BEAR1, a bHLH Transcription Factor, Controls Salt Response Genes to Regulate Rice Salt Response

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Abstract

Transcription factors play important roles in plant abiotic stresses. However, there are a large number of TF functions in rice salt response need to be characterized. Our study identified a new transcription factor, BEAR1, acts as a regulator of rice salt response. We used artificial microRNA to generate *BEAR1* knockdown transgenic rice, and CRISPR to generate *BEAR1* gene editing mutant lines. Both knockdown lines and mutant lines showed salt sensitive phenotypes, compared to NIP. Transient expression in rice protoplast was used to confirm the subcellular localization of BEAR1. The transcription activity of BEAR1 was detected by yeast system. *BEAR1* was induced by salt stress and dominantly expressed in root at seedling stage and spikelet at mature stage. BEAR1 regulating rice salt response by controlling expression levels of many salt-related genes, including salt response marker genes and ion transporter genes. The study revealed that BEAR1 was a regulator of rice salt response.

Keywords BEAR1 · Rice · Seedling stage · Salt tolerance · Transcription factor

Introduction

Salt stress influences plant growth and development during the whole life cycle, which can result in growth retardation, membrane damage, ion balance turbulence, lipid peroxidation, ROS accumulation (Yang and Guo 2018). High salt level can be monitored by GIPC (glycosyl inositol phosphorylceramide) in the plant cell membrane. MOCA1, as a glucuronosyltransferase for GIPC, plays vital role in salt-induced membrane depolarization, and Ca^{2+} signal

transduction. The Ca^{2+} signal can be sensed by Ca^{2+} -binding protein SOS3 and SCaBP8 in roots and shoots, respectively. SOS3/SCaBP8 then recruits and activates SOS2, a protein kinase. The activated form of SOS2 can phosphorylate plasma membrane Na^+/H^+ anti-porter SOS1, vacuolar Na^+/H^+ anti-porter NHX and vacuolar H^+ -ATPase. The SOS pathway is vital for cytoplasmic Na^+/K^+ homeostasis and plant adaptation under salt stress (Zhu 2016).

Salt stress also can rapidly induce ROS generation, which is harmful for plant cell. Hence, the scavenging of ROS plays important role in the plant adaptation formation in high salt circumstance (Miller et al. 2010). Many proteins were considered as a sensor of redox state in plant cell, which include heat shock transcription factor Hsf4a, S-sulfocysteine synthase CS26, thylakoid-associated thioredoxin-type protein ACHT1, protein kinase p46-MAPK, glutathione peroxidases GPX3 and others (Bermudez et al. 2010; Dangoor et al. 2012; Livanos et al. 2014; Miao et al. 2006; Miller and Mittler 2006). ROS scavengers participate in active-oxygen-induced cell damage repair. NCA1, a chaperone protein, can interact with CAT2 and increase the enzyme activity of H_2O_2 degradation (Li et al. 2015).

Transcription factors, as well as other gene expression-regulating components, are vital for plant adaptation to abiotic stresses (Yamaguchi-Shinozaki and Shinozaki 2006).

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Many subfamilies of transcription factors are well known as abiotic stress regulators, including AP2/ERF, NAC, WRKY, MYB and bHLH (Huang et al. 2012). The conserved bHLH domain in bHLH subfamily consists of 50–60-amino acid residue, which can be divided into basic amino acid residue at N terminus and helix–loop–helix domain at C terminus. The E box (CANNTG), especially G box (CACGTG), is the core binding motif of bHLH (Atchley et al. 1999; Carrettero-Paulet et al. 2010; Massari and Murre 2000). Several bHLH transcription factors are discovered in plant salt stress response, such as AtbHLH39, AtbHLH112, NtbHLH123, VvbHLH1, CsbHLH1, PebHLH35, etc. (Cui et al. 2018; Dong et al. 2014; Wang et al. 2016; Xu et al. 2014; Zhai et al. 2016; Zhao et al. 2018).

Salt stress is one of the most common environmental stimuli in rice cultivation. There is a large difference in the whole process of rice growth and development. The most two sensitive stages are seedling stage and flowering stage (Todaka et al. 2012). Dozens of salt tolerant genes were cloned and characterized, function as signal transduction, transcription regulation, ion transportation, metabolic pathway and others (Ganie et al. 2019; Kumar et al. 2013; Todaka et al. 2012; van Zelm et al. 2020). Negative transcription regulators, such as OsIDS1, OsWRKY1, OsWRKY45-2, participate in salt stress response by repressing target gene expression (Cheng et al. 2018, 2021; Qiu et al. 2007, 2008). Positive transcription regulators, such as OsCOIN, OsDREB2A, OsMYB2, OsbZIP71, OsbZIP23, increase the ability of rice salt adaptation by promoting the accumulation of Osmotic protective substances and antioxidant, increasing Na^+/K^+ transporter activity, enlarging biomass (Liu et al. 2014a, b; Liu et al. 2007; Mallikarjuna et al. 2011; Xiang et al. 2008; Yang et al. 2012).

Here, we report BEAR1, a bHLH transcription factor, plays important role in rice salt stress response. The knockdown lines of *BEAR1* showed remarkably salt sensitive phenotypes and confirmed by CRISPR lines. BEAR1 was localized in nuclear and had a transcription activation function. The expression pattern was detected at both seedling stage and mature stage. *BEAR1* expression level was induced by NaCl treatment. The expression levels of stress response marker genes were differentially regulated between NIP and BEAR1 mutant material.

Results

Identification of BEAR1 as a Regulator of Rice Salt Tolerance

We screened the whole transcription factors for salt stress response (Cheng et al. 2018). In addition to an AP2/ERF family protein IDS1, we also found BEAR1, a bHLH TF,

was regulated by salt stress signal. To confirm the potential role of BEAR1 in rice salt tolerance, we created artificial microRNA (amiRNA) construct using pNW55 as a backbone vector. We used WMD3 (Web MicroRNA Designer) to calculate the best artificial microRNA target site, followed by designing 4 primers to amplify mimic miRNA528 with designing artificial microRNA sequence (TCCATCAATGGCGCCTAGAGCG). As shown in Fig. 1A, the amiRNA target site was located in the 3rd exon of *BEAR1* genomic DNA. The amiRNA sequence was perfectly matched the target site. The strategy of artificial pri-miRNA generation was presented in Fig. 1B. *OsaMIR528* gene was used as a template. The sequence was subcloned into plant binary vector pCAMBIA1302 and we named the construct pBEAR1-ami. The pBEAR1-ami transgenic lines were generated using the rice *ssp. japonica* cv Nipponbare (NIP) as background. Based on the artificial microRNA knockdown function to target gene, we checked the expression level of *BEAR1* in all transgenic lines. *BEAR1* level in 3 transgenic lines, BEAR1-ami2, BEAR1-ami8, BEAR1-ami9 were decreased to 9–15% compared to NIP (Fig. 1C).

The 3 transgenic lines were selected for further salt treatment. 3-week-old seedlings of NIP and knockdown lines in pots were washed with 150 mM NaCl and recovered by water. As shown in Fig. 2A, all of the 3 amiRNA lines were much more sensitive to salt, seedling growth were greatly obstructed and plant heights were significantly lower than NIP (Fig. 2B). After recovery with water, NIP rejuvenated rapidly while most seedlings of 3 amiRNA lines were dead, the survival rates showed in Fig. 2C. Osmotic reagents concentration and plasma membrane integrality were important features of salt tolerance. We measured the content of proline and malondialdehyde (MDA) in NIP and amiRNA lines. As shown in Fig. 2D and E, proline decreased and MDA increased in knockdown lines when compared with NIP. These results illuminated that BEAR1 was a positive regulator of rice salt tolerance.

Characterization of BEAR1 as a Transcription Factor

To confirm BEAR1 as a transcription factor, we made a BEAR1–YFP fusion construct by the gateway LR reaction. Rice protoplasts were isolated from etiolated seedlings and BEAR1–YFP fusion proteins were expressed by PEG-mediated transient expression. NLS-mCherry was used as nucleus location marker. As shown in Fig. 3A, BEAR1 was co-localized with nucleus marker protein in rice protoplast. To verify the transcription activity of BEAR1, we constructed BEAR1 into pGBKT7, which was named pBEAR1, and transformed into yeast cells AH109. As shown in Fig. 3B, BEAR1 demonstrated transcription activation factor as positive control (ck+).

Fig. 1 Generation of BEAR1 amiRNA lines. **A** Selection of amiRNA target site. The target site on diagram of BEAR1 genomic DNA was presented. **B** The strategy of artificial microRNA generation. Four BEAR1 target site-specific primers (I, II, III and IV) were designed as protocol and 3 fragments were amplified using P-F and II, I and IV, III and P-R. Then, the fragments were used as templates to amplify

the full length of artificial microRNA using P-F and P-R as primers. **C** The knockdown efficiency of 3 amiRNA lines. Seedling materials of NIP and 3 amiRNA lines were harvested. After RNA extraction and reverse transcription, real-time qPCR assays were performed and *ACT2* was used as the internal control. Error bars represent SD ($n = 3$)

Spatiotemporal and Stress Expression Pattern of BEAR1

Since BEAR1 was verified as a transcription factor, we wanted to discover the expression patterns of *BEAR1* gene in rice. Total RNA was isolated from different tissues of developmental stages, such as root and shoot at the juvenile stage, root, stem, leaf spikelet and seed at reproductive stage. As shown in Fig. 3C, *BEAR1* was dominantly expressed in root at seedling stage, which was 3 times compared with seedling shoot. At reproductive stage, *BEAR1* was mostly expressed in spikelets. Due to the roles in rice salt response, we checked the BEAR1 expression levels in salt stress treatment. We found that, with the salt treatment lasting, *BEAR1* was constantly induced with expression peak at 6 h NaCl treatment (Fig. 3D).

Generation of BEAR1 CRISPR Lines

To confirm the salt sensitive phenotypes of BEAR1 amiRNA knockdown lines, we created BEAR1 mutant lines by CRISPR–Cas9 method. Using CRISPR-GE (<http://skl.scau.edu.cn/>) online, we screened the best target site of *BEAR1*.

As shown in Fig. 4A, *BEAR1* genomic DNA diagram was presented and the target site (GGCTCTCGTCGACCGTATCA) at the 3rd exon, which corresponding to 132–151 bp in BEAR1 coding sequence. At the protein level, the target site located at 44–50 a.a. of BEAR1 protein, which was outside of the bHLH domain (Fig. 4B). Following constructs generation, *Agrobacterium*-mediated transformation was used to create BEAR1 CRISPR lines. We sequenced the lines and two independent lines were selected for further experiments. As shown in Fig. 4C–E, one line with 1nt deletion at the target site, was named BEAR1-M1; another line, with 1nt insertion, was named BEAR1-M2. The single peak represented that two lines were homozygous lines. To test if the RNA level was changed in mutant line, we extracted total RNA and examined *BEAR1* expression by real-time qRT-PCR. As shown in Fig. 4F, *BEAR1* was decreased in two mutant lines, which may be caused by NMD pathway mRNA decay initiated by pre-mature stop codon in *BEAR1* mRNA.

Fig. 2 The salt sensitive phenotype of BEAR1 amiRNA lines. **A** Salt sensitive phenotype of BEAR1 amiRNA lines. The seedlings of NIP and amiRNA-mediated BEAR1 knockdown lines ami2, ami8 and ami9 were treated with or without 150 mM NaCl for 10 days followed by recovery with water for another 10 days. **B** The statistical results of seedling heights under salt treatment. Error bars represent SD ($n \geq 30$). **C** The statistical results of survival rates after recovery from water. Error bars represent SD ($n \geq 30$). **D** and **E** The proline and MDA (malondialdehyde) contents of NIP and amiRNA lines with or without salt treatment. Error bars represent SD among three independent replicates. **, $P < 0.01$ (Student's test)

Fig. 3 Function and expression pattern characterization of BEAR1. **A** Subcellular localization of BEAR1. BEAR1 was fused with YFP and NLS (nuclear localization sequence) was fused with mCherry. After transient expression in protoplast, the fluorescence signals were detected by confocal. **B** Identification of the transcription activation function of BEAR1. Yeast-one-hybrid assay was used to examine the transcription activity of BEAR1. VP16 was used as positive control (ck+); empty vector pGBK was used as negative control (ck-); pBEAR1 showed blue as ck+. **C** Gene expression pattern of *BEAR1*

at different stages. Tissues at different stages were collected. After RNA extraction and reverse transcription, real-time qPCR assays were performed and *ACT2* was used as the internal control. **D** *BEAR1* expression pattern under salt treatment. 10-day-old seedlings were treated with 150 mM NaCl with the time course. After RNA extraction and reverse transcription, real-time qPCR assays were performed and *ACT2* was used as the internal control. Error bars represent SD ($n=3$)

Confirmation of BEAR1 as a Salt Regulator by CRISPR Lines

Since two CRISPR lines BEAR1-M1 and BEAR1-M2 were generated and confirmed by sequencing, we used them for NaCl treatment, while NIP as a control. The seeds were germinated in water and transferred to the soil for growing 3 weeks, followed by different concentration salt stress. As shown in Fig. 5A, after 10 days treatment, the mutant lines had salt sensitive phenotype with 75 mM NaCl and

150 mM NaCl stress. We could see from Fig. 5B, with NIP was almost unaffected, the growth of M1 and M2 were suppressed to 63% of height at 75 mM NaCl condition, compared to normal condition. After 10 days recovery with water, the survival rates showed most significant at 150 mM NaCl condition (Fig. 5C). We measured the content of proline and MDA in NIP and mutant lines. The proline contents decreased in mutant lines M1 and M2, as well as MDA contents increased in mutant lines when compared with NIP, under salt condition (Fig. 5D and E).

Fig. 4 Generation of BEAR1 CRISPR lines. **A** CRISPR target site on *BEAR1* genomic DNA. The gRNA was designed on the third exon of *BEAR1* genomic DNA. **B** The corresponding mutation site of *BEAR1* protein. **C–E** Sequencing confirmation of BEAR1 CRISPR lines. **C** The wild-type NIP sequence of target site; **D**, the edited sequence with deletion in BEAR1-M1 CRISPR line; **E** the edited

sequence with insertion in BEAR1-M2 CRISPR line. **F** BEAR1 expression levels in NIP and CRISPR lines. 10-day-old seedlings were harvested. After RNA extraction and reverse transcription, real-time qPCR assays were performed and *ACT2* was used as the internal control. Error bars represent SD ($n=3$)

Differentially Regulated Response of Root and Leaf Under Salt Stress

Due to the different response mechanism of shoot and root to salt stress, we harvested seedling root and seedling leaf samples which were bearing salt stress. Total RNA was extracted and the cDNA libraries were generated by standard protocols. After sequencing, the RAW data were trimmed and clean data. Following quality control, the clean data were mapped to rice reference genome by HISAT2. Principal component analysis (PCA) showed that replicates in the same group were clustered together (Fig. 6).

We first checked genes expression pattern between NIP and M1 under salt stress. The volcano map showed that the

differentially expressed genes in seedling root part were 675, in which 295 genes were up-regulated and 380 genes were down-regulated (Fig. 7A). Meanwhile, the differentially expressed genes in seedling leaf part were 1886, and down-regulated genes were 1482 which accounted for the majority of total differentially expressed genes (Fig. 7B). Cluster analysis of differential genes was performed to gather genes with similar expression patterns. These genes had similar functions or participate in same metabolic pathways and signal pathways. In the heat map, red indicates high expression and blue indicates low expression. Cluster analysis of differential genes was presented as heat map and showed in Fig. 7C and D. All differentially expressed genes, in seedling root part and seedling leaf part, are listed in Table S2

Fig. 5 Confirmation of BEAR1 salt response by CRISPR lines. **A** The salt phenotypes of BEAR1 CRISPR lines. The seedlings of NIP and BEAR1 CRISPR mutant lines BEAR1-M1 and BEAR1-M2 were treated with different concentration of NaCl for 10 days. **B** The statistical results of seedling heights under salt treatment. Error bars represent SD ($n \geq 30$). **C** The statistical results of survival rates after recovery from water. Error bars represent SD ($n \geq 30$). **D** and **E** the proline and MDA contents of NIP and CRISPR lines under salt treatment. Error bars represent SD among three independent replicates. **, $P < 0.01$ (Student's test)

and Table S3. From these data, we could conclude that M1 mutant line was sensitive to salt due to the genes down-regulation under salt stress especially the shoot part of rice.

We also analyzed seedling root samples and seedling leaf samples separately. As shown in Fig. 8A, most differential expressed genes were overlapped between NIP-root-NaCl/NIP-root-H₂O and M1-root-NaCl/M1-root-H₂O, which mean that under salt stress, most gene expression pattern were same between NIP and M1 CRISPR line. Meanwhile,

in seedling leaf tissue, we could not find a dominant section of differential genes (Fig. 8B), which was consistent with Fig. 7.

Since we showed that the difference of gene expression pattern between root tissue and leaf tissue of NIP and M1 mutant line, we performed KEGG analysis. As shown in Fig. 8C and D, the number of KEGG enrichment of differential genes in leaf tissue was more than root tissue, which 58 genes were classed into the biosynthesis of secondary metabolites in leaf,

Fig. 6 Principal component analysis of samples. All samples were harvested with or without 250 mM NaCl treatment for 3 h. Total RNA was extracted and cDNA libraries were generated, followed by next-generation sequencing. Clean data were processed and principal component analysis was performed to show repeatability of replicates

comparing with 24 genes classed into metabolic pathways in root. Indicated by red asterisk, there were 4 pathways with more than 10 genes were clustered in leaf tissue while only 2 pathways were presented in root tissue.

Due to the transcription activation function of BEAR1 and most differential genes were down-regulated in seedling shoot part, we assumed that these down-regulated genes may be the target or affected genes of BEAR1. We pay special attention to the Gene ontology (GO) analysis of these genes. Figure 9A showed the GO analysis of down-regulated genes in M1–root–NaCl/NIP–root–NaCl comparison. We could see that the most clustered section in BP (biological processes), MF (molecular function) and CC (components of cell) were 44 genes, 36 genes and 46 genes, corresponding to cellular component organization, oxidoreductase activity and cell periphery, respectively. At leaf tissue, the down-regulated genes were classified by GO analysis and red asterisk indicated the section which had more than 100 genes clustered (Fig. 9B). The most clustered section in BP, MF and CC were 320 genes, 720 genes and 532 genes, respectively. These data demonstrated that the salt sensitive phenotype of CRISPR lines maybe caused by the response differential genes in leaf tissue.

Salt Response Marker Genes were Differential Expressed in NIP and CRISPR Lines

To find the molecular mechanism of BEAR1 in rice salt response, we performed real-time qRT-PCR for checking salt

response genes and regulators. As shown in Fig. 10A, many salt response genes, such as *DREB1F*, *LEA3*, *bHLH120* etc., were slightly increased in mutant lines M1 and M2, while the genes were increased dramatically in NIP under salt stress. The expression levels of ion transporter genes, *HAK6* and *NHX4*, were also lower in mutant lines.

We proposed a model for the function of BEAR1 in rice salt response. After receiving the salt stress signal, BEAR1 protein controlled the gene expression level of salt response signaling cascade on one hand, and regulated the Na^+/K^+ balance and membrane stabilization genes on the other hand (Fig. 10B).

Discussion

From the rice transcription factor database, we identified BEAR1 as a salt response gene under salt stress. We generated BEAR1 amiRNA construct and created BEAR1 amiRNA lines, of which ami2, ami8 and ami9 lines were selected for salt treatment. We discovered the salt sensitive phenotype of under salt treatment and recovery by water. The results were confirmed by CRISPR lines of BEAR1.

We also verified the nuclear localization by transient expression in rice protoplast. The transcription activation assay was performed in yeast system, and VP16 was used as positive control. Clearly, BEAR1 had the transcription activation function, but weaker than VP16. Many salt response marker genes, such as *SNAC1*, *LEA3*, were differential regulated between NIP and amiRNA lines when suffering salt stress.

The bHLH family was the second largest family in plant world, and there were 176 members in rice which were consist of 22 subfamilies (Li et al. 2006). Many of bHLH proteins played roles in salt tolerance. In *Arabidopsis*, *AtNIG1*, *AtbHLH122* and *AtMYC2* over-expression confers increased salt tolerance. These proteins were regulator Na^+/H^+ antiporter, such as *AtNHX1* and *AtNHX6* (Kim and Kim 2006; Krishnamurthy et al. 2019; Liu et al. 2014a, b; Verma et al. 2020). In rice, *OrbHLH001*, which was a gene from Dongxiang wild rice (*Oryza rufipogon* Griff.), enhanced rice salt tolerance by regulating the expression level of *OsAKT1* (Li et al. 2010). *ZmbHLH55* in maize and *SlbHLH22* in tomato improved plant salt tolerance by over-expression (Waseem et al. 2019; Yu et al. 2021). From Fig. 4, we could find that BEAR1 may share the same molecular mechanism of salt response as *OrbHLH001*, *AtbHLH122* and *AtMYC2*.

In conclusion, we found a new gene, which participated in rice salt response from transcription factor screening. BEAR1 was verified as a salt regulator in rice and the SNP of BEAR1 in cultivated varieties can be used as a molecular marker for rice salt tolerance breeding.

Fig. 7 Volcano and heat map of differential genes under salt stress. **A** and **B** the volcano map showed that the differentially expressed genes in seedling root part (**A**) and leaf part (**B**). Green dot represents down-regulated genes, red dot represents up-regulated genes. **C** and **D** the heat map showed the expression patterns of transcripts in seedling root part (**C**) and leaf part (**D**). Red indicates high expression and blue indicates low expression. The expression levels of all transcripts were rescaled by $\log_{10}$

Fig. 8 Venn diagram and KEGG enrichment of root and leaf under salt stress. **A** and **B** Venn diagram of the overlap of differential expression genes of NIP and BEAR1 CRISPR lines seedling root and

leaf with NaCl treatments. **C** and **D** KEGG enrichment of seedling root and leaf under salt stress. Red asterisk represented more than 10 genes were clustered

Materials and Methods

Plant Material and Salt Treatment

The rice seeds were immersed into water for imbibitions and keep at 37 °C for 2 days for germination. After the appearance of coleoptiles, the germinated seeds were transferred to soil in the pots under 13 h–28°C–light/11 h–24°C–dark program in a greenhouse.

To compare the difference of salt tolerance phenotype between NIP (*Oryza. Sativa* L.spp.japonica, var Nipponbare) and mutants materials, the 3-week-old rice seedlings were treated with different concentration of NaCl for 10 days and recovered to normal condition by replacing the salt solution with water for another 10 days. The seedling height and survival rate were recorded and calculated. The malondialdehyde (MDA) and proline contents were measured before recovery, according to the standard determination methods (Abraham et al. 2010; Li et al. 2011).

Fig. 10 Salt response marker genes were down-regulated in BEAR1 mutant lines. **A** Salt-related genes expression in NIP and CRISPR lines under salt treatment. 10-day-old seedlings were treated with 150 mM NaCl for 3 h. After RNA extraction and reverse transcription, real-time qPCR assays were performed and *ACT2* was used as the internal control. Error bars represent SD ($n=3$). **B** A model of BEAR1 function in rice salt response. When rice suffered salt stress, the signal was perceived and transduced to BEAR1. As a transcription activator, BEAR1 induced two class genes, one was in charge of salt response and signal cascade, another was effector genes such as Na^+/K^+ balance Membrane stabilization. All these genes facilitated rice salt response

Gene Clone and Vector Construction

The cDNA library of rice seedling was used to clone *BEAR1* gene. The coding sequence was amplified using primers (Table S1) followed by cloning into the gateway entry vector pENTR-D-TOPO. The destination vectors, pGWB542 and pDEST-GBKT7, were used to generate constructs for subcellular localization and transcription regulation experiments by LR reaction.

Transgenic Plants Generation

To generate artificial microRNA knockdown lines, pN580 was used as backbone and the whole procedure was described (Ossowski et al. 2008). The generated construct, named as BEAR1-PN580, was transformed into *Agrobacterium* to infect rice calli. The transgenic plants were named as BEAR1-ami. BEAR1 mutant materials were generated by CRISPR–Cas9 system (Yan et al. 2015). The detailed protocol was followed to generate gene editing constructs,

named BEAR1-M1 and BEAR1-M2. The *BEAR1* gene editing plants were obtained by *Agrobacterium*-mediated transformation as BEAR1-ami transgenic plants.

BEAR1 Subcellular Localization

For localization assay, *BEAR1* was cloned into destination vector pGWB542, the protoplast of rice seedling was isolated by previously described method (Yang et al. 2014). The BEAR1–EYFP fusion proteins were transiently expressed in rice protoplast for 16 h and the fluorescent signals were detected by Zeiss LSM 710 inverted confocal microscope.

Transcription Activity Assay

BEAR1 from gateway entry vector was introduced into destination vector GBKT7, which was named as pBEAR1. pGBK-VP16 was used as positive control (CK+) and pGBKT7 was used as negative control (CK-). Constructs were transferred into yeast strain AH109, respectively. The

transcription activity assay was performed as described in the Clontech yeast protocol PT3024-1.

Deep Sequencing and Data Analysis

Total RNA was extracted separately from seedling root and leaf with or without 250 mM NaCl treatment for 3 h. The RNA-seq libraries were constructed using Illumina whole transcriptome analysis kit with standard protocol and quantified the concentration by Qubit 2.0. The library insert size was detected by Agilent 2100. The RNA libraries were sequenced on HiSeq2500 to generate high throughput raw reads. The clean reads were obtained by trimming the raw reads and quality control, followed by mapping to reference genome IRGSP-1.0 using HISAT2. The differentially expressed genes (DEGs) were calculated by DESEQ2 with P value ≤ 0.05 and fold change ≥ 2 .

RNA Extraction and Gene Expression Analyses

Total RNA was extracted from 2-week-old seedlings treated with 300 mM NaCl for 3 h or water as a control. cDNA libraries were synthesized from total RNA. Real-time qPCR was performed on the ABI QuantStudio5 system using ACT2 as a control. Primers used in this study are presented in Table S1.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12374-022-09347-4>.

Author contributions YT and ML conducted the study, collected, and analyzed the data. TC designed the study and methods and collected the funds. XZ and MC provided technical assistants. TC prepared manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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