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Original Research

A Cloned Gene *HuBADH* from *Hylocereus undatus* Enhanced Salt Stress Tolerance in Transgenic *Arabidopsis thaliana* Plants

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Abstract

Background: Betaine aldehyde dehydrogenase (BADH) catalyzes the synthesis of glycine betaine and is considered to be a type of osmoregulator, so it can play a role in plants' responses to abiotic stresses. **Methods**: In this study, a novel *HuBADH* gene from *Hylocereus undatus* (pitaya) was cloned, identified, and sequenced. The full-length cDNA included a 1512 bp open reading frame that encoded a 54.17 kDa protein consisting of 503 amino acids. Four oxidation-related stress-responsive marker genes (*FSD1*, *CSD1*, *CAT1*, and *APX2*) were analyzed by Quantitative real-time reverse transcription (qRT-PCR) in wild type (WT) and transgenic *A. thaiana* overexpression lines under NaCl stress. **Results**: HuBADH showed high homology (79–92%) with BADH of several plants. The *HuBADH* gene was genetically transformed into *Arabidopsis thaliana* and overexpressed in transgenic lines, which accumulated less reactive oxygen species than WT plants, and had higher activities of antioxidant enzymes under NaCl stress (i.e., 300 mM). All four marker genes were significantly upregulated in WT and *HuBADH*-overexpressing transgenic *A. thaliana* plants under salt stress. Glycine betaine (GB) content was 32–36% higher in transgenic *A. thaliana* lines than in WT in the control (70–80% in NaCl stress). **Conclusions**: Our research indicates that *HuBADH* in pitaya plays a positive modulatory role when plants are under salt stress.

Keywords: pitaya; salt stress; betaine aldehyde dehydrogenase; *HuBADH* gene; physiological analysis; transgenic *Arabidopsis thaliana*

1. Introduction

Abiotic stresses, such as salinity, extremely low or high temperatures, and drought, influence plant growth and development, so they are a major challenge for sustainable agricultural development because they can reduce crop yield [1,2]. Soil-based salinity has become a worldwide problem because of poor irrigation systems, salt infiltration, water pollution, reduced rainfall, and other environmental factors, so the areas affected by saline stress are likely to increase [3–5]. Salinization has affected an estimated 400 million ha of land around the world, or about 3% of the globe's arable land [6]. Salt stress interferes with osmotic balance and ion homeostasis in plants, decreasing photosynthetic activity, inducing metabolic dysfunction, and finally resulting in decreased crop production, so many agricultural lands and crops suffer from the secondary effects of salinization [7]. Plants employ a range of mechanisms to respond to salt stress, including minimizing the amount of salt absorbed via roots and the partitioning of salt at cellular and tissue levels to prevent its accumulation in the cytosol of physiologically functional leaves [8]. The accumulation of compatible solutes is one such important mechanism. Comptible solutes, including amino acids, sugar alcohols, quaternary ammonium compounds, and tertiary sulphonium compounds vary depending on the plant species [9,10]. Glycine betaine (GB) is an important compatible solute for achieving salinity tolerance in many plants [11]. In plants, GB is abundant in the Graminaceae, Asteraceae, Malvaceae, and Amaranthaceae [9]. GB can induce plant stress tolerance by increasing the levels of expression of stress resistance genes, stress signal transduction, enhancing the activities of antioxidant enzymes, protecting cell osmotic pressure, maintaining cell membrane integrity, as well as protecting the photosystem II (PSII) complex [12]. GB achieves this by maintaining a high Na^+:K^+ ratio by regulating osmotic balance and reducing the toxic effects of ions on the cell's structures [13]. GB can decrease adverse effects of drought and salinity stresses and increase photosynthetic efficiency under stress [14]. The exogenous application of GB increased the activity of antioxidant enzymes in *Axonopus compressus* [14].

Betaine aldehyde dehydrogenase (BADH) is a key enzyme related to the biosynthetic pathway of GB because it oversees the second step in the GB biosynthetic pathway,

and the introduction of its gene through transgenetics has fortified the tolerance of various plant species to abiotic stresses [12]. BADH is coded by multifunctional genes that can enhance stress tolerance an improve the productivity and longevity of plants under stress by protecting their photosynthetic apparatus [12]. As one example, *LrAMADH1* in *Lycium ruthenicum* increased GB content under salt stress [15].

Salt stress also induces the accumulation of reactive oxygen species (ROS), a high level of which may cause molecular damage, including to proteins, DNA and lipids, or ultimately result in cell death [16–18]. ROS can also increase the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), thereby decreasing oxidative stress [19]. By revealing the molecular mechanisms underlying salinity tolerance in plants, it may be possible to improve the tolerance of crops to salinity [20].

Increased tolerance to abiotic stresses by BADH-overexpressing transgenic plants has been reported for various plant species. Overexpression of the BADH gene in *Atriplex hortensis* (*AhBADH*) improved the salt tolerance of transgenic *Poncirus trifoliata* [21]. Overexpression of the *Ammopiptanthus nanus* BADH gene (*AnBADH*) in *Arabidopsis thaliana* and *Escherichia coli* enhanced salt and drought tolerance [22,23]. Ectopic overexpression of the *ALDH21* gene from *Syntrichia caninervis* transformed into tobacco conferred salt and drought stress tolerance [24]. The *Suaeda liaotungensis SlBADH* gene improved plants' salinity tolerance [25]. A *BADH* gene (*AmBADH*) from *Atriplex micrantha* increased salt tolerance in transgenic maize [26]. Rice *OsBADH1* and *OsBADH2* increased salt tolerance at various growth stages, with *OsBADH1* overexpression during germination and in seedlings while *OsBADH2* was overexpressed at the reproductive stage [27]. Several plants have more than one *BADH* gene paralog, including *Spinacia oleracea* [28], *Hordeum vulgare* [29], *Glycine max* [30] and *Oryza sativa* [27].

Hylocereus undatus (pitaya) belongs to the Cactaceae family [31,32]. Three pitaya varieties with red-skinned fruit and white flesh, all native to Central and South America [33], have been industrialized [34]. These are now widely cultivated in tropical and subtropical areas of the world, particularly in Asian countries including Vietnam, the Philippines, Malaysia, Thailand and China [35]. At present, the main regions of pitaya cultivation in China include Guangxi, Hainan, Yunnan, Guangdong, Fujian and Taiwan [36]. Pitaya can tolerate different abiotic stresses, including cold, heat, drought, nutritionally poor soil [37,38], and salt [39,40]. Pitaya is thus an outstanding plant species to mine genes related to drought and salt tolerance. Some research on pitaya has focused on the biosynthesis of betaine and the formation of pigments [41,42], as well as on antioxidant and radical-scavenging capacity [43]. A transcriptomic analysis identified several key genes in the betaine biosynthetic pathway [44].

Pitaya plants typically display high salt tolerance [45]. To investigate the function of the *HuBADH* gene in salt stress, we cloned the *HuBADH* gene from pitaya and transformed it into *A. thaliana* for the first time. We found that transgenic *A. thaliana* plants harboring the overexpressed *HuBADH* gene showed significantly higher salt tolerance than wild type (WT) plants. Our results will be useful for investigating the detailed function of the *HuBADH* gene in salt tolerance and for exploring a new method to develop abiotic stress-resistant pitaya.

2. Materials and Methods

2.1 Plant Material and Culture Conditions

The model plant *A. thaliana* (ecotype Col-0) was used for ectopic gene expression. Seeds were surface sterilized in 1 mL of 70% (v/v) ethanol for 15 min and washed three times with autoclaved water. *A. thaliana* seeds were placed on agar-solidified Murashige and Skoog (MS) medium [46], and kept at 4 °C in the dark for 3 d. Plates were then transferred to a climate-controlled growth chamber where plants were grown for 4 or 5 d at 22 °C in a 16-h photoperiod. WT and transgenic *A. thaliana* seedlings were transferred to pots with nutrient soil for 2 weeks. Pots were placed in a growth chamber at 22 °C and grown in light (100 μmol m^{-2} s^{-1}; 16-h photoperiod). Pitaya seedlings were cultured in a growth chamber at 25 °C in a 16-h photoperiod, and at 70–80% relative humidity (light intensity: 100 μmol m^{-2} s^{-1}). Adult pitaya plants were grown in a greenhouse of South China Botanical Garden (Guangzhou, Guangdong, China) under ambient conditions.

2.2 Analysis of the HuBADH Sequence

DNAMAN 7.0 software was used to align the sequences of HuBADH and BADH proteins from other plants (Lynnon Biosoft Corp., San Ramon, CA, USA) [47]. Protein sequences of proteins homologous to *H. undatus* BADH (MK160492), *Amaranthus hypochondriacus* BADH (AAB70010), *Sesuvium portulacastrum* BADH (AEK98521), *Spinacia oleracea* BADH (XP_021837164), *Tamarix hispida* BADH (AIL24123), and *A. thaliana* BADH (AAG51938), were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/). A phylogenetic tree was built by MEGA v7.0.14 software (https://www.megasoftware.net/) [48] using the neighbor-joining (NJ) method [49]. Bootstrap values were assessed as 1000 replicates. The conserved domain of the HuBADH protein was analyzed according to the conserved domains within the protein coding nucleotide sequence (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The sequences for protein HuBADH was submitted to ExPASy (https://web.expasy.org/protparam/) to determine MWs and theoretical pIs. All BADH proteins similarity were further identified by BlastP in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

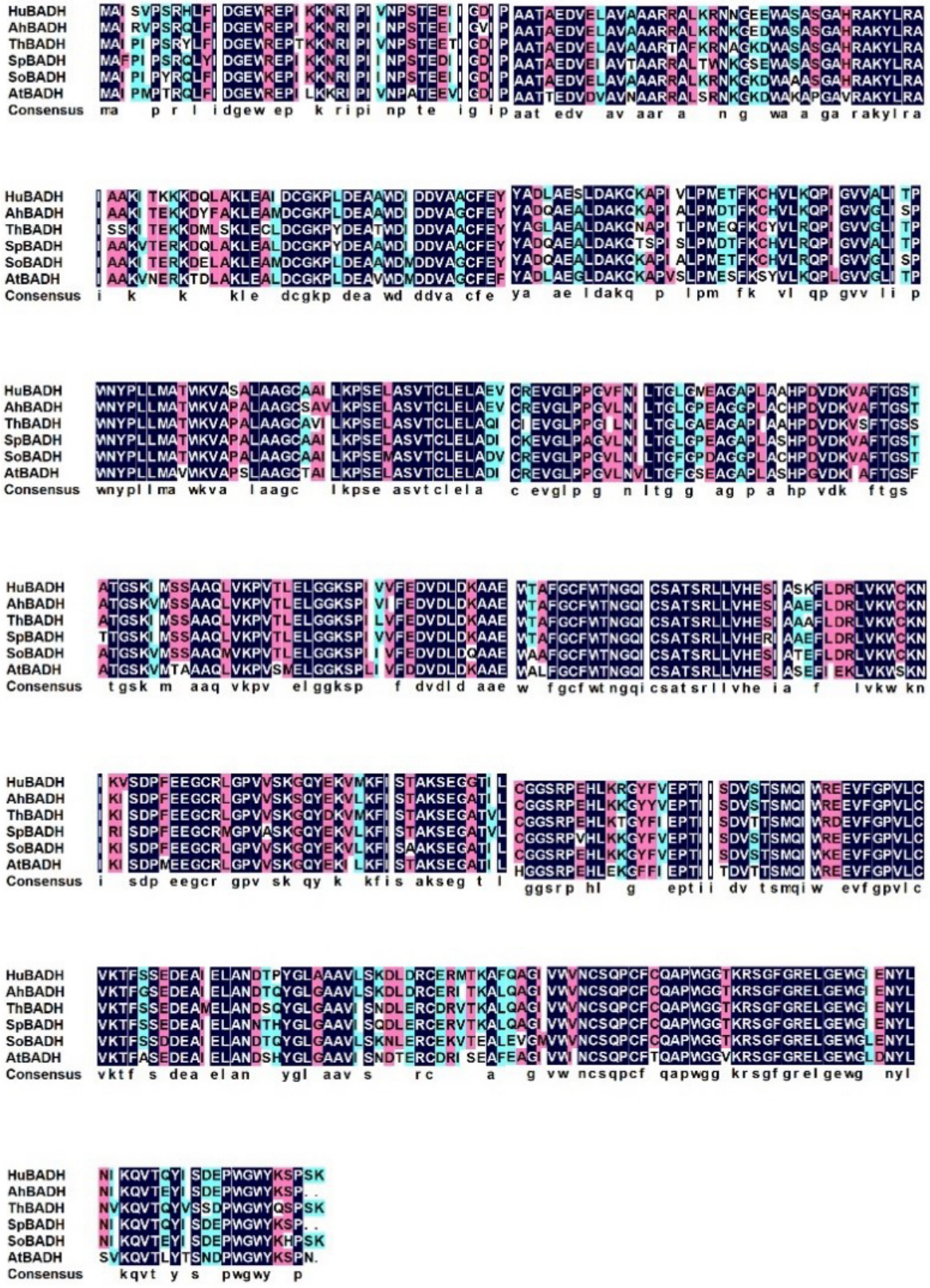

Fig. 1. Multiple sequence alignment analysis of the BADH protein amino acids. Multiple alignment of betaine aldehyde dehydrogenase (BADH) amino acid sequences performed in DNAMAN 7.0. Protein sequences of BADH homologues (in order of listing) from *Hylocereus undatus* (MK160492), *Amaranthus hypochondriacus* (AAB70010), *Tamarix hispida* (AI L24123), *Sesuvium portulacastrum* (AEK98521), *Spinacia oleracea* (XP_021837164), and *Arabidopsis thaliana* (AAG51938).

2.3 Vector Construction and Genetic Transformation

To obtain a plasmid construct of the *HuBADH* gene, its full-length ORF was amplified by Real-time reverse transcription PCR (RT-PCR) from pitaya seedlings using specific primers (**Supplementary Table 1**). The polymerase chain reaction (PCR) product of the *HuBADH* gene was inserted into the pCAMBIA1302 vector to obtain *35Spro::HuBADH* recombinant plasmids and sequence. The constructed vector was mobilized to *Agrobacterium tumefaciens* EHA105 by a 42 °C heat shock in a water bath (ZX-S24, Southeast Yicheng Laboratory Equipment Co., Ltd., Beijing, China), then used to transform *A. thaliana* using the floral dip method [50]. *HuBADH*-overexpressing (OE) transgenic *A. thaliana* lines were cultured on MS medium with 50 μg mL^{-1} kanamycin (1162GR005 Yibaishun Technology Co., Ltd., Guangdong, China) to select resistant lines [51]. After 5 d, *A. thaliana* seedlings (T0 generation) with true green leaves were selected as transformants and transplanted to pots containing soil (WT-NTT-5, Witte, Fuller, Germany). Seeds of T1 and T2 generations were screened on MS medium with 50 μg mL^{-1} kanamycin (1162GR005). Positive transgenic lines were selected when the resistant : sensitive segregation ratio was 3:1. Positive transgenic (T0, T1, T2) and WT lines were identified using PCR with the following procedure: stage 1 (94 °C for 3 min); stage 2 (30 cycles of 94 °C for 10 s, 55 °C for 30 s, 72 °C for 2 min); stage 3 (72 °C for 5 min). PCR products were detected on 1% agarose under ultraviolet light. DL2000 is used as control (MYDEER, Guangzhou Anbang biotechnology Co. Ltd. Guangzhou, China) PCR primers are listed in **Supplementary Table 1**.

2.4 RNA Extraction and qRT-PCR

To detect the expression level of *HuBADH*, total RNA was isolated from the leaves of *A. thaliana* plants, and from the roots, stems, petals, calyces, and squamas of adult pitaya plants with the Eastep® Super Total RNA Extraction Kit (Promega, Beijing, China). RNase-free DNase I (Promega) was used to degrade residual genomic DNA, and 1 μg of DNA-free RNA product was synthesized from first-strand cDNA by the GoScript^{TM} Reverse Transcription Mix (Promega). Products were diluted five-fold for quantitative real-time reverse transcription PCR (qRT-PCR). The Eastep qPCR Master Mix Kit (Promega) was used to perform qRT-PCR. qRT-PCR was conducted with Eastep qPCR Master Mix Kit (Promega) on a Roche Light Cycler 480 Real-time PCR System (Roche, Basel, Switzerland). Thermal cycling was 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. Melting curve analysis was achieved with the following program after 40 PCR cycles: 95 °C for 15 s, 72 °C for 5 min. *A. thaliana Actin2* and pitaya *HuEF-I*α were used as internal controls. Dissociation kinetic curves were established at the end of each qRT-PCR run. All reactions were executed in triplicate for three biological replicates. Relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method [52].

2.5 Semi-qRT-PCR

Total RNA from the leaves of 14-d-old WT and three OE transgenic lines were extracted and purified as described above. Semi-qRT-PCR was performed with the following protocol: stage 1 (95 °C for 5 min); stage 2 (35 cycles of 94 °C for 10 s, 57 °C for 30 s, 72 °C for 2 min); stage 3 (72 °C for 5 min). *AtUBQ10* (At4g05320) from *A. thaliana* was used as the internal control. PCR products were detected on a 1% agrose gel under ultraviolet light. The primers designed for semi-qRT-PCR are listed in **Supplementary Table 1**.

2.6 Salt Stress Treatment of Pitaya Seedlings and Transgenic A. thaliana Lines

Pitaya seedlings (14-d-old) were treated with 300 mM NaCl (S805277-500 g; Macklin, Beijing Ruizhi Hanxing, Beijing, China) for different periods of time (0, 3, 6, 9, 12, and 24 h). *HuBADH* expression levels were then analyzed. To assess the percentage of seed germination, more than 200 seeds of homozygous transgenic *A. thaliana HuBADH*-overexpressing (OE) lines and WT were sown onto MS medium containining 0 or 120 mM NaCl for 3 d. Seeds were also sown onto MS medium with 300 mM NaCl, but they did not germinate (data not shown), so this treatment was not used in further assays. Germination percentage was assessed after growth in the presence of 120 mM NaCl for 3 d. For the *in vitro* assay, 5-d-old transgenic *A. thaliana* seedlings were transplanted to MS medium containining 0 or 120 mM NaCl for 15 d. Root length and fresh weight was investigated after culture for 15 d. In the salt stress assay, 5-d-old transgenic and WT *A. thaliana* seedlings were transplanted to pots containing sterilized soil (WT-NTT-5) for 14 d under normal conditions (no salt stress). Seedlings were irrigated with 300 mM NaCl every 3 d. Treatments lasted for a total of 21 d to maintain long-term salt stress. Survival percentage was investigated after 21 d.

2.7 Assessment of CAT and SOD Content

To assess biochemical traits under salt stress, 5-d-old *A. thaliana* seedlings, including the WT and transgenic lines, were transplanted to pots with nutrient soil for 14 d under normal conditions (no salt stress). Both WT and transgenic lines were watered with 0 or 300 mM NaCl, respectively for 24 h. The spectrophotometer used for all biochemical measurements (AS11D-H, Asone, Merrill Biochemical Technology Co., Ltd. Shanghai, China).Twenty leaves of 10 *A. thaliana* lines were sampled for both 0 or 300 mM NaCl treatments. CAT and SOD assay kits (CAT: BC0205; SOD: BC0175. Solarbio Science and Technology Co. Ltd., Beijing, China) were used to measure of CAT (E.C. 1.11.1.6) and SOD (E.C. 1.15.1.1) activities, respectively. Each assay used 0.1 g of fresh leaves per 1 mL of extract and activity was determined by following the man-

ufacturer's intructions. CAT activity was determined at 240 nm [53] while SOD activity was determined at 560 nm [54].

2.8 Assessment of Proline and MDA Content

Proline content was measured according to the Zhang *et al.* [55] method. Briefly, fresh leaves (0.1 g) were homogenized in 1 mL of extraction buffer of a Solarbio Science and Technology Co. Ltd. kit (BC0295), and proline content was determined at 520 nm by following the manufacturer's intructions.

Malondialdehyde (MDA) content were measured using the Liu *et al.* [56] method. Briefly, fresh leaves (0.1 g) were homogenized in 1 mL of extraction buffer of a Solarbio Science and Technology Co. Ltd. kit (BC0025) and MDA content was determined at 532 and 600 nm by following the manufacturer's intructions.

2.9 Assessment of H_2O_2 and O_2^- Content

Hydrogen peroxide (H_2O_2) content was measured using the Tiryaki *et al.* [57] method. Briefly, fresh leaves (0.1 g) were homogenized in 1 mL of extraction buffer of a Solarbio Science and Technology Co. Ltd. kit (BC3590), and H_2O_2 content was determined at 415 nm by following the manufacturer's intructions.

Superoxide radical (O_2^-) content was measured using the Cai *et al.* method [58]. Briefly, fresh leaves (0.1 g) were homogenized in 1 mL of extraction buffer of a Solarbio Science and Technology Co. Ltd. kit (BC1295), and Superoxide radical (O_2^-) content was determined at 530 nm by following the manufacturer's intructions.

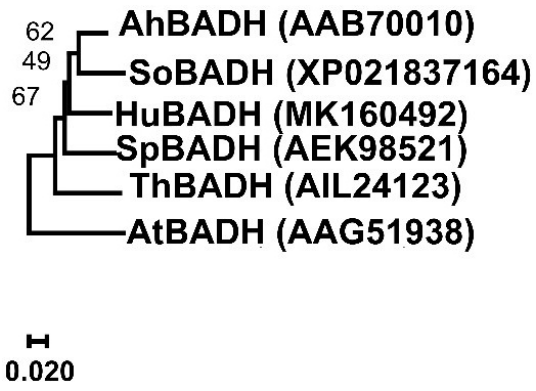

Fig. 2. Phylogenetic tree analysis comparing HuBADH and BADH proteins from other plants. The phylogenetic tree of BADH subfamily proteins was developed in MEGA7.0 software. These BADH proteins include *Hylocereus undatus* (MK160492), *Amaranthus hypochondriacus* (AAB70010), *Sesuvium portulacastrum* (AEK98521), *Spinacia oleracea* (XP_021837164), *Arabidopsis thaliana* (AAG51938), and *Tamarix hispida* (AIL24123). GenBank accession numbers of these proteins are indicated in parentheses.

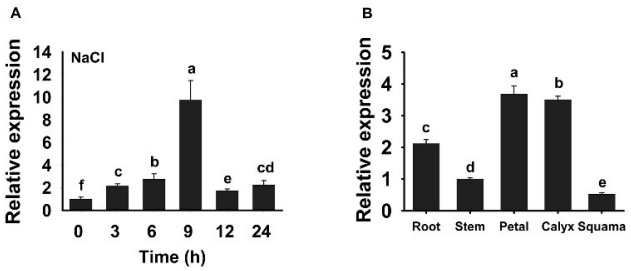

Fig. 3. Expression analysis of *HuBADH* of 14-d-old pitaya seedlings treated with 300 mM NaCl. (A) *HuBADH* expression levels at 0, 3, 6, 9, 12, and 24 h after salt treatment. The value at 0 h served as the control. (B) *HuBADH* expression levels in different tissues. *HuEF1-α* was used as the internal standard. Different lower-case letters above error bars indicate significant differences at $p < 0.05$ (ANOVA followed by Duncan's multiple range test). Data are shown as the means (20 seedlings for each experiment) and SD of three biological replicates.

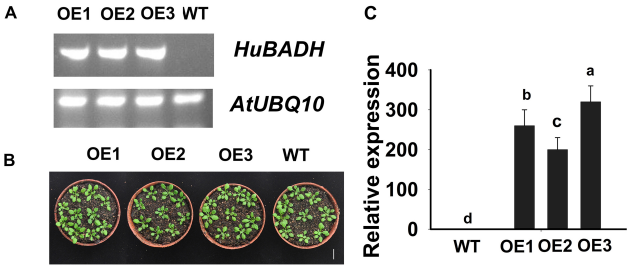

Fig. 4. Expression levels of three *HuBADH*-overexpressing (OE) transgenic *A. thaliana* lines identified by semi-qRT-PCR. (A) Semi-qRT-PCR analysis of the *HuBADH* gene in wild-type (WT) and three *35S::HuBADH* transgenic (OE) *A. thaliana* lines. *AtUBQ10* served as the internal control. (B) 15-d-old *A. thaliana* seedlings of WT and *HuBADH* OE lines. (C) qRT-PCR analysis of *HuBADH* in three transgenic *A. thaliana* OE lines and WT. In (C), different lower-case letters above error bars indicate significant differences at $p < 0.05$ (ANOVA followed by Duncan's multiple range test). Data shown as the means (20 seedlings for each experiment) and SD of three biological replicates. Bar in (B) = 2 cm.

2.10 Assessment of GB Content

GB content was measured by the Wang et al. method [59]. Briefly, fresh leaves (0.1 g) were homogenized in 1 mL of extraction buffer of a Solarbio Science and Technology Co. Ltd. kit (BC3135), and GB content was determined at 525 nm by following the manufacturer's intructions.

2.11 3,3'-Diaminnobenzidine (DAB) Staining

To detect O_2^- and H_2O_2 *in situ*, 30 fresh terminal leaves for each of 35-d-old WT and transgenic *A. thaliana* OE lines growing in control and salt stress (300 mM NaCl) treatments were soaked in a solution of 1 mg/mL

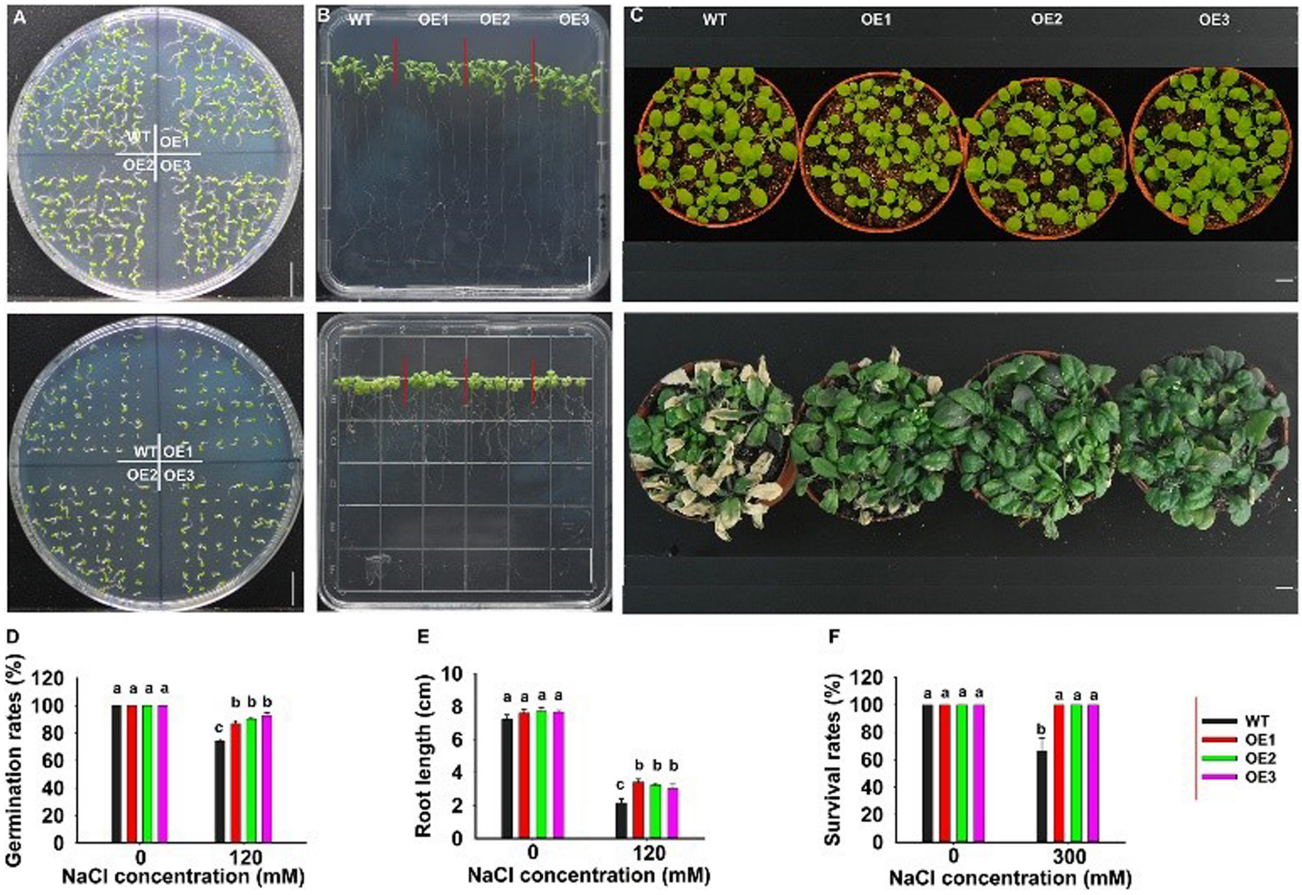

Fig. 5. Overexpressing *HuBADH* in transgenic *A. thaliana* improved tolerance to salt stress and enhanced seed germination. (A) Seed germination of *HuBADH*-overexpression (OE) transgenic *A. thaliana* lines on MS medium with 0 (above) and 120 mM NaCl (below) for 3 d, when germination rates were measured (D). (B) Four-d-old transgenic *A. thaliana* and WT seedlings were grown on MS medium with 0 (above) and 120 mM NaCl (below) for 15 d. Root length (E) and fresh weight (F) were measured. (C) Five-d-old *HuBADH* OE transgenic *A. thaliana* and WT seedlings were transplanted in plastic pots for 14 d, then treated with 300 mM NaCl for 21 d, when survival was measured (G). (D–G) Different lower-case letters above error bars indicate significant differences at $p < 0.05$ (ANOVA followed by Duncan's multiple range test). Mean values (>200 seeds per line for each experiment) and SD of three biological replicates are shown. Bars (A–C) = 1 cm.

3,3'-diaminnobenzidine (DAB) (1024, Nanjing Jiancheng, China) for 10 h then washed in 95% ethanol, as suggested by a previous protocol [60].

2.12 Statistical Analysis

All data were plotted in Sigmaplot 12.5 (Systat Software Inc., San Jose, CA, USA) [61]. Data were analyzed by Duncan's multiple range test ($p < 0.05$) in SPSS version 20.0 software (IBM Corp., Armonk, NY, USA).

3. Results

3.1 Phylogenetic Analysis of the HuBADH Protein and Isolation of the HuBADH Gene

Among several BADH homologs that were identified in a previous pitaya transcriptomic analysis, *HuBADH* was shown to be upregulated in a salt stress treatment [45]. The BADH open reading frame (ORF) was 1512 bp long, it encoded 503 amino acids (aa), had a molecular weight of 54.792 KDa, and a theoretical pI of 5.75. BlastP results indicated that HuBADH had 89.2% similarity with BADH of *Sesuvium portulacastrum*, 92.0% similarity with BADH of *Amaranthus hypochondriacus*, 88.3% similarity with BADH of *Spinacia oleracea*, 88.3% similarity with BADH of *Tamarix hispida*, and 79.2% similarity with BADH of *A. thaliana* (Fig. 1).

Using MEGA7.0 software, a NJ-based phylogenetic tree of these BADH sequences was constructed to evaluate the phylogenetic relationships among *Hylocereus undatus* (MK160492), *Amaranthus hypochondriacus* (AAB70010), *Tamarix hispida* (AIL24123), *Sesuvium portulacastrum* (AEK98521), *Spinacia oleracea* (XP_021837164), and *A. thaliana* (AAG51938) (Fig. 2). The results indicate that HuBADH is likely a member of the BADH family.

3.2 Bioinformatics Analysis of the HuBADH Protein

Since BADH catalyzes the last biosynthetic step, i.e., the transfer of betaine aldehyde into betaine, we evaluated the HuBADH protein in pitaya. The BADH protein sequences from other plants were aligned. The HuBADH protein contains a fairly conserved domain F-G-C-F-W-T-N-G-Q-I-C-S-A-T-S-R-L-L-V-H-E (Fig. 1), which depicts dehydrogenation, and several residues are denoted as being related to catalytic and NAD^+-binding sites.

3.3 Expression Patterns of the HuBADH Gene under NaCl Stress

To appreciate the expression levels of the *HuBADH* gene in response to 300 mM NaCl, qRT-PCR was performed using total RNA from 14-d-old pitaya seedlings as the template. In the 300 mM NaCl treatment, the level of *HuBADH* transcript was initially upregulated, peaking at 9 h, and declining thereafter (Fig. 3A). This result indicates that *HuBADH* responds to salt stress. *HuBADH* gene expression levels, which were determined in different tissues of pitaya at the flowering stage, were highest in petals and the calyx, and lowest in the squama (Fig. 3B).

3.4 Overexpression of HuBADH in Transgenic A. thaliana Enhanced Tolerance to Salt Stress

The overexpression of *HuBADH* in *A. thaliana* was achieved by transforming this gene into *A. thaliana* lines using an *Agrobacterium*-based floral dip method [50], driven by the *CaMV*-35S promoter. Five positively transgenic *A. thaliana* lines were selected on MS medium with 50 μg/mL kanamycin (**Supplementary Fig. 1**) using PCR and a kanamycin-resistance assay [51]. Homozygous lines (T2-T3) were examined. The expression levels of three *HuBADH* OE transgenic *A. thaliana* lines with a higher expression level were identified by semi-qRT-PCR (Fig. 4A). There were no differences in the phenotype (size, flowering time, color, leaves number) between WT and the three OE transgenic lines (Fig. 4B). *A. thaliana* OE lines showed relatively higher expression levels of *HuBADH* than WT *A. thaliana* in normal (non-salt stressed) conditions (Fig. 4C). These results indicate that *HuBADH* was successfully transformed into the *A. thaliana* genome and expressed normally.

To appreciate the function of *HuBADH* in seed germination under salt stress, all seeds of transgenic *A. thaliana* OE and WT plants were sown on MS medium supplemented with 0 or 120 mM NaCl for 3 d. Under salt stress, WT plants showed 74.5 $\pm$ 1.29% seed germination whereas transgenic *A. thaliana* OE lines OE1, OE2 and OE3 displayed 89.25 $\pm$ 2.21%, 90.25 $\pm$ 1.26% and 90.75 $\pm$ 2.22% seed germination, respectively. Seed germination of transgenic *A. thaliana* was at least 16% higher than that of WT seeds exposed to 120 mM NaCl, although no significant differences were found between WT and OE lines in the control (0 mM NaCl) treatment after 3 d (Fig. 5A,D).

The root length and fresh weight of transgenic *A. thaliana* OE and WT seedlings were assessed after they were transplanted for 15 d on MS medium supplemented with 0 and 120 mM NaCl. Transgenic *A. thaliana* seedlings displayed significantly longer roots and higher fresh weight than WT seedlings under salt stress but no differences were observed between WT and transgenic OE lines in the control (no salt stress) (Fig. 5B,E,F). These results indicate that *HuBADH* OE transgenic *A. thaliana* lines experienced an obvious increase in salt tolerance during seed germination and at the seedling stage.

To further assess whether transgenic *A. thaliana* OE lines could enhance salt stress tolerance at a later stage, in the flowering period, 5-d-old WT and transgenic *A. thaliana* seedlings were cultivated in salt-free conditions for 14 d. Thereafter, WT and OE lines were watered with a 300 mM NaCl solution for 21 d. Transgenic *A. thaliana* OE lines showed significantly higher tolerance to salt stress than WT (Fig. 5C,G). While survival of transgenic *A. thaliana* OE1, OE2 and OE3 lines was 100%, that of WT plants was only 66.67 $\pm$ 9.07%. Under normal (non-salt stressed) growth conditions, in contrast, there were no significant differences in survival percentage between transgenic *A. thaliana* OE lines and WT plants (Fig. 5C,G). These results indicate that transgenic *A. thaliana* OE lines showed greater salt tolerance than WT plants under salt stress, even during the flowering period.

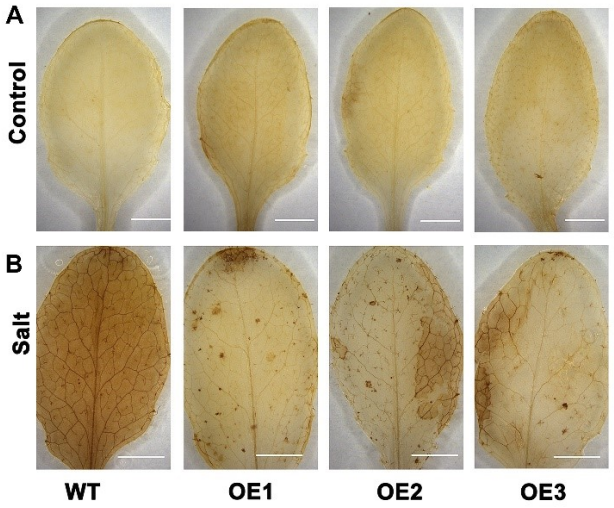

Fig. 6. Detection of ROS accumulation in the leaves of *HuBADH*-overexpressing *A. thaliana* and wild-type (WT) seedlings under salt stress. (A) ROS detected by DAB staining in 35-d-old WT and transgenic seedlings under control conditions. (B) ROS detected under salt stress. For the salt stress treatment, plants grown in pots were irrigated with 300 mM NaCl for 24 h. Bars = 0.5 cm.

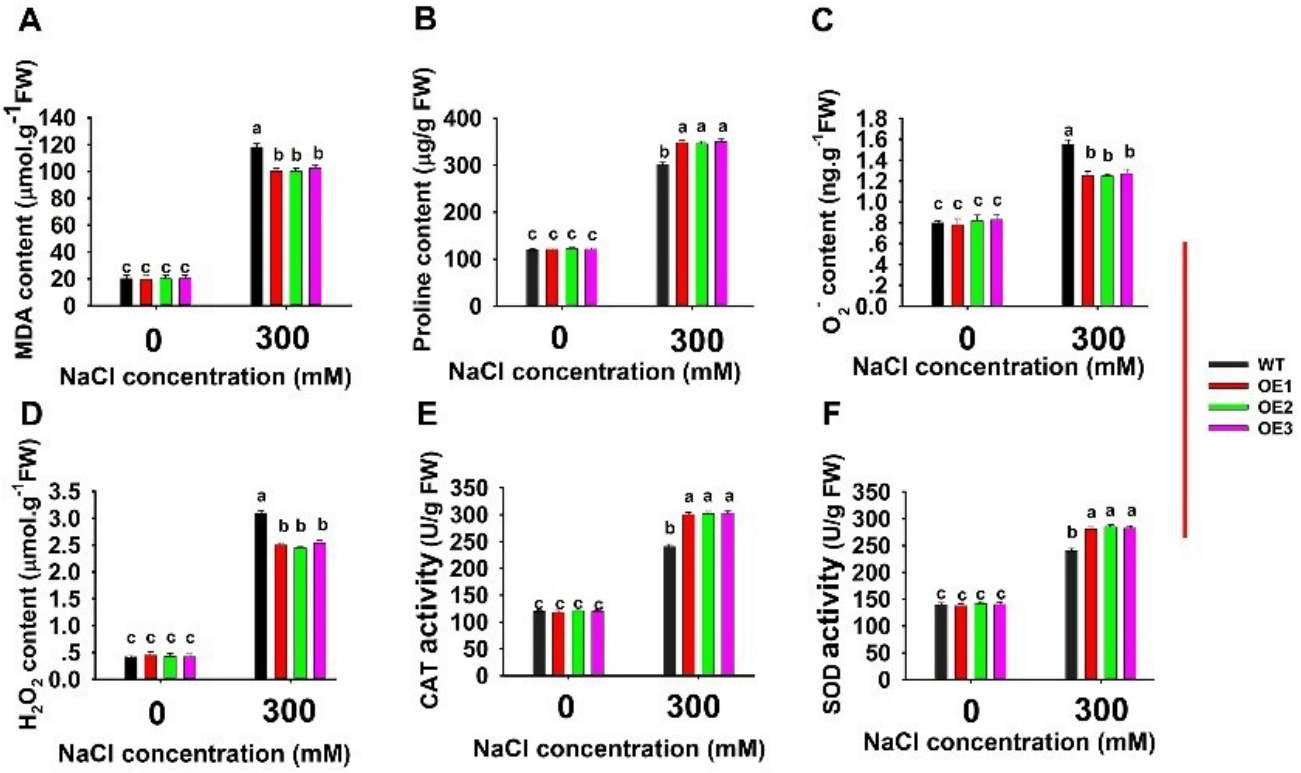

Fig. 7. Activities of two antioxidant enzymes and different physiological parameters in the salt stress treatment (300 mM NaCl) in 35-d-old transgenic *A. thaliana* (*HuBADH*-overexpressing; OE) and wild-type (WT) plants. (A) MDA content. (B) Proline content. (C) O_2^- content. (D) H_2O_2 content. (E) CAT activity. (F) SOD activity after treatment for 24 h. Different lower-case letters above error bars indicate significant differences at $p < 0.05$ (ANOVA followed by Duncan's multiple range test). Mean values (10 seedlings for each experiment) and SD of three biological replicates are shown.

3.5 HuBADH-Overexpression Lines Eliminated Reactive Oxygen Species

To study the function of *HuBADH* in oxidative stress, we assayed the level of O_2^- and H_2O_2 accumulation in the leaves of *HuBADH* OE transgenic *A. thaliana* plants (Fig. 6A,B). Transgenic *A. thaliana* OE1, OE2, and OE3 lines accumulated less O_2^- and H_2O_2 than WT in the 300 mM NaCl treatment (Fig. 6B) whereas there were no significant differences in the levels of O_2^- and H_2O_2 between WT and OE plants in the control (0 mM NaCl) treatment (Fig. 6A).

To further verify the possible physiological mechanisms mediated by *HuBADH*, several physiological indices, such as the content of H_2O_2, proline, O_2^-, the activities of antioxidant enzymes, and the content of MDA were measured in WT and transgenic *A. thaliana* plants in the 0 and 300 mM NaCl treatments (Fig. 7A–F). Compared to WT, H_2O_2, O_2^- and MDA contents were significantly less in transgenic *A. thaliana* plants exposed to salt stress, but not significant in the control (0 mM NaCl) (Fig. 7A,C,D). Proline content and activities of antioxidant enzymes (SOD; CAT) were significantly higher in transgenic *A. thaliana* plants than in WT in the 300 mM NaCl treatment (Fig. 7E,F), but there were no differences in proline content or in the activities of both antioxidant enzymes between WT and OE1, OE2, and OE3 lines in the no-stress (0 mM NaCl) treatment (Fig. 7). This indicates that the overexpression of *HuBADH* in transgenic *A. thaliana* reduced the accumulation of ROS by decreasing MDA, H_2O_2 and O_2^- contents and by increasing the activities of two antioxidant enzymes and proline content in transgenic *A. thaliana* lines under salt stress, suggesting that the transgenic *A. thaliana* OE lines are salt tolerant, unlike WT plants (Fig. 7B).

To further clarify the possible functional molecular mechanisms of the *HuBADH* gene under salt stress, four oxidation-related stress-responsive marker genes (*FSD1*, *CSD1*, *CAT1*, and *APX2*) were analyzed by qRT-PCR in WT and transgenic *A. thaliana* lines exposed to 300 mM NaCl. All four marker genes were significantly upregulated in WT and transgenic *A. thaliana* plants under salt stress (Fig. 8A–D). This result indicates that *HuBADH* induced the expression of oxidative stress-responsive genes, leading to improved salt stress tolerance of transgenic plants.

GB content in WT and transgenic lines was determined in control (0 mM NaCl) or salt stress (300 mM NaCl) treatments. In the control, transgenic lines OE1, OE2 and OE3 showed a 32.20, 34.96 and 36% increase, respectively

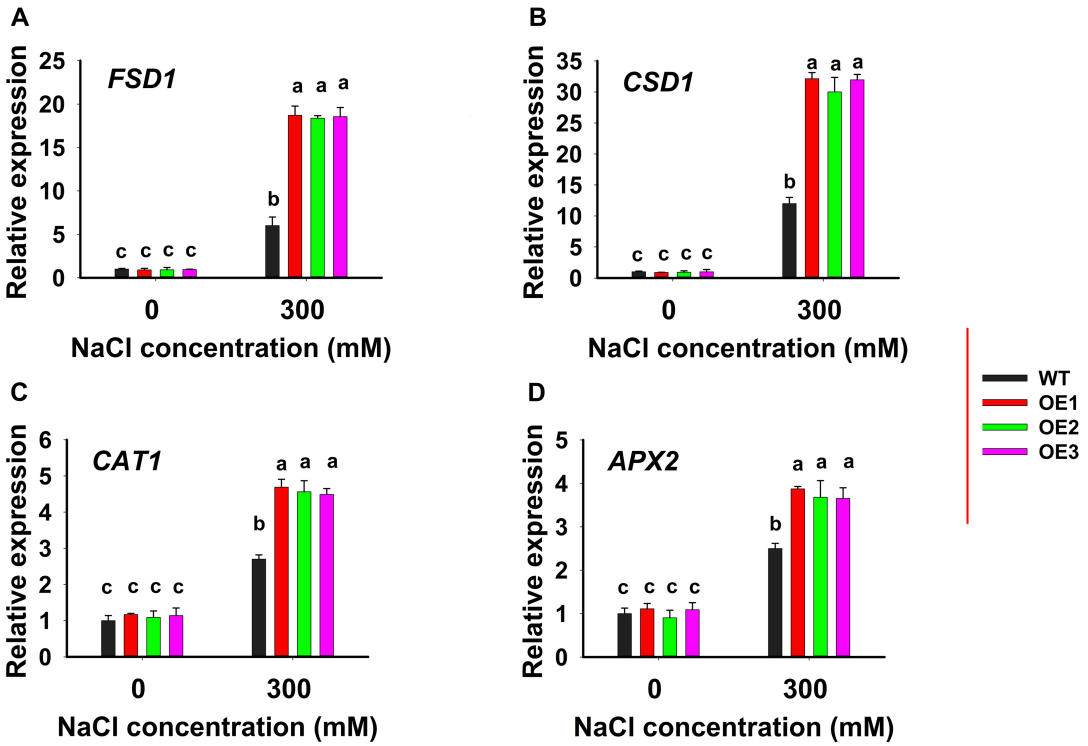

Fig. 8. Oxidation-related genes were analyzed by qRT-PCR in 15-d-old transgenic *A. thaliana* (*HuBADH*-overexpressing; OE) and wild-type (WT) plants under salt stress (300 mM NaCl) for 24 h. (A) *FSD1*. (B) *CSD1*. (C) *CAT1*. (D) *APX2*. Different lower-case letters above error bars indicate significant differences at $p < 0.05$ (ANOVA followed by Duncan's multiple range test). Mean values (10 seedlings for each experiment) and SD of three biological replicates are shown.

in GB content relative to WT. After 24 h of exposure to 300 mM NaCl, GB content in OE1, OE2 and OE3 increased 70.00, 73.87 and 80.97% compared to WT, respectively (Fig. 9).

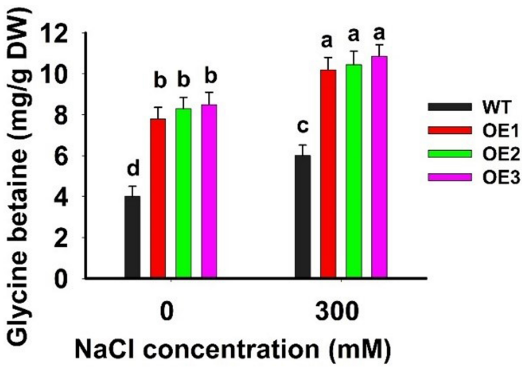

Fig. 9. Glycine betaine content in *HuBADH*-overexpressing transgenic *Arabidopsis thaliana.* Five-d-old *HuBADH* OE transgenic *A. thaliana* and WT seedlings were transplanted in plastic pots for 14 d, then treated with 300 mM NaCl for 24 h. Glycine betaine content was measured in control (0 mM NaCl) and salt-stressed (300 mM NaCl) conditions. Different lower-case letters above error bars indicate significant differences at $p < 0.05$ (ANOVA followed by Duncan's multiple range test). Mean values (10 seedlings for each experiment) and SD of three biological replicates are shown.

4. Discussion

A previous study profiled the metabolites of two species of pitaya (*H. undatus* and *H. polyrhizus*) to compare their antioxidant activities and betalain biosynthesis [62]. Plants must efficiently adapt their growth and development to stressful conditions. Salt stress, a globally impactful abiotic stress [63], negatively impacts plant growth and development, although some plants have developed regulatory mechanisms permitting them to adapt to adverse environments [64,65]. To date, only a few salt stress-related genes have been identified in pitaya, such as *HuCAT3* [66], *miR396b-GRF* [67] and *HuERF1* [51]. The exact functions of BADH proteins in pitaya remain unkown, but they have been found to play a major function in modulating the response of plants to different abiotic stresses, including drought in *Nicotiana tabacum* [68], heat in *Hylocereus polyrhizus* [69], cold in *Hordeum vulgare* [70] and salt in *Leymus chinensis* [71].

In our study, a new gene named *HuBADH* was cloned from pitaya for the first time. Sequence alignment and phylogenetic analysis of HuBADH and related proteins in other plants indicated that HuBADH is highly homologous to AhBADH from *Amaranthus hypochondriacus* (Figs. 1,2). Bioinformatics analysis showed that the BADH protein had

a fairly conserved domain F-G-C-F-W-T-N-G-Q-I-C-S-A-T-S-R-L-L-V-H-E (Fig. 1). Therefore, we deduced that the BADH motif was highly conserved, not only in terms of its amino acid sequence, but also biological and biochemical roles.

The overexpression of stress-related genes endowing plants with salt resistance is a popular method to enhance the salt stress tolerance of crops. The expression of *HuBADH* was stimulated by salt treatment (Fig. 3A). Therefore, *HuBADH* plays a role in salt tolerance. *HuBADH* was strongly expressed in the petals and calyx of pitaya plants, but was weakly expressed in the squama (Fig. 3B). Also in pitaya, *HuERF1* was strongly expressed in the roots [51] but *miR396-GRF* was weakly expressed in the roots, relative to stems [67]. In summary, the genes involved in salt stress have different expression patterns in different tissues in pitaya.

Semi-qRT-PCR and qRT-PCR analyses showed that *HuBADH* expression levels were significantly higher in transgenic *A. thaliana* OE lines than in WT plants (Fig. 4), suggesting that *HuBADH* is a candidate gene to improve salt stress tolerance in pitaya.

The level of *HuBADH* expression increased under salt stress (Fig. 3A). Molecular, morphological and physiological analyses revealed a relationship between *HuBADH* overexpression and salt stress tolerance (Fig. 5A–G). At the germination stage, transgenic seeds overexpressing *HuBADH* had significantly higher germination rates on MS medium containing NaCl than WT seeds (Fig. 5A,D). At the seedling stage, the salt stress tolerance of transgenic *A. thaliana* was enhanced (Fig. 5B,E,F). At the flowering stage, transgenic *A. thaliana* plants showed enhanced tolerance to salt stress, as demonstrated by significantly higher survival relative to WT plants (Fig. 5C,G). These findings indicate that *HuBADH* plays a modulatory role in pitaya salt stress resistance and/or tolerance.

When ROS overaccumulates, there is interference with cellular homeostasis, and this induces oxidative stress in mitochondria, leading to their dysfunction [71]. ROS can be reduced in the ROS-scavenging pathway [72]. H_2O_2 is one form of ROS, so improving the expression of genes related to the ROS-scavenging pathway is one way to prevent the accumulation of H_2O_2 [73]. In our study, the accumulation of ROS was significantly lower in transgenic *A. thaliana* lines harboring the *HuBADH* gene than in WT plants exposed to salt stress (Fig. 6). In addition, four genes involved in the ROS-scavenging pathway were upregulated in transgenic *A. thaliana* plants after exposure to salt stress (Fig. 8). This result indicates that *HuBADH* enhanced salt stress tolerance by regulating the expression of at least these four stress-responsive marker genes.

HuBADH-OE transgenic plants had higher tolerance to salt stress than WT plants under saline conditions. The levels of GB were higher in transgenic lines than WT lines in plants growing in control or salt-stressed conditions (Fig. 9). The level of NaCl in plants could be determined in future research.

5. Conclusions

BADH is a central enzyme in the biosynthetic pathway of GB. Overexpression of the *BADH* has been shown to increase tolerance to different abiotic stresses in a range of plants. The *HuBADH* gene from pitaya was cloned. Its full-length cDNA had an ORF of 1512 bp that encodes a 54.17 KDa protein with 503 amino acids. When the *HuBADH* gene was genetically transformed into *A. thaliana*, transgenic *HuBADH*-OE lines displayed tolerance to salt (300 mM NaCl). Transgenic *A. thaliana* accumulated less ROS than WT plants and showed higher activities of two antioxidant enzymes (SOD, CAT) in the NaCl treatment. Four oxidation-related stress-responsive marker genes (*FSD1*, *CSD1*, *CAT1*, and *APX2*) were significantly upregulated in WT and transgenic *A. thaliana* plants under salt stress. The level of GB was 32–36% higher in transgenic *A. thaliana* lines than in WT in the control, and 70–80% higher in NaCl stress. Our research indicates that *HuBADH* in pitaya plays a positive role in modulating the negative impact of salt stress, so it may be an ideal candidate to increase salt tolerance in pitaya breeding programs.

Abbreviations

APX2, Ascorbate peroxidase; BADH, Betaine aldehyde dehydrogenase; CAT, Catalase; CSD1, Copper/zinc superoxide dismutase; DAB, 3,3'-Diaminobenzidine; FSD1, Fe superoxide dismutase; GB, Glycine betaine; O_2^-, Superoxide anion; ORF, Open reading frame; qRT-PCR, Quantitative real-time reverse transcription PCR; ROS, Reactive oxygen species; SOD, Superoxide dismutase; RT-PCR, Real-time reverse transcription PCR.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

YQ and QN designed and performed the experiments including HuBADH gene cloning and genetic transformation; ZB provided materials and assisted YQ and QN with experimental execution; WQ was also involved with experimental design and conducted the statistical analyses; YQ, JATdS and GM co-wrote and edited all versions of the manuscript; GM and JATdS supervised the project, and provided scientific advice and guidance. All authors contributed to editorial changes in the manuscript. All authors read and approved the manuscript for publication. All authors take responsibility for the accuracy and integrity of the findings.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2804078.

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