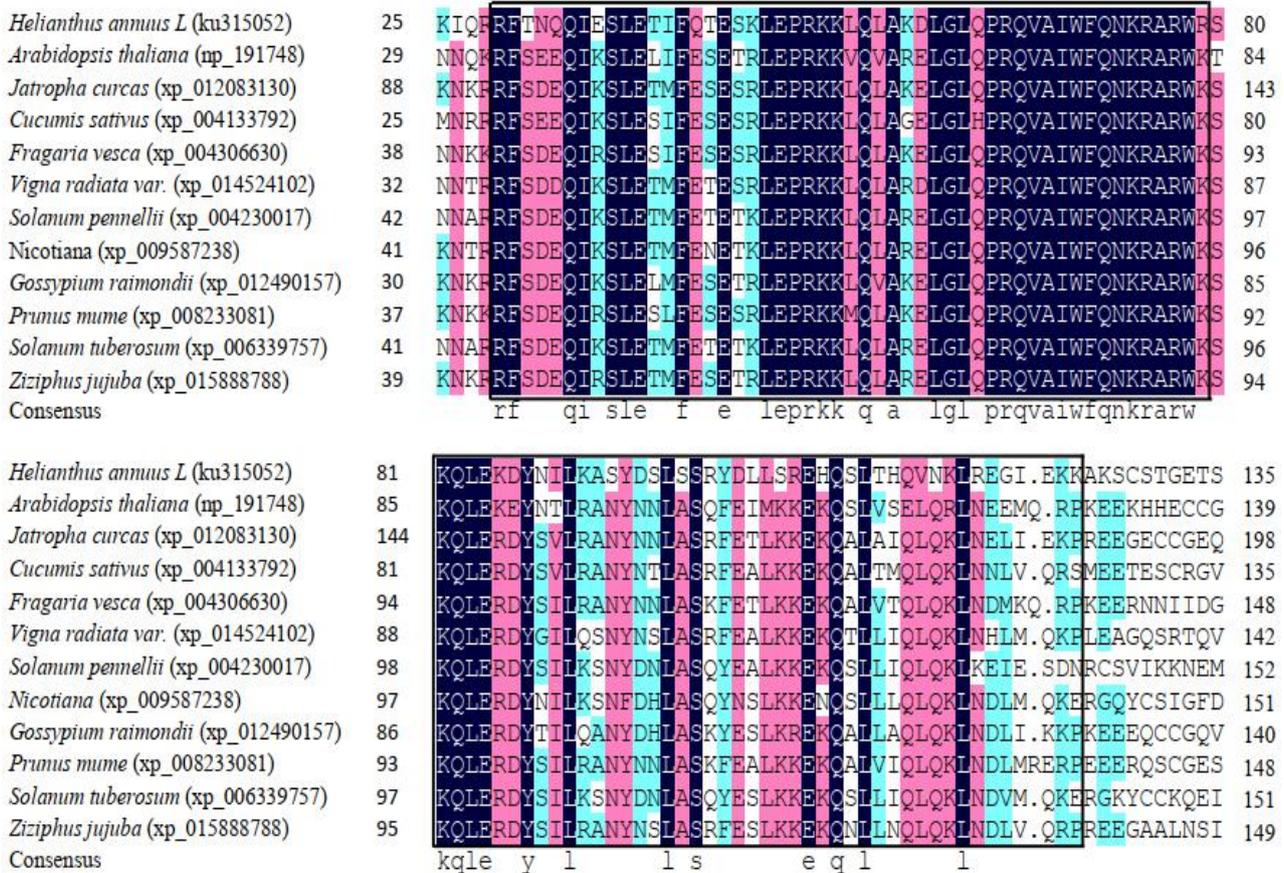


Figure 4 The sequence alignment analysis of HD domains and Zip domains HB-12 of sunflower with some homologous proteins in some other plants

Note: Box above: Homeodomain (HD); Box below: homeobox associated leucine zipper domain (HALZ)

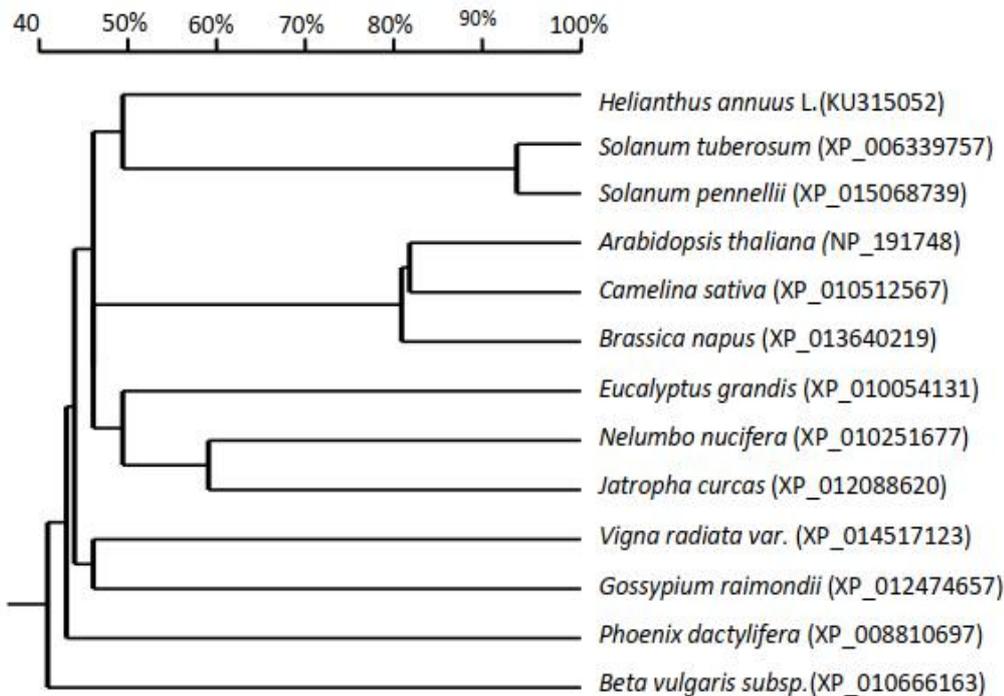


Figure 5 Phylogenetic analysis of HB-12 between sunflower and other species

1.2 Amplification of *HB-12* gDNA sequence and gene structure analysis

A gDNA sequence corresponding to the full-length cDNA of *HB-12* was amplified from gDNA template using GSF and GSR primers. A band of about 800 bp was obtained (Figure 2, Lane 1). Sequencing results showed that the coding region (ORF) length from ATG to TAA is 652 bp, including two exons and one intron. The lengths of the two exons are 344 bp and 229 bp, whereas the length of the intron is 79 bp. The GenBank sequence accession number is KU315053.

1.3 Expression characteristics of *HB-12* in response to abiotic stress

1.3.1 Effect of abscisic acid on *HB-12* expression

Real-time PCR analysis showed that the overall *HB-12* expression increased with exogenous ABA concentration and treatment time (Figure 6). Under 5 $\mu\text{mol/L}$ ABA treatment, *HB-12* expression increased gradually and was 1.61 and 2.52 folds higher than the control at 6 h and 24 h time points, respectively. At 48 h, *HB-12* expression increased significantly and was 7.75 folds higher than the control. A similar expression trend was observed under 10 $\mu\text{mol/L}$ ABA treatment. Specifically, *HB-12* expression was 2.03, 2.84, and 7.60 folds higher than the control at 6 h, 24 h and 48 h, respectively. The expression levels of *HB-12* increased gradually after treatment with 50 $\mu\text{mol/L}$ ABA and were 2.73 and 3.74 folds higher than the control at 6 h and 24 h time points, respectively. However, the expression gradually decreased after 24 h and was 2.94 folds higher than the control at 48 h. These results indicate that the sunflower *HB-12* gene participates in response to ABA stress, and high ABA concentrations inhibit its expression.

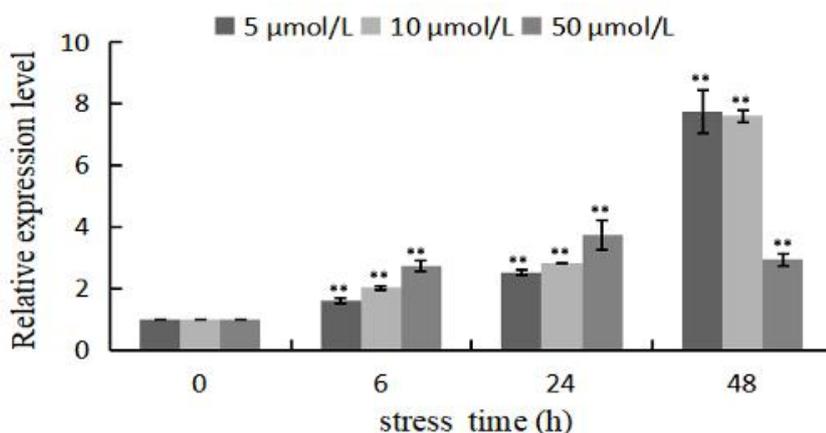


Figure 6 Expression of sunflower *HB-12* gene under ABA stress

Note: Significant differences compared with 0 h ($p < 0.01$)

1.3.2 Effect of NaCl stress on *HB-12* expression

Sunflower *HB-12* gene exhibited similar expression trends at all time points following treatment with different NaCl concentrations (120, 150, and 180 mmol/L). Notably, the expression peaked at 24 h time point in all NaCl treatments. However, variations in *HB-12* expression were observed between different NaCl treatments (Figure 7). Under 120 mmol/L NaCl stress, *HB-12* expression level increased gradually, reaching 1.61 and 7.93 folds higher than the control at 6 h and 24 h time points, respectively. However, the expression decreased gradually after 24 h (the peak) and was 2.47 folds higher than the control at 48 h. Under 150 mmol/L NaCl stress, *HB-12* expression did not increase significantly at 6 h, and was only 1.19 folds higher than the control. However, the expression increased significantly afterwards and peaked at 24 h. At this point, the expression was 16.91 folds higher than the control. Notably, *HB-12* expression decreased gradually after 24 h and was 3.95 folds higher than the control at 48 h. *HB-12* expression was down-regulated at 6 h time point following treatment with 180 mmol/L NaCl, and its expression was 0.69 folds that of the control. However, the expression increased to 29.45 folds higher than the control at 24 h, which was the peak. Overall, *HB-12* expression trend exhibited an initial increasing phase that continued until 24 h, then a decreasing phase, except for 180 mmol/L NaCl treatment, which led to an initial decrease and then a substantial increase in the expression.

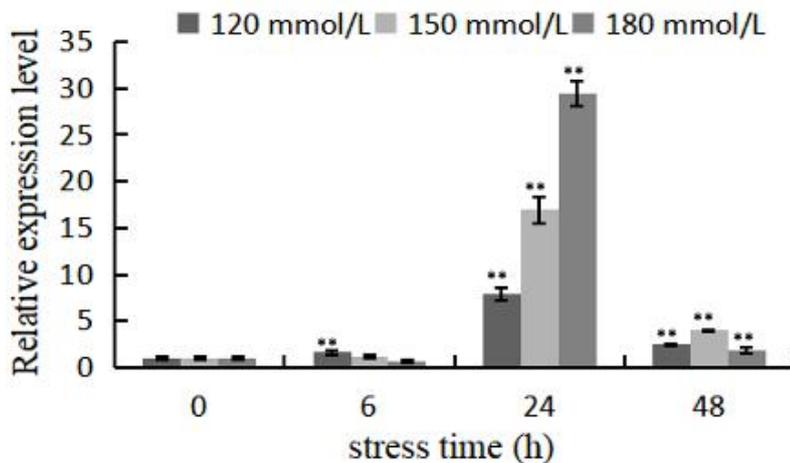


Figure 7 Expression of sunflower *HB-12* gene under NaCl stress
 Note: Significant differences compared with 0 h ($p < 0.01$)

1.3.3 Effect of drought stress on *HB-12* expression

Real-time PCR analysis showed that *HB-12* expression followed a similar trend under different PEG600 treatments at various time points 0~48 h (Figure 8). *HB-12* expression did not increase significantly at the beginning drought stress treatment (0~6 h), but gradually increased with time. Under 5% PEG treatment, *HB-12* expression was 1.02, 3.65, and 6.01 folds higher than the control at 6 h, 24 h, and 48 h, respectively. Furthermore, 10% PEG treatment increased *HB-12* expression by 0.95, 3.47, and 7.11 folds higher than the control at 6 h, 24 h and 48 h, respectively. *HB-12* expression was substantially higher than the control at 6 h, 24 h and 48 h time point following 20% PEG treatment, and was 1.79, 10.75 and 13.06 folds higher than the control.

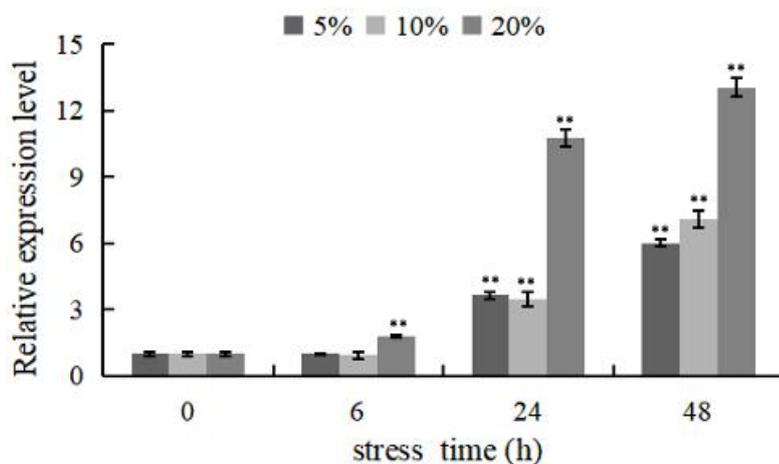


Figure 8 Expression of *HB-12* gene of sunflower under PEG stress
 Note: Significant differences compared with 0 h ($p < 0.01$)

1.3.4 Organ-specific *HB-12* expression

Real-time PCR analysis showed that *HB-12* is expressed in roots, hypocotyls, and leaves of sunflower under NaCl, PEG600, and exogenous ABA induction. However, *HB-12* expression varied significantly between the different organs under different stress conditions (Figure 9). Under NaCl stress, *HB-12* expression in the leaves was 5.18 folds higher than in the roots and lowest in the hypocotyls. Notably, *HB-12* expression did not vary significantly between the roots and hypocotyls. Under exogenous ABA treatment, *HB-12* expression was lowest in the roots and did not vary significantly between the hypocotyls and leaves. Specifically, *HB-12* expression was 2.44 and 2.33 folds higher in the hypocotyls and leaves than in the roots. PEG stress significantly increased *HB-12* expression in the leaves, followed by hypocotyls, 7.76 and 3.23 folds higher than in the roots. These results indicate that *HB-12* expression varies significantly between different organs of the sunflower.

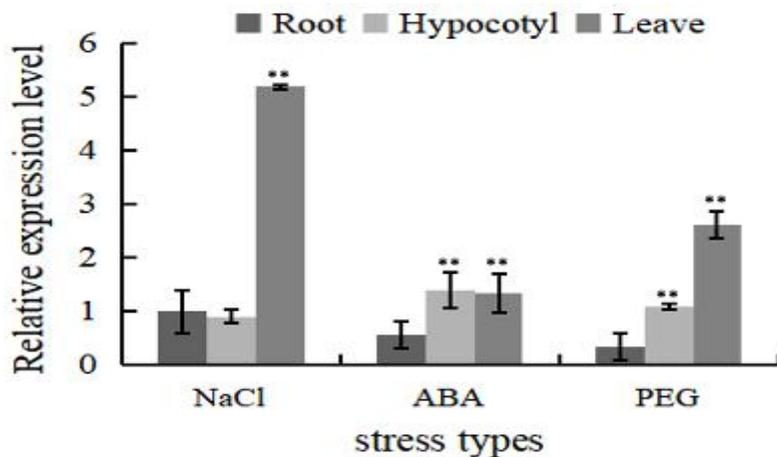


Figure 9 Expression of *HB-12* gene of sunflower in various organs
 Note: Significant differences compared with root ($p < 0.01$)

2 Discussion

HD-Zip I is the first subfamily of HD-Zip with the most typical HD-Zip domain. Besides, HD-Zip I is the largest subfamily of HD-Zip and an important member of HB superfamily. So far, several HD-Zip I class transcription factors have been identified and cloned from many plant species. HD-Zip I class transcription factors have conserved HD and LZ domains and DNA-specific binding sites. They code for proteins that are mainly involved in response to biotic and abiotic factors, hormones, light signals, and regulation of organ development in plants. Both *athb7* and *athb12* are strongly induced by water deficit and abscisic acid (ABA) in *A. thaliana*. As the mediators of negative feedback of ABA signaling in response to water deficit, they play a critical function in drought response (Valdés et al., 2012). HD-Zip I transcription factor *MsHB2* from *alfalfa* is induced by NaCl and ABA stress and inhibits the growth of transgenic *Arabidopsis* under NaCl and ABA stresses. It is speculated that *MsHB2* may regulate salt stress through the ABA signaling pathway in *alfalfa* (Li et al., 2014). HD-Zip I transcription factor *Oshox22* from Rice regulates drought and salt tolerance through ABA signaling. Notably, drought and salt stress increase *Oshox22* expression significantly (Zhang et al., 2012).

At present, only two HD-Zip transcription factors (*Hahb-4* and *Hahb-10*) have been identified in sunflower, which is relatively less than those identified in *Arabidopsis* and rice. The transcription factor *Hahb-4* belongs to the sunflower (*Helianthus annuus*) HD-Zip I subfamily that can bind to the target sequence CAAT(A/T) ATTG *in vitro* (Palena et al., 1999). *Hahb-4* and *GUS* chimeric gene expression in transgenic *Arabidopsis* revealed that reporter gene expression is induced by water, high salt, drought, and ABA at specific developmental stages (Dezar et al., 2005a; Dezar et al., 2005b). *Hahb-4* is up-regulated in the dark and disappears soon after the plants are exposed to light. This reduces the transcription of photosynthesis-related genes, indicating that *Hahb-4* potentially participates in the complex regulatory mechanism of photosynthesis of other factors in the dark (Manavella et al., 2008b). *HaHB-4* overexpressing *Arabidopsis* lines, transformed plants show a specific phenotype that includes a strong tolerance to water stress and a shorter stems and internodes, rounder leaves and more compact inflorescences than their nontransformed counterparts (Dezar et al., 2005a). *Hahb-4* is regulated by water and ABA transcription, and its overexpression in *Arabidopsis* improves water tolerance (Manavella et al., 2006). In the same study, it was speculated that *HaHB-4* may be a new component of ethylene signal transduction pathway and can significantly induce the delayed aging process. Transgenic plants overexpressing *Hahb-4* were not sensitive to exogenous ethylene and thus underwent senescence relatively later, showing no typical triple reaction. Compared with the wild-type plants, *Hahb-4* overexpression significantly inhibited the expression of genes related to ethylene synthesis, such as *ACO* and *SAM*, and those related to ethylene signal transduction such as *ERF2* and *ERF5*. Expression analysis of *Hahb-4* in sunflower revealed that *Hahb-4* transcript level increases in mature and senescent leaves following ethylene treatment. Transient expression of *Hahb-4* in sunflower leaves confirmed that the gene regulates ethylene-related genes, suggesting that *Hahb-4* participates in a new conservative mechanism related to ethylene-mediated senescence. These functions are believed to improve dehydration tolerance.

Manavela et al. (2008a) showed that *Hahb-4* expression in sunflower plants is strongly induced by behavioral attack, mechanical damage, or treatment with MeJA, ET. Promoter fusion analysis in *Arabidopsis* and sunflower further showed that these treatments induce the expression and regulation of *Hahb-4* at the transcriptional level. In the transiently transformed sunflower plants, the expression of *Hahb-4* was up-regulated at the transcriptional level, involving JA biosynthesis and defense-related processes, indicating that *Hahb-4* expression can improve the transcription level of defense-related genes. Heterotopic expression of *Hahb-4* in *Arabidopsis* resulted in higher JA content. Compared with the control plants before and after injury, JA and ET levels decreased after injury and bacterial infection, indicating that *Hahb-4* coordinates the production of plant hormones during biological stress response and mechanical injury. This is achieved through positive regulation of JA and ET production and negative regulation of ET sensitivity and SA accumulation. The transcription factor *Hahb-10* belongs to the sunflower (*Helianthus annuus*) HD-Zip II subfamily (Rueda et al., 2005; Dezar et al., 2011). Evidence shows that *HaHB-10* is mainly expressed in mature leaves, and its expression is higher in etiolated seedlings and seedlings growing under light, indicating that *Hahb-10* expression promotes early plant maturation and flowering. Upregulation of *Hahb-10* was observed in the seedlings treated with gibberellin. Notably, *PsbS* (a light-regulated gene) as *Hahb-10* target gene showed different responses to light conditions in transgenic plants and wild-type plants, indicating that *Hahb-10* potentially participates in gibberellin signal transduction pathway in a signal cascade that controls light quality and quantity in plants (Rueda et al., 2005). The transcription factor *Hahb-10* is related to the genes that induce flowering, *Hahb-10* is expressed in the flowering leaves and stamens of sunflower and induces early flowering in transgenic *Arabidopsis* plants (Dezar et al., 2011). Overexpression of *Hahb-10* in *Arabidopsis* induces specific flowering transcription factors *FT*, *FUL*, and *SEP3* and inhibits biological stress-related genes such as *PR1*, *PR2*, *ICS1*, *AOC1*, *EDS5*, and *PDF1-2a*. The expression of *Hahb-10* and flowering genes *HASEP3* and *HAFT* are up-regulated following SA, JA, and pathogen treatment. The expression levels of SA and JA in transgenic *Arabidopsis* expressing *Hahb-10* were similar to the non-transgenic control plants. However, SA levels increased in the transgenic plants after injury treatment and decreased after pathogen treatment, indicating that *Hahb-10* facilitates two different processes in plants. The transition from growth to flowering induced the accumulation of specific flowering genes and plant hormones under biological stress.

There are four different intron/exon patterns in HD-Zip I gene: *ATHB52/54* has no intron, *ATHB21/40/53/22/51* has 1 intron inserted after the 4th leucine codon in LZ domain, *ATHB3/20/13/23/5/6/16/1/7/12* has 1 intron inserted after the 5th leucine codon in LZ domain, and *ATHB1* has extra intron inserted in the coding region of the 1st helix of HD (Henriksson et al., 2005). The HD domain sequence of HD-Zip I protein is highly conserved, but the homology of N-terminal and C-terminal sequences is very low, and the LZ domain sequences of different proteins are quite different (Ariel et al., 2007).

In this study, sequence analysis revealed that the cloned sunflower HB-12 transcription factor protein contains HD and LZ domains plus a DNA-specific binding site, qualifying it as a sunflower HD-Zip I transcription factor. Cluster analysis indicated that HB-12 belongs to HD-Zip I transcription factors alongside *ATHB-12* and *ATHB-7* of *Arabidopsis*. Analysis of genomic DNA and its corresponding cDNA sequence alignment revealed that the sunflower *HB-12* gene contains an intron inserted after the 4th leucine codon of LZ domain. Expression analysis showed that *HB-12* is induced by exogenous ABA, NaCl, and drought in sunflower seedlings, suggesting that *HB-12* may participate in ABA signal transduction or stress regulation in sunflower. Protein sequence alignment analysis showed significant differences in other regions except for the HD-Zip domain, indicating differences in the stress regulation mechanism of HD-Zip I transcription factors in plants. Therefore, there is a need to conduct further studies on the role of *HB-12* in stress regulation to understand the mechanism of sunflower stress resistance.

3 Materials and Methods

3.1 Materials

The seeds of sunflower variety Neikuiza 4 (P50) were provided by Sunflower Research Group, Institute of Crop Research, Inner Mongolia Academy of Agriculture and Animal Husbandry Sciences. Plant total RNA extraction

reagent (RNAiso Reagent), DNA Polymerase (Tks Gflex), DNA A-Tailing Kit, DNA Ligation Kit Ver.2.1, cDNA Synthesis kit (M-MLV), and PrimeSript RT reagent Kit with gDNA Eraser (Perfect Real Time), *LA Taq*, SYBR Premix EX *TaqII*(Tli RNaseH plus) were purchased from TakaRa. SMARTer[®]5'/3'RACE Kit and High Pure PCR Product Purification Kit were products of Roche. 2×Pfu *Taq* PCR Mix was purchased from Nanjing Bordi Biotech Co., Ltd. Plant total DNA Extraction Kit, pGM-T Cloning Kit were purchased from Tiangen Biotech (Beijing) Co., Ltd. Other reagents were imported or acquired locally. Only analytical grade reagents were used. Primer synthesis and sequencing were performed by Genscript (Table 1).

Table 1 Primer sequence

Name	Number	Sequence (5'-3')
5'RACE primer	Outer SP1	ATCACACTCCAAATCATCCCAA
	Inner SP2	AGTTTCCCCTGTGCTACAGCTT
3'RACE primer	SP3	AGAGCATCAGTCATTAACCCACCAG
ORF and gDNA primer	GSF	CCTCTTGGTCACATCCTTC
	GSR	TATGACATCCCCTCAAGGCT
qRT-PCR primer	QSF	AGTTTCACTGCCTTCTTCA
	QSR	CGAGTTTGGATTCCGGTCT
18S rRNA primer	Q18SF	AGAAACGGCTACCACATCCA
	Q18SR	TTGTTATTTATTGTCACTACCTCCC

Note: SP3, GSF, QSF, and Q18SF are the upstream primers; SP1, SP2, GSR, QSR and Q18SR are the downstream primers

3.2 Sunflower seedlings under stress treatment

Sunflower P50 seeds were planted and the seedlings were cultured in 1/2 Murashige and Skoog (MS) nutrient solution for 4~5 d, then treated with 120 mmol/L NaCl for 3 d (Sun et al., 2015b), and total RNA from tissue samples of young leaves were used to clone full-length cDNA and gDNA sequences of the target gene. Furthermore, treated with 1/2 MS nutrient solution containing: 0, 5, 10 and 50 μ mol/L ABA; 0, 120, 150 and 180 mmol/L NaCl; and 0, 5%, 10% and 20% PEG6000 for 6 h, 24 h and 48 h time points. The roots, hypocotyls, and young leaves of different treatments were frozen in liquid nitrogen and stored at -70°C for subsequent gene expression analysis.

3.3 Total RNA extraction

The RNAiso Reagent was used to extract total RNA from sunflower leaves treated with 120 mmol/L NaCl for 3 d according to the manufacturer's instructions. The purity and integrity of the extracted RNA were examined using 1% agarose gel electrophoresis. Exactly 5 μ L of the total RNA per sample was used for the gel electrophoresis.

3.4 Cloning of terminal sequence of target gene

The 5' RACE downstream primers SP1, SP2, and 3' RACE upstream primer SP3 were designed and synthesized based on the obtained candidate gene sequence with a length of 612 bp (Unigene551_All) (Table 1). The first cDNA was synthesized by reverse transcription of total RNA using SMARTer[®] RACE 5'/3'Kit. The Tks Gflex DNA Polymerase was used for rapid amplification of cDNA ends (RACE). (1) The reverse transcription reaction mix contained 1 μ g Total RNA, 1 μ L 5'-CDS/3'-CDS Primer and 3.5 μ L ddH₂O. The initial reaction conditions were 72°C for 3 min and 42°C for 2 min, after which the following components were added: 5×First-Strand Buffer 2 μ L, SMARTer IIA oligo (24 μ mol/L) 0.5 μ L, DTT (100 mmol/L) 0.25 μ L, dNTP (20 mmol/L) 0.5 μ L, RNase Inhibitor (40 U/ μ L) 0.25 μ L, SMARTScribe Reverse Transcriptase (100 U/ μ L) 1 μ L, 5.5 μ L ddH₂O and 45 μ L Tricine-EDTA buffer. The final reaction conditions were 42°C for 90 min, then 70°C for 10 min. (2) 5' RACE Outer PCR reaction mix contained: 1 μ L 5'-terminal reverse transcription reaction solution, 25 μ L 2×Gflex PCR Buffer (contain Mg²⁺ and dNTP), 1 μ L Tks Gflex DNA Polymerase (1.25 U/ μ L), 5 μ L UPM (10 μ mol/L) Primer, 1 μ L Outer SP1 (10 μ mol/L), and topped up to 50 μ L with ddH₂O. The reaction conditions were as follows: 94°C for 1 min, 98°C for 10 s, 55°C for 15 s, and 68°C for 60 s. The inner PCR reaction system was as follows 1 μ L Outer PCR Reaction solution, 25 μ L 2×Gflex PCR Buffer (contain Mg²⁺ and dNTP), 1 μ L Tks Gflex DNA Polymerase (1.25 u/ μ L), 1 μ L UPM (10 μ mol/L) Primer, 1 μ L Inner SP2 (10 μ mol/L), and topped up to 50 μ L

with ddH₂O. The reaction conditions were the same as those of the Outer PCR reaction. (3) 3' RACE Outer PCR reaction system contained: 1 µL 3'-terminal reverse transcription reaction solution, 25 µL 2×Gflex PCR Buffer (contain Mg²⁺ and dNTP), 1 µL Tks Gflex DNA Polymerase (1.25 U/µL), 5 µL UPM (10 µmol/L) Primer, 1 µL SP3 (10 µmol/L), and topped up to 50 µL with ddH₂O. The reaction conditions were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min, then final extension at 72°C for 10 min. The PCR products (10 µL per sample) were run on 1% agarose gel electrophoresis. (4) High Pure PCR Product Purification Kit was used to recover and purify 5' and 3' RACE products. After adding "A", the purified products were connected with pGM-T and transformed into TOP10 competent cells. The positive clones were selected and sequenced.

3.5 Cloning and bioinformatics analysis of full-length cDNA sequence of target gene

The full-length cDNA sequence was obtained by splicing 5' and 3' RACE sequences with known sequences. A pair of specific primers GSF and GSR were designed according to the full-length sequence. The total RNA was used as a template to amplify the full-length cDNA using RT-PCR with LA *Taq*. The PCR reaction system contained the following: 5 µL 10×LA PCR Buffer II, 8 µL dNTP Mixture, 1 µL GSF, 1 µL GSR, 1 µL cDNA, 0.5 µL LA *Taq*, and topped up to 50 µL with ddH₂O. The reaction conditions were 94°C for 1 min, followed by 35 cycles of 98°C for 10 s, 64°C for 30 s, 72°C for 3 min, and final extension at 72°C for 10 min. The PCR products were run on 0.8% agarose electrophoresis and purified by gel cutting. The purified PCR products were then subjected to TA cloning and sequencing. The ORF of full-length cDNA sequence was analyzed using online analysis software (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Another online software (http://us.expasy.org/tools/pi_tool.html) was used to estimate the theoretical molecular weight and isoelectric point of the protein. Blast alignment of nucleotide sequence and deduced amino acid sequence was performed using online software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and then the conserved domain of protein was predicted and analyzed. Online analysis software (<http://www.cbs.dtu.dk/services/TMHMM-2.0>) was used to analyze transmembrane domains. The deduced amino acid sequences were compared with those of reported species using DNAMAN software, and then phylogenetic analysis was performed. ProtComp 9.0 software (<http://linux1.softberry.com/berry.phtml>) was used to predict the subcellular localization of the target gene.

3.6 Acquisition of target gene genomic DNA sequence and gene structure analysis

Total DNA was extracted from sunflower leaves treated with 120 mmol/L NaCl for 3 d using the plant total DNA extraction kit according to the manufacturer's protocol. The genomic DNA (gDNA) fragment corresponding to cDNA was amplified using GSF and GSR specific primers, the reaction system and conditions were the same as those of cDNA sequence amplification with LA *Taq*. The PCR products were purified, cloned, and sequenced. To analyze the gene structure, the cDNA sequence was compared with the gDNA sequence using DNAMAN software.

3.7 Expression analysis

The qRT-PCR primers QSF and QSR were designed according to ORF sequence of the target gene. *HB-12* expression in various organs under different treatments was analyzed using qRT-PCR. Total RNA was extracted from each sample using RNAiso Reagent. The total RNA concentration was measured using UV spectrophotometer, and then sample concentrations were standardized to 200 ng/µL. The total RNA was reverse transcribed into single-strand cDNA using PrimeSript RT reagent Kit with gDNA Eraser, according to the manufacturer's instructions. The qRT-PCR reaction was performed using LightCycler 480 (Roche). The reaction system was as follows: 10 µL SYBR Premix Ex *Taq*II, 0.5 µL (10 µmol/L) each of the upstream and downstream primers, 1 µL cDNA, and topped up to 20 µL with ddH₂O. The reaction conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Each sample was analyzed in triplicate, and each experiment was repeated thrice. The relative expression of the target gene was calculated using the 2^{-ΔΔC_t} method (Sun et al., 2015b). Sunflower *18S rRNA* gene was used as an internal reference gene.

Authors' contributions

SRF conceived and designed the study, performed sequence analysis, drafted and revised the manuscript. ZYF and GSC conducted the experiments, collected and analyzed data. YHF, LSP, NH, and AYL participated in the experiments and data analysis. All authors read and approved the final manuscript.

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References

- Ariel F.D., Manavella P.A., Dezar C.A., and Chan R.L., 2007, The true story of the HD-Zip family, *Trends Plant Sci.*, 12(9): 419-426
<https://doi.org/10.1016/j.tplants.2007.08.003>
PMid:17698401
- Byrne M.E., 2006, Shoot meristem function and leaf polarity: the role of class III HD-ZIP genes, *PLoS Genet.*, 2(6): 785-790
<https://doi.org/10.1371/journal.pgen.0020089>
PMid:16846251 PMCid:PMC1484593
- Ciarbelli A.R., Ciolfi A., Salvucci S., Ruzza V., Possenti M., Carabelli M., Fruscalzo A., Sessa G., Morelli G., and Ruberti I., 2008, The *Arabidopsis* homeodomain-leucine zipper II gene family: diversity and redundancy, *Plant Mol. Biol.*, 68(4-5): 465-478
<https://doi.org/10.1007/s11103-008-9383-8>
PMid:18758690
- Dezar C.A., Gago G.M., González D.H., and Chan R.L., 2005a, *Habb-4*, a sunflower homeobox-leucine zipper gene, is a developmental regulator and confers drought tolerance to *Arabidopsis thaliana* plants, *Transgenic Res.*, 14(4): 429-440
<https://doi.org/10.1007/s11248-005-5076-0>
PMid:16201409
- Dezar C.A., Fedrigo G.V., and Chan R.L., 2005b, The promoter of the sunflower HD-Zip protein gene *Habb4* directs tissue-specific expression and is inducible by water stress, high salt concentrations and ABA, *Plant Sci.*, 169(2): 447-456
<https://doi.org/10.1016/j.plantsci.2005.04.008>
- Dezar C.A., Giacomelli J.I., Manavella P.A., Ré D.A., Alves-Ferreira M., Baldwin I.T., Bonaventure G., and Chan R.L., 2011, *HAHB10*, a sunflower HD-Zip II transcription factor, participates in the induction of flowering and in the control of phytohormone-mediated responses to biotic stress, *J. Exp. Bot.*, 62(3): 1061-1076
<https://doi.org/10.1093/jxb/erq339>
PMid:21030388
- Elhiti M., and Stasolla C., 2009, Structure and function of homeodomain-leucine zipper (HD-Zip) proteins, *Plant Signal. Behav.*, 4(2): 86-88
<https://doi.org/10.4161/psb.4.2.7692>
PMid:19649178 PMCid:PMC2637487
- Frank W., Phillips J., Salamini F., and Bartels D., 1998, Two dehydration-inducible transcripts from the resurrection plant *Craterostigma plantagineum* encode interacting homeodomain-leucine zipper proteins, *Plant J.*, 15(3): 413-421
<https://doi.org/10.1046/j.1365-3113X.1998.00222.x>
PMid:9750352
- Henriksson E., Olsson A.S., Johannesson H., Johansson H., Hanson J., Engström P., and Söderman E., 2005, Homeodomain leucine zipper class I genes in *Arabidopsis*. Expression patterns and phylogenetic relationships, *Plant Physiol.*, 139(1): 509-518
<https://doi.org/10.1104/pp.105.063461>
PMid:16055682 PMCid:PMC1203399
- Itoh J.I., Hibara K.I., Sato Y., and Nagato Y., 2008, Developmental role and auxin responsiveness of class III homeodomain leucine zipper gene family members in rice, *Plant Physiol.*, 147(4): 1960-1975
<https://doi.org/10.1104/pp.108.118679>
PMid:18567825 PMCid:PMC2492597
- Johannesson H., Wang Y., and Engstrom P., 2001, DNA-binding and dimerization preferences of *Arabidopsis* homeodomain-leucine zipper transcription factors *in vitro*, *Plant Mol. Biol.*, 45(1): 63-73
<https://doi.org/10.1023/A:1006423324025>
PMid:11247607
- Li M.N., Long R.C., Yang Q.C., Shen Y.X., Kang J.M., and Zhang T.J., 2014, Cloning and function analysis of a salt-stress-induced HD-Zip transcription factor *MsHB2* from alfalfa, *Scientia Agricultura Sinica.*, 47(4): 622-632
- Meijer A.H., Scarpella E., Dijk E.V., Rueb S., Harrington S.E., McCouch S.R., Schilperoort R.A., and Hoge J.H., 1997, Transcriptional repression by Oshox1, a novel homeodomain leucine zipper protein from rice, *Plant J.*, 11(2): 263-276
<https://doi.org/10.1046/j.1365-3113X.1997.11020263.x>
PMid:9076993

- Mattsson J., Söderman E., Svenson M., Borkird C., and Engström P., 1992, A new homeobox-leucine zipper gene from *Arabidopsis thaliana*, *Plant Mol. Biol.*, 18(5): 1019-1022
<https://doi.org/10.1007/BF00019223>
PMid:1349836
- Morelli G., and Ruberti I., 2002, Light and shade in the photocontrol of *Arabidopsis* growth, *Trends Plant Sci.*, 7(9): 399-404
[https://doi.org/10.1016/S1360-1385\(02\)02314-2](https://doi.org/10.1016/S1360-1385(02)02314-2)
- Manavella P., Arce A., Dezar C., Bitton F., Renou J., Crespi M., and Chan R., 2006, Cross-talk between ethylene and drought signalling pathways is mediated by the sunflower *Hahb-4* transcription factor, *Plant J.*, 48: 125-137
<https://doi.org/10.1111/j.1365-313X.2006.02865.x>
PMid:16972869
- Manavella P.A., Dezar C.A., Bonaventure G., Baldwin I.T., and Chan R.L., 2008a, *HAHB4*, a sunflower HD-Zip protein, integrates signals from the jasmonic acid and ethylene pathways during wounding and biotic stress responses, *Plant J.*, 56(3): 376-388
<https://doi.org/10.1111/j.1365-313X.2008.03604.x>
PMid:18643970
- Manavella P.A., Dezar C.A., Ariel F.D., Drincovich M.F., and Chan R.L., 2008b, The sunflower HD-Zip transcription factor *HAHB4* is up-regulated in darkness, reducing the transcription of photosynthesis-related genes, *J. Exp. Bot.*, 59(11): 3143-3155
<https://doi.org/10.1093/jxb/ern170>
PMid:18603614
- Olsson A., Engstrom P., and Soderman E., 2004, The homeobox genes *ATHB12* and *ATHB7* encode potential regulators of growth in response to water deficit in *Arabidopsis*, *Plant Mol. Biol.*, 55(5): 663-677
<https://doi.org/10.1007/s11103-004-1581-4>
PMid:15604708
- Palena C.M., Gonzalez D.H., and Chan R.L., 1999, A monomer-dimer equilibrium modulates the interaction of the sunflower homeodomain leucine-zipper protein Hahb-4 with DNA, *Biochem. J.*, 341: 81-87
<https://doi.org/10.1042/bj3410081>
PMid:PMC1220332
- Rueda E.C., Dezar C.A., Gonzalez D.H., and Chan R.L., 2005, *Hahb-10*, a sunflower homeodomain-leucine zipper gene, is regulated by light quality and quantity, and promotes early flowering when expressed in *Arabidopsis*, *Plant Cell Physiol.*, 46(12): 1954-1963
<https://doi.org/10.1093/pcp/pci210>
PMid:16215272
- Schena M., and Davis R.W., 1992, HD-Zip proteins: Members of an *Arabidopsis* homeodomain protein superfamily, *Proc. Natl. Acad. Sci. USA*, 89: 3894-3898
<https://doi.org/10.1073/pnas.89.9.3894>
PMid:1349174 PMid:PMC525597
- Son O., Hur Y.S., Kim Y.K., Lee H.J., Kim S., Kim M.R., Nam K.H., Lee M.S., Kim B.Y., Park J., Park J., Lee S.C., Hanada A., Yamaguchi S., Lee I.J., Kim S.K., Yun D.J., Eva S., and Cheon C.I., 2010, *ATHB12*, an ABA-inducible homeodomain-leucine zipper (HD-Zip) protein of *Arabidopsis*, negatively regulates the Growth of the inflorescence stem by decreasing the expression of a gibberellin 20-oxidase gene, *Plant Cell Physiol.*, 51(9): 1537-1547
<https://doi.org/10.1093/pcp/pcq108>
PMid:20668225
- Sahu B.B., and Shaw B.P., 2009, Isolation, identification and expression analysis of salt-induced genes in *Suaeda maritime*, a natural halophyte, using PCR-based suppression subtractive hybridization, *BMC Plant Biol.*, 9(1): 69-94
<https://doi.org/10.1186/1471-2229-9-69>
PMid:19497134 PMid:PMC2702304
- Sun R.F., Zhang Y.F., Guo S.C., Yu H.F., Li S.P., Nie H., Qiao H.L., and An Y.L., 2015a, Analysis on transcriptome of sunflower under salt stress based on RNA-Seq technology, *Molecular Plant Breeding.*, 13(12): 2736-2742
- Sun R.F., Zhang Y.F., Guo S.C., Yu H.F., Li S.P., Qiao H.L., Nie H., and An Y.L., 2015b, Cloning and expression analysis of ACC oxidase gene (*HaACO1*) from sunflower (*Helianthus annuus* L.), *China Biotechnology.*, 35(9): 21-27
- Vernoud V., Laigle G., Rozer F., Meeley R.B., Perez P., and Rogowsky P.M., 2009, The HD-ZIP IV transcription factor *OCL4* is necessary for trichome patterning and anther development in maize, *Plant J.*, 59(6): 883-894
<https://doi.org/10.1111/j.1365-313X.2009.03916.x>
PMid:19453441
- Vollbrecht E., Veit B., Sinha N., and Hake S., 1991, The developmental gene *Knotted-1* is a member of a maize homeobox gene family, *Nature*, 350: 241-243
<https://doi.org/10.1038/350241a0>
PMid:1672445

- Valdés A.E., Övernäs E., Johansson H., Rada-Iglesias A., and Engström P., 2012, The homeodomain-leucine zipper (HD-Zip) class I transcription factors *ATHB7* and *ATHB12* modulate abscisic acid signalling by regulating protein phosphatase 2C and abscisic acid receptor gene activities, *Plant Mol. Biol.*, 80(4): 405-418
<https://doi.org/10.1007/s11103-012-9956-4>
PMid:22968620
- Wang Y.J., Li Y.D., Luo G.Z., Tian A.G., Wang H.W., Zhang J.S., and Chen S.Y., 2005, Cloning and characterization of an HD-Zip I gene *GmHZ1* from soybean, *Planta.*, 221(6): 831-843
<https://doi.org/10.1007/s00425-005-1496-6>
PMid:15754189
- Zhang S., Haider I., Kohlen W., Jiang L., Bouwmeester H., Meijer A.H., Schlupepmann H., Liu C.M., and Ouwerkerk P.B., 2012, Function of the HD-ZipI gene *Oshox22* in ABA-mediated drought and salt tolerances in rice, *Plant Mol. Biol.*, 80(6): 571-585
<https://doi.org/10.1007/s11103-012-9967-1>
PMid:23109182