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Research Paper

Isolation and analysis of LmDREB2 gene from Leymus muticaulis Tzvelev (Poaceae: Trticeae)

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ABSTRACT: *The dehydration responsive element binding proteins (DREB) are important and specific plant transcription factors responding to stress conditions including drought, salt and low temperature. It has been generally accepted that DREB can regulates the expression of a number of abiotic stress-related genes in down stream of the stress signal transduction pathways. In this paper, a DREB-like gene, named LmDREB2 (accession No. JQ755244), was cloned from Leymus muticaulis* Tzvelev. *The structure analysis showed that LmDREB2 contained a 795 bp ORF, encoding a protein of 264 amino acids with predicted molecular weight of 29.01 kD and a isoelectric point of 7.72. The predicted protein sequence contained one conserved AP2 domain, which is the typical characteristic of DREB transcription factors. Based on the sequences similarity, LmDREB2 is classified into DREB2 subfamily. The deduced protein contained a putative acidic activation domain and an AP2 DNA binding domain of 64 amion acids with three-stranded anti-parallelβ-sheet and anα-helix.*

KEYWORDS- *Leymus muticaulis; LmDREB2; Bioinformation analysis*

I. INTRODUCTION

Plants are exposed to environmental stresses such as drought, high salt and low temperature, which cause adverse effects on the growth of plants and quality and quantity of their products. Plants respond to abiotic stresses with a series of physiological and biochemical changes. A Number of genes that respond to abiotic stresses have been identified [1-5]. A group of these genes encode transcription factors that are involved in further regulation of gene expressions that contribute to stress tolerance. Previous research indicated that dehydration responsive element (DRE)-binding transcription factor plays an important role in regulating the expression of downstream stress-responsive gene with a DRE *cis*-acting element in their promoter regions [6,7]. DREB transcription factor belongs to AP2/ethylene response element binding protein (EREBP) family that was characterized by a conserved AP2/EREBP DNA-binding domain and functioned in plant development, stress

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responses, or hormone responses. DRE *cis*-element responsive to drought, cold and salinity stress was identified in stress-inducible genes *RD29A* and *COR15a* in *Arabidopsis* [8], followed by the identification of a great number of complementary DNA (cDNA)-encoding DRE-binding proteins [6,9-12]. These proteins specifically bind to the DRE/CRT element with the core sequence CCGAC and activate the transcription of target stress-inducible genes. Drought, salinity, or cold stress signals are first perceived by the receptors present on the membrane of plant cells and then transduced downstream to switch on the stress-induced genes with DRE *cis*-elements. Expressions of the target genes result in physiological and biochemical changes of plants and finally, enhance the stress tolerances[13]. One DREB transcription factor can regulate the expression of several genes' response to various abiotic stresses. It is expected to improve the stress tolerance of plants by *DREB* gene transfer.

Leymus muticaulis Tzvelev is an important species of *Leymus* Hochst (Triticeae:Poaceae), which is an important genetic resource to Triticeae crops and forage grass. *L. muticaulis* normally grows on grasslands and meadows which has a strong drought, cold, alkaline and disease resistance, insect resistance, resistance to the capacity of sand. In this study, a new DREB transcription factor was cloned and characterized, which would lay down the basis for further research on the tolerance of *Leymus* to abiotic stresses and Triticeae crops and forage grass improvement by genetic engineer.

II. MATERIALS AND METHODS

2.1. Plant Material

Leymus muticaulis seeds (PI440324) was were kindly provided by American National Plant Germplasm System (Pullman, Washington, USA). The plant and voucher specimen of the material have been deposited at the perennial nursery and Herbarium of the Triticeae Research Institute, Sichuan Agriculture University, China (SAUTI).

2.2. Preparation of total RNA, cDNA synthesis and DNA extraction

Total RNA was isolated by using the RNeasy kit (Tiangen) according to the manufacture's instructions. The RNA preparations were subjected to DNase digestion in the presence of recombinant ribonuclease inhibitor. RNA was extracted with phenol and precipitated in ethanol. Equal amounts of 2 μg of total RNA were reverse transcribed into cDNA in 20 μl reactions containing 50 mM Tris-HCl (pH8.3), 75 mM $MgCl_2$, 10 mM DTT, 50 μM dNTP, 200 U SuperScript™ III Reverse Transcriptase (Invitrogen) and 50 pmol Olig-T(15)nucleotides for 60 min at 37 ℃ and a final denaturation step at 95 ℃ for 5 min. PCR amplifications were carried out in a PTC-240 thermocycler (Genetic Technologies, MJ Research, USA).

RT-PCR were conducted using independently isolated total RNAs with the following thermal cycling parameters: 94 ℃ for 30 s, 60 ℃ (63 ℃, or 66 ℃ depending on different primer pairs) for 1 min, and 72 ℃ for 2 min. various numbers of PCR cycles were tested to ensure that the reactions had not reached the plateau. The RT-PCR products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide. Images were photographed and captured by Gel Doc 2000™ (Bio-Rad, USA). Total DNA was extracted from the seeding leaves by the cetyltrimethylammonium bromide (CTAB) method.

2.3. Full-length cDNA and DNA cloning and sequence analysis

To generate the full-length cDNA and DNA sequences of *L. muticaulis* DREB2, a pair of primers were designed (DREB2-F: 5'-ATGTCCAGGAAGAAGAAAGTGC-3'; DREB2-R: 5'-CTATTGCTCCACGTGACTACAACC-3') according to *PpDREB2* (AY553331). The PCR program was as

follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 1 min, and ended by extension of 72°C for 7 min. The PCR product was purified and cloned into pMD19-T vector (Takara) followed by sequencing.

Sequence alignments were performed by the software DNAMAN with the database released in GenBank. The secondary structures of AP2 domain were analyzed by SMART server (http://coot.embl-heidelberg.de/SMART/). The 3D structure was predicted by CPHmodels-2.0 server (http://www.genome.cbs.dtu. dk/services/CPHmodels-2.0 Server-3D.htm). The neighbor-joining (NJ) phylogenetic tree was constructed based on a Clustal W amino acid alignment generated with the Mega 5.0 method and using 1 000 bootstraps to estimate the node strength.

III. RESULTS AND DISCUSSION

3.1. Isolation of the Full-Length cDNA of *LmDREB2*

To generate the full-length cDNA and DNA sequences of *L. muticaulis* DREB2, a pair of primers were designed (DREB2-F: 5'-ATGTCCAGGAAGAAGAAAGTGC-3'; DREB2-R: 5'-CTATTGCTCCACGTGACTACAACC-3') according to *PpDREB2* (AY553331). The two PCR reactions resulted in fragments of the same length (Fig. 1), which indicated that there was no intron in *LmDREB2*. The *LmDREB2* cDNA sequence and deduced amino acid sequence were submitted to the National Center for Biotechnology Information (NCBI) GenBank, and the accession number was JQ755244.

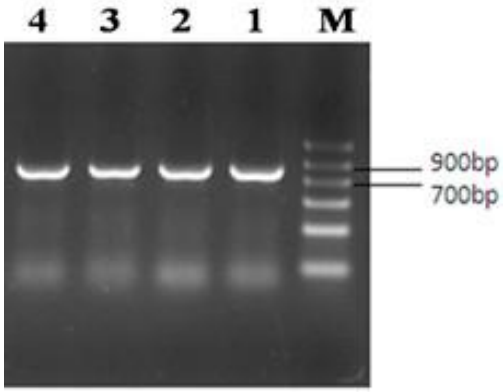

Fig.1 Genomic DNA structure analysis of *LmDREB2*

PCR products of *LmDREB2* gene by using the same gene specific primers and different templates. 1, 2. genomic DNA as the template; 3, 4. cDNA as the template; M. marker Ⅱ

3.2. Sequence Analysis of *LmDREB2*

Sequence analysis indicated that the full-length cDNA contained an open reading frame (ORF) of 795 bp encoding a putative protein of 264 amino acids (Fig. 2) with a predicted molecular weight of 29000.21 Da and a isoelectric point of 7.72. Figure 3 showed the 3D structure of *LmDREB2* predicted by CPHmodels-2.0 server (http://www.genome.cbs.dtu.dk/ services/CPHmodels-2.0 Server-3D.htm). Analysis of the deduced protein revealed that it contained a conserved AP2 domain of 64 amino acids and an acidic C-terminal region that may act as an activation domain for transcription. The secondary structures of AP2 domain were analyzed by Predict Protein (http://www.predictprotein.org/) and the result showed that this domain contained three pieces of β-sheets and one α-helix, which is a typical characteristic of AP2/EREBP protein. Previous report indicated that in DREB proteins, AP2 domain is characterized by a conserved valine in the 14th position and a

glutamic acid in the 19th position, which may play important roles in recognition of the DNA-binding sequence, whereas further research showed that E19 may not be as important as V14 for the recognition of the DNA-binding sequence [14]. AP2 domain of *LmDREB2* had the conserved V14, but there was a leucine in the 19th position.

```
1    ATGTCCAGGAAGAAGAAAGTGCGCAGGAGAAGCACTGGTCCCGAT
      M  S  R  K  K  K  V  R  R  R  S  T  G  P  D
46   TCGGTTGCTGAAACCATCAAGAAGTGGAAGGAGCAAAACCAGAAG
      S  V  A  E  T  I  K  K  W  K  E  Q  N  Q  K
91   CTCCAGCAAGAGAATAGATCCCGGAAAGCACCGGCCAAGGGTTCC
      L  Q  Q  E  N  R  S  R  K  A  P  A  K  G  S
136  AAGAAAGGGTGCATGGCAGGGAAAGGAGGTCCAGAGAATTCAAAC
      K  K  G  C  M  A  G  K  G  G  P  E  N  S  N
181  TGCGCTTACCGCGGTGTGAGGCAGAGGACGTGGGGCAAATGGGTT
      C  A  Y  R  G  V  R  Q  R  T  W  G  K  W  V
226  GCTGAGATCCGTGAGCCCAACCGTGGCAACCGGCTGTGGCTTGGT
      A  E  I  R  E  P  N  R  G  N  R  L  W  L  G
271  TCATTCCCTACCGCAGTCGAAGCTGCACGTGCATATGATGATGCG
      S  F  P  T  A  V  E  A  A  R  A  Y  D  D  A
316  GCAAGGGCAATGTATGGCGCCAAAGCACGTGTCAACTTCTCAGAG
      A  R  A  M  Y  G  A  K  A  R  V  N  F  S  E
361  CAGTCCCCAGATGCCAATTCTGGTTGCACGCTGGCACCTCCATTG
      Q  S  P  D  A  N  S  G  C  T  L  A  P  P  L
406  CTGATGTCTAATGGGGCAACCGCTGCGTCACACCCTTCTGATGGG
      L  M  S  N  G  A  T  A  A  S  H  P  S  D  G
451  AAGGATGAATCGGAGTCTCCTCCTCCTCTTATTTCAAATGCGCCT
      K  D  E  S  E  S  P  P  P  L  I  S  N  A  P
496  ACAGCTGCGCTGCATCAGTCTGATGCTAAGGATGAGTCTGAGTCT
      T  A  A  L  H  Q  S  D  K  D  E  S  E  S  P
541  GCAGGGACCGTGGCACATAAGGTGAAAACAGAAGTGAGCAATGAT
      A  G  T  V  A  H  K  V  K  T  E  V  S  N  D
586  TTGAGAAGTACCCATGAGGAGCACAAGACCCTGGAAGTATCCCAA
      L  R  S  T  H  E  E  H  K  T  L  E  V  S  Q
631  CCAAAAGGGAAGGCTTTACATAAAGAAGCGAACGTAAGTTATGAT
      P  K  G  K  A  L  H  K  E  A  N  V  S  Y  D
676  TACTTCAACGTCGAGGAAGTTCTTGACATGATAATTGTGGAATTG
      Y  F  N  V  E  E  V  L  D  M  I  I  V  E  L
721  AGTGCTGATGTAAAAATGGAAGCTCATGAAGAGTACCAAGATGGT
      S  A  D  V  K  M  E  A  H  E  E  Y  Q  D  G
766  GATGATGGGTTTAGTCTTTTCTCATATTAG GGTTTTAGCTATGAGGGTTGTAGTCACGTGGAGCAATAG
      D  D  G  F  S  L  F  S  Y  *
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Fig.2 Nucleotide sequence and deduced amino-acid sequence of *LmDREB2* cDNA.

The EREBP/AP2 domain is double underline, the basic region in the N-terminal, a predicted nuclear localization signal is shown by underlines

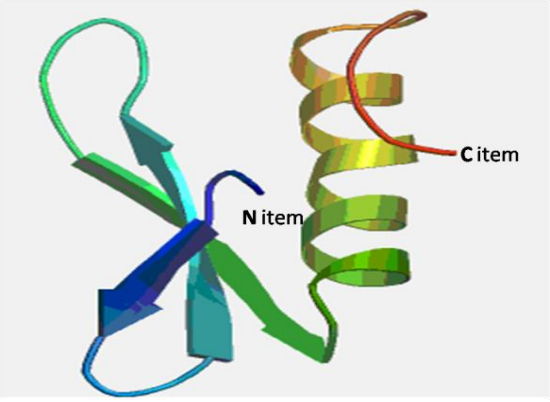

Fig. 3 The 3D structure of *LcDREB2* was predicted by CPHmodels-2.0 server.

The deduced amino acid sequence of *LmDREB2* and its AP2 domain were both aligned with other reported AP2/EREBP proteins in the NCBI database (Fig. 4). Phylogenetic analysis showed that *LmDREB2* had higher homology with DREB proteins from monocots but had lower homology with those from dicots.

Furthermore, it had the highest homology with DREB protein from *Hordeum brevisbulatun* because they both belonged to Triticeae (Fig. 5).

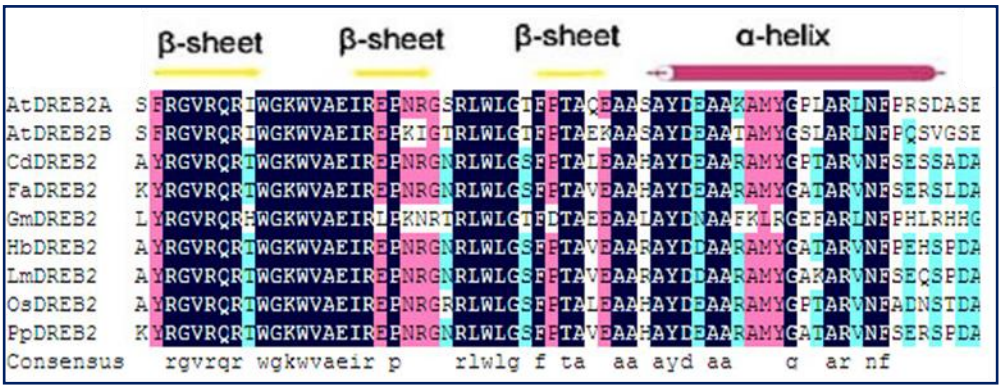

Fig.4 Comparison of the AP2 amion-acid sequences of DREB2 proteins isolated from plants

GeneBank accession number are : *OsDREB2*, *Oryza sativa*, AF300971; *CdDREB2*, *Cynodon dactylon*, AY462118; *FaDREB2*, *Festuca arundinacea*, AY426639; *PpDREB*2, *Poa pratensis*, AY553331; *HbDREB2*, *Hordeum brevisbulatun*, AY728807; *LmDREB2*, *Leymus muticaulis*, JQ755244; *AtDREB2A*, *Arabidopsis thaliana*, AB007790; *AtDREB2B*, *Arabidopsis thaliana*, AB007791; *GmDREB2*, *Glycine max* , AAQ57226.

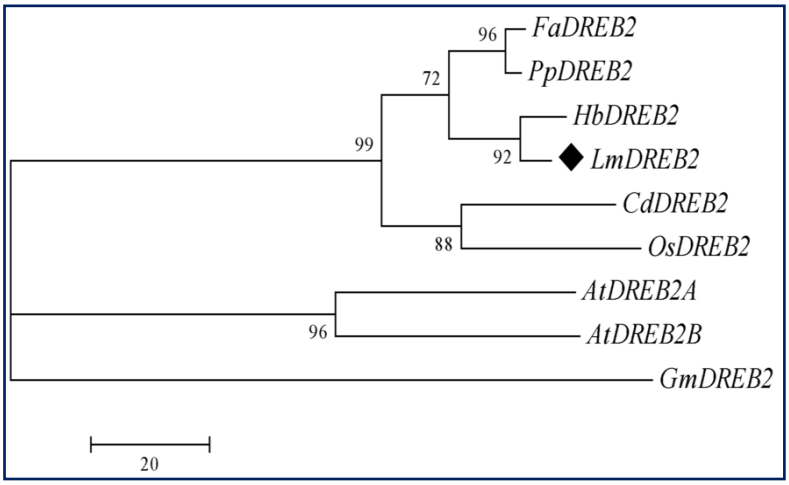

Fig.5 Phylogenetic analysis of DREB2 protein isolated from plant

The gene resources and their GenBank accession number are seen in Fig.4

3.3. Subcellular localization and phosphorylation site prediction of *LmDREB2*

Subcellular localization of *LmDREB2* was predicted by software WoLF PSORT. The results indicated that *LmDREB2* is a nuclear-localized protein possibly functioning as a transcription factor. phosphorylation site prediction of *LmDREB2* was predicted by software NetPhos2.0 Server. The results indicated that 9 serine sites, 4 threonine sites and 4 tyrosine sites were phosphorylated (Fig. 6).

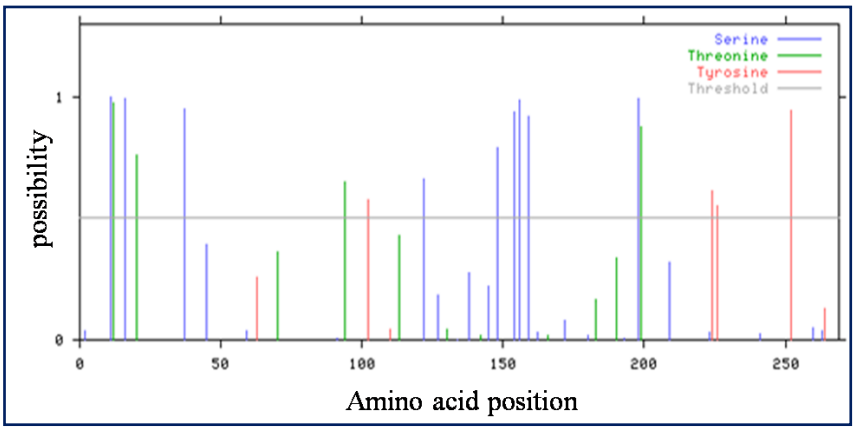

Fig.6 The analysis of phosphorylation site

IV. CONCLUSION

A great number of cDNA encoding DREB transcription factors have been identified in various plants, such as *AtDREB* in Arabidopsis, *OsDREB* in rice, *TaDREB1* in wheat, and *GmDREB* in soybean [6,10,15,16]. In this study, a new member of DREB gene family, named *LmDREB2* was isolated from the plant *L. muticaulis* and characterized. Sequence analysis showed that the putative protein contained an AP2 domain of 64 amino acids that is essential to DREB proteins, and there was a typical NLS in the N-terminal region. AP2 domain is generally characterized by a conserved valine (V) in the 14th position and a glutamic acid (E) in the 19th position. Whereas, *GmDREB2* protein has a valine at the 14th position and a leucine at the 19th position. Further research showed that E19 may not be as important as V14 for the recognition of the DNA-binding sequence. So far, many DREB proteins containing a leucine at the 19th position of AP2 domain have been isolated from different plants, such as DREB3 protein from *Aloe officinalis* (GenBank accession No. DQ211835), *BpDREB* from *Broussonetia papyrifera* (DQ211836), and *ZmDREB1* from *Zea mays* (AF448789), etc. The 19E may have different significance in different DREB proteins. However, the 14V is the key point in most of DREB proteins to define their DNA-binding specificity and ability [17].

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