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 I 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; Ciarbelli 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 OD260 to OD230 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 homebox domain (HD) sequence and homebox 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).

![RNA and RACE amplification of HB-12 in sunflower](image1)

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

![ORF cDNA and gDNA amplification](image2)

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

![Full-length nucleotide sequence of HB-12 cDNA](image3)

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 μmol/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 μmol/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 μmol/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.

![Expression of sunflower HB-12 gene under ABA stress](image)

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.
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.

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.
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 (Manavela 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 (Manavela 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.
Manabela 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 PrimeScript 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| ATCACACTCCAAAAATCATCCCCAA        |
|                 | Inner SP2| AGTTTCCCTGTGCTACACCTT            |
| 3′RACE primer   | SP3      | AGACGACATGCTATCAAACACAG          |
| ORF and gDNA primer | GSF      | CCTCTTGTCATCATCCTTC             |
|                 | GSR      | TATGACATCCCCCTCAAGGCT            |
| qRT-PCR primer  | QSF      | AGTTTCCTGCTTCCTTCA               |
|                 | QSR      | CGAGTTTGGATCGGTGCT               |
| 18S rRNA primer | Q18SF    | AGAAACGGCTACACATCCA              |
|                 | Q18SR    | TTGGTATTATTGTCACACTCC            |

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 μmol/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 μL 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 (Unigene5551_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 μg Total RNA, 1 μL 5′-CDS/3′-CDS Primer and 3.5 μL 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 μL, SMARTer IIA oligo (24 μmol/L) 0.5 μL, DTT (100 mmol/L) 0.25 μL, dNTP (20 mmol/L) 0.5 μL, RNase Inhibitor (40 U/μL) 0.25 μL, SMARTScribe Reverse Transcriptase (100 U/μL) 1 μL, 5.5 μL ddH2O and 45 μL 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 μL 5′-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 Outer SP1 (10 μmol/L), and topped up to 50 μL 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 μL Outer PCR Reaction solution, 25μL 2×Gflex PCR Buffer (contain Mg2+ and dNTP), 1 μL Tks Gflex DNA Polymerase (1.25 u/μL), 1 μL UPM (10 μmol/L) Primer, 1 μL Inner SP2 (10 μmol/L), and topped up to 50 μL
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℃ for 5 min, followed by 35 cycles of 94℃ for 30 s, 68℃ for 30 s, and 72℃ for 3 min, then final extension at 72℃ 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℃ for 1 min, followed by 35 cycles of 98℃ for 10 s, 64℃ for 30 s, 72℃ for 3 min, and final extension at 72℃ 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.pthtml) 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 PrimeScript 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 TaqII, 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℃ for 30 s, followed by 40 cycles of 95℃ for 15 s and 60℃ 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⁻ΔΔCT 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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