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Isolation and functional characterization of a salt responsive transcriptional factor, LrbZIP from lotus root (*Nelumbo nucifera* Gaertn)

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Abstract Basic leucine zipper transcription factor (bZIP) is involved in signaling transduction for various stress responses. Here we reported a bZIP transcription factor (accession: JX887153) isolated from a salt-resistant lotus root using cDNA-AFLP approach with RT-PCR and RACE-PCR method. Full-length cDNA which consisted of a single open reading frame encoded a putative polypeptide of 488 amino acids. On the basis of 78, 76, and 75 % sequence similarity with the *bZIP*s from *Medicago truncatula* (XP_003596814.1), *Carica papaya* (ABS01351.1) and *Arabidopsis thaliana* (NP_563810.2), we designed it as *LrbZIP*. Semi quantitative RT-PCR results, performed on the total RNA extracted from tips of lotus root, showed that *LrbZIP* expression was increased with 250 mM NaCl treatment for 18 h. Effects of low temperature on the expression of *LrbZIP* was also studied, and its expression was significantly enhanced with a 4 °C treatment for 12 h. In addition, *LrbZIP* expression was strongly induced by treatment with exogenous 100 μM ABA. To evaluate its function across the species, tobacco (*Nicotiana tabacum* L.) was transformed with *LrbZIP* in a binary vector construct. Transgenic plants exhibited higher resistance as compared with the control according to the results of the root growth, chlorophyll content and electrolyte leakage when exposed to NaCl treatment. In addition, *LrCDPK2*, *LrLEA,* and *TPP* also showed enhanced expression in the transgenic plants. Overall, expression of *LrbZIP* was probably very important for salt-resistant lotus root to survive through salt stress.

Keywords Lotus root · *LrbZIP* · cDNA-AFLP · NaCl · Expression

Introduction

Salinity affects about 800 million hectares of land worldwide, comprising nearly 7 % of the world's total land area and one-third of irrigated land [1]. Most crop plants are sensitive to saline concentrations that raise the electrical conductivity of saturated soil extracts above 4 dS m^{-1} [2]. NaCl, the dominant salt in nature, elicits two primary effects on plants: osmotic stress and ionic toxicity [3]. Osmotic stress reduces the ability of plants to take up water and minerals. It not only reduces the growth rate in proportion to the salinity level, but also the tiller numbers in plants [4, 5]. Ion toxicity inhibits a variety of processes such as K^+ absorption, vital enzyme reactions, protein synthesis and photosynthesis [6, 7]. Secondary effects include the production of reactive oxygen intermediates (ROSs) [3].

Recently, it was shown that salinity affected plant growth through various pathways, with the involvement at the level of genome, transcriptome, proteome, metabolome, and ionome [8–10]. It is reported that the responses to salt stress at physiological and biochemical levels are different in cultivated species and wild species [11]. Therefore, it is imperative to have a clear understanding of this

Libao cheng and Shuyan Li are the first co-authors and have equal contribution to this research study.

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tolerance mechanism at molecular level [3], especially cloning the genes involved in salt tolerance, to improve salt resistance in plants, through molecular engineering strategies [12]. Many genes responding to salt stress have been documented from different plants, and most of them are also involved in various stress responses [13–18]. Transcription factors, which regulate the down-stream gene expression, are important in almost all biological processes. Basic region/leucine zipper motif (bZIP) transcription factors are involved in various metabolisms, such as defense response, signaling transduction and flower development [19, 20].

Constitutive expression of maize *ABP9*, which enodes a bZIP transcription factor in *Arabidopsis* leads to remarkably enhanced tolerance to multiple stresses [21]. *SlAREB*, a member of the ABF/AREB subfamily, encodes a bZIP transcription factor in *Solanum lycopersicum*. Overexpression of this gene improves the survival rate of tobacco plants in drought stress [22]. Evidence shows that bZIP can enhance survival in salt stress in transgenic plants through mediating physiological and biochemical metabolism. Constitutive expression of *ThbZIP1* in tobacco improves the activity of both peroxidase (POD) and superoxide dismutase (SOD), and increases the content of soluble sugars and soluble proteins [23]. However, overexpression of *OsABI5* (a bZIP transcription factor) in rice enhances the sensitivity to salt stress, suggesting that different expression profiles of *bZIP* regulates plant adaptation to stress response. At present, most reports on stress responses show that bZIP transcription factors regulate stress response through mediating endogenous abscisic acid [24, 25]. Further study testifies that bZIP transcription factors may interact with specific ABA-responsive *cis*-acting elements (ABRE) and promotes transcription of down-stream genes [26]. The ABA adaptive responses leading to stress adaptation can be divided into two broad categories: ABA-dependent and ABA-independent pathways [27]. Therefore, it can conclude that some bZIPs should be involved into ABA-dependent pathway to respond to stresses [28, 29].

Lotus root (*Nelumbo nucifera* Gaertn), which originated from India and China, is an aquatic herb vegetable and a member of the family *Nymphaeaceae* [30]. It is one of the oldest dicot plants in the world with many features of monocot plants, and has been widely grown in China, Japan, and other Southeast Asian counties for multiple purposes [31]. The products of lotus root such as fresh, salted and boiled rhizomes, lotus root starch, drinks, teas, and lotus seeds are very popular in the daily diet because of its richness in nutrients including starch, proteins, vitamins and mineral substances [32, 33]. Therefore, China is already exporting the processed products of lotus root to Japan, Korea, Europe, and the United States as a kind of off-season vegetable. In addition, nodus nelumbinis rhizomatis, germ, stamens and lotus root stems are also used as important ingredients in the traditional medicine [34–36].

Production of lotus root has already been affected with increasing concentration of salinity in soil. Commonly cultivated species of lotus root are sensitive to salt stress, while wild type species show more resistance with unknown mechanisms at molecular level. Therefore, isolation and functional identification of salt-tolerance genes from wild type species is expected to improve salt resistance of cultivated species. RNA fingerprinting method, cDNA-AFLP provides detailed characterization of gene expression in a wide range of biological processes without prior sequence information. This technique helps to carry out a comprehensive and systematic analysis on the transcriptome of organism, and successfully separates differentially expressed genes [37]. Using cDNA-AFLP approach, we identified a bZIP gene from a salt-tolerant species of lotus root. Because its sequence showed high similarity with bZIPs from other organisms, we named it *LrbZIP*. The expression profiles and possible functions of the gene were also investigated.

Materials and methods

Plant material and growth conditions

Almost all cultivated species of lotus root are sensitive to salt stress, therefore, in this experiment, a salt tolerant wild species (W08124) of lotus root was used to isolate salt tolerant genes. The lotus root was planted 10 cm deep in water in the pots in spring, with average temperature 25 °C/day and 17 °C/night during the whole growth season. Several stolons developed and elongated in proper order in each plant about 20 days of plantation, and then plants were treated with 250 mM NaCl for 24 h with the light regime set at the normal growth conditions, and the tips of stolon were used as sample to extract RNA.

cDNA-AFLP

Total RNA was extracted from tips of lotus root treated with NaCl for 24 h for cDNA-AFLP. M-MLV RTase cDNA synthesis kit (Takara, Japan) was used to synthesize cDNA. cDNA-AFLP was performed according to Lang et al. and Cheng et al. [38, 39]. 50 ng of each ds-cDNA sample was incubated with *Eco*RI and *Mse*I enzymes for 24 h, and the fragments were ligated to adapters for amplification (*Eco*RI-F: 5′-CTCGTAGACTGCGTACC-3′ and *Eco*RI-R: 5′-AATTGGTACGCAG TCTAC-3′; *Mse*I-F: 5′-GACGATGAGTCCTGAG-3′ and *Mse*I-R: 5′-TACT CAG GACTCAT-3′). A primer pair (forward 5′-GACTGC GTACCAATTC-3′ and reverse 5′-GATGAGTCCTGAGT

AA-3′) was designed for pre-amplification. Sequences of the selective primers used were: forward 5′-GACTGCGTA CCAATTCACC-3′ and reverse 5′-GATGAGTCCTGAGT AACTT-3′. Pre-amplification was performed with 20 μl reaction mixture including 0.2 mM dNTPs, 0.2 μM non-selective primers, 1 mM $MgCl_2$, 0.8 U Taq polymerase (Tiangen, China), and 2 μl ligated cDNA fragments. The PCR reaction consisted of 30 cycles: 94 °C for 5 min; 94 °C for 1 min; 56 °C for 1 min; 72 °C for 1 min, and a final extension at 72 °C for10 min. For the selection of gene, 4 μl pre-amplification products were used as template in the reaction, and concentrations of all the other reagents used were same as mentioned above. The PCR program consisted of 35 cycles: 94 °C for 2 min; 94 °C for 1 min; 58 °C for 1 min; 72 °C for 1 min, and the final extension at 72 °C for 10 min. The PCR products were separated on a 6 % polyacrylamide sequencing gels (40 cm-long, 0.25-mm spacer thickness) containing 6 % polyacrylamide gel solution (BMA, Rockland, ME, USA), 7.0 M urea and 1.0 TBE (10 TBE: 89 mM Tris, 89 mM boric acid and 2 mM EDTA). Final AFLP reaction products were mixed with a 2 μl of loading buffer (95 % deionized formamide, 20 mM EDTA, pH 7.5, 1 % bromophenol blue), denatured for 10 min in boiling water, and then transferred to ice before loading. The mix solution was run at 80 W until the bromophenol blue reached the bottom. DNA fragments were visualized by silver staining according to the Silver Sequence™ DNA Sequencing System Technical Manual (Promega, USA) [40].

Full length amplification by RACE-PCR

The PAGE gel was treated with diluted water two times. All differentially expressed bands were cut from the gel with a surgical blade and eluted into centrifuge tube with 40 μl sterile distilled water. The centrifuge tube was placed into boiling water for 10 min, and then centrifuged for 5 min. The supernatant was used as template for following sub-cloning. 2 μl from the above supernatant was used as template for re-amplification using selective amplification primers (forward 5′-GACTGCGTACC AATTCA CC-3′ and reverse 5′-GATGAGTCCTGAGTA ACTT-3′.). PCR product was purified with a PCR purification kit (Tiangen, China) and cloned into PMD18-T vector and sequenced.

To amplify full length gene, RACE-PCR was performed using Clontech SMART ™ RACE mix. RNA was extracted from the tips of lotus root by using plant RNA extract mix (Tiangen, China). DNase was added to remove any DNA contaminations. For the first cDNA strand synthesis, ~2–3 μg RNA was used with RNA first strand mix (Promega, USA). 20 μl PCR reaction mixture consisted of 0.2 mM dNTP, 0.2 μM of forward and reverse primers, 1 mM $MgCl_2$, 0.5 U of Taq polymerase (Tiangen, Beijing, China), and 2 ng cDNA fragments. Primer sequences used include: forward 5′- ATGGGGAATACATGTGTTG -3′ and reverse 5′-TCAGCCAAGCTTTAAGGC -3′. The PCR program consisted of 35 cycles: 94 °C for 2 min; 94 °C for 30 s; 52 °C for 30 s; 72 °C for 60 s and the final extension at 72 °C for 10 min.

Expression analysis of *LrbZIP*

Four-leaf old seedlings of wild type lotus root in pots were exposed to 250 mM NaCl, low temperature (4 °C), and then total RNA was extracted from 100 mg tips after 0, 6, 12, 18, 24, and 30 h time intervals treatment. Semi quantitative qRT-PCR was performed to determine the expression of *LrbZIP*. To ensure the reproducibility of the results, these experiments were repeated three times. For plant hormone treatments, 100 μM ABA was applied on 4-leaf old seedlings under normal conditions. Total RNA was extracted from the tips of treated seedlings after time intervals of 0, 6, 12, 18, 24, and 30 h, respectively. Semi quantitative RT-PCR was carried out to study the gene expression. In all these Semi quantitative RT-PCR experiments, β-actin was used as internal standard (forward 5′-ACGCGTATGAAGTCAGTTGT-3′ and reverse 5′-TTTATGGGGAT CAGCTGGT-3′ primers). The PCR program consisted of 30 cycles: 94 °C for 2 min; 94 °C for 30 s; 56 °C for 30 s; 72 °C for 60 s and the final extension at 72 °C for 10 min. the PCR product was identified by 1 % agro gel.

Functional study in transgenic tobacco

Tobacco (*Nicotiana tabacum* L.) was used to study the function of *LrbZIP*. Tobacco seeds were surface-sterilized in 30 % sodium hypochlorite for 20 min and rinsed six times with sterile water before putting on the medium for germination. Plant growth conditions were set at 25 °C with a diurnal cycle of 16 h light/8 h darkness and a light intensity of 150 $m^{-2}s^{-2}$. Full-length *LrbZIP* cDNA was ligated into a binary vector (pSN1301) under the control of CaMV 35S promoter, and then inserted into *Agrobacterium tumefaciens* strain GV3101. Tobacco transformation was carried out by *A. tumefaciens*-mediated leaf disc method [41]. Leaf segments were co-cultivated with *A. tumefaciens* harboring the binary vector pSN1301, followed by regeneration under the selection medium containing kanamycin (100 μg ml^{-1}). Timentin (400 mg l^{-1}, Agri-Bio) was added in the regeneration/selection medium in the early process to eliminate Agrobacteria. Regenerated leaves were excised and transferred to MS agar medium for root development, containing the same concentration of kanamycin as used in the regeneration medium for root development.

Screening for transgenic plants

Plants from transgenic tobacco were transferred in the pots and grown in a greenhouse to obtain the seeds of self-pollinated T_0 progeny. Transgenic plants were identified by screening on a medium containing hygromycin B (20 μg ml^{-1}) and PCR method. T_2 plants were used for further stress treatment. T_2 plants (per pot with the same soil moisture) including transgenic plants and wild type plants at the two-leaf stage were first applied to 250 mM NaCl. Fresh and dry weight of the treated plants (20 plants each) was measured after 20 days of treatment. Root growth was also studied after exposing the two-leaf old plants of both types of plants to different concentrations of NaCl (50 mM, 100 mM, and 250 mM) for 20 days, and then the root length was measured.

Analysis of chlorophyll content and electrolyte leakage in transgenic and wild type plants

Transgenic tobacco and wild type plants treated with 250 mM NaCl for 20 days, were used to study the content of chlorophyll. Tobacco seeds were surface-sterilized in 30 % sodium hypochlorite for 20 min and rinsed six times with sterile water before putting on the medium for germination, and then transferred into pots. Plant growth conditions were set at 28 °C with a diurnal cycle of 16 h light/8 h darkness and a light intensity of 150 $m^{-2}s^{-2}$. About 10 plants (per pot with the same soil moisture) including the transgenic line 1 and line 2, and wild type plants at the four-leaf stage were treated with 250 mM NaCl. The leaves of these plants were collected three weeks after NaCl exposure. Chlorophyll were isolated and determined using the methods of Hiscox and Israelstam (1979) [42].

For electrolyte leakage measurements, seedlings of four-leaf stage transgenic as well as wild type plants were harvested after salt treatment and washed with deionized water to remove surface-adhered electrolytes. The tubers containing three plantlets were incubated at 25 °C by in the growth chambers and set the thermostat at dark for the first 24 h. The deionized water was added into the tubes and kept at room temperature for 2 h, and then Solution conductivity of about 20 ml of solution was measured using a conductivity meter (Value A). Samples were boiled for 20 min and the final electrical conductivity (Value B) was obtained. Electrolyte leakage was calculated as following: Electrolyte leakage (%) = (A/B) × 100. Above experiments were repeated three times.

Expression study of stress related genes by RT-PCR

Semi-RT-PCR analysis was performed to study the expression of five novel genes associated with stress response with four leaf old seedlings. Total RNA was extracted using RNA extraction mini kit (QIAGEN, Germany) from wild type and transgenic plants (line 1 and line 2). *DNase*I was used to digest DNA during the RNA extraction process to eliminate DNA contamination. A total of 1–2 μg of RNA was used in cDNA synthesis according to the manufacturer's instructions (Promega, USA). Possible targeted genes were selected from bibliographical information [22]. According to the cDNA sequences in NCBI databases, primers used for five genes relevant to stress response were designed which are as follows: *NtCDPK1*: forward primer 5′-CGTTGAGGA ATTAGC ACAGG-3′, reverse primer; 5′-CGAATAGTCACACCAT GCAA-3′; *NtCDPK2*: forward primer 5′-CACGATCGGG AAGTTGTTG-3′, reverse primer 5′-TGACCTCTCGCTT AACATCCT-3′, *NtCDPK3*: forward primer 5′-AGGAAG TA GGAAGAGGGCATT-3′, reverse primer 5′-TCTTCA CCTCCCTTCTCACA-3′. *NtLEA*: forward primer 5′-CTT TCTCTAACTCCAAACTCATC-3′, reverse primer 5′-AA ATTTAACTTTATTAGAAGGTCA-3′; trehalose-6-phosphate phosphatase (TPP): forward primer 5′-AAGACATC ACGGGAGCAAAG-3′, reverse primer: 5′-CCCTTGTCC CAGTTAAGCAC-3′; Tobacco *actin* gene was used as internal standard with the primer sequences; forward primer 5′-GCCGTGACCTAAC TGATAACC-3′, reverse primer 5′-GCTCCTGCTCGTAGTCAAGA-3′. The PCR reaction consisted of 30 cycles: 94 °C for 5 min; 94 °C for 1 min; 56–59 °C for 1 min; 72 °C for 1 min, and a final extension at 72 °C for10 min.

Results

Several differentially expressed genes were isolated by cDNA-AFLP method from lotus root after exposure to NaCl for 24 h (Fig. 1a). All these gene fragments were cloned and sequenced, and most of which were ribosomal protein gene (data not shown). Only one band containing 725 bp DNA fragment was identified to show high similarity to *bZIP* of other species. Semi quantitative RT-PCR was used to confirm the respective gene through the study of its expression. Analysis of expression profile testified that this gene fragment was obviously induced by salt (Fig. 1b, c). Primary analysis of this fragment showed high sequence similarity with some previously reported bZIP genes from other organs.

Full length of this gene was amplified with RACE-PCR method, which was found to be 1,464 bp consisting of a single open reading frame, encoding a putative polypeptide of 488 amino acids. The deduced protein contained a conserved domain of bZIP1 super family and DOG1 super family. When compared with the existing protein sequences in NCBI database, this gene showed 78 %, 76 %, 75 %

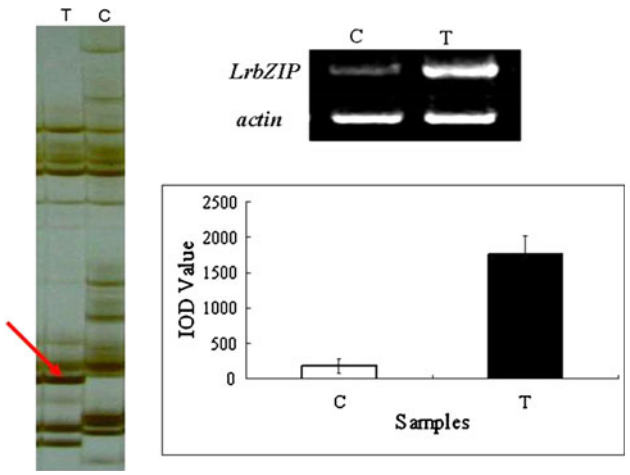

Fig. 1 Isolation of *LrbZIP* from lotus root. **a** A differentially expressed gene was identified through cDNA-AFLP method from the total RNA extracted from the tips of salt-tolerant lotus root after exposure to 250 mM for 24 h (pointed with an arrow in the figure). **b** Semi quantitative RT-PCR was performed to ascertain that the gene was induced by salt stress (C: control; T: sample treated with salt stress). **c** Quantitation of RT-PCR product was determined by IOD value through a UVP Bioimaging system. The level of expression of LrbZIP treated with salt stress (T) was five times higher as compared with that of control (C)

sequence similarity with the *bZIPs* from *Medicago truncatula* (XP_003596814.1), *Carica papaya* (ABS01351.1), and *Arabidopsis thaliana* (NP_563810.2) (Fig. 2). On the basis of this similarity, we named it as *LrbZIP*. In addition, molecular evolutionary analysis also indicated that the origin of *LrbZIP* was very close to the bZIP of maize and rice (Fig. 3).

Expression of *LrbZIP*

To study the pattern of *LrbZIP* mRNA accumulation in lotus root, total RNA was extracted from tips at different time intervals after NaCl treatment. Semi quantitative RT-PCR was performed to evaluate the expression of *LrbZIP*. Results showed that *LrbZIP* expression was induced by NaCl treatment and its expression was increased after exposure to NaCl for 18 h (Fig. 4a). The expression of *LrbZIP* to low temperature (4 °C) and ABA (100 μM) was also analyzed. *LrbZIP* was responsive to low temperature, and the expression was strongly enhanced after treatment of 12 h, and then declined at 30 h (Fig. 4b). Exogenous ABA significantly induced the *LrbZIP* expression after 24 h of treatment under normal growth conditions (Fig. 4c).

Functional study in tobacco

The transgenic tobacco plants carrying *LrbZIP* were confirmed by RT-PCR method with primer designed in low homologous region of nucleotide sequence between *LrbZIP* and *NtbZIP*. The expression of *LrbZIP* was detected in transgenic plants when grown at 25 °C, whereas no expression was observed in the wild type plants (Fig. 5a). After selection, the transgenic tobacco plants were exposed to 250 mM NaCl for 20 days, and wild type plants with the same treatment were used as control. Results clearly demonstrated that transgenic plants with overexpression of *LrZIP* showed better growth as compared with wild type plants (Fig. 5b). For further confirmation, fresh and dry weight of salt treated transgenic plants and control plants were measured, which showed that transgenic plants had higher fresh and dry weights than the control (Fig. 5c).

Effects of different concentrations (50, 100, and 250 mM) of NaCl on roots of transgenic tobacco plants and wild type plants were also investigated. No significant effects were observed on the root growth of both transgenic and wild type plants under normal growth condition. But inhibition of root metabolism and growth was observed in the wild type plants exposed to salt concentrations of 50, 100, and 250 mM of NaCl concentrations (Fig. 6). Both transgenic and wild type plants were used to study the chlorophyll and electrolyte leakage after NaCl treatment. It was observed that transgenic plants (line 1 and line 2) had significantly higher chlorophyll as compared with the wild type seedlings when exposed to NaCl stress (Fig. 7a). However, the electrolyte leakage was lower in transgenic plants as compared with that of wild type plants (Fig 7b). Above experiments were further confirmed that transgenic plants with overexpression of LrbZIP enhanced the tolerance to salt stress.

Expression of stress related genes in transgenic and wild type plants

A few stress-responsive genes in tobacco were selected as candidate target genes for this transcription factor, and homologous sequences (*LEA*, *CDPKs,* and *TPP*) were searched in the databases for tobacco. Most of these genes have been reported to be induced by different stresses in the literature. After 24 h of salt treatment, total RNA was extracted from leaves and the expression of these genes was analyzed with semi RT-PCR method. The expression of three genes (*LEA*, *CDPK*2, and *TPP*) in wild type plants was lower or undetectable, whereas the expression level was enhanced in transgenic plants transformed with 35S-LrbZIP after exposure to salt stress. In addition, we observed that the expression of *CDPK1* and *CDPK2* did not have any significant change after NaCl treatment. These results indicate that the expression of LrbZIP in tobacco could improve the expression of certain genes, which further provided evidence for the involvement of this transcription factor in stress responses in the salt stress-resistant species of lotus root.

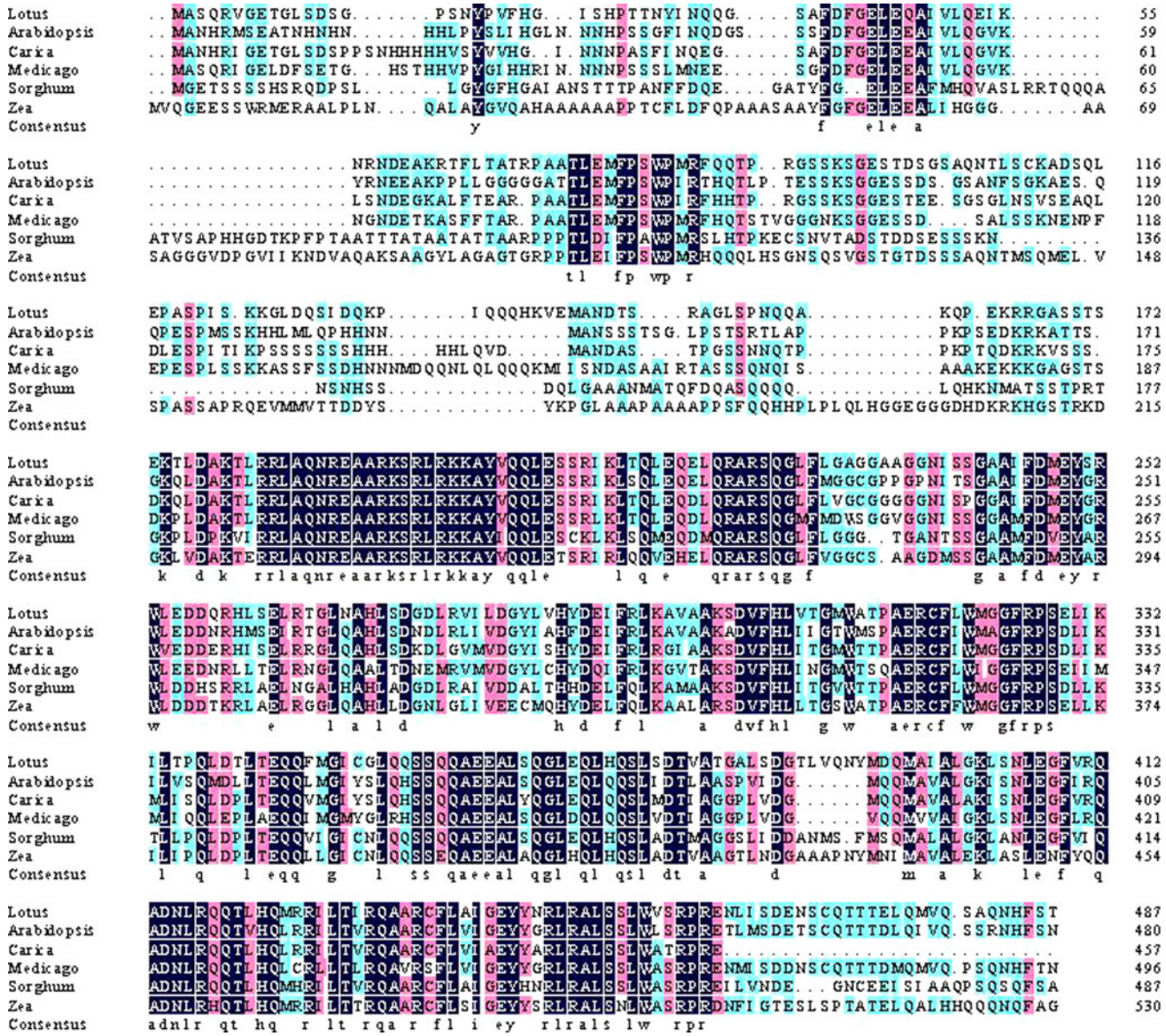

Fig. 2 Protein sequence similarity between LrbZIP and bZIPs in other species

Discussion

Isolation of *LrbZIP* with cDNA-AFLP approach

Salt tolerance of crops must be increased to sustain food production in many regions of the world. If all the other agronomic constraints are overcome, even then subsoil salinity remains a major limitation to agriculture in all semi-arid regions. Various approaches have been used to improve the salt tolerance of plants by introducing the genes for salt tolerance into adapted cultivars [14]. Research has been carried out with the aims to exploit variation in salt tolerance within lotus root and its progenitors or close relatives to produce new cultivars with more tolerance than common lotus root cultivars. Therefore, isolation of salt resistant genes becomes very important for engineering salt-tolerant species to enhance agricultural production.

cDNA-AFLP approach is a powerful gel-based genome-scale transcript profiling technique to generate gene expression profiles [37]. Many stress-induced genes, for example, cold acclimated genes in leaves of *Citrus unshiu* and salt-induced gene in soybean, have been discovered using this method [38, 43]. In this study, a transcription factor, *LrbZIP* was isolated from salt-tolerated lotus species by cDNA-AFLP (Fig. 1), which showed 78, 76, and 75 % sequence similarity with the *bZIPs* from *M. truncatula*, *C. papaya,* and *A. thaliana* (Fig. 2).

bZIPs are normally classified into 19 groups of homologues according to their highly conserved domains [44],

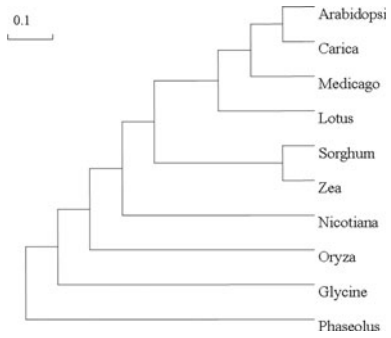

Fig. 3 Phylogenetic tree was obtained by using ClustlWand Mega 4.0. Ten bZIPs were selected from plants which are as follows: bZIP (NP.563810) from Arabidopsis; bZIP (ABS01351) from Carica; bZIP (XP_003606112) from Medicago; bZIP (XP_002450285.1) from Sorghum; bZIP (NP 0011104893.1) from Zea; bZIP (AAF06696.1) from Nacotiana; bZIP (BAB72064.1) from oryza; bZIP (ABP8823.1) from Glycine; bZIP (ADK74993.1) from Phaseolus

and their diversity probably results from their independent ways from different ancients [45]. Although there is a large variation in amino acid sequence within a subfamily among organisms, these changes usually occur outside the conserved domains of bZIP, which indicates that this kind of changes might facilitate new interactions with signal transduction and coactivator–corepressor proteins [46]. Our molecular phylogenetic analysis showed that the origin of LrbZIP lies very close to the bZIPs of *M. truncatula*, *C. papaya,* and *A. thaliana*. (Fig. 3). Functions of these *bZIPs* in response to different stresses have already been identified [47]. In addtition, The LrbZIP belongs to bZIP1 superfamily (the S-group bZIPs) according to the conserved domain of deduced protein. bZIP1 was shown to be linked to the SnRK1 (The sucrose non-fermenting 1-related protein kinase) signal cascade. SnRK is homologous of SNF1 and AMP-activated protein kinase (AMPK), which widely exists in plant and involves in a variety of signaling pathways, and is believed as a switch in plant response to stress and other metabolism [48]. Evidence shows that both rice (*Oryza sativa*) and *Arabidopsis* (*A. thaliana*) SnRK1 activities critically influence the expression of stress-inducible and lead to the induction of stress tolerance [49, 50]. Thus LrbZIP might be playing a critical role in lotus root to adapt to different stresses.

Expression of *LrbZIP*

Plant growth substances or hormones are involved in signal transduction [51]. To elaborate the role of plant hormones in the induction of *LrbZIP* expression, we exogenously exposed lotus root to the ABA. ABA is a small, lipophilic plant hormone that modulates plant growth, seed maturation, dormancy, and adaptive responses to environmental stresses [52]. It is ubiquitous in lower and higher plants and its biosynthetic and catabolic pathways have been elucidated. Physiological responses to ABA are brought about by changes in gene expression.

Hundreds of genes in various species have been shown to be responsive to ABA [53]. Recent genome-wide expression profiles have revealed that over a thousand genes are either up- or down regulated by ABA in *Arabidopsis* [54]. It has been reported that application of the plant hormone-ABA has resulted in increasing stress tolerance both in herbaceous and woody species [55]. ABA plays an important role in adaptive responses to various abiotic stresses [52]. Depending on its involvement, the ABA adaptive responses can be divided into two broad categories: ABA-dependent and ABA-independent pathways [27]. Here in this study, ABA induced the expression of *LrbZIP* (Fig 4c), which showed that expression of this gene belonged to ABA dependent pathways. Many bZIP subfamilies are found to interact with G-ABRE or G-box

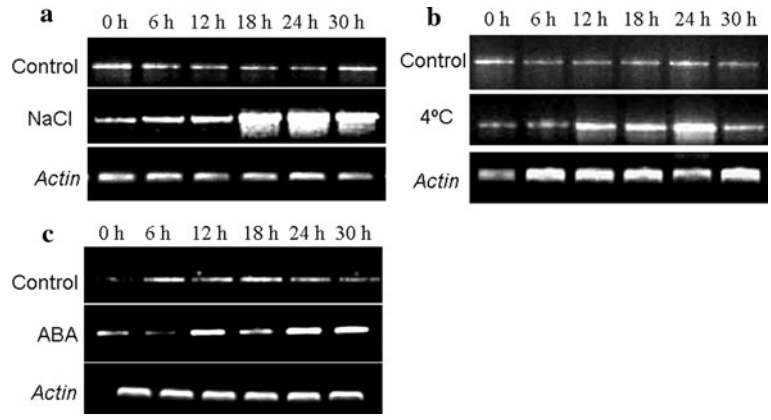

Fig. 4 Expression of *LrbZIP* in tips of lotus root. Total RNA was extracted from tips after 0, 6, 12, 18, 24, and 30 h time intervals of stresses and hormone treatment. DNaseI was added to eliminate DNA contamination. Semi quantitative RT-PCR was used to study the gene expression. Three experiments were replicated and *actin* was used as internal standard. **a** *LrbZIP* expression in tips with 250 mM salt treatment; **b** *LrbZIP* expression with low temperature treatment (4 °C). **c** *LrbZIP* expression with exogenous 100 μm ABA treatment

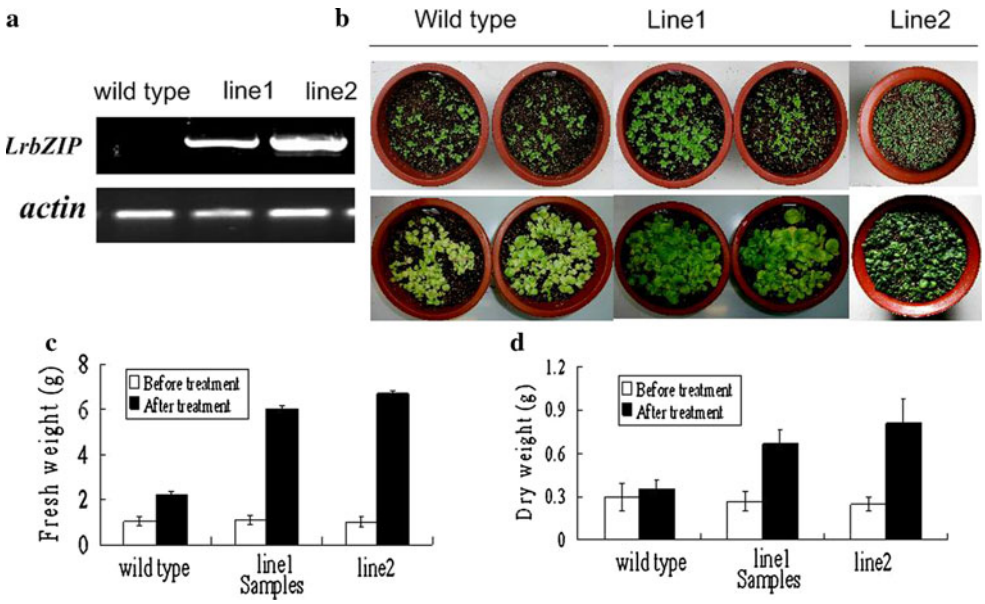

Fig. 5 Study of *LrbZIP* function in the transgenic and wild type plants, when exposed to salt stress. Transformed tobacco plants with *LrbZIP*, inserted in a binary vector construct under a 35S promoter. Two-leaf old seedlings of transgenic and wild type plants were treated with 250 mM NaCl, and measured the dry and fresh weight of plants as survival index under salt stress

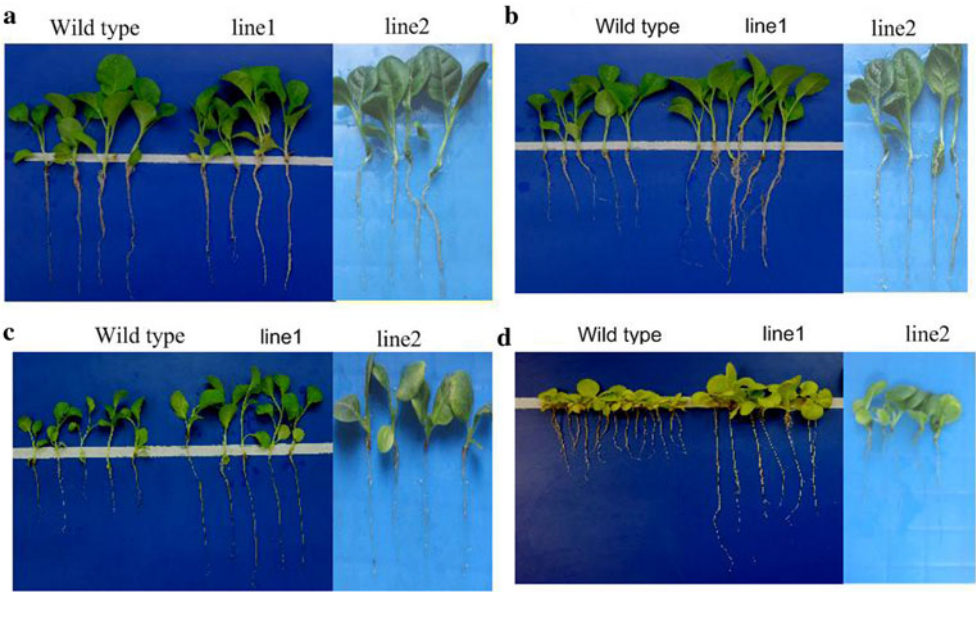

Fig. 6 Growth of transgenic and wild type plants subjected to salt stress. Four-leaf old seedlings of transgenic and wild type plants were treated with different concentrations of NaCl for a period of 20 days, and then growth conditions were investigated. **a** Treated with 0 mM NaCl; **b** treated with 50 mM NaCl; **c** treated with 100 mM NaCl and **d** treated with 250 mM NaCl

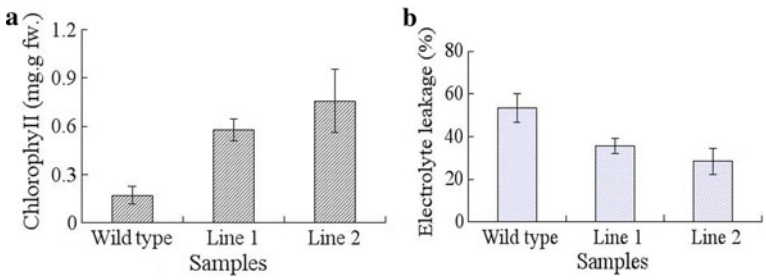

Fig. 7 Chlorophyll content and electrolyte leakage in transgenic plants and wild type plants after salt streatmen. Transgenic tobacco and wild type plants were treated with 250 mM NaCl for 20 days. The chlorophyII content and electrolyte leakage were measured in both type of plants. **a** Chlorophyll content in transgenic plants and wild type plants; **b** electrolyte leakage in transgenic plants and wild type plants

sequence [56]. However, until now only the ABA-responsive element binding protein subfamily of bZIP transcription factors has been documented to be involved in ABA and stress responses [57]. From the expression characteristic to salt, low temperature and ABA (Fig. 4a, b, c), we conclude that *LrbZIP* should belong to AREB subfamily of bZIP transcription factors.

Functional analysis of *LrbZIP*

We transformed tobacco plants with *LrbZIP* under a 35S promoter to study its response to stresses. After selection, the transgenic tobacco plants were exposed to NaCl treatment. Wild type plants with the same treatment were used as control. Results demonstrate that transgenic plants with overexpression of *LrbZIP* showed better growth as compared with the control (Fig. 5). In addition, transgenic tobacco plants treated with salt showed better root growth than that of wild type plants (Fig. 6), suggesting that expression of *LrbZIP* probably helps salt-tolerant species of lotus root to adapt to salt stress.

Overexpression of transcription factors enhances the tolerance of transgenic plants to various abiotic stresses [58]. *LrbZIP* has been testified to belong to AREB/ABF bZIP transcription factor according to expression profile, because in plant kingdom, only the AREB/ABF bZIP transcription factor is ABA-responsive gene which regulates plant abiotic stress responses [57, 59]. Until now, four types of AREB/ABF bZIP transcription factors are found to be induced by exogenous ABA and other stresses [34]. *StABF1*, a potato AREB/ABF bZIP transcription factor, is induced by ABA and salt stress [60]. The expression of *OsABF1* in rice shoots and roots is also observed to be enhanced by anoxia, salinity, drought and oxidative stress, cold and ABA. *Osabf1-1* shows more sensitivity to drought and salt stress as compared with that of wild type plants, suggesting that *OsABF1* is involved in abiotic stress responses and ABA-dependent pathways in rice [52]. In addition, *ABF2* over expression in plants enhanced their resistance to drought, salt, heat, and oxidative stress [62].

Uno et al. [24] found that AREB2 is responsive to exogenous ABA, drought and salinity stresses. Further study testifies that AREB2 and AREB1 have largely overlapping functions to stress conditions according to expression patterns and subcellular localization [63]. For ABF3 and ABF4, constitutive expression of these two genes in plant confers positive regulatory roles in ABA and stress responses [64], and down-regulated ABF3 or ABF4 lead to insensitive to ABA and drought stress [62]. Unlike other ABFs, plants with constitutive expression of ABF3 leads to reprogramming of the drought response through enhancing expression of some drought response genes without growth inhibition or visible phenotypic alterations [65, 66]. We found that transgenic tobacco plants overexpressing *LrbZIP* showed enhanced survival to salt stress. However, transgenic and wild type plants showed no obvious difference after recovery from low temperature treatment (data not shown), suggesting that the roles of AREB/ABF bZIP transcription factors might be different in different stresses.

Aside from the *LrbZIP*, several salt-resistance genes have already been identified in different organs in literature. Transgenic *Arabidopsis* plants with *AtSOS1* display enhanced salt tolerance [67]. An antiporter AtNHX1 induced into *Arabidopsis* leads to increase both Na^+ accumulation and Na^+ tolerance [68], giving similar results in tomato and brassica [69, 70]. At the same time, grain yield is also enhanced when AtNHX1 is overexpressed in wheat in moderately saline soils [71]. Wu et al. [72] found that constitutive expression of a cotton orthologue, GhNHX1, markedly increased salt tolerance in tobacco. Transgenic rice with wheat LEA genes PMA80 and PMA1959 shows enhanced salt tolerance in glasshouse tests [73]. Above reports raise the exciting possibility that salt tolerance can be engineered into important crop plants through the transfer and appropriate expression of a single gene. Therefore, improvement in the salt resistance of cultivated species of lotus root with our identified *LrbZIP* will be possible in near future.

Expression of stress related genes in transgenic plants

Several classes of calcium-sensing proteins have been identified in plants and many extracellular signals elicit changes in the cellular Ca^{2+} concentrations in plants [73, 74]. Decoding of these calcium signals is performed by protein kinases, such as the CDPKs, that mediate cellular responses either directly by changing enzymatic activities via protein phosphorylation or indirectly by changing gene expression patterns [75]. NtCDPK1, 2, and 3 are membrane associated protein kinases in tobacco. Although calcium binding is a necessary prerequisite for CDPK activation, our data indicate that kinase phosphorylation is also mechanistically involved for the CDPK to become biologically functional. The phosphorylation of the NtCDPKs by unidentified upstream kinase(s) was observed to be stimulus-dependent, suggesting that these kinases will also be activated during a biotic or abiotic stimulation [76]. Expression of *NtCDPK2* is enhanced and it is phosphorylated when treated with osmotic stress [74]. *NtCDPK3*, which shares high similarity with *NtCDPK2*, has also been found to be involved in the plant defence responses [77]. Therefore it is very important to study the expression of Ca^{2+} dependent proteins *CDPK1*, *CDPK2,* and *CDPK3*, in transgenic tobacco plants transformed with *LrbZIP* when exposed to salt stress. Results show that there was no

significant change in the expression of *NtCDPK1* and *NtCDPK3* in transgenic or control plants. However, the expression of *NtCDPK2* was enhanced in transgenic plants as compared with the wild type plants (Fig. 8). Therefore it can be concluded that overexpression of *LrbZIP* in transgenic plants induced the expression of *NtCDPK2*, which contributed in their survival in salt stress.

Late-embryogenesis abundant (LEA) proteins are a family of hydrophilic proteins which are involved in stress tolerance in plant kingdom [78]. LEA proteins not only accumulate in seeds but also in vegetative tissues after exogenous ABA treatment or environmental stresses such as chilling, freezing, drought, and salinity stimulation [79]. A series of experiments with recombinant *Saccharomyces cerevisiae* illustrated that increased stress tolerance is directly attributable to the accumulation of LEA proteins. Xiao found that Overexpression of a LEA gene in rice improves drought resistance in transgenic plants compared with wild type plants under the field conditions. At the same time, plants transformed with a Thellungiella LEA gene can improve salt-tolerance in Arabidopsis, which suggests that LEA may participate in response to stresses [80]. Yeasts harbouring tomato Le4 (LEA II), barley HVA1 (LEA III) or tomato Le25 (LEA II) genes were studied under various stresses, and over-expression of these proteins increases abiotic stress tolerance [81–83]. Blackman et al. (1991) [84] demonstrated that the increased LEA protein level might reduce the electrolyte leakage after desiccation and subsequent rehydration. Over-expression of barley *HVA1* (a late embryogenesis abundant protein gene) confers tolerance to salt stress in transgenic rice [85]. In addition, transgenic plants overexpressing Rab16A, a Group 2 lea gene families, exhibited significantly increased tolerance to salinity, and the transgenic plants show normal growth, morphology, seed production, delayed development of damage symptoms, lesser chlorophyll loss under stress conditions [86].

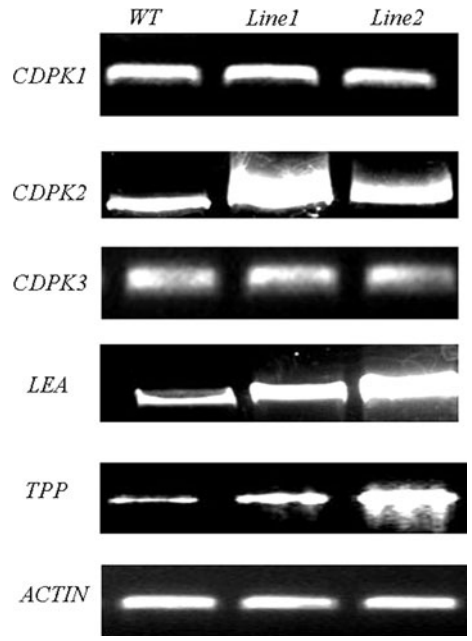

Fig. 8 Expression of stress-related genes in transgnic plants and wild type plant after salt stress treatment. The expression of five novel genes (*CDPK1*, *CDPK2*, *CDPK3*, *LEA* and *TPP*) associated with stress response was studied by semi RT-PCR method with four leaf old tobacco seedlings. Tobacco *actin* gene was used as internal standard

The above reports suggest that different LEA proteins may have unique contributions to cellular protection against stresses, and may increase plant adaptability under extreme environments. We found that expression of *TPP* was enhanced in transgenic plants (Fig. 8). TPP encodes an enzyme involved in the synthesis of disaccharide trehalose, which is believed to play an important role in synthesis of sugar, regulation of various metabolic reactions and stress protectant in a variety of organisms [87].. A mutant deficient in the trehalose-6-phosphaste was sensitive to salinity, suggesting that a role of trehalose as a secondary solute involved in plant adaptation to salt stress [88]. Wingler (2002) [89] reports that TPP provides a protective role when plant is in osmotic stress situations. Further study found that overexpression of TPP gene in rice can improve the tolerance to salt stress and low temperatures [90, 91]. In this experiment, improvement in the expression of *NtLEA* and *NtTPP* in transgenic plants containing LrbZIP, might aid tobacco to adapt to salt stress.

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