Rapid Identification of Enhanced Drought and Salt Tolerances 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). 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 monoxygenase (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) (Hasthansombut et al., 2011), Atriplex micrantha (Di et al., 2015) and Suaeda 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 mM 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 Brassica 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% HgCl2 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’- ATGGCGATTCCGATGCTACTC-3’; BADH-2: 5’- TTAGTTGGGAGATTGTACCAT-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 were 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 BamH 1 and Sac 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 T0 generation. The T0 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 T0 plants were self-pollinated to obtain the T1 generation; consequently, the T1 plants were then self-pollinated to obtain the T2 generation. The homozygous lines in T2 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 gene sequence in the selected plants was verified by PCR with the forward primer annealed to the 35S (35S-F: 5’- CCTTCCGAAGACCTTTCC-3’) and the reverse primer annealed to the coding region (BADH-JC: 5’- GTCCCCACAATCTCAATGTT-3’). The sample with a 500 bp band was identified positive.

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Table 1: Primer sequences for qPCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>BnBADH1-pqCR-F</td>
<td>5’-AGAAAGGTGACCTGGTTA-3’</td>
</tr>
<tr>
<td>BnBADH1-pqCR-R</td>
<td>5’-TTCTGACCATCCAATGCA-3’</td>
</tr>
<tr>
<td>actin-F</td>
<td>5’-TGGTGAAGGCTGTTTGC-3’</td>
</tr>
<tr>
<td>actin-R</td>
<td>5’-GGAGAAAAACACCTGTA-3’</td>
</tr>
</tbody>
</table>

Fig. 1: Strategy for construction of overexpressing vector of pB1121-BnBADH1

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 mM mannitol and MS+ 200 mM NaCl, respectively. 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 band was then fused into pEASY-T, followed by induced 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 decapeptide (VTLELGKSP). Cysteine residue was also found, which was considered related to catalysis of this enzyme. Moreover, the QLFIDGE (Fig. 2b) sequence was also found.

Fig. 2: Isolation and analysis of BnBADH1

a) Full length of BnBADH1 CDS, M. DNA marker DL, 2000; b) full length of BnBADH1 c) Amino acid sequence alignment with Arabidopsis (ALDH10A8). Red dotted lined boxes indicated important sites
d) Phylogenetic tree analysis of BnBADH1 gene among species: Medicago sativa (GenBank: AFS33786.1), Spinacia oleracea (GenBank: AAB41696.1), Lycium barbarum (GenBank: AAC99195.1), Hordeum brevisubulatum (GenBank: AAS66641.1), Triticum urartu (GenBank: EMB48376.1), Atriplex canescens (GenBank: AFG28557.1), Pandanus amaryllifolius (GenBank: ARJ05824.1) and Arabidopsis (GenBank: ALDH10A8)

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 mM NaCl was added to the soil. Over the first few hours (2-8 h), there was no obvious difference in 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 T₀ 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 mM mannitol. On the MS medium, overexpressing BnBADH1 Arabidopsis showed no difference to WT (data not shown); while on the MS+ 200 mM 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.

The similar thing happened in the case of 200 mM 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

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

<table>
<thead>
<tr>
<th>Arabidopsis plants</th>
<th>Control</th>
<th>200 mM mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic plants</td>
<td>25.1</td>
<td>13.2</td>
</tr>
<tr>
<td>with BnBADH1 gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild plants</td>
<td>25.2</td>
<td>6.7</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Arabidopsis plants</th>
<th>Control</th>
<th>200 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic plants</td>
<td>21.2</td>
<td>7.7</td>
</tr>
<tr>
<td>with BnBADH1 gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild plants</td>
<td>21.0</td>
<td>3.5</td>
</tr>
</tbody>
</table>
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 \textit{BnBADH1} had significantly increased expression at this point. At the 5th day (120 h), because of the desiccation of the soil, the \textit{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 \textit{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 \textit{BnBADH1} expression. As to the salt stress, within 20 h after 200 mM NaCl was added into the soil, the \textit{BnBADH1} expression did not change much. But it got an increased expression at 20 h. It showed that \textit{BnBADH1} responded salt stress quicker than drought stress.

Since the mRNA expression level of \textit{BnBADH1} was increased under drought or salt stress, we wondered that whether overexpression of \textit{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 \textit{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 \textit{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 \textit{BnBADH1}. Thus the target band would get much shorter and it cost less time to conduct the PCR. In the certified transgenic \textit{Arabidopsis}, under the control of 35S promoter, the \textit{BnBADH1} was necessarily overexpressed in transgenic \textit{Arabidopsis}. Under the drought (200 mM mannitol) or salt (200 mM NaCl) stress, both \textit{BnBADH1 Arabidopsis} and WT grew with inhibition; but the \textit{BnBADH1 Arabidopsis} got longer roots than WT under both stress, showing the enhanced tolerances conferred by \textit{BnBADH1} overexpression. It also could be seen that the inhibition of salt (200 mM NaCl) stress was more significant than drought (200 mM mannitol) stress. This might be due to the limited regulating ability of \textit{BnBADH1}: after all, it was only a harmless osmotic regulator. In many reported studies, overexpression of endogenous or exogenous \textit{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 \textit{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 \textit{BnBADH1} gene isolated from rapeseed were investigated by qPCR under drought or salt stress. When this \textit{BnBADH1} gene was induced into \textit{Arabidopsis}; it was overexpressed and the transgenic \textit{Arabidopsis} plants observed more drought and salt tolerances.

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**References**


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