Isolation and Characterization of the Betaine Aldehyde Dehydrogenase Gene in *Ophiopogon japonicus*

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Abstract: Betaine aldehyde dehydrogenase (BADH) catalyzes the last step in the synthesis of the glycine betaine from choline. The *BADH* gene from turfgrass *Ophiopogon japonicus* has not been reported. In this study, we first isolated the full length cDNA of betaine aldehyde dehydrogenase gene (*OjBADH*) from *O. japonicus* using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Rapid Amplification of cDNA Ends (RACE) techniques. The *OjBADH* gene (GenBank accession number: DQ645888) has 1785 nucleotides with the 5' untranscribed region (UTR) of 63 nucleotides, 3' UTR of 219 nucleotides, and an open reading frame of 1503 nucleotides. This gene encodes a polypeptide of 500 amino acids. It shares a high homology with *BADH* genes of other *Chenopodiaceae* species. The putative protein includes a conservative region of phosphofructokinase, aldehyde dehydrogenase, and glutamy phosphoric acid reductase. Overexpression of *OjBADH* in transgenic tobacco plants demonstrated 2-2.5 folds increase of glycine betaine content and 60-85% increase of survival rate under salt tolerance. These results suggested that the *O. japonicus BADH* gene may be used to engineer plants for salt stress tolerance.

Keywords: Betaine aldehyde dehydrogenase, Choline, Gene cloning, *Ophiopogon japonicas*, Turfgrass.

INTRODUCTION

Betaine aldehyde dehydrogenase (BADH) catalyzes the last step (Fig. **1**) in the synthesis of the osmoprotectant glycine betaine from choline [1-3]. Many bacteria, plants and animals accumulate glycine betaine under water or salt stress conditions [1, 4-6]. BADH is a specific enzyme for production of glycine betaine [1, 7-9]. BADHs have been purified from several species [10-13]. The promoter of betaine aldehyde dehydrogenase gene has been isolated from *Suaeda liaotungensis* K., and the analysis of the promoter sequence has revealed the existence of several putative *cis*-elements, such as TATA-box, CAAT-box, GC-motif, EIRE, MRE, WUN-motif, heat shock element (HSE) and ABRE [14]. Studies showed that BADHs from sugar beet [15] and humans [16, 17] also catalyze the oxidation of v-aminoaldehydes such as 3-aminopropionaldehyde and 4-aminobutyraldehyde, which are intermediates in putrescine and polyamine degradation.

Glycine betaine is an important osmoprotectant that is produced in response to salt and other osmotic stresses [1, 18-20]. Overexpression of *BADH* results in increase of tolerance of salt and osmotic stresses in many organisms [21-23]. Using *Agrobacterium tumefaciens* strain LBA4404 carrying a binary vector pBin438 and a leaf regeneration system, Jia *et al.* [20] demonstrated that overexpression of the *BADH* gene cloned from *Atriplex hortensis* in a salt-sensitive to mato cultivar, Bailichun, improved salt tolerance. Wu *et al.* [24] reported that the *Suaeda liaotungensis* betaine aldehyde dehydrogenase gene improved salt tolerance of transgenic maize mediated with minimum linear length of DNA fragment. After 15 days of treatment, 73.9–100% of the transgenic seedlings survived and grew well. However, more than 90% wild-type seedlings wilted and showed loss of chlorophyll. Only 8.9% of the wild-type plants survived but gradually died after salt stress [24].

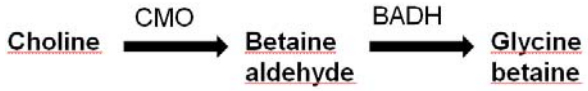

Fig. (1). Production of glycine betaine in higher plants. CMO, choline monooxygenase; BADH, betaine aldehyde dehydrogenase.

O. japonicus [(L.F) Ker-Gawl] is an evergreen perennial herb and a shade tolerant plant in the family of Liliaceae. *O. japonicus* has great stress tolerance and is adapted to a wide range of moisture conditions, and retains green color even in cold season. It is widely distributed and cultivated in many areas of China [9]. Environmental stress tolerance is one of the most important factors limiting *O. japonicus* growth and survival. Increasing environmental stress tolerance has been one of the major objectives in *O. japonicus* breeding programs. Cloning of the *BADH* gene from turfgrass *O. japonicus* has not been reported. Although many BADH genes have been isolated from other species [5, 11-13], it may not be functioning efficiently. To determine the phylogenetic relation of the BADH gene derived from *O. japonicus* with other available BADH genes and to identify the specific function of the *O. japonicus* BADH gene in environmental stress tolerance of turfgrass, we have cloned and character-

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ized the *O. japonicus BADH* gene in this investigation. We isolated a full-length cDNA sequence of *BADH* gene from *O.japonicus* through RT-PCR and RACE, and expressed the *O. japonicus BADH* gene in transgenic tobacco, which demonstrated the increased tolerance to salt. Our results suggested that the *O. japonicus BADH* gene may be used to engineer plants for salt and other osmotic stresses tolerance.

MATERIALS AND METHODS

Plant Material

O. japonicus plants were collected from Longtoushan Forestry Station in Hebei province and were planted in a laboratory nursery of Beijing Forestry University. *E. coli* (JM109), expression strains (*E. coli* BL21) and expression vector [pET-30a(+)] were preserved in -80℃, cloning vector (pMD-18T) was purchased from TAKARA company. The reagents and primers included Advantage™ RT-for-PCR Kit (TAKARA); BD SMART™ RACE cDNA Amplification Kit (CLONETECH); TIANgel Maxi Purification Kit, Taq DNA Polymerase, Pfu DNA Polymerase, and TRNzol Total RNA (Beijing TIANGEN); and Restriction endonucleases (NEB), DEPC (Promega). Primers were synthesized by Shanghai Sangon and other reagents for analysis were all made in China.

Isolation of the *OjBADH* cDNA

The total RNA was extracted from the leaves of one-year-old plants by TRNzol RNA reagents. Using the total RNA as template, the first-strand cDNA was synthesized by Advantage™ RT-for-PCR Kit according to the manufacturer's protocols. Based on the cDNA sequences of *Spinacia oleracea* L., *Beta vulgaris* L., *Atriplex hortensis* Golosk., *Hordeum vulgare* L., *Sorghum bicolor* Pers, *Amaranthus hypochondriacus* L., *Avicennia marina* (Forsk.)Vierh., *Suaeda liaotungensis* Kitag., and *Atriplex centralasiatica* Iljin published in GenBank (http://www.ncbi.nlm.nih.gov/), higher homology region was selected and degenerate primers were designed as F1: 5'-CGHGCBATYGCTGCTAAG -3' and R1: 5'-TKGTACCANCCCCAYTGYTCAT-3'. The middle segment was obtained and sequenced by using the reverse transcribed cDNA as a template for the PCR amplification. Then, 5'RACE primer closed to the 5' terminus and 3'RACE primer close to the 3' terminus were designed as F2 5'-CAGAATCAAGGGTTTCCAGTTTAAC-3' and R2 5'-CCCCGTTATTAGTAAAGGACA according to the results of sequencing, respectively. RACE was performed using the BD SMART™ RACE cDNA Amplification Kit User Manual. The full-length sequence was obtained by stitching the three fragments from PCR amplification using DNAMAN software. Then, the specific primer at the end of 5' terminus F3 was designed as 5'-GCCAACGTAAGCCTATCGTT CCCATACCCG-3'. A pair of gene specific primers F：5'-AACGGATCCATGGCGTTCCCAATTCCTGC-3'; R: 5'-ACCGAGCTCTCAAGGAGCCTTGTACCATCCCC-3' were designed after the coding region was identified according to the complete sequence. The complete BADH cDNA was amplified using primers F and R. The PCR reaction was performed at 94℃ for 4 min, then at 94℃ for 30 s, 59℃ 30 s, 72℃ for 1.5 min for 30 cycles and 72℃ for 10 min. The coding fragment was amplified using these primers and the complete cDNA as a template and then ligated into pMD-18T named as pMD-18T-MD*BADH*. Database searches for sequence homology and comparisons were performed with various web based analytical tools compiled at the website http://www.sdsc.edu/ResTools/. The phylogenetic tree was constructed using the ClustalW (http://www.ebi.ac.uk/clustalw). We use ClustalW to create a multiple alignment of the protein sequences of the selected orthologs. Save the output file of the ClustalW output and run the program without changing the parameter settings to create the phylogenetic tree.

Construction of the *OjBADH* cDNA Expression Vector

The 1.785kb fragment of the *O. japonicus BADH* gene was cloned into the expression vector pBI121 by following the protocol previously described [25] to generate expression vector pBI-*OjBADH-GUS*. To construct the *OjBADH* cDNA expression vector, the *OjBADH* cDNA was amplified using primer R and primer F from plasmid pMD-18T-*OjBADH*. The PCR product was purified and blunted with Klenow fragment. Plasmid pBI121 were digested with restriction endonucleases *BamHI* and blunted with Klenow fragment. After agarose gel electrophoresis, the long fragment (vector fragment from plasmid pBI121) and short fragment (*OjBADH* fragment amplified from pMD-18T-*OjBADH*) were purified and ligated. The vector has only one restriction endonucleases *BamHI* site in its T-DNA region. After transformation with competent cells, the positive clones were confirmed by PCR using primers F：5'-AACGGATCCATG GCGTTCCCAATTCCTGC-3'；R：5'-ACCGAGCTCTC AAGGAGCCTTGTACCATCCCC-3'. The *OjBADH* DNA fragments in plant expression vector pBI-*OjBADH-GUS* (Fig. **5**) were sequenced and the orientation of the *OjBADH* gene in pBI121 was determined by PCR and sequencing of PCR products before used for plant transformation.

Expression of *OjBADH* in Tobacco

For regeneration of *OjBADH* transgenic tobacco plants, plasmid pBI-*OjBADH-GUS* DNA were precipitated, adsorbed to gold particles (1μm), and delivered to target calli using a BioRad PDS-1000/He device by following the protocol (BioRad, USA). Plates containing the target tissues were placed 6 cm below the stopping mesh. Particle acceleration was done according to BioRad PDS-1000/He device at 1100 psi. Sixteen hours after particle bombardment, the calli were transferred to regeneration medium as previously [26].

PCR and Southern Blot Analysis

For molecular characterization of transgenic plants, genomic DNA from young leaves of putative transgenic plants (three-month-old) was extracted and used as template for PCR amplification. The primers were R2 and F2. The expected size of PCR product is 680bp. The PCR reaction was performed at 94℃ for 4 min, then at 94℃ for 30 s, 59℃ 30 s, 72℃ for 1.5 min for 30 cycles and 72℃ for 10 min. PCR products were visualized on 1.0% agarose gel. For Southern blot analysis, genomic DNA was isolated from PCR positive transgenic plants. After digested with *Bam*HI, DNA was blotted onto a nylon membrane positively charged by capillary transfer with 20×SSC, hybridized with DIG-labeled DNA probe at 58℃, and washed at high stringency (2×SSC, 0.1% SDS, at 15-25℃ for 10min, then 0.5×SSC, 0.1% SDS at 65-68℃ for 30 min) (Sambrook and Russell 2001). Immunological detection was according to the protocol of DIG

DNA Labeling and Detection Kit (Roche). The DIG-labelled DNA probes were made from the pBI-*OjBADH-GUS* plasmid use the PCR DIG Probe Synthesis Kit (Roche, Cat. No. 1636090). The PCR mixture includes 5 μl PCR buffer, 5 μl DIG labelling mix, 1 μl primer R, 1 μl primer F, 1.25 μl template (10 ng/μl). 0.75 μl enzyme mix, and 36 μl sterile water. The total volume is 50 μl and PCR was run by following the manufacturer's instruction.

Northern Blotting

Leaves of one-year old *OjBADH* transgenic tobacco plants were picked for total RNA extraction by TRNzol reagents. The RNA was transferred onto nylon film *via* high salt (20×SSC) solution after electrophoresis in 1% agarose gel and denatured by 6% formaldehyde [25]. Northern blotting analysis was performed by using the coding fragment of the *BADH* gene as a probe, and labeled with DIG High Prime DNA Labeling and Detection Starter Kit I. Northern blot analysis of the *O. japonicus BADH* mRNA derived from *O. japonicus* plant 7 days after treated with 400 mmol/l NaCl and non-treated control plant was carried out as previously described standard protocol [25], using the coding fragment of the *BADH* gene as a probe as above.

Salt Tolerance Analysis

Salt-tolerance experiments were carried out as described previously [18, 27]. Transgenic plants grown for three months were subjected to salt-shock treatment, which was administered by watering transgenic plants with 400 mM NaCl 4 times a day for 2 days, and then returned to normal growth conditions [plants were grown in plastic pots filled with a perlite/peatmoss/vermiculite (1:1:1 v/v) mixture located in a greenhouse at 25°C with 16 h of light (50 $\mu molm^{-2}\ s^{-1}$) and 8 h of dark]. Survival rate was determined 15 days after treatment by dividing the survival plants with the total tested plants for each treatment. We only evaluate the tolerance to salt in the 7 transgenic F0 plants. Experiments were repeated three times, and each replicate consisted of 30–90 plants, data represent the mean ± SD.

Glycine Betaine Analysis

The measurement of betaine level was conducted as before [10, 18] in three months old tobacco plants, which were with 400 mM NaCl 4 times a day for 2 days, and then returned to normal growth conditions as salt tolerance experiments. Betaine level was measured at 1, 4, 7, 10, and 13 days after salt stress. Experiments were repeated three times, and each replicate consisted of 3–5 plants, data represent the mean ± SD.

RESULTS

Isolation the Full-Length cDNA of the *O. japonicus BADH* Gene

Betaine aldehyde dehydrogenase (BADH) catalyzes the last step (Fig. **1**) in the synthesis of the osmoprotectant glycine betaine from choline in higher plants. The fragment of the *O. japonicus BADH* cDNA, obtained by RT-PCR, was about 1250bp (Fig. **2A**), the 5'end is 360bp (Fig. **2B**), and the 3'end is 680bp (Fig. **2C**). Therefore, the full length *BADH* cDNA assembled by software DNAMAN was 1785bp (Fig. **2D**) with a 63bp for the 5' UTR, a 219bp for the 3' UTR and a polyA signal-AATTAA. The open reading frame (ORF) was 1503bp, which encodes a 500-amino acid polypeptide. The *BADH* cDNA sequence of *O. japonicus* was submitted to the GenBank database and the registration number is DQ645888. The nucleotide sequence and its encoding amino acid sequence were shown in Fig. (**3**). Northern blot analysis of the *O. japonicus BADH* mRNA derived from *O. japonicus* plant 7 days after treated with 400 mmol/l NaCl and non-treated control plant demonstrated that 400 mmol/l NaCl salt stress induced the increase of *OjBADH* mRNA (Fig. **2E**).

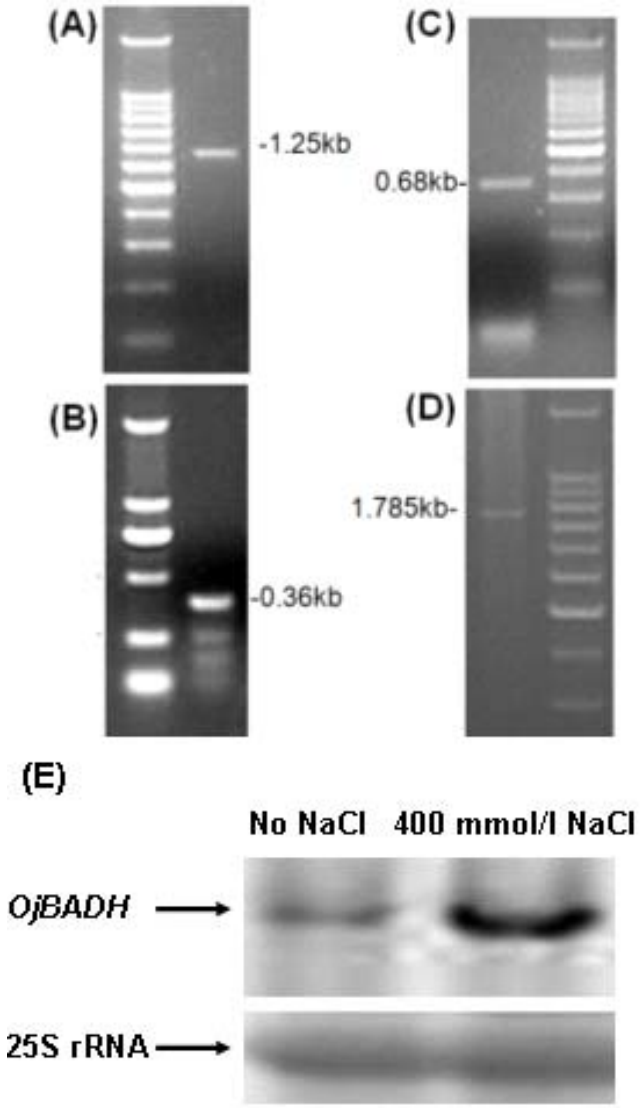

Fig. (2). PCR products of the *BADH* gene.

(**A**) EST of *BADH*, obtained with degenetrate primers, (**B**) The 5' end of *BADH* and (**C**) the 3' end of *BADH* obtainded with RACE technique. (**D**) The complete cDNA of the *BADH* gene amplified with primers derived from the sequene obtained by means of RACE 5' and 3'. (**E**) Northern blot analysis of the *O. japonicus BADH* mRNA derived from *O. japonicus* plant 7 days after treated with 400 mmol/l NaCl and non-treated control plant. Low panel: tobacco 25Sr RNA.

The complete *BADH* sequence homology comparison was done between *O. japonicus* and other reported species. The result showed that *O. japonicus BADH* gene and that of

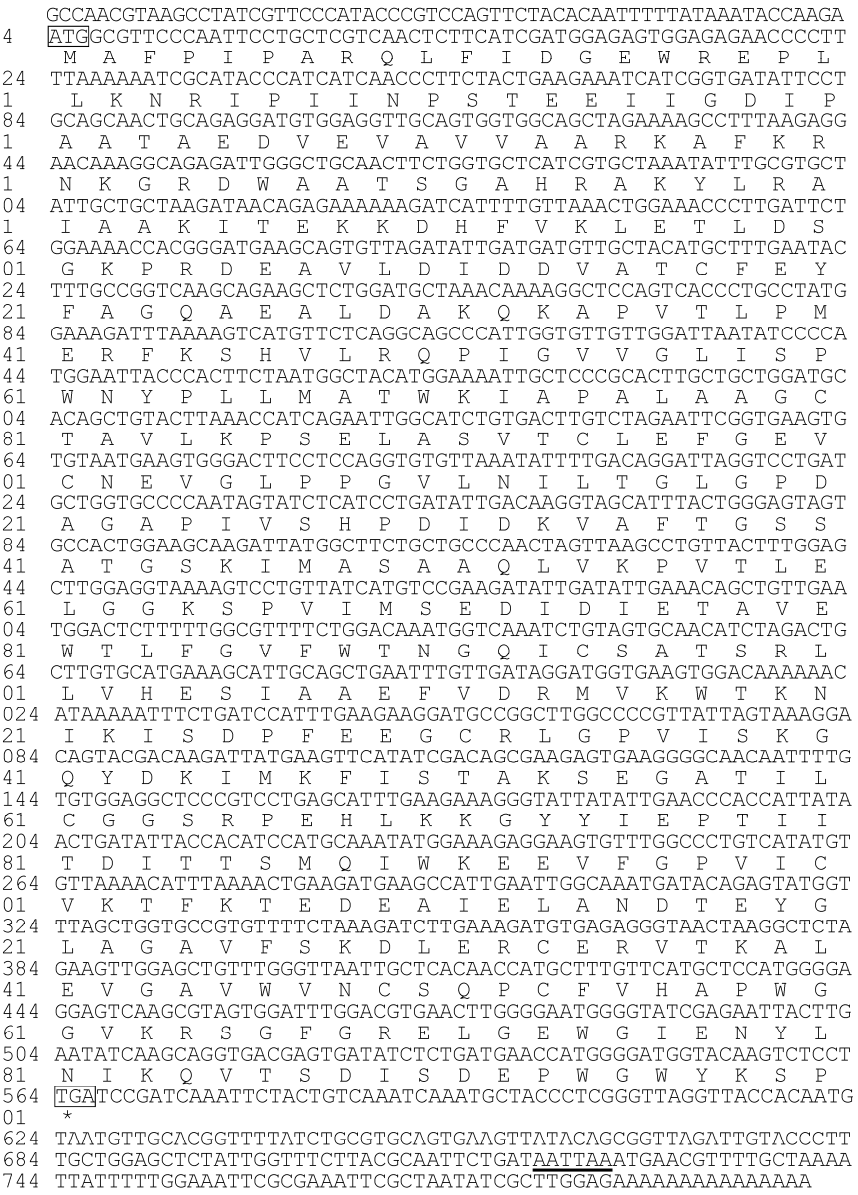

Fig. (3). Nucleotide sequence of the *O. japonicus BADH* gene. The amino acid sequences are below the nucleotide sequence of the *O. japonicus BADH* gene. Start codon and stop codon are indicated by boxes. The region before the start codon is the 5'UTR. The 3'UTR follows the stop codon. The polyA signal-AATTAA was underlined.

the Chenopodiaceae plants had a high homology. It was as high as 98% with *Atriplex hortensis*, 89% with *Spinacia oleracea L*. and 86% with *Beta vulgaris*. The homology with *Suaeda* genus plants, rice, *Arabidopsis thaliana* plants are more than 70% (Fig. **4**). Functional domains of the amino acid sequence encoded by *BADH* ORF were analyzed. It included the conservative region of phosphofructokinase, aldehyde dehydrogenase family and glutamy phosphoric acid reductase, which are conserved in other BADH genes derived from *Spinacia oleracea* L., *Beta vulgaris* L., *Atriplex hortensis* Golosk., *Hordeum vulgare* L., *Sorghum bicolor* Pers, *Amaranthus hypochondriacus* L., *Avicennia marina* (Forsk.)Vierh., *Suaeda liaotungensis* Kitag., and *Atriplex centralasiatica* Iljin. These functional domains play important role in salt stress tolerance.

Overexpression of *OjBADH* Gene in Tobacco

To generate transgenic plants, expression vector pBI-*OjBADH-GUS* was delivered to target issue using a BioRad PDS-1000/He device. Transient GUS expression was

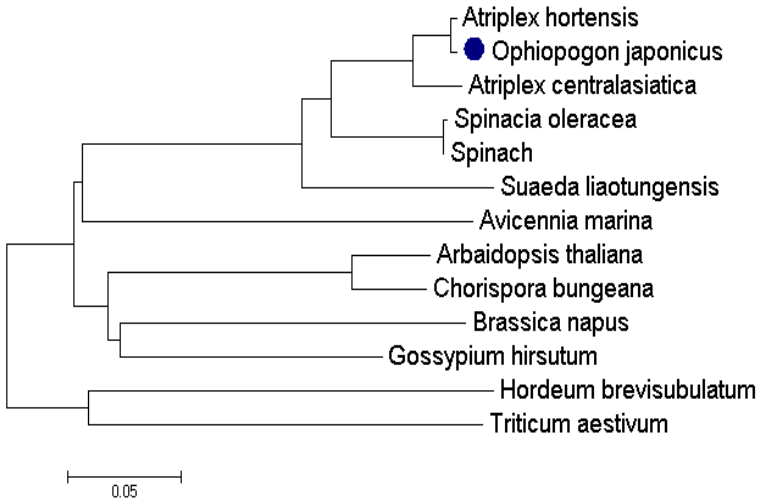

Fig. (4). Phylogenetic tree of *BADH* genes includes the *O. japonicus BADH* gene and *BADH* genes from twelve other plant species including *Atriplex hortensis* (DQ497233.1), *Ophiopogon japonicas* (DQ645888.1), Atriplex centralasiatica (AE017353.1), *Spinacia oleracea* (FJ595952.1), Spinach (M31480.1), *Suaeda liaotungensis* (AF359282.), *Avicennia marina* (AB043540.1), *Arabidopsis thaliana* (AY062987.1), *Chorispora bungeana* (AY804192.1), *Brassica napus* (AY351634.1), *Gossypium hirsutum* (AY461804.2), *Hordeum brevisubulatum* (AY188952.1), *Triticum aestivum* (AY050316.1). The dot (●) indicates the *O. japonicus BADH* gene. The scale bar represents the protein distance.

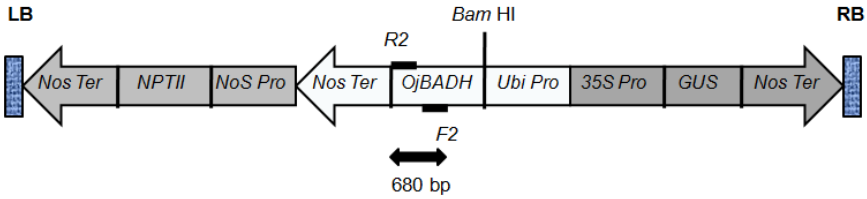

Fig. (5). Linear plasmid map indicating the localization of the different genes (*NPTII* neomycin phosphotransferase gene, *OjBADH O. japonicus* betaine aldehyde dehydrogenase gene, *GUS* β-glucuronidase), promoters (*Nos pro* the promoter from nopaline synthase gene, *35S Pro* the cauliflower mosaic virus 35S promoter, *Ubi Pro* ubiquitine promoter), terminator (*Nos Ter* the terminator from nopaline synthase gene), and T-DNA borders (*LB* left border, and *RB* right border). The arrows indicate the translation orientation of the genes. The probe used in Southern blot analysis of transgenic plants is the PCR fragment of *OjBADH* amplified using primers R2 and F2, *BamHI* was used to digest the genomic DNA isolated from transgenic plants, and their positions are indicated immediately above. Binding sites of PCR primers R2 and F2 are shown as black rectangles.

detected 40 h after particle bombardment (data not shown). Calli at different growth stage were tested for transformation and GUS staining. Calli grown on fresh medium for less than two weeks have high frequency of positive GUS staining (up to 19%). Calli grown on fresh medium for more than three weeks have low frequency of positive GUS staining (about 7%). The frequency of positive GUS staining calli is about 7-19%. Selection was initiated six days after particle bombardments. Calli were transferred onto the regeneration medium containing 200 mg/l kanamycin for two months. After transgenic shoots were generated on medium containing 200 mg/l kanamycin, transgenic shoot clusters were transferred onto kanamycin-free rooting medium to regenerate transgenic plants. Nine putative transgenic lines were obtained and putative transgenic plants were produced from seven of them. Three-month-old putative transgenic plants were used to evaluate by means of PCR, Southern and Northern in tobacco transgenic plants. Transgenic lines were confirmed by PCR (Fig. **6A**), Southern blotting (Fig. **6B**), and northern blotting analysis (Fig. **6C**). Transgenic plants with one copy of the transgene were selected for stress resistant assay.

O. japonicus BADH Gene Improved Salt Tolerance

For salt tolerance analysis, transgenic tobacco plants were watered with 400 mM NaCl for 48 h at room temperature, then return to normal growth condition. The phenotype of WT and transgenic lines after salt stress was shown in Fig. (**7A**). The transgenic plant has normal stem and leaves and the control has wilted leaves and stop to grow. Under non-stress conditions, transgenic plants have normal stem and leaves as the wild type control. They have the same growth rate. Survival rate was measured 15 days after treatment (Fig. **7B**). Stress resistance analysis of *OjBADH* transgenic tobacco plants demonstrated the improved salt tolerance under stress of 400 mM NaCl (Fig. **7B**). Survival rate (%) of transgenic lines (BADH3, BADH6, BADH8, and BADH9) under stress of 400 mM NaCl has been significantly increased compared to the control (Fig. **7B**). Thirty wild type plants were used as control plants and the experiments were

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