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Full Length Article

Rapid Identification of Enhanced Drought and Salt Tolerance in *Arabidopsis* Conferred by *BnBADH1* Gene

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Abstract

Identifying and applying salt or drought tolerant genes is a quick method of enhancing crop tolerance to environmental stresses. In this study, a full-length new betaine aldehyde dehydrogenase gene from *Brassica napus* (*BnBADH1*) was isolated by homologous cloning method according to the sequence of *Arabidopsis BADH1* gene. *BnBADH1* was 1506 bp in length and it theoretically encoded a hypothetical protein with 501 aa; sharing 90.2% identity with its ortholog in *Arabidopsis* (GenBank: ALDH10A8) in nucleotide sequence and 93.4% identity in amino acid sequence. The mRNA expression levels of *BnBADH1* under drought or salt stress was investigated by quantitative real-time PCR. Under drought stress, the expression level of *BnBADH1* mRNA began to decrease slightly from 12 h to 72 h; but it suddenly increased at 96 h and reached a peak at 120 h; after that, its expression reduced drastically from 144 h to 192 h. While under salt stress, there was no obvious difference in expression level at the first 8 h (2–8 h); but mRNA increased sharply at 20 h. Moreover, the *BnBADH1* gene was induced into *Arabidopsis* and overexpressed. Drought and salt stress tolerance was enhanced in *Arabidopsis* as was expressed by higher root length of transgenic plants compared with its wild plants. It is the first report which highlighted the expressions of newly isolated gene *BnBADH1* from rapeseed in transgenic *Arabidopsis* to enhance drought and salt stresses tolerance.

Keywords: Abiotic stresses; Betaine aldehyde dehydrogenase; *Brassica napus*; Gene expression level; Transgenic plants

Introduction

Plants often suffer from various environmental stresses like drought, salinity, cold and heat. Drought and salt stresses significantly limit the geographical distribution of crops and cause reductions in their yield and quality (Jia *et al.,* 2002; Wu *et al.,* 2008; Farooq *et al.,* 2018). Drought stress affects 50% of rice production globally (Bouman *et al.,* 2005; Wang *et al.,* 2018). It inhibits the photosynthesis, affects the respiration and absorption of nutrients, restrains the cell division and disturbs plant metabolism (Farooq *et al.,* 2009; Djebbar *et al.,* 2012). Salinity is another abiotic stress, which imposes osmotic stress, ion stress and further secondary stresses, then consequently decreases the crop yields (Munns and Tester, 2008; Farooq *et al.,* 2015, 2017a). Wang *et al.* (2003) predicted that by the middle of this century, half of all cultivable lands may suffer from serious salinization. Several approaches have been suggested to mitigate salt or drought stress including irrigation and soil amelioration; nonetheless identification and application of salt or drought tolerance genes provide possible solution (Hussain *et al.,* 2018; Farooq *et al.,* 2017b).

Confronting the drought or salt stress, higher plants have evolved several morphological, physiological and biochemical strategies to reduce adverse effects (Farooq *et al.,* 2009; Li *et al.,* 2008). Among them, biosynthesis of osmotic regulators such as proline, mannitol and betaine has vital role. Betaine protects biomacromolecules from denaturation under high electrolyte concentration, maintains cellular turgor without disturbing cellular structures and functions (Zhang *et al.,* 2007; Wang *et al.,* 2008). It existed widely in animals, plants and microorganisms; while in higher plants, the osmotic regulator betaine is synthesized *via* two oxidation reactions: choline → betaine aldehyde → betaine. In this reaction two important enzymes are involved successively: choline monooxygenase (CMO) catalyzes the prior reaction and betaine aldehyde dehydrogenase (BADH) catalyzes the latter (Sakamoto and Murata, 2000). Thus BADH is considered as a key enzyme, playing an important role in betaine biosynthesis.

To date, *BADH* gene from spinach (*Spinacia oleracea*) (Zhang *et al.,* 2011), rice (*Oryza sativa*) (Hasthanasombut *et al.,* 2011), *Atriplex micrantha* (Di *et al.,* 2015) and *Suaeda*

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liaotungensis (Wu *et al.,* 2008) etc. was cloned and functionally analyzed. Di *et al*. (2015) isolated a *BADH* gene from *A. micrantha*, and then introduced it into maize (*Zea mays* L.) to enhanced salt tolerance. Likewise, Zhang *et al.* (2011) found that the spinach *BADH* gene enhanced both drought and salt tolerances in transgenic potato (*Solanum tuberosum*). In another study, Jia *et al.* (2002) reported enhanced salt tolerance (120 m*M* salt concentration) in salt-sensitive tomato by introducing a *BADH* gene from *A. hortensis*. It was also well known that *BADH* has no signal peptide and its expression product location varies from species to species. In spinach (Shen *et al.,* 2001) it was located in chloroplast stroma while in rice (McNeil *et al.,* 1999) it was located in lysosome. However, *BADH* from *Brassica napus* (rapeseed) is seldom reported.

Rapeseed is an important oil crop in China, which provides ~60% of its vegetable oil supply. Drought stress affects yield components in rapeseed, such as seed number per pod, pod number per plant, seed number per plant and yield per plant, thus further affects the yield (Gunasekera *et al.,* 2009; BirunAra *et al.,* 2011). In the rapeseed main planting area of China, the seasonal drought usually causes 15–50% yield reduction and even fail in yielding. Traditionally, breeding tolerance cultivars is a good way of yield stability. Recently, with the development of molecular biotechnology, it is a better method to improve the tolerance of crops to abiotic stresses by molecular skills, including identifying the related genes and understanding their expression model and functions.

In this study, *BnBADH1* from rapeseed was cloned and sequenced, followed by primary bioinformatics analysis. Its mRNA expression level under drought or salt stress was investigated by qPCR. Further the enhanced salt and drought tolerances in model transgenic *Arabidopsis* by inserting *BnBADH1* gene were also studied. This study provided a candidate gene from rapeseed potentially capable to induce salt and drought tolerance in plants.

Materials and Methods

Plant Materials and Culturing Conditions

Arabidopsis thaliana (Columbia) and *B. napus* (JR9) seeds were kept in Crop Research Institute, Sichuan Academy of Agricultural Sciences. After immersed in water at 4°C for 3 days, *Arabidopsis* seeds were surface sterilized in 75% ethanol for 1 min and 0.1% $HgCl_2$ for 10 min successively, followed by rinse with sterile distilled water at least three times. Then the seeds were germinated on solid MS medium (Murashige and Skoog, 1962) or in soils with vermiculite and peat in climatic chamber under normal conditions: 23°C, 16 h light/8 h dark cycle and 60% humidity.

The Cloning of *BnBADH1*

Total RNA was extracted from leaves of 20-days old rapeseed seedlings using RNA extraction kit (TianGen Company, China), followed by reverse transcription with Reverse Transcriptase kit (TianGen Company, China). Thus, the total cDNA was obtained. Then PCR was performed with specific primers BADH-1 and BADH-2 (BADH-1: 5'-ATGGCGATTCCGATGCCTACTC-3'; BADH-2: 5'-TTAGTTGGGAGATTTGTACCAT-3'), which were designed by Primer Premier 5.0 with homologous cloning method, according to the sequence of *Arabidopsis BADH1* gene (Genbank: ALDH10A8). The PCR cycling procedure consisted of 2 min at 95°C, 36 cycles for 30 s at 95°C, 30 s at 53°C and 1 min at 72°C, and a final 15-min extension at 72°C. The PCR products were then purified from agarose gel using gel extraction and purification kit.

Expression of *BnBADH1* Gene in Rapeseed under Drought or Salt Stress

Plump and full rapeseed seeds were selected to determine the transcriptional expression of *BnBADH1* gene under drought stress. Twenty days after germination in soils under normal conditions mentioned above, watering was stopped to the seedlings. The reverse transcription was performed as described above and then the qPCR was operated. Specific primers to detect the transcriptional expression of *BnBADH1* gene was designed, while *ACTIN* gene worked as internal control (Table 1). The qPCR cycling procedure was slightly modified from Chai *et al.* (2012). All of the cycle threshold (Ct) values of *BnBADH1* amplification were normalized by their corresponding internal control. Three repetitions were conducted. Microsoft Office software was used to analyze the data.

Transformation of *Arabidopsis*

The *BnBADH-1* fragment was cut from pEASY-T-*BADH1* with B*am*H 1 and S*ac* 1 restriction sites and fused into the binary vector pBI121-*BADH1* in sense orientation under the control of the 35S promoter (Fig. 1) to ensure its overexpression. The recombinant vector pBI121-*BnBADH1* was introduced into *Arabidopsis via Agrobacterium*-mediated inflorescence-dip method (Clough and Bent, 1998). The seeds harvested after the dip was defined as T_0 generation. The T_0 seeds were harvested and then plated on MS medium with 50 mg/L kanamycin (Kan). The seeds which developed green euphylla and long root were identified as Kan-resistant. The T_0 plants were self-pollinated to obtain the T_1 generation; consequently, the T_1 plants were then self-pollinated to obtain the T_2 generation. The homozygous lines in T_2 were screened by both Kan-resistance and PCR analysis.

Total DNA from *Arabidopsis* leaves was extracted from three-week-old seedlings by CTAB method (Allen *et al.,* 2006) and then was used for PCR analysis. The presence of the *BnBADH1* sense sequence in the selected plants was verified by PCR with the forward primer annealed to the 35S (35S-F: 5′-CCTTCGCAAGACCCTTCCTC-3′) and the

Table 1: Primer sequences for qPCR

Primer	Sequence
BADH-qPCR-F	5'-AGAAAGGTGTGACCGCGTAA-3'
BADH-qPCR-R	5'-CAAACCCACTGCGTTTGACT-3'
actin-F	5'-TGGTGAAGGCTGGTTTTGCT-3'
actin-R	5'-TTCTGACCCATCCCAACCAT-3'

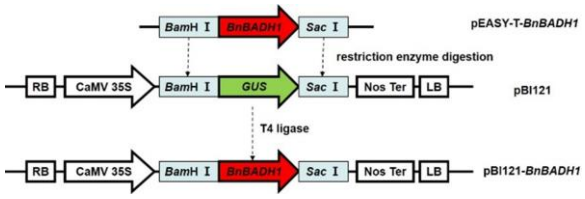

Fig. 1: Strategy for construction of overexpressing vector of pBI121-*BnBADH1*

reverse primer annealed to the coding region (BADH-JC: 5'-GTCCCACAACTCCAATAGGTT-3'). The sample with a 500 bp band was identified positive.

Drought or Salt Tolerance Assay with Transgenic *Arabidopsis*

After immersed in water for three days at 4°C, seeds of both wild type and transgenic *Arabidopsis* were plated on MS, MS+ 200 m*M* mannitol and MS+ 200 m*M* NaCl, respectably. The seedlings were planted in horizontal lines and the plates were placed vertically, in order to observe and measure the root length, which was a measured as a main index for drought tolerance.

Statistical Analysis

Three replicates were carried out and the data were subjected to analysis of variance (ANOVA) using Microsoft Office software and means were separated following least significant difference (LSD) test.

Results

Cloning of *BnBADH1* and Its Sequence Analysis

Based on the RT-PCR, a band with expected size was obtained (Fig. 2a). The fragment was then fused into pEASY-T, followed by inducing into *E. coli* and positive clones screening. After sequenced, the fragment was analyzed and it was found that *BnBADH1* had 1506 bp in length and it theoretically encoded a hypothetical protein with 501 aa, with molecular weight of 55 kDa and isoelectric point of 5.16. Sequence analysis showed that *BnBADH1* shared 90.4% identity with *Arabidopsis BADH* gene (GenBank: ALDH10A8) in nucleotide sequence and 93.4% identity in amino acid sequence (Fig. 2b). Further analysis showed that *BnBADH1* contained a special decapeptide (VSMELGGKSP), which had a difference with 2 amino acids from the regular highly-conserved

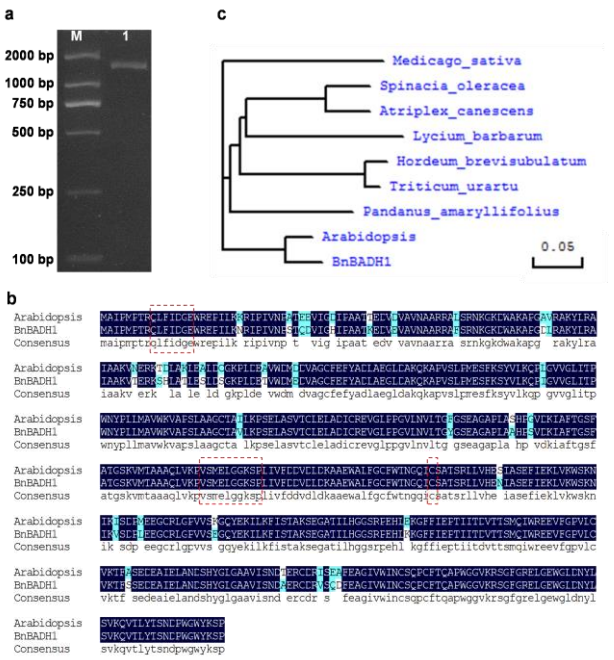

Fig. 2: Isolation and analysis of *BaBADH1*

a) Full length of *BnBADH1* CDS. M: DNA marker DL 2000; 1: full length of *BnBADH1*

b) Amino acid sequence alignment with *Arabidopsis* (ALDH10A8). Red dotted lined boxes indicated important sites

c) Phylogentic tree analysis of *BADH* gene among species: *Medicago sativa* (GenBank: AFS33786.1), *Spinacia oleracea* (GenBank: AAB41696.1), *Lycium barbarum* (GenBank: ACQ99195.1), *Hordeum brevisubulatum* (GenBank: AAS66641.1), *Triticum urartu* (GenBank: EMS48376.1), *Atriplex canescens* (GenBank: AFG28557.1), *Pandanus amaryllifolius* (GenBank: ARR95824.1) and *Arabidopsis* (GenBank: ALDH10A8)

decapeptide (VTLELGGKSP). Cysteine residue was also found, which was considered related to catalysis of this enzyme. Moreover, the QLFIDGE (Fig. 2b) sequence was also found. The phylogentic tree analysis (Fig. 2c) showed that *BnBADH1* had the closest evolutionary relationship with *Arabidopsis* compared with other plants.

The Expression of *BnBADH1* in Rapeseed under Drought or Salt Stress

In order to reveal the expression regulation under drought stress, we stopped watering the soil to create the drought environment. Compared with normal conditions (0 h, Fig. 3a), the expression level of *BnBADH1* mRNA began to decrease slightly from 12 h to 72 h; but it suddenly increased at 96 h and reached a peak (1.8 fold of control) at 120 h; after that, its expression reduced drastically from 144 h to 192 h. In other words, expression level of *BnBADH1* mRNA was obviously higher in the 4th and 5th day after ceasing watering; in other time, it was lower than control (0 h).

As to the salt stress, leaves were sampled every 2 h after 200 m*M* NaCl was added to the soil. Over the first few hours (2–8 h), there was no obvious difference in

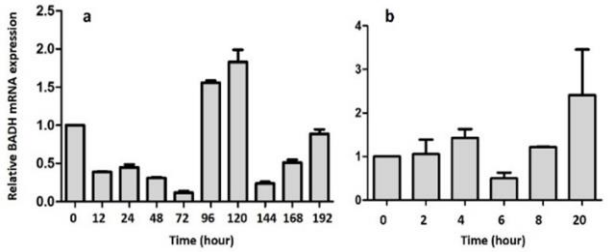

Fig. 3: The mRNA expression of *BnBADH1* under drought or salt stress by qPCR
a: *BnBADH1* relative expression in leaves 0, 12, 24, 48, 72, 96, 120, 144, 168, 192 h after cease of watering
b: *BnBADH1* relative expression in leaves 0, 2, 4, 6, 8, 20 h after adding 200 m*M* NaCl in soil

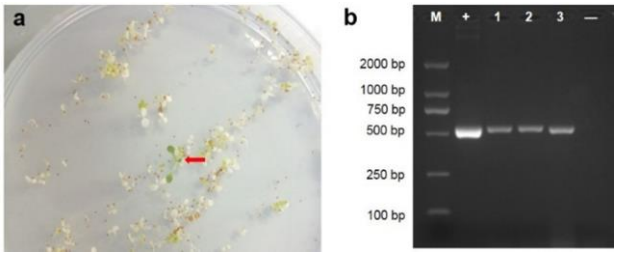

Fig. 4: Identification of positive *BnBADH1* transgenic *Arabidopsis* lines
a: Kan-resistance to identify positive seedlings. As the red arrow pointed, the seedling with green euphylla and long root was considered positive
b: PCR identification of positive seedlings. M: DNA marker DL 2000; 1-3: three identified positive seedlings; +: agrobacterium with pBI121-*BADH1* as positive control; -: *Arabidopsis* WT as negative control

expression level. However, the expression level of *BnBADH1* mRNA increased sharply at 20 h, reaching 2.4 fold of the control at 0 h (Fig. 3b).

Identification of Transgenic *Arabidopsis*

Three individual Kan-resistant lines from T_0 generation were selected, which had green euphylla and long root (Fig. 4a); whereas the Kan-sensitive ones got discolored cotyledons and shortened root. These Kan-resistant plants were further checked by PCR, when they were transplanted in soil and grew taller. The presence of the 500 bp band (Fig. 4b) was identified, which was consistent with positive control (*Agrobacterium* containing pBI121-*BADH1* vector); whereas the WT got no such band. This confirmed the success of the transgenic *Arabidopsis* achievement.

Overexpression of *BnBADH1* Enhanced Drought or Salt Tolerance in Transgenic *Arabidopsis*

After immersed in water at 4°C for 3 days, seeds of both transgenic *Arabidopsis* and wild type were placed on MS medium of 0 and 200 m*M* mannitol. On the MS medium, overexpressing *BnBADH1* *Arabidopsis* showed no difference to WT (data not shown); while on the MS+ 200 m*M* mannitol medium (Fig. 5a, Table 2), the *BnBADH1* grew significantly better: they (13.2 mm) had about 98% longer roots than WT (6.7 mm), though both of them showed restrained growth under the simulant drought environment.

Table 2: Root length (mm) of transgenic *Arabidopsis* and WT on MS medium with 200 m*M* mannitol for 7 days

Arabidopsis plants	Control	200 m*M* mannitol
Transgenic plants with *BnBADH1* gene	25.1	13.2
Wild plants	25.2	6.7

Table 3: Root length (mm) of transgenic *Arabidopsis* and WT on MS medium with 200 m*M* NaCl for 5 days

Arabidopsis plants	Control	200 m*M* NaCl
Transgenic plants with *BnBADH1* gene	21.2	7.7
Wild plants	21.0	3.5

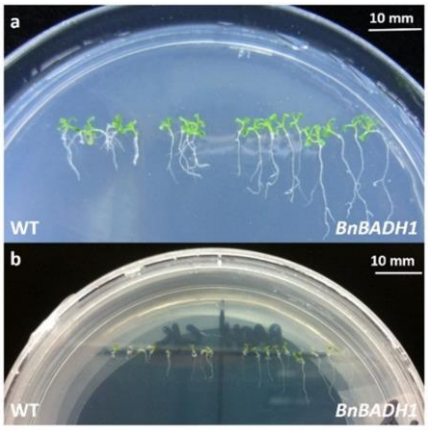

Fig. 5: Enhanced tolerance to drought or salt stress
a: transgenic *Arabidopsis* and WT on MS+ 200 m*M* mannitol 7 days after germination
b: transgenic *Arabidopsis* and WT on MS+ 200 m*M* NaCl 5 days after germination

The similar thing happened in the case of 200 m*M* NaCl treatment. The transgenic *Arabidopsis* and WT only differed from each other under salt stress. The restrained growth phenomenon was more obvious than in drought stress, but *BnBADH1* plants were always stronger than WT (Fig. 5b, Table 3): it had 120% longer root (7.7 mm) than WT (3.5 mm), indicating enhanced tolerance to NaCl stress.

Discussion

The *BnBADH1* from rapeseed was first cloned and analyzed by bioinformatics. Besides physiochemical properties, some important conserved sequences were studied: its core sequence VSMELGGKSP was exactly the same as of *Arabidopsis*, but 2 aa difference from some other species. Although it is not confirmed whether this affects the enzymatic activity, at least it indicated that *BnBADH1* had the closer evolutionary relationship with *Arabidopsis* than other plants. The QLFIDGE atypical signal peptides were

also found in 5'-terminal, implying that *BnBADH1* was probably located in the chloroplast (Weretilnyk and Hanson, 1990).

In order to investigate the response to drought stress, mRNA expression level of *BnBADH1* was determined. We stopped watering the soil to simulate the drought environment. At the first 3 days, the mRNA expression level decreased slightly, this might be because the evaporation of the originally residual water in soil cost some time. From 96 h on, the soil became very dry, and the plants started to suffer water deficiency indeed. This could explain the reason why *BnBADH1* had significantly increased expression at this point. At the 5th day (120 h), because of the desiccation of the soil, the *BnBADH1* continued to overexpress to maintain osmotic pressure and its expression reached the peak level. But from the 6th day to onward, the expression of *BnBADH1* decreased again. It perhaps was due to the damage of the whole plant resulting from the long-term water deficiency. In other words, metabolisms in plants were disrupted in this case, including the recession of *BnBADH1* expression. As to the salt stress, within 20 h after 200 m*M* NaCl was added into the soil, the *BnBADH1* expression did not change much. But it got an increased expression at 20 h. It showed that *BnBADH1* responded salt stress quicker than drought stress.

Since the mRNA expression level of *BnBADH1* was increased under drought or salt stress, we wondered that whether overexpression of *BnBADH1* could enhance tolerance to drought or salt stress. Thus the transgenic experiment was performed then. A Kan-resistance fragment was introduced into plants together with *BnBADH1*, so the positive seedlings should be able to survive on MS+ Kan medium. To avoid the false positive result, PCR was used to confirm the selection. Although the full length of *BnBADH1* was 1506 bp, another pair of primers was designed for convenience: this forward primer (35S-F) annealed to 35S promoter and a reverse primer (BADH-JC) annealed to the 500 bp position (instead of the end) of *BnBADH1*. Thus the target band would get much shorter and it cost less time to conduct the PCR. In the certified transgenic *Arabidopsis*, under the control of 35S promoter, the *BnBADH1* was necessarily overexpressed in transgenic *Arabidopsis*. Under the drought (200 m*M* mannitol) or salt (200 m*M* NaCl) stress, both *BnBADH1 Arabidopsis* and WT grew with inhibition; but the *BnBADH1 Arabidopsis* got longer roots than WT under both stress, showing the enhanced tolerances conferred by *BnBADH1* overexpression. It also could be seen that the inhibition of salt (200 m*M* NaCl) stress was more significant than drought (200 m*M* mannitol) stress. This might be due to the limited regulating ability of *BnBADH1*: after all, it was only a harmless osmotic regulator. In many reported studies, overexpression of endogenous or exogenous *BADH* gene also enhanced tolerances in plants (Jia *et al.,* 2002; Wu *et al.,* 2008; Liu *et al.,* 2011; Zhang *et al.,* 2011; Di *et al.,* 2015).

There were many indexes to determine the abiotic tolerances, such as biomass, dry weight, chlorophyll content, conductivity and so on. Here in this study, root length was chosen as the most visible and convenient index. So more experiments verifying *BnBADH1* is still undergoing. Although application of transgenic (or GMO) crops remains controversial, a new gene from rapeseed enhancing drought and salt stresses was theoretically investigated here in this study. It provided an understanding of rapeseed endogenous gene that could probably be utilized in directional breeding in future. After all, the ultimate goal is to obtain new cultivars resistant to abiotic stresses.

Conclusion

The expression models of *BnBADH1* gene isolated from rapeseed were investigated by qPCR under drought or salt stress. When this *BnBADH1* gene was induced into *Arabidopsis*; it was overexpressed and the transgenic *Arabidopsis* plants observed more drought and salt tolerance.

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