IDENTIFICATION, CLONING AND IN-SILICO CHARACTERIZATION OF DROUGHT INDUCIBLE *OsDREB2A* TRANSCRIPTION FACTOR FROM INDICA RICE VARIETY

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ABSTRACT

DREB proteins are transcription factors belong to the AP2/ERF (APPETALA2/ Ethylene Responsive Elements Binding Factor) domain containing gene family which mediates abiotic stress responsive cellular signal transduction pathway. DREBs have been broadly categorized into DREB1 and *DREB2* proteins based on their responses to abiotic stresses. *OsDREB2A* has been identified and characterized in rice as the homolog for *DREB2A* which interact with *cis*-acting DRE/CRT (Dehydration responsive element/C-repeat) elements and activate the expression of downstream genes involved in dehydration and salinity stresses. In this study we identified, cloned and in-silico characterized the *OsDREB2A* Indica homolog sequence which exhibits N-terminal sequence variation from japonica sequence while maintaining the basic structural and functional sequence similarity, which is necessary for the activity of the protein. Structure prediction and analysis was performed to determine the structural and functional features of the *OsDREB2A* Indica gene. Multiple sequence analysis of twenty *DREB2A* like complete cDNA sequences of family Poaceae was conducted to interpret their phylogenic relationships.
Key words: DRE, *OsDREB2A*, Dehydartion stress, Phylogenetic, Indica Rice, Insilico analysis.

[I] INTRODUCTION

Dehydration and extreme temperature are crucial abiotic stresses which pose serious constraints upon growth and development of plants and sustainable crop productivity. To survive under such extreme conditions plants must respond and adapt to stresses at molecular and cellular level by increased responses generated through physiological and biochemical processes. Several genes and gene families have been identified in many plant species which respond to adverse

environment effects [2,4,11,8]. Various stresses of plants are regulated by multigenic signaling networks that are largely comprised of transcription factors (TFs) that activate downstream signaling cascades of signal transduction pathway [17].

The *Arabidopsis* genome hosts more than 1550 transcription factors belonging to 39 transcription factor families and nearly 5% of the *Arabidopsis* genome capacity contributes to transcription factors [14,9,5]. A single transcription factor regulate the expression of many target genes by binding to the specific sites in the promoter region [25,6]. The *Arabidopsis* genome contains several large transcription factor families which have higher number of individual TF members such as MYB, MADS, Basic Helix-Loop-Helix (bHLH) And APETALA2 (AP2)/Ethylene Response Element Binding Protein (EREBP) [10]. Genome wide database analysis suggests the presence of approximately 121 ERF proteins in the *Arabidopsis* genome. Proteins containing ERF/AP2 domains are known as transcriptional activators [3,7] which specifically bind to DRE/CRT (A/GCCGAC) sequences of the promoter region of the responsive genes and activate the transcription [6,8].

DREB (CBF1, DREB1A, and *DREB2A*) genes have been isolated in yeast-one hybrid screening [6,3] and identified as transcription factors belong to ERF/AP2 (Ethylene Responsive-element Binding factor /APETELA2) domain gene family [16]. The ERF protein family shares a conserved 58-59 amino acid domain that can bind to DRE/CRT *cis*-elements in the promoter regions of the drought and low temperature responsive genes. Two types of trans-acting DREB transcription factors have been identified as DREB1 and *DREB2* based on transient expression in *Arabidopsis* protoplast assay [6,3]. Expression of DREB1 transcription factors have been identified in response to early and transient cold stress [13,15,24] while *DREB2* proteins confer

resistance under osmotic stress by inducing target genes under drought and salinity stresses [24,6,16]. Although both DREB1 and *DREB2* proteins binds to DRE elements in the promoter regions of the stress responsive genes, DREB1 only regulates the expression of cold responsive genes while *DREB2* specifically express drought and salinity responsive regulatory genes. Dubouzet *et al*[16], in 2003 showed the existence of two cDNA clones homologous to DREB1A (DREB1B, DREB1C) and one cDNA clone homologous to *DREB2A* (*DREB2*B) in *Arabidopsis*. Recent findings revealed the existence of three novel DREB1 type (DREB1D, DREB1E, DREB1F) and six novel *DREB2* type (*DREB2*C, *DREB2*D, *DREB2*E, *DREB2*F, *DREB2*G, *DREB2*H) genes in *Arabidopsis* genome, although expression levels of these genes under different stress conditions are low [15].

Genome wide conserved sequences analysis in rice indicates the existence of DREB homologs. There are seven DREB1 type homologs (OsDREB1A, 1B, 1C, 1D, 1E, 1F and 1G) [16] and six *DREB2* type (*OsDREB2A*, 2B, 2C, 2D, 2E and OsAB14) [24] in rice. Over expression of OsDREB1A in *Arabidopsis* under CaMV35S promoter has shown increased tolerance to drought, cold and salt stresses [16]. Chen *et al.*[21], in 2008 has shown that OsDREB1E, OsDREB1F and OsDREB1G also improve drought, salt and low temperature tolerance in rice. Os*DREB2* types in rice are orthologoues to *DREB2* genes in *Arabidopsis* and confer drought and salt tolerance [6,12,20]. *OsDREB2A* has been identified as a strong candidate gene in the drought responsive pathway and whose poor sensitivity for Abscisic Acid (ABA) suggested it may be involved in the ABA independent pathway [16]. *Arabidopsis DREB2A* and *OsDREB2A* show high levels of homology in the N-terminal region and the AP2/ERF domain suggests similarity in the expression and function of both genes.

OsDREB2A was first identified in japonica rice cultivars and later studies were concentrated on indica rice cultivars. Sequence analysis showed that Japonica cultivar *OsDREB2A* cDNA differ from indica cultivar *OsDREB2A*. To study the sequence properties and features of Indica *OsDREB2A* with respect to *OsDREB2A* Japonica sequence, we isolated, cloned and sequenced the *OsDREB2A* coding region from Indica rice. The novel sequence was used to compare with Japonica sequences to find putative changes in the coding region at nucleotide and amino acid levels. Analysis of the sequence properties and features, which predicted the putative function of the protein, was conducted to determine the sequence as a transcription factor. The putative structure of the Indica rice sequence was developed to determine the structural variations in the active domain level. *DREB2A* Sequence analysis and comparisons were carried out with twenty poaceae family *DREB2* homologs to determine the chemical, structural, functional and evolutionary conserved features of newly identified sequence.

[II] MATERIAL AND METHODS
2.1 Plant materials and DNA extractions
Salinity tolerant Indica rice (*Oryza sativa*) cultivar pokkali variety was used for DNA extraction along with 17 other drought resistant (10 varieties) and sensitive (7 varieties) varieties using miniprep C-TAB method. Leaf blades of three weeks old seedlings were used for plant DNA extraction and Gene Quant (Pharmacia Biotech) spectrophotometer was used for DNA quantification. Agarose gels (1%) were used to determine the quality of DNA and 50ng/µl aliquots was made from each original DNA extract.

2.2 Genome data screening and primer designing
Primers were designed to amplify coding region from salinity tolerant Pokkali variety assuming that both Indica and Japonica cultivars exhibit sequence similarities. PCR amplification of *OsDREB2A* from pokkali variety was carried out using japonica sequence specific primers. PCR results showed that the *OsDREB2A* sequence in Indica cultivar varies from Japonica sequence. The Chromosome 1 complete sequence of Beijing Indica was retrieved from Beijing genomics institute's RIS database. The complete sequence of the Beijing Indica Chromosome 1 was screened for *OsDREB2A* coding region, resulting in a new coding sequence for *OsDREB2A* and respective sequence specific primers were designed to amplify the gene.

Indica cultivar specific primers (reference sequence Genbank HM807364.1)

Forward primer (BamH1site added)
5'-TAT**GGATCC**ATGCTGTTTCGATTTGTGTC- 3'
Reverse primer (ECoR1site added)
5'TT**GAATTC**CTAATAGGAGAAAAGGCTAAACCCA-3'

Japonica cultivar specific primers (reference sequence Genbank FN556368.1)

Forward primer (BamH1site added)
5'-TCG**GGATCC**ATGGAGCGGGGGGGAG-3'
Reverse primer (ECoR1site added)
5'-ATT**GAATTC**CTAATAGGAGAAAAGGC-3'

2.3 PCR amplification
The *OsDREB2A* coding region was PCR amplified using both Indica and Japonica sequence specific primers for all the 17 varieties (Figure 1). Genomic DNA from Indica rice was PCR amplified with 10 X PCR buffer (genshung bio) 2.5µl, 3.5mM dNTP (genshung bio) 2.5 µl, 2.5 µM primers (forward and reverse primer mix) and 0.2 µl of Taq DNA Polymerase (genshung bio) (10U/µl). DNA amplification with both japonica and Indica primers were done using standard PCR protocol with annealing temperature 55^0C. PCR cycle was 94^0C initial denaturing 4min, with 35 cycles of 94^0C 1 min; 55^0C 1min; and 72^0C 1 min. final extension was 72^0C for 7 min. Agarose gel

(2%) electrophoresis was done with100bp DNA ladder to determine the expected band sizes.

2.4 Sequencing, cloning and sequence analysis of Open reading frame

OsDREB2A Indica sequence was amplified from the pokkali variety and cloned in to pUC18 in BamH1 and EcoR1 restriction sites (figure 2) followed by Sequencing of *OsDREB2A* gene. The coding region of Indica *OsDREB2A* was sequenced from 3 resistant and 3 sensitive varieties were compared. The Indica OsDREB2A coding region was analyzed to study thesequence features and the properties of the AP2/ERF DNA binding domain. The gene was analyzed for distribution of hydrophilic properties along the sequence with twenty other *DREB2* like coding regions from Poaceae family. Conserved and varied regions along the gene were examined using BioEdit and Mega 4 sequence analysis tools. The properties at the amino acid level were determined using ExPASSy protein tools and structure prediction and comparison was done for the DNA binding domain to analyze the differences between Japonica and Indica ORFs.

The Phylogenetic relationship of twenty selected sequences was analyzed to study the conserved nature of the sequences. An unrooted tree for 1000 bootstrap value was drawn to elaborate the phylogenetic relationship between poaceae family *DREB2* type members.

[III] RESULTS AND DISCUSSION

To examine the variation of *OsDREB2A* gene sequence between Indica and japonica cultivars, the gene sequence was isolated and analyzed. First the *OsDREB2A* japonica sequence was identified from NCBI genbank database (GenBank Accession No: AF300971.2) and PCR primers were designed to amplify the coding region.

The same primers were used to amplify the Indica *OsDREB2A* coding region from genomic DNA of pokkali Indica variety. No amplification was observed though optimization was done changing

annealing temperatures using gradient PCR (Range 55^0C - $60\ ^0$C) and other reaction conditions (dNTP concentration, primer concentration and initial genomic DNA concentration). Primers were redesigned for *OsDREB2A* japonica sequence to identify whether variation is at the N-terminal region or C-terminal region. One primer set was designed with forward primer covering starter codon of the gene and the reverse primer covering middle sequence of the gene. A second primer set was designed with forward primer covering the middle sequence of the gene and reverse primer covering the stop codon region (this was done on an assumption that the DNA binding domain and nuclear localization signal is located in middle and towards the N-Terminal region therefore it must be conserved and variation could occur at ends of the ORFs). With the amplification of second primer set's expected band the variation was confirmed to be at the N-Terminal region. Primers used to amplify the gene from seventeen Indica rice varieties confirmed that the new gene is common to Indica genome (Figure 1). New Indica *OsDREB2A* (846bp) (Figure 2) gene sequence was cloned in to pUC18 vector and sequence was published in the NCBI database (Genbank Accession No: JQ341059.1). Sequencing of the OsDREB2A coding region from 3 sensitive and 3 resistant varieties showed that the gene exhibits no polymorphism between varieties.
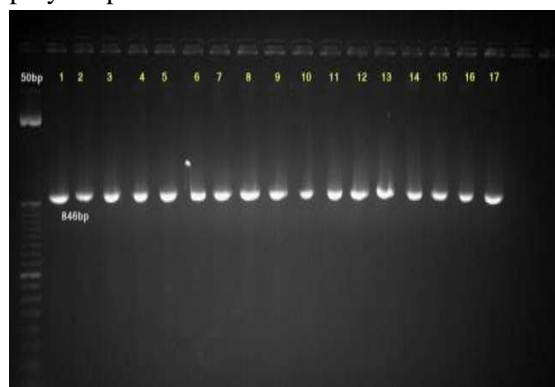

Figure 1: PCR amplification of Indica *OsDREB2A* coding region with Indica specific primers using 17 Indica rice varieties including Pokkali variety (lane 8)
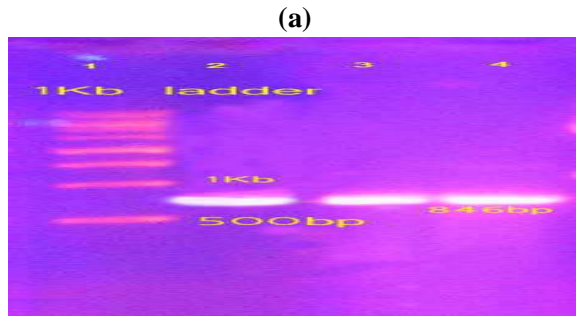
(a)

Figure 2: (a) PCR amplification of Indica *OsDREB2A* coding region from pokkali variety;

The analysis of Chromosome 1 of the complete sequence of Beijing Indica variety showed that the *OsDREB2A* Indica sequence contains variation at the N-Terminal region. The variation was observed starting from the starter codon and extends up to 66th nucleotide at the N-terminal, thereby changing first 22 amino acids.

3.1 Analysis of *OsDREB2A* Indica gene sequence

The Nuclear localization Signal (NLS) and the DNA binding domain were within the new gene as in *OsDREB2A* japonica and sequence variation was exterior to the NLS regions. After first 66 nucleotides the rest of the nucleotides are identical to the *OsDREB2A* japonica sequence, which demonstrate that most of the features are conserved in the gene, although some changes can be expected at the structural and functional level. These variable and conserved features were true even at the putative amino acid level. After first 22 amino acids the rest of the sequence was identical to the japonica sequence except at the 665th nucleotide which changes Aspergine in Japonica in to Threonine in Indica. The Japonica sequence (825bp) is much shorter than the Indica sequence (846bp). This length variation has also occurred due in the N-terminal. In the *OsDREB2A* japonica sequence, the nuclear localization signal starts from 51st nucleotide and extends up to 171st nucleotide. The NLS of the New Indica *OsDREB2A* gene was found at the N-terminal starting from 69th nucleotide, immediate next to

the N-terminal variation, and extending up to the 162nd nucleotide (Figure 3). The NLS of the Indica *OsDREB2A* is identical to the japonica *OsDREB2A* gene. As in the *OsDREB2A* Japonica gene, *OsDREB2A* Indica sequence has an intronless coding region which excludes the necessity for the use of cDNA strand for gene expression studies.

3.2 Analysis of physical and chemical properties of *OsDREB2A* protein

OsDREB2A indica sequence has PI value 5.87 which is slightly acidic where Japonica *OsDREB2A* has theoretical PI of 5.77 and Grand Average Hydropathicity Index (GRAVY) of the new Indica *OsDREB2A* was calculated as -0.670, which indicate that the gene is hydrophilic in nature and exhibit features of a putative transcription factor. Hydrophilicity distribution along the ORF of the new Indica *OsDREB2A* was analyzed with twenty other *OsDREB2A* related genes (Figure 5) (Kyte and Doolittle, 1982). All these sequences showed a similar pattern of hydrophilicity suggesting the conserved nature of the homology *DREB2* type transcription factors, regardless of their origin. Similar hydropathy profiles in all twenty sequences show that Os*DREB2* type sequences share high degree of homology at sequence level and in protein chemistry.

Figure 3: The amino acid sequence of Indica *OsDREB2A* transcription factor. Amino acids of the DNA binding domain are underlined by a single line. The amino acids of the nuclear localization signal are underlined using double lines.

A multiple sequence alignment was conducted with respect to *Arabidopsis* thaliana *DREB2A* amino acid sequence (O82132), in order to identify nuclear localization signal and the DNA binding domain of Indica *OsDREB2A* amino acid sequence and found nuclear localization signal share higher level of similarity in amino acid sequence.
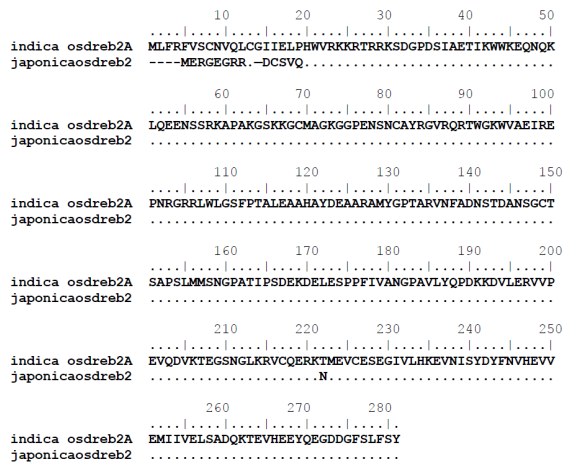
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                10        20        30        40        50
            ....|....|....|....|....|....|....|....|....|....|
indica osdreb2A  MLFRFVSCNVQLCGIIELPHWVRKKRTRRKSDGPDSIAETIKWWKEQNQK
japonicaosdreb2  ----MERGEGRR.-DCSVQ...............................

                60        70        80        90        100
            ....|....|....|....|....|....|....|....|....|....|
indica osdreb2A  LQEENSSRKAPAKGSKKGCMAGKGGPENSNCAYRGVRQRTWGKWVAEIRE
japonicaosdreb2  .................................................

               110       120       130       140       150
            ....|....|....|....|....|....|....|....|....|....|
indica osdreb2A  PNRGRRLWLGSFPTALEAAHAYDEAARAMYGPTARVNFADNSTDANSGCT
japonicaosdreb2  .................................................

               160       170       180       190       200
            ....|....|....|....|....|....|....|....|....|....|
indica osdreb2A  SAPSLMMSNGPATIPSDEKDELESPPFIVANGPAVLYQPDKKDVLERVVP
japonicaosdreb2  .................................................

               210       220       230       240       250
            ....|....|....|....|....|....|....|....|....|....|
indica osdreb2A  EVQDVKTEGSNGLKRVCQERKTMEVCESEGIVLHKEVNISYDYFNVHEVV
japonicaosdreb2  ......................N..........................

               260       270       280
            ....|....|....|....|....|....|....|....|
indica osdreb2A  EMIIVELSADQKTEVHEEYQEGDDGFSLFSY
japonicaosdreb2  ..............................
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Figure 4: sequence comparison between the indica and japonica *OsDREB2A* amino acid sequences. Conserved positions of the japonica *OsDREB2A* sequence with respect to indica *OsDREB2A* sequence are shown in dots. Variation in the initial 21 amino acids is clearly visible.
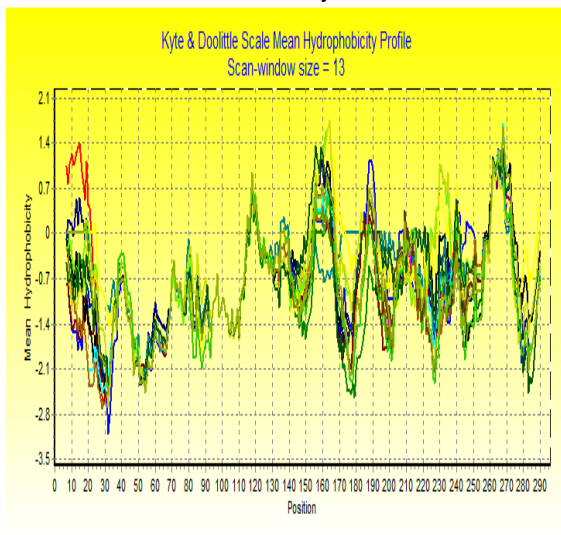

Figure 5: Hydrophathy profile variation for Os*DREB2* like sequences from 20 species of family Poacea (Kyte and Doolittle mean hydrophobicity scale developed using Bioedit sequence analysis tool)

Based on the Kyte and Doolittle Scale of Mean hydrophobicity profile all 20 DREB sequences exhibited similar hydrophylicity variation along the sequence (Figure 5) suggesting all the sequences possess conserved hydropathy profile which is very crucial for the function of the protein. Generally all the proteins are showing mean negative value for hydrophobicity indicating the protein's affinity for hydrophilic molecules such as DNA which suggest their DNA binding ability. DNA binding domains (82-141 amino acids in Indica *OsDREB2A*) of all the twenty proteins have mean negative values for hydrophylicity suggesting the conserved nature of the chemistry of proteins which facilitate the DNA binding activity. High mean hydrophilicity observed in the n-terminal and C terminal regions of all the proteins regardless of the variations at those regions in amino acid sequences. Conserved high mean hydrophilicity in the protein may be necessary for the sequence to function as a transcription factor and remain within the nucleus once transcribed to trigger the downstream signaling genes. C-terminal regions of the sequences exhibit higher degree conserved nature including the "LFSY" chain termination signal, are more conserved as expected for regions that are crucial for the protein's activity.
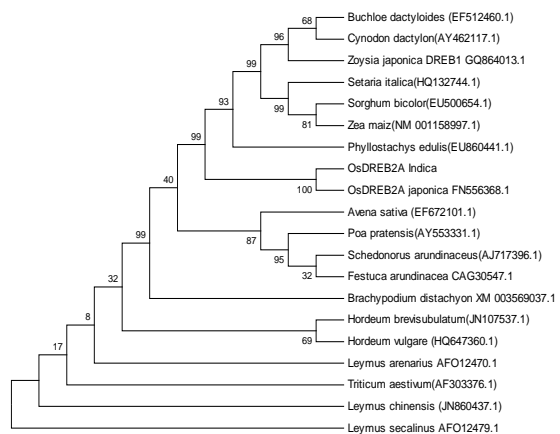

Figure 6: unrooted consensus tree with 1000 bootstrap value for 20 *DREB2* like sequences from Family Poaceae (plotted using MEGA4 sequence analysis tool)

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An unrooted bootstrap consensus tree was plotted (figure 6) for all the 20 *DREB2A* related sequences of family poaceae using the maximum parsimony method for 1000 bootstrap values with 1001 randomly added trees. According to the tree results the *OsDREB2A* gene was found to be having higher similarity with *OsDREB2A* japonica and can be predicted as an ortholog protein which has been diversified while speciation. from the comparison it is not possible to predict which gene is descended from the other. An unrooted bootstrap consensus tree was plotted with 11replication random addition tree option and it was found that the Indica *OsDREB2A* is likely orthologous to Japonica *OsDREB2A*. Tree results infer all rice sequences have developed before the *Sorghum bicolor* and *Zea maize* DREB like proteins in the evolution. The *DREB2* like proteins of Sorghum *bicolor* and *Zea maize* share higher similarity in their sequence and behave as orthologous sequences.

3.4 Prediction of *OsDREB2A* protein structure

The SWISS MODEL workspace and Phyre2 server was used for the DNA binding domain analysis and protein structure prediction and analysis.
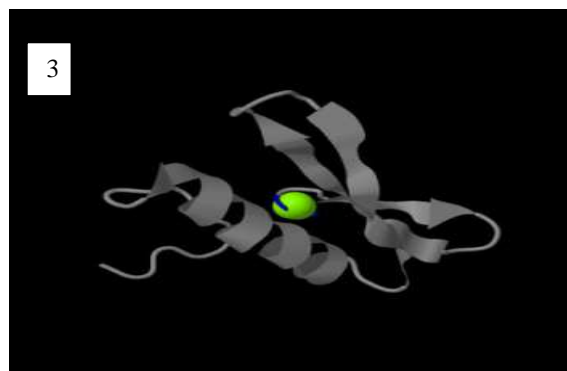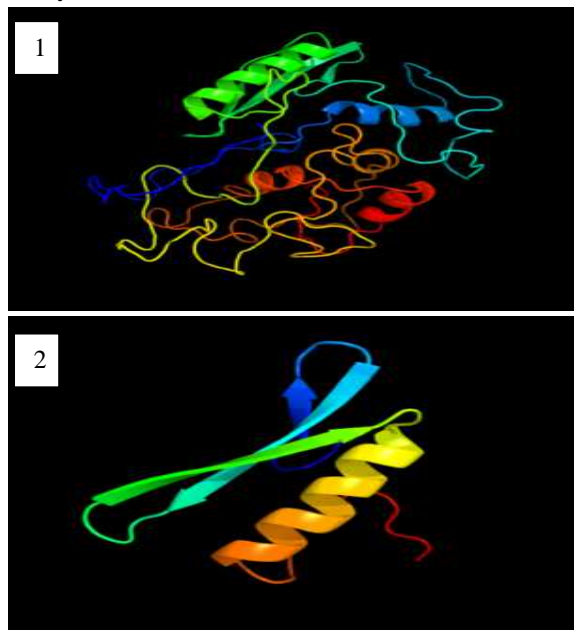

Figure 7: (1) complete structure prediction of the Indica *OsDREB2A* protein with DNA binding domain colored in green (2) predicted structure for the GCC box DNA binding domain of the *OsDREB2A* Indica (3) Predicted ligand binding site of the *OsDREB2A* Indica Domain (phyre2 server and 3DLigandSite)

Based on Swiss model workspace and Phyre2 server DNA binding domain is 99.9% similar to 1gccA template which is GCC box binding domain. This DNA binding domain has 3 discrete regions of ß-pleated sheets followed by a distinct α-helix (figure 7). The Phyre2 server has predicted the *OsDREB2A* domain structure with 99.9% confidence. The DNA binding domain of both japonica and Indica sequences has predicted 82nd Alanine residue and 83rd Tyrosine residues which are very crucial in DNA binding activity. In *OsDREB2A* Indica sequence, N-terminal has ß-strand followed by a stretch of α-helix which was not predicted in *OsDREB2A* japonica.

[IV] CONCLUSION

We isolated the coding region of the OsDREB2A Indica sequence and compared it with the OsDREB2A Japonica sequence and found significant sequence variation at the N-terminal region. Sequence differences were observed from the starter codon of OsDREB2A Japonica and extended for 66 nucleotides. Sequence analysis using Bioedit and MEGA4 sequence analysis tools suggests the sequence variation doesn't affect the

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basic structure and function of the protein. Features like the DNA binding domain and NLS region are outside the variable region. The OsDREB2A nucleotides and amino acid sequences exhibit 100% sequence homology in the rest of the sequence including NLS region, DNA binding domain and LFSY chain termination signal. Sequences from 20 DREB2 like proteins from Poaceae family show that all the DREB2 like proteins share a higher level of homolgy at NLS region, DNA binding domain and at LFSY chain termination signal. This suggests that such regions have been conserved during evolution due to their importance in sequence conformation and function as a dehydration responsive transcription factor.

Protein structure prediction and analysis revealed that the DNA binding domain architecture of the protein is conserved in both Indica and Japonica genes. Structural variability at the N-terminal indicates that it may play crucial part in the Indica gene. The 82nd Alanine residue and the 83rd Tyrosine residues in the DNA binding domain have been found to be ligand binding sites and crucial for the activity of the protein.

The DREB2 like sequences from all the 20 species from family Poacea share similar mean hydrophylicity distribution along the sequences and all the sequences show low hydrophilicity at the NLS region and DNA binding domain which helps the protein to interact with other proteins.

An Unrooted maximum parsimony tree with1000 bootstrap value for 20 DREB2 like amino acid sequences from Poaceae family show OsDREB2A Indica and Japonica genes are orthologous and may have speciated even before Sorghum *bicolor* and *Zea maize* DREB2s.

[IV] ACKNOWLEDGEMENT

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