Cloning and Expression of An Alcohol Dehydrogenase from *Lotus japonicus* and Characterization of LjADH1

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**Abstract**

Alcohol dehydrogenase (ADH), usually using NAD⁺ and NADP⁺ as coenzymes, widely exists in many organisms, it plays extremely important roles in growth, development and stress resistance in plants. In this research, we used diploid *Lotus japonicus* MG20 (originated from Miyako Island of Japan) as plant material to identify the characteristics of alcohol dehydrogenase gene. Based on the conservative sequences of ADH, The ADH homologous gene was cloned from *Lotus japonicus* MG20 cDNA, whose full length was 1,143 bp in length encoding 380 amino acids. Homologous analysis showed that the amino acid sequence of the cloned gene was highly homologous with plant zinc-binding ADH family proteins. We have this gene named as *LjADH1* and ligated the gene into the prokaryotic vector pQE30 and yeast expression vector pYES2 to make the recombinant vectors of *LjADH1*. Under the optimum conditions of expression, the Histag fusion proteins were highly expressed with 1.12 mg/mL in *E. coli* M15 and yeast INVScl. Under the optimum conditions of expression, the His-tag fusion proteins were highly expressed with 1.12 mg/mL in *E. coli* and 48.2 U/mg ADH activity examined by the method of Vallee and Hoch. We found that *LjADH1* over expressed in prokaryotic cells can increase the recombinant strains’ tolerance to H₂O₂ stress, while *LjADH1* expressed in recombinant yeast can promote growth of the recombinant yeast under the stress of H₂O₂ and some heavy metal salts such as CuCl₂ and CdCl₂ except for NiCl₂. In this research we have preliminary clues that *LjADH1* is a member of zinc-binding ADH family proteins in plant and that has some functions for resistance to abiotic stresses.

**Keywords** *Lotus japonicus*; Alcohol dehydrogenase; *LjADH1* (GenBank Accession No.: JN165714); Prokaryotic expression; Yeast expression; Abiotic stress

**Background**

Alcohol dehydrogenases (ADH, EC 1.1.1.1) are a group of dehydrogenase enzymes that have extensive zymolysate and catalyze the interconversion between alcohols and aldehydes. With the reduction of nicotinamide adenine dinucleotide (NAD⁺ to NADH), ADH facilitate the dehydrogenation of primary alcohols or secondary alcohols, aldehydes and ketones to lose their hydrogen. In this reaction, the NAD⁺ gains hydrogen and become NADH.

ADH widely occurs in all kinds of organs in plant, which involves in development, maturation and senescence of fruits (Chervin et al., 1999), as well as in the resistant responses to stresses. ADH can be induced to express by dehydration, cold (Dolferus et al., 1994) and oxygen deficit (Newman and Vantoai, 1992), as well as ADH has some links to Ca²⁺ signal transduction (Hwa-Jee and Ferl, 1999). Most of ADH in plants have the structures with the length of amino acid sequence from 370 to 380, which evenly consist of three folding structures, helix, folding and curling. There is little difference of secondary structure in ADH among the plant species, particularly in the parts of conservative function domains. Even though there is a few differences in amino acid sequences among individuals, the difference in folding status of peptide chain is usually very small, which indicates highly conservative secondary structures should maintain the functional stabilities. Meanwhile, the highly conservation of ADH sequences in all kinds of species indicated that the ADH play an important role in plant growth, so that any slight mutation in ADH will result in death of plant.
The study of ADH gene was initiated in corn (Zea mays). ZmADH1 gene and ZmADH2 gene were cloned successfully from corn (Gerlach et al., 1982). The 82% homology of the deoxyribonucleic acid (DNA) was between two genes whereas 87% homology in the sequences of amino acids, which indicated that both of the genes possible derive from the common ancestor although the location of the genes in the genome are at different chromosomes. ZmADH1 was located at chromosome 1 and ZmADH2 was located at chromosome 4.

So far, there are some reports on ADH1 and ADH2 genes cloned in grape (Vitis vinifera), potato (Solanum tuberosum), rice (Oryza sativa) and wheat (Triticum monococcum). However, only one ADH gene, AtdADH1, was reported in Arabidopsis, which located at chromosome 1, whose sequences of nucleotides in genome distributed from 28 980 288 to 28 982 311. While, there was no any annotation about AtdADH2. In Lotus, a model plant of legume, the LcADH1 (Gen- Bank ID: AAO72531.1) had been identified from tetraploid species, Lotus corniculatus, whereas there was no any report about the gene, LjADH1 or LjADH2 cloned from Lotus japonicus, a popular diploid species so far.

Lotus plant belongs to the genus Lotus L. in subfamily of Papilionoideae of Leguminose family. Lotus japonicus, a diploid species has been instead of tetraploid species of Lotus corniculatus used for genetic study because of small genome size of about 470 Mb, diploid genome with 6 haploid chromosomes (2n=12) and a short life cycle of about 2 to 3 months. With the completion of whole genome sequenced, Lotus japonicus was reported in Arabidopsis. This species, Lotus japonicus, has been a convenient model plant for the studies of genome and genetics in legumes, particularly in reference to rhizobial and arbuscular mycorrhizal symbiosis since in the early 1990s (Handberg and Stougaard, 1992). Based on the conservative characteristics of ADH genes, we used MG20, a Lotus japonicus germplasm originated from Miyako Island of Japan (Kawaguchi, 2000), as experimental materials to clone homologous ADH gene from Lotus japonicus cDNA, to express the gene in E. coli and yeast and to characterize the enzyme in this study.

1 Results and analysis
1.1 Cloning and sequence analysis of LjADH1
In this research we designed a pair of primers based on the information of LcADH1 to amplify the ADH analog using cDNA from Lotus japonicus MG20 as template. The analog was cloned from MG20 germplasm and then sequenced, which was 1 143 bp in length encoding 380 amino acids. The further sequence alignment indicated that the cloned sequence had 99% homology to an unannotated Lotus japonicus sequence deposited in the GenBank (GenBank ID: CAG 30579.1). We confered the sequence as ADH1 of Lotus japonicus named as LjADH1 (GenBank Accession No.: JN165714).

Multiple sequence alignment was conducted among putative LjADH1 protein and other ADH proteins from Zea mays, Triticum monococcum, Oryza sativa, Arabidopsis thaliana, Lotus corniculatus, Aegilops speltoides, Vitis vinifera, Solanum tuberosum and Miscanthus transmorrisonensis, respectively (Figure 1), the results showed that the cloned protein sequence had more than 95% homologous with other ADH sequences except MtADH (80%). The analysis indicated that the cloned sequence has common ADH structures of plant ADH including zinc-binding sites, ADH_N domain and NADB_Rossmann conservative functional domain. Phylogenetic analysis further demonstrated that ADH gene from Lotus japonicus shared the same evolutionary branch with AtADH of Arabidopsis thaliana (Figure 2).

1.2 Construction of prokaryotic expression vector
In order to express the LjADH1 gene in E. coli, We employed the prokaryotic expression vector pQE30, which has a 6 histidine tag, little influence on targeted proteins and easy to be purified (Wang et al., 2010; Liu et al., 2010; 2011), to make the heterologous expression construct pQE30-LjADH1. The construct was validated by digestion of BamH I and Sac I to produce 3.4 Kb and 1.1 Kb fragments by agarose gel electrophoresis (Figure 3), those fragments in length, were in accord with the size of pQE30 and LjADH1 sequence.

1.3 Expression of fusion protein and concentration determination
We transformed pQE30-LjADH1 into E. coli M15 strains to express LjADH1 fusion protein in E. coli and optimized the conditions of fusion protein expression. The result showed that fusion proteins were expressed by inducing of IPTG at 0.1 mmol/L of IPTG concentration, with the optimum induction time 3 h to 4 h and induction temperature at 30°C (Figure 4). The concentration of purified fusion protein 6×His-LjADH1

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was measured at 1.12 mg/mL by the BCA method, the concentration of 6×His-LjADH1 under the optimum expression conditions.

1.4 Enzymatic activity of fusion LjADH1 protein

We proposed the expressed fusion protein of LjADH to be a kind of dehydrogenase, therefore we employed the Vallee and Hoch method to measure enzymatic activity (Vallee and Hoch, 1955). The principle of this method is that NADH and NAD⁺ have their own maximum absorption peaks at 340 nm and 260 nm, respectively, so as NAD⁺ was converted to NADH, the maximum absorption peaks at 260 nm will be shifted to 340 nm. The enzymatic activity of LjADH1 was calculated following the formula, $U/mg = E_{340} \times 3.1 / (6.2 \times E_{W})$, where $E_{340}$ is the extinction coefficient at 340 nm and $E_{W}$ is the weight of the sample.
Figure 2 Phylogenetic tree of ADH in plants

Figure 3 Identification of recombinant plasmid pQE30-LjADH1 by restriction enzymes BamH I and Sac I
M: λ DNA Marker; 1: pQE30-LjADH1 digested by restriction enzymes BamH I and Sac I

Figure 4 SDS-PAGE analysis of fusion protein expressed with time gradient under induction of 0.1 mmol/L IPTG
M: Protein molecular marker; 1: No induction; 2: 30 min induction; 3: 60 min induction; 4: 120 min induction; 5: 180 min induction; 6: 240 min induction; 7: 300 min induction; 8: 360 min induction

here $E_{340}$ is the absorbance increase within 5 minutes at 340 nm (absorbency units 0.001), $E_w$ is the weight (mg) of enzyme per mL enzyme solution. In this re-

search we measured that absorbance change of Lj-ADH1 in a minute was 0.108, so LjADH1 enzymatic activity came out 48.2 U/mg (figure 5).

Figure 5 The absorbance of recombinant fusion protein at 340 nm

1.5 Resistant analysis of the prokaryotic fusion protein
We added H2O2 into E. coli M15 recombinant strain which harboring pQE30-LjADH1 and reference strain with pQE30. The reference strain grew better than that of recombinant strain in absence of H2O2. This phenomena might be due to the effect of overexpressed heterologous proteins during bacteria growth stages. Whereas when we added H2O2 into recombinant strain solution at 1 mmol/L H2O2 final concentration, the recombinant strain obviously grew better than that of the reference strain (Figure 6). The results suggested that alcohol dehydrogenase might have some functions in the survival capability of prokaryotes under oxidative stress.

1.6 Construction of eukaryotic expression vector pYES2-LjADH1
In this research, we constructed a yeast expression construct named pYES2-LjADH1, The construct was validated by digestion of restriction enzymes BamH I and Xba I to produce 5.9 Kb and a 1.1 Kb fragment by agarose gel electrophoresis(Liu et al., 2010, Wang et al., 2010) (figure 7A), those fragments in size were in accord with the size of pYES2 and LjADH1 sequence. that was confirmed by digestion of restriction enzymes BamH I and Xba I. The recombinant strains were also identified by PCR to confirm that the pYES2-LjADH1 were transferred into yeast (figure 7B).

1.7 Resistant analysis of LjADH1 protein expression in yeast
In order to understand how do the LjADH1 protein response to different ion stresses, we inoculated the
recombinant yeast strain with pYES2-LjADH1 and wild type strain pYES2 in the YPG media with different ion concentrations, respectively (Figure 8). The responses showed that the growth status between the recombinant and its wild type yeast had very similar in the YPG medium without any treatment, but the recombinant yeast grew better than the wild type in the YPG media with final concentrations of 10 mmol/L CuCl$_2$, 100 μmol/L CdCl$_2$, 150 μmol/L CdCl$_2$, and 3.5 mmol/L H$_2$O$_2$, growth status observation after 2~3 days incubation at 30℃ concentration of 1.8 mmol/L NiCl$_2$. The results indicated that the LjADH1 protein has some resistant functions to oxidative stress in yeast. With reference to evidence of the recombinant yeast growth in the concentration of 3.5 mmol/L H$_2$O$_2$ we considered that LjADH1 might have certain resistant functions to abiotic stresses. There was a report that the plants will lack of resistance to waterlogging while the ADH gene in plant is going to silence, whereas overexpressing ADH alone might not yet improve the plant resistance to waterlogging (Ismond et al., 2003). Therefore the functions of LjADH1 in plants need to be further studied in future.

2 Conclusions

In this research, we cloned alcohol dehydrogenase gene from *Lotus japonicus* MG20, and considered that the gene encodes a typical zinc-binding alcohol dehydrogenase, named as *LjADH1*.

In this research, the gene was ligated to pQE30 expression vector with a 6×His tag, and a batch of non-denaturalized proteins was expressed in *E. coli* and purified by Ni$^{2+}$-NTA gel column under the optimum induced expression conditions. The enzymatic activity of the LjADH1 was determined by method proposed by Vallee and Hoch, which demonstrated strong alco-
hol dehydrogenase activity. In respect to stress tolerance of fusion proteins over-expressed in the *E. coli* and yeast, the recombinant strains grew well than that of the wild type. While, *LjADH1* gene was ligated into the yeast vector pYES2 to over-express *LjADH1* gene in yeast cells, the result showed that the growth status of the recombinant yeast harboring pYES2-LjADH1 were much better than that of the wild type with pYES2 under the stresses of 10 mmol/L CdCl$_2$, 150 μmol/L CdCl$_2$ and 3.5 mmol/L H$_2$O$_2$, respectively, but except for 1.8 mmol/L NiCl$_2$. It was obvious that the LjADH1 protein over-expressed in yeast might increase the yeast cells’ tolerance to abiotic stresses. In this research we have preliminary clues that LjADH1 is a member of zinc-binding ADH family proteins in plant and that has some functions for resistance to abiotic stresses.

### 3 Materials and Methods

#### 3.1 Materials used in this research

The *Lotus japonicus* MG20 seeds were kindly provided by Dr. Da Luo (Professor of Sun Yat-Sen University, P. R. China) and were deposited in Hainan Institute of Tropical Agricultural Resources (HITAR, China). The seeds were sanitized and germinated in greenhouse for 24 h at 30°C prior to planting in flowerpots (5×7 cm) with 12 h/12 h day/night cycle at 26°C, 80% humidity. Two weeks later, the seedlings were collected and cleaned for ready.

The bacteria and plasmids used in this research including *E. coli* JM109, Yeast strain INVSc1 (Clontech), *E. coli* M15, pMD18-T (TaKaRa), pQE30 (Qiagen), pYES2 (Clontech) were deposited in the lab of Alkali Soil Natural Environmental Science Center (ASN ESC), Northeast Forestry University. Enzymes and chemicals including restriction enzyme, EX Taq TM, T4 DNA ligase, kanamycin *et al.* were purchased from Takara Company.

#### 3.2 cDNA synthesis, Gene cloning and sequence analysis

Total RNA from MG20 was extracted using Biozol Total RNA Extraction kit (Biomiga). The first strand cDNA of *Lotus japonicus* MG20 was synthesized by RT-PCR using RNA (AMV) reverse transcription kit (Takara) by RT-PCR following the procedures as: 30°C, 10 min; 42°C, 15 min; 50°C, 15 min; 55°C, 15 min; 60°C, 15 min; 90°C, 10 min; 5°C, 5 min; for 30 cycles.

A pair of primers was designed according to known *LeADH1* gene, forward primer was given as 5'-TAG CTATGTGACACCAGCT-3', reverse primer as 5'-AACTCAGTCCCCAAATAGGG-3'. ADH analogous gene was amplified from *Lotus japonicus* MG20 cDNA under the procedures as follows: pre-denaturation at 95°C for 3 min, followed by 30 cycles (95°C 30 sec, 52°C 30 sec and 72°C 2 min), and ended at 72°C for 10 min. The PCR products were separated by agarose gel electrophoresis and ligated into pMD 18-T vector (Takara), then transformed into *E. coli* JM109 strain. The positive clones were selected to be sequenced at Beijing Genomic Institute, China. Sequence analysis was conducted online in NCBI for Blast and phylogenetic analysis.

### 3.3 Construction of prokaryotic expression vector

We designed a pair of primers with the restriction sites of *Bam* H I and *Sal* I by Primer Premier (Version 5.0), forward primer with *Bam* H I as 5’-GGGATCCA TGTCGACCACAGCT-3’, and reverse primer with *Sal* I as 5’-CGGAGCTCACACATCATGTCTTTTG-3’, respectively. The plasmid for sequencing was used as templates to amplify the gene following the procedures as pre-denaturation at 95°C for 3 min, followed by 30 cycles (95°C 30 sec, 54°C 30 sec and 72°C 2 min), and ended at 72°C for 10 min. The PCR products were recovered to ligated into pMD18-T vector named as pMD18T-LjADH1, and then transformed into *E. coli* JM 109 strains by the approach of thermal stimulation. Meanwhile the recombinant plasmids and pQE30 vectors were extracted to be digested by *Sac* I and *Bam* H I, respectively, and then have the LjADH1 ligated to the pQE30 vector to make recombinant vector, pQE30-LjADH1. We transformed pQE30-LjADH1 into *E. coli* JM109 competent cells. Monoclonal was randomly picked up for PCR amplification to identify whether the gene transformed into *E. coli* M15 strains by *Bam* H I and *Sac* I digestion.

### 3.4 Expression of fusion protein and concentration determination

The recombinant *E. coli* M15 strains harboring pQE30-LjADH1 were inoculated into resistant medium and incubated overnight at 37°C and 200 r/min shaking culture. Then 200 μL overnight incubated strains was added into 5 mL LB liquid medium containing 100 μg/mL ampicillin and 25 μg/mL kanamycin to continue shaking culture at 37°C. While the OD$_{600}$ value
reached 0.6, IPTG was added with a final concentration of 0.1 mmol/L to induce target fusion protein. Under the shaking culture at the speed of 200 r/min at 30°C, the recombinant strains were collected after induced for 0 min, 30 min, 60 min, 120 min, 180 min, 240 min, 300 min and 360 min, respectively. The supernatant was discarded by centrifugation at 13 000 r/min at 4°C for 1 min, and precipitate was re-suspended with PBS, then added equivalent 2×SDS sample buffer, denatured for 5 min at 100°C and cooled on ice bath for 5 min, finally centrifugated at 13 000 r/min at 4°C for 5 min. Took about 20 μL of the samples used for SDS-PAGE electrophoresis.

Protein concentration was measured by the BCA method (Walker, 1994). 37.5 μL (8 mg/mL) BSA was added into 262.5 μL ddH2O to make protein standard solution with final BSA concentration 1 mg/mL, took 150 μL solution diluted 7 times consecutively to prepare BSA diluting standard solution with the concentrations at 500 μg/mL, 250 μg/mL, 125 μg/mL, 62.5 μg/mL, 31.25 μg/mL, 15.625 μg/mL, and 7.813 μg/mL, respectively. The purified fusion proteins was also diluted and then took 100 μL diluted standard solutions respectively to mix with 2 400 μL CBB (Coomassie Brilliant Blue), the OD595 value was read three times by spectrophotometer using ddH2O as reference.

3.5 Enzymatic activity of Fusion protein
Enzymatic activity of fusion protein was measured by the method of Vallee and Hoch (Vallee and Hoch, 1955), 150 μL pyrophosphate buffer, 50 μL substrate solution and 100 μL solution of NAD+ were mixed to a cuvette. The cuvette was placed in water bath at 37°C for 5 min and then added 10 μL purified fusion protein solution preheated under the same conditions, immediately count the time and read the increase of absorbance at 340 nm every 1 min interval in the successive 5 min until the values of absorbance is stable.

3.6 Resistant analysis of the fusion protein expressed in E. coli
Employ Echave’s method to be slightly modified to analyze the prokaryotic resistance (Echave et al., 2003) in this research. 2.5 mL recombinant strain solution and its reference respectively cultured overnight until the OD600 reached 0.3, then added 22.5 mL LB media containing 100 μg/mL ampicillin and 25 μg/mL kanamycin cultured at 37°C. then 0.1 mmol/L IPTG was added to dilute ten times, The cultured media were divided into two groups, one group of the recombinant added H2O2 with a final concentration of 1 mmol/L and the other group added water as control, the OD600 values were measured in every 30 min to calculate the resistant activity.

3.7 Construction of yeast expression vector
The primers were designed based on LcADHI sequence information, forward primer as: 5’-GGGATCC ATGTCGACCACACAGCT-3’ with BamHⅠ site, and reverse primer as: 5’-CGTCTAGAACACATCATGG TTTTGTG-3 with XbaⅠ site. pMD18T-LjADH1 (with restriction sites BamHⅠ and XbaⅠ ) and pYES2 (Clontech) vectors were digested with BamHⅠ and XbaⅠ. The products of digestion were recovered to ligate into pYES2 vector, the recombinant was named pYES2-LjADH1. then transformed into the yeast INVScI (Clontech) by LiAc/PEG chemical transformation method (Gietz et al., 1995).

3.8 Resistant analysis of LjADH1 protein expressed in yeast
The recombinant INVScI strains harboring pYES2 and pYES2-LjADH1 respectively were cultured in liquid SC-U medium at 30°C overnight culture until the OD600 reached 0.6. Then diluted them to 10^-1 times, 10^-2 times, 10^-3 times, 10^-4 times and 10^-5 times, respectively with YPG medium. Picked up 5 μL strain solution in the pYES2-LjADH1 and pYES2 medium in each dilution concentration, dropped on YPG solid medium which containing 10 μmol/L CuCl2, 1.8 mmol/L NiCl2, 100 μmol/L CdCl2, 150 μmol/L CdCl2 and 3.5 mmol/L H2O2, respectively cultured at 30°C for 2 days.

Authors’ contributions
TZ and SKL designed and conducted this experiments. RYL, PTG and DGZ participated the experiment design, data analysis; XJF is the person who takes charge of this project, including experiment design, data analysis, writing and modifying of the manuscript. All authors have read and approved the final manuscript.

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