Plant Tissue Cult. & Biotech. **24**(1): 111-120, 2014 (June)

PTC&B

Cloning and Characterization of Alcohol Dehydrogenase (*Adh*) Promoter Region for Expression Under Submergence and Salinity Stress

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Key words: Stress inducible promoter, Alcohol dehydrogenase (*Adh*), Cloning

Abstract

The characterization of promoter is important for developing stress tolerant crops as well as understanding the role of promoters in regulating gene expression. The current study was initiated with an aim to characterize the *Adh* promoter under salinity and submergence stress in rice calli. The upstream regions (~1kb) of the *Adh* gene was amplified from the genomic DNA of *Arabidopsis* (Columbia Ecotype). The amplified product was then cloned successively into an entry and promoter-characterization binary destination vector having the reporter gene *β-glucuronidase* (*GUS*) by applying Gateway Technology. A positive clone was confirmed by applying PCR, restriction digestion and sequencing. The construct was then transformed into *Agrobacterium tumefaciens* LBA4404 strain and rice calli infected with the latter. In both salt and submergence stresses, *Adh* could selectively express *GUS* gene activity up to two-fold compared to control.

Introduction

Salinity and drought stresses are prevalent in approximately one fifth of the entire world (Qadir et al. 2008) and one third of the total cultivable land area of Bangladesh (Rahman 2001). However, plant adaptation to environmental stresses is dependent on the initiation of cascades of molecular networks involved in stress sensitivity, signal transduction, and the expression of specific stress related genes and metabolites. Engineered genes that preserve the function and structure

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of cellular components can enhance tolerance to stress (Vinocur and Altman 2005). Therefore, solutions to facilitate crop adaptation to abiotic stress conditions (drought and salinity) need to be developed.

Transgenic approach has become the choice for development of abiotic stress tolerant plants (Arzani 2008, Bhatnagar et al. 2008, Tuteja et al. 2012). Functional genomics have allowed the selection of genes of interest for conferring resistance (Hilson et al. 2004). Genetic engineering allows control of the timing, tissue-specificity, and expression level of the introduced genes for their optimal function. *CaMV35S* is frequently used as a constitutive promoter to control the gene expression in conventional transgenic approaches (Hull et al. 2000, Kasuga et al. 2004). This, however, frequently results in suboptimal growth and yield penalty under normal conditions (Wang et al. 2003). Furthermore, the efficacy of the *CaMV35S* promoter in monocots is reported to be less than optimal (Wang et al. 1997). Therefore, isolation and characterization of inducible promoters are a pre-requisite to transgenic approaches aiming to produce stress tolerant crops (Corrado and Karali 2009).

The basic findings on stress-responsive promoters have led to a major shift in the paradigm for producing genetically engineered stress-tolerant crops (Katiyar et al. 1999). Some stress inducible promoters such as peroxidase (POD), responsive to dehydration 29A (RD29a) and dehydration-responsive element-binding protein 1(DREB1)(Kim et al. 2003, Shinwari et al. 1998, Yamaguchi and Shinozaki 1993) have already been characterized (Rerksiri et al. 2013). *Alcohol dehydrogenase* (*Adh*) gene is reportedly induced by abiotic stresses such as hypoxia, drought, and cold stresses (Dolferus et al. 1994) and is expressed mainly in roots (Hossain et al. 1996). In addition, it has also been found that *GUS* gene expression under *Adh* promoter is higher and root specific in *Arabidopsis* (Chung and Ferl 1999). Therefore, *Adh* promoter can be a good candidate as a stress inducible promoter to maximize crop production through a transgenic approach. If found correctly inducible, the goal would be to hook it upstream to transgenes already cloned earlier (Emran et al. 2010, Amin et al. 2012, Islam et al. 2010, Razzaque et al. 2014).

In the current study, *Adh* promoter was cloned from *Arabidopsis thaliana* (Columbia ecotype) in an attempt to characterize its efficiency in salinity and submergence stress response. Calli generated from tissue culture responsive rice landrace, Binnatoa, was transformed with the cloned *Adh* promoter and *GUS* assay was conducted to measure the expression of *Adh* during submergence and salt stress.

Materials and Methods

Arabidopsis thaliana seeds were germinated to grow up to maturity. DNA was isolated using the CTAB method (Stewart and Via 1993). The extracted DNA was quantified using Nanodrop® spectrophotometer ND-1000 (Thermo Fisher Scientific Inc. Waltham, MA, USA). Target size (1009 bp) from *Adh* promoter was amplified by PCR with target specific primers (Table 1). PCR reaction program for amplifying ~1kb *Adh* promoter was optimized as follows: Initial denaturation was at 95°C for 5 min and 35 cycles of denaturation at 95°C for 1 min, annealing at 61.5°C for 1 min, extension at 72°C for 2.10 min followed by a final extension at 72°C for 10 min. A final concentration of 2.3 mM $MgCl_2$, 0.1 mM dNTPS, 0.3 μM of each primer and 1 unit of recombinant Taq polymerase (Invitrogen, Carlsbad, CA, USA) was used. The forward primer was designed adding *CACC* overhang to ensure compatibility with *pENTR/D-TOPO* vector.

PCR amplicons were gel extracted using Qiaquick Gel extraction kit (Qiagen, Hilden, Germany) and quantified through nanodrop. Cloning reaction into *pENTR/D-TOPO* vector (Invitrogen, Carlsbad, CA, USA) was then initiated following manufacturer's protocol. The *pENTR_Adh* plasmid construct was transformed into *E. coli DH5α* competent cells through heat shock employing standard protocols (Sambrook et al. 1989). Successful cloning into *pENTR* was then confirmed by PCR, restriction digestion with *NdeI* restriction enzyme (NEBr inc.,Ipswich, MA,USA) followed by final confirmation by direct sequencing of the insert with gene specific primers.

Cloned *pENTR* vector was then used to recombine the desired sequence of *Adh* promoter into the destination vector (*pHGWFS7.0*) using the Gateway® LR recombination reaction (Invitrogen, Carlsbad, CA, USA). The LR reaction was carried out following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The Gateway technology allows taking advantage of the site specific recombination properties of bacteriophage lambda to provide a rapid and highly efficient way to recombine target sequences to multiple vector systems (Invitrogen, Carlsbad, CA, USA). The destination vector (*pHGWFS7.0*) used here is efficient for promoter expression analysis (Karimi et al. 2002). Site specific recombination properties of Gateway system allowed recombination of the *Adh* promoter from the *pENTR_Adh* to the target destination vector (*pHGWFS7.0*). The successful recombination would place the *Adh* promoter immediately upstream of the *GUS* gene. Positive colonies were determined by PCR with gene specific primers and restriction digestions with *NdeI* (NEBr inc., Ipswich, MA, USA). Finally, *Agrobacterium tumefaciens* (*LBA4404*) was electroporated with the constructed *pHGWFS7.0_Adh* using standard protocols (Sambrook et al. 1989).

Positive colonies were authenticated by PCR reactions with target-specific primers (Table 1).

Table1. List of primers used in the study and corresponding sequences.

Primer	Sequence
ADH_F	5′-CACCGAGCTCTGTTCTTGAGGAGCTTG-3′
ADH_R	5′-GGACTAGTTGTTAGTTTTGTGTGATTGTG-3′

Agrobacterium containing the designed construct was then streaked and cultured in YM media for initiating *Agrobacterium* mediated transformation following the protocol discussed by Islam et al. (2009). Rice calli were regenerated from the landrace Binnatoa for the transformation. Seeds of Binnatoa were collected from Bangladesh Rice Research Institute (BRRI). Callus induction and pre-incubation were done following the method mentioned by Islam et al. (2009).

Rice calli infected with *Agrobacterium tumefaciens* containing the *pHGWFS7.0_Adh* construct was kept in dark at 28°C for 72 hrs. After 72 hrs of co-cultivation calli were washed with double distilled H_2O mixing carbenicillin at 250 mg/l. Then the infected calli were transferred to MS induction media containing 2,4-D at 10 mg/l and kept in dark for another 24 hrs. The following day after incubation, 200 mM salt stress (NaCl) was applied to the calli for salinity assay. Three replicates and four individuals from each replicate were used for this study. Samples were collected at two time intervals, one was at 5 hrs after stress and other was at 10 hrs. *GUS* activity in the control and transformed calli were measured histochemically (Jefferson 1987) using the indigogenic substrate 1 mg/ml X-gluc (5-bromo, 4-chloro, 3-indolyl ß-D-glucuronide) (Carl Roth GmbH+ Co., Karlsruhe, Germany). The calli were then washed three times in 50 mM phosphate buffer and incubated in X-gluc for 3 days at 37°C. After incubation calli were decolorized by 99 per cent ice cold ethanol. In this experiment untransformed calli were used as negative control and transformed tobacco leaf with *GUS* was used as positive control. For the submergence assay, similar numbers of infected and recovered calli were kept under double distilled H_2O up to 10 hrs. Samples were collected at two time intervals, 5 and 10 hrs. The calli were subjected to *GUS* assay in X-gluc. Data were collected at the mentioned time intervals. Scoring of the *GUS* expression was based on color intensity (described in Fig. 2A). Statistical analysis was performed applying t test.

Results and Discussion

A single PCR program was applied to amplify the targeted sequence of the *Adh* promoter. The target sequence was 1009 bp upstream of the start codon of the *Adh* gene and was amplified from the genomic DNA of *Arabidopsis* (Fig. 1A). The target upstream 1009 bp has already been reported to show root specific expression (Hossain et al. 2006). PCR products were gel purified and cloning reaction initiated by incubating with the *pENTR/D-TOPO* vector. *pENTR/D-TOPO* is designed to facilitate rapid, directional TOPO® cloning of blunt end PCR products for entry into the Gateway® system. The system was adopted for this experiment since inserts can be cloned in correct orientation with efficiencies equal to or greater than 90% (Invitrogen, Carlsbad, CA, USA). Cloned *pENTR/D-TOPO* was transformed into *E. coli* and incubated O/N at 37°C. Colonies were observed on LB plate containing the antibiotic kanamycin. Plasmid was isolated from the colonies found after the cloning reaction. Initially, six colonies were used for plasmid isolation (Fig. 1B).

Isolated plasmids were then confirmed by *Adh* specific PCR (Fig. 1C) and restriction digestion with *NdeI* (Fig. 1D). The digested product by *NdeI* was expected at 3580 bp which was found positive for one of the plasmids (Fig. 1D). Further confirmation of the *Adh* cloning into *pENTR* was performed by sequencing. M13 primers and *Adh* specific primers (Table 1) sets were used to get the sequences and the BLAST hit indicated 100% match with the desired fragments.

The *Adh* promoter was transferred into the gateway destination vector *pHGWFS7.0* (Karimi et al. 2002) from the entry clone *pENTR_Adh* by LR recombination reaction. This recombination reaction creates an expression clone for plant transformation and contains the selectable marker for transformation into both bacteria and plants and is compatible as a vector for *Agrobacterium*. Following LR recombination reaction and transformation, colonies were observed on the LB plate containing the antibiotic spectinomycin/streptomycin at 25 and 20 mg/l, respectively. Plasmids were isolated from those colonies (Fig. 1E) and positive clones were confirmed by PCR using insert specific primers (Fig. 1G) and restriction digestion by *NdeI* enzyme (Fig. 1F). The desired band sizes from the digestion were expected at 5.8, 3.8, 2.9 and 0.850 kbp. The constructed destination vector *pHGWFS7.0_Adh* was transformed into *LBA4404* strain of *Agrobacterium* by electroporation. The transformed plate was kept at 28°C for 72 hrs and positive colonies were screened out by PCR with insert specific primers. The insert was amplified to the expected size, 1 kbp **(**Fig. 1H-I)**.** The confirmed *Agrobacterium* was then used for rice calli transformation.

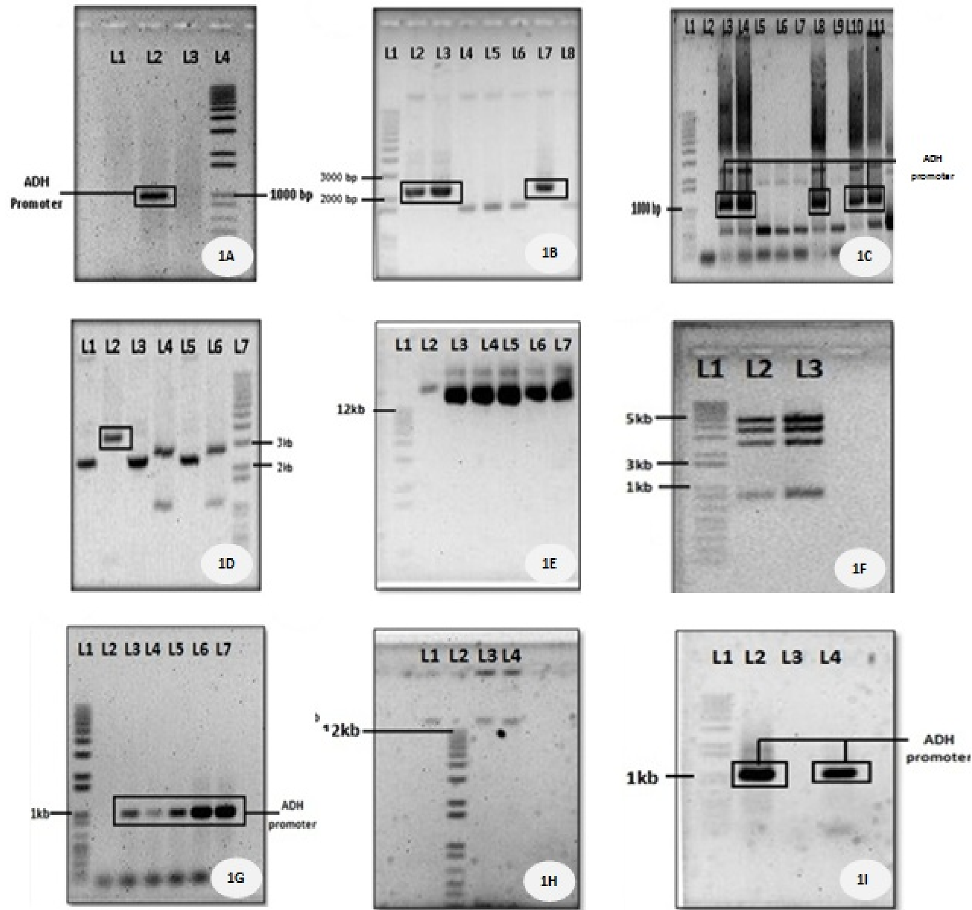

Fig.1. Amplification, cloning and confirmation of the *Adh* promoter from *Arabidopsis thaliana.* 1A: Target size was amplified using sequence-specific primers. L2, the amplified *Adh* promoter sequence, L4: 1kb+ DNA ladder. 1B: Plasmids isolated from the cloned *pENTR* vector. L1: 1kb+ DNA ladder, L2-L7: Plasmids of different size. L2, L3 and L7 showed plasmids of correct size. 1C: *Adh* amplification from the plasmid, L1: 1kb+ DNA Ladder, L2: Negative control, L3-L4: Positive control and L5-L11: PCR products. L8, L10 and L11: Amplicons of the right size. 1D: Restriction digestion (RD) of cloned *pENTR_Adh* plasmid. *NdeI* for a single cut fragment, L7:1kb+ DNA ladder, L1-L6: Digested samples, L2: Positive sample with true single cut. 1E: Plasmid isolation from *pHGWFS7.0_Adh* vector. L1:1kb+ DNA Ladder, L2-L6: Plasmid isolated after LR recombination with promoter insert L7: Plasmid without *Adh* Promoter. 1F: Restriction digestion with *NdeI*, L1: 1Kb+ DNA ladder, L2-L3 samples digested with *NdeI* with fragments at the expected sizes. 1G: PCR amplification. L1: 1kb+ DNA ladder, L3-L7: PCR amplification of 1kb inserts. 1I: Plasmid isolation from *Agrobacterium* after electroporation, L2:1kb+ DNA ladder, L1, L3 and L4: Isolated plasmid. 1I: PCR amplification. L1:1kb+ DNA ladder, L2-L4: PCR amplicons of right size.

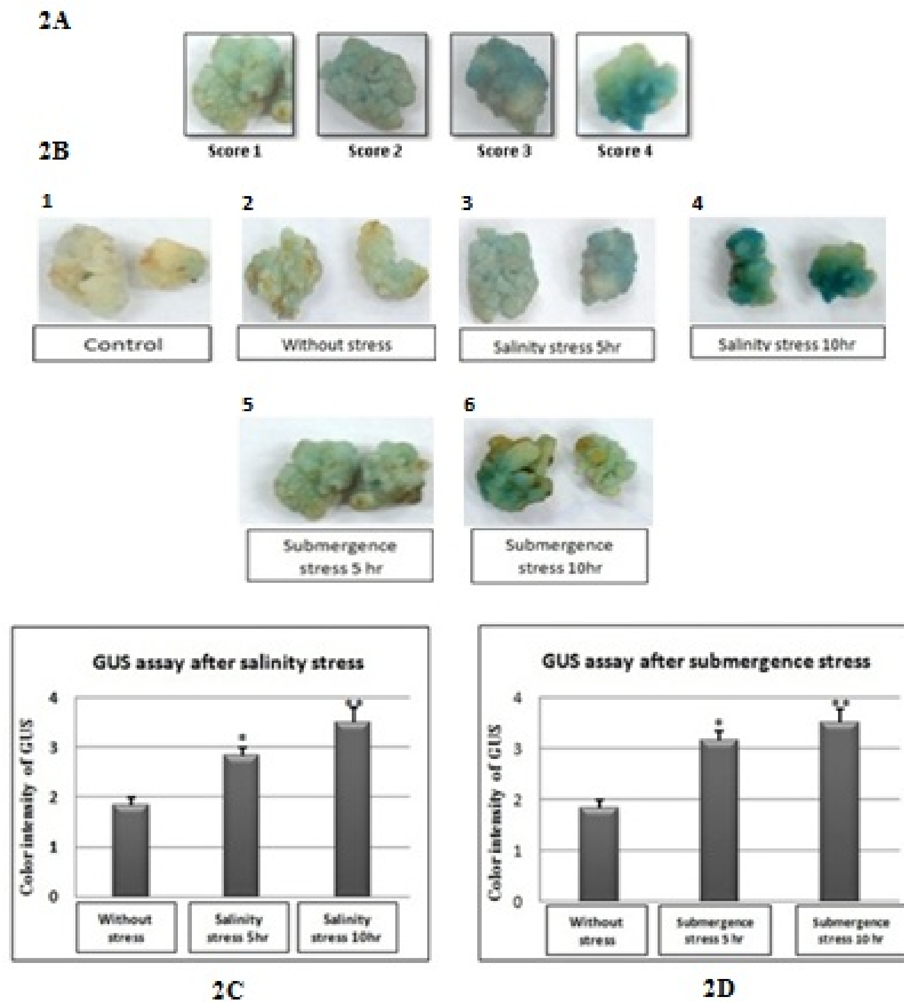

Fig. 2. *GUS* expression analysis (histochemical) in rice calli driven by the *Adh* promoter and bar diagram representing the *Adh* promoter activity measured by histochemical *GUS* assay. 2A: Samples were scored from 1 to 4 based on the color intensity. 2B: Histochemical *GUS* assay in different samples. Note that stressed (NaCl and submergence stress) samples showed color with incresed intensity (2B: 3-6) compared to control (2B: 1) and without stress (2B: 2). Highest intensity was observed in samples treated with both stresses for 10 hrs (2B: 4 and 6). Fig. 2C: *Adh* expression in both salinity (NaCl) and submergence (Fig. 2D) stress was significantly higher than the control explant and the explant without stress. Each bar represents the mean ± SE (n=3). *Indicates the significance difference between *Adh* induced expression of *GUS* at the probability of $p \leq 0.05$.

** Indicates the significance difference between *Adh* induced expression of *GUS* at the probability of $p \leq 0.01$.

Binnatoa calli were used for the transformation with *Agrobacterium* containing the *pHGWF7.0_Adh* binary vector. Tissue culture responsive Binnatoa calli could easily be transfected and *GUS* expression was examined to observe

the activity of the *Adh* promoter. Control wild type explant gave no color and calli with *Adh* promoter without stress showed poor expression. The stressed explants showed up to two fold better expression compared to control and no-stress explants. (Fig 2B. 1-6).

In salinity stress screening, *GUS* activity was two-fold higher in 10 hrs stress and in 5 hrs stress the expression was one and a half-fold higher compared to the control explants (Fig. 2C). In addition, *GUS* activity in submergence screening was found similar to salinity stress. The expression of *GUS* after 10 hrs was also two-fold higher and 5 hrs stress induced *GUS* expression was one and a half-fold higher compared to control **(**Fig. 2D**)**.

The study conducted here shows the inducibility of the *Adh* promoter under abiotic stresses like salinity and submergence. These results can be further fine-tuned using quantitative measures of *GUS* activity as well as checking induction in the specific tissues of plants regenerated from the positive calli.

Acknowledgement

The authors would like to thank the Ministry of Science and Technology, Govt. of the People's Republic of Bangladesh for funding this project.

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