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Molecular cloning and *in silico* analysis of *DREB*-like gene in watermelon

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ABSTRACT

The objective of this study was to identify and characterize sequences from watermelon related to drought tolerant genes. In order to achieve this, publicly available *Cucumis melo REB* gene (*Cme-DREB1*) sequence was retrieved from NCBI database and gene-specific primers were synthesized. A fragment of 274 bp corresponding to DREB-like gene was amplified from genomic DNA of watermelon. PCR product was cloned in *E. coli* vector (pDrive) and validated through sequencing. The BLAST search showed 99 and 97% homology to *CmeDREB1* gene and *C. sativus DREB3*-like (*CsDREB3*-like) gene, respectively. Furthermore, it showed 87 to 93% homology with rest of DREB genes available in the public database. Full length watermelon *DREB*-like gene (*C. lanatus DREB*-like, *ClaDREB*-like) was identified from cucurbit genomic database and investigated through *in silico* analysis. The intronless structural organization of *ClaDREB*-like gene was found with a conserved AP2/ERF domain.

Key words: Drought tolerance, *DREB*, *in silico* analysis, watermelon.

INTRODUCTION

Watermelon [*Citrullus lanatus* var. *lanatus* (Thunb.) Matsumura & Nakai] is the third most important vegetable fruit crop. It belongs to the Cucurbitaceae family. Watermelon is a diploid (2n = 2x = 22) as well as in certain cases a tetraploid species (seedless) in nature. The genome size of the watermelon is 425 MB with 23,440 predicted genes (Guo *et al.*, 3). The contribution of watermelon in total vegetable production is 7% worldwide. China is the major producer of watermelon in the world. In India, it is grown predominantly in the arid and hot region and river basin areas of the country.

The watermelon and its wild relatives (for example *Citrullus collosynthis*) have the great potential to withstand severe water deficit conditions. Due to its deep root system and high accumulation of compatible solutes in root and aerial parts, watermelon serves as a suitable model system for understanding drought tolerance mechanism of C3 plants (Yoshimura *et al.*, 14). Several abiotic stress tolerant genes have been isolated and characterized in watermelon. The enhanced expression of molecular chaperones as well as higher accumulation of numerous drought responsive metabolic enzymes was reported in the roots of wild watermelon under drought stress treatment (Yoshimura *et al.*, 14). Watermelon accumulates high concentrations of citrulline, glutamate and arginine (DRIP-1 homologue) in place of proline and glycinebetaine in its aerial parts, consequently, which leads to the scavenging of hydroxyl radicals and induction of drought tolerant genes (Kawasaki *et al.*, 6; Akashi *et al.*, 2). cDNA sequences encoding *COR15b* and *KIN1* genes were isolated from leaves of watermelon. The expression analysis of these genes revealed their involvement in the dehydration tolerance (Kang *et al.*, 5). The *Ccrboh*, a drought-responsive gene, encoding the respiratory burst oxidase homologue (rboh) was cloned and investigated in a wild relative of watermelon, *Citrullus colocynthis* for drought responsiveness (Si *et al.*, 10). Moreover, the transcriptome analysis of *C. colocynthis* revealed an array of differentially expressed drought responsive genes (Wang *et al.*, 13). In addition to watermelon, 131 AP2/ERF gene family was identified and characterized in cucumber (Hu, 4).

Watermelon has been sequenced recently (Guo *et al.*, 3) and is a good source of agronomically important traits, however, the availability of information about genomic resources developed for understanding drought tolerance mechanism is still very less. Therefore, the present study was planned to isolate and characterize the drought tolerance related gene sequences in watermelon, specifically, the *DREB*-like gene from watermelon.

MATERIALS AND METHODS

Five seeds of watermelon line DRB-669 (red pulp black seeded) were germinated on a moist germination paper at the temperature of 24°C. The seedlings at true two-leaf stage were selected for DNA isolation. The seedlings were immersed in liquid nitrogen and DNA was isolated according to CTAB method with

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some modification. The DNA was treated with *RNase A* for removing of RNA molecules and purified using phenol: chloroform and ammonium acetate method. The integrity of DNA was checked on 0.8% agarose gel followed by quantification using NanoDrop 1000 (Thermo Scientific, USA).

The available *CmeDREB1* (Genbank accession No. AB125974.1) gene was retrieved from NCBI database and gene-specific primer pair (F- 5'CCCAGCCCTGAAATGGCTGCT3' & R- 5' TCGTTCCCAGAGCCGGCAAC3') was designed using Primerquest software of IDT (http://eu.idtdna.com/Primerquest/Home/Index). For amplification of *ClaDREB*-like gene, PCR assay was performed in 20 µl of reaction containing 1.0 µl DNA (100 ng/µl), 1.0 µl of each forward and reverse primer (10 mM), 1.0 µl dNTPs (10 mM), 0.5 µl *Taq* DNA polymerase, 2.5 µl 10 × buffer with $MgCl_2$ (Takara) and 13 µl of nuclease free water (Hi-Media, Mumbai). The thermal cycler machine (Eppendorf) was used for amplification with the following programme: an initial denaturation of 5 min. at 95°C; 35 cycles of 1 min. at 95°C, 30 sec. at 55°C and 2 min. at 72°C followed by a final extension of 10 min. at 72°C. The PCR products were analyzed on 1.0% agarose gel. The desired size products were cloned in pGEM T vector (Promega) and validated through sequencing.

The validation of partial *ClaDREB*-like gene sequence and protein homology was carried out using BLAST programme of NCBI Server (http://www.ncbi.nlm.nih.gov/). The partial sequence of *ClaDREB*-like gene was used as a query search in cucurbit genomic database (http://www.icugi.org/) for mining of full length gene. The full length genomic sequence of *ClaDREB*-like gene was used for structural analysis using Gene Structure Display Server (http://www.gsds.cbi.pku.edu.cn). Multiple protein sequence alignment and phylogenetic relationship of *ClaDREB*-like protein with publicly available database was established using MEGA 6.0 (Tamura *et al.*, 12). The phylogenetic tree of proteins was constructed using the Neighbor-Joining model of MEGA 6.0. The conserved domain AP2/ERF was identified at the NCBI web site (http: //www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi.). Nucleotide sequence alignment between *ClaDREB*-like gene and other cucurbit *DREB* genes was carried out using CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The 1.5 kb upstream sequence of the identified gene was retrieved form watermelon database (http://www.icugi.org/cgi-bin/ICuGI/genome/index.cgi/watermelon) and analyzed for drought stress responsive *cis*-regulatory elements using PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

RESULTS AND DISCUSSION

Based on accessible *Cucumis melo DREB1 (CmeDREB1)* gene sequence at NCBI, the *DREB-like* gene was amplified in watermelon using designed gene-specific primers. PCR amplification was carried out to amplify a fragment of 274 bp size and was confirmed by gel electrophoresis (Fig. 1). To verify that, the amplified product belongs to *DREB* gene of *C. melo*, the resultant fragment was cloned in pGEM T vector and was sequenced. The obtained putative sequence of *ClaDREB*-like gene was analyzed with BLAST tool for sequence homology confirmation. The *ClaDREB*-like gene showed 99 and 97% sequence homology with *CmeDREB1* gene and *CsDREB3* gene, respectively.

Full length *ClaDREB*-like gene was identified from cucurbit genomic database using the cloned putative *ClaDEB*-like gene sequence as a query search. The BLAST search revealed a locus namely *Cla022212* on chromosome No. 8 showing maximum sequence similarity with putative fragment. The explored 651

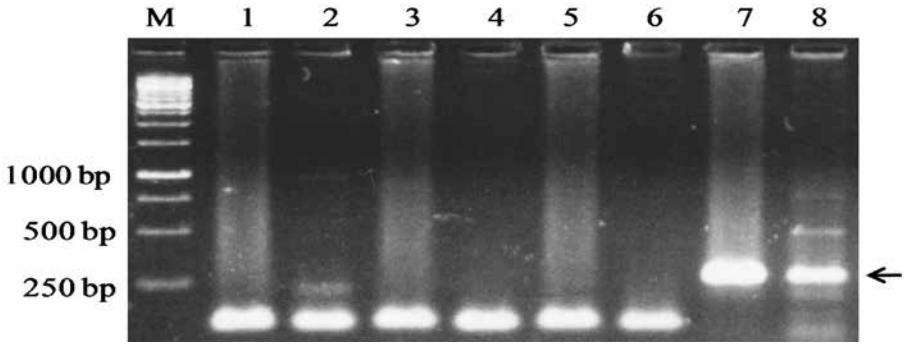

Fig. 1. PCR amplification of *DREB*-like gene in watermelon. Lane M indicates standard marker, 1.0 kb DNA ladder. Lane 1-6 indicates amplification of *Osmotin* and *ERF* gene in watermelon. Lane 7 and 8 indicate amplification of *DREB*-like gene in *C. melo* (positive control) and watermelon, respectively. Arrow at the right side of gel indicates fragment of 274 bp corresponding to *DREB*-like gene.

bp sequence encoding *ClaDREB*-like gene was retrieved from database and deduced into amino acid sequence. The deduced amino acid sequence was further analyzed in BLASTp of NCBI database with default parameters. Twenty two DREB proteins homologous from different plant species matched with ClaDREB-like protein with homology ranging from 83.98% (*CsDREB3*) to 48.79% (*VvDREB3*) [Table 1]. Since DREB3 protein matched most with ClaDREB-like protein, therefore, DREB3 protein of other plants were taken for analysis. Additionally, the BLASTp analysis indicated close proximity of *ClaDREB*-like to *CsDREB3* at amino acid based similarity rather than with *CmeDREB1* gene as mentioned in initial analysis. The reason could be that the partial *ClaDREB*-like gene sequence covered only a part of the *CmeDREB1*gene with maximum homology, while at protein level it was found similar with CsDREB3 protein because of the contained conserved protein domains (AP2/ERF *etc.*).

To obtain insights into the structural organization of the *ClaDREB*-like gene, full length CDS and genomic sequences of the gene was used as an input sequence in the Gene Structure Display Server 2.0. The structural analysis by software showed the *ClaDREB*-like gene is intronless. It was also compared with structural organization of *CsDREB3* and *CmeDREB3* gene (Fig. 2A). Though, the *ClaDREB*-like gene of watermelon is comparatively shorter than *CsDREB3* and *CmeDREB3* gene due to occurrence of some deletions at different position in the gene (data not shown), however, the intronless organization of all the three analyzed genes showed conserved evolutionary relationship between them.

To investigate the AP2/ERF domain in *ClaDREB*-like gene, predicted protein was analyzed in CDD of NCBI database. A domain with signature motif *YRGVRMRNWGKWVSEIREPRKKSRIWL GTFPSPEMAARAHDVAALSIKGNSAILNFPEL* was identified in *ClaDREB*-like protein sequence (Fig. 2B).

Table 1. Amino acid sequence homology of ClaDREB-like protein with DREB3 protein of other plants.

Subject ID	% identity	Alignment length	Mismatches	e-value	Bit score
CsDREB3	83.98	206	24	1e-96	272
CmeDREB3	83.5	206	25	4e-90	255
FvDREB3	60.09	223	49	9e-85	243
PmDREB3	59.35	214	40	2e-79	229
EgDREB3	56.31	222	71	9e-75	218
MnDREB3	59.72	216	51	1e-73	214
CsiDREB3	62.98	208	57	3e-73	212
MdDREB3	57.01	221	74	5e-73	213
CsaDREB3	52.99	234	76	9e-73	212
AtDREB3	52.99	234	76	1e-71	208
NnDREB3	59.9	207	60	3e-71	207
PeDREB3	59.9	197	59	7e-70	205
GmDREB3	56.73	208	63	7e-70	202
BrDREB3	58.25	194	64	1e-69	204
CaDREB3	59.67	181	54	1e-69	201
BnDREB3	60.56	180	53	5e-65	191
VvDREB3	48.79	248	70	7e-64	190
StDREB3	62.01	179	44	2e-63	187
BvDREB3	52.28	241	67	8e-63	187
GsDREB3	61.99	171	46	7e-62	183
SlDREB3	59.36	187	46	1e-61	182
NsDREB3	60.73	191	34	3e-61	181

Abbreviation: *Cs* (*Cucumis sativus*); *Cme* (*Cucumis melo*); *Fv* (*Fragaria vesca*); *Pm* (*Prunus mume*); *Eg* (*Eucalyptus grandis*); *Mn* (*Morus notabilis*); *Csi* (*Citrus sinensis*); *Md* (*Malus domestica*); *Csa* (*Camelina sativa*); *At* (*Arabidopsis thaliana*); *Nn* (*Nelumbo nucifera*); *Pe* (*Populus euphratica*); *Gm* (*Glycine max*); *Br* (*Brassica rapa*); *Ca* (*Cicer arietinum*); *Bn* (*Brassica napus*); *Vv* (*Vitis vinifera*); *St* (*Solanum tuberosum*); *Bv* (*Beta vulgaris*); *Gs* (*Glycine soja*); *Sl* (*Solanum lycopersicum*); *Ns* (*Nicotiana sylvestris*).

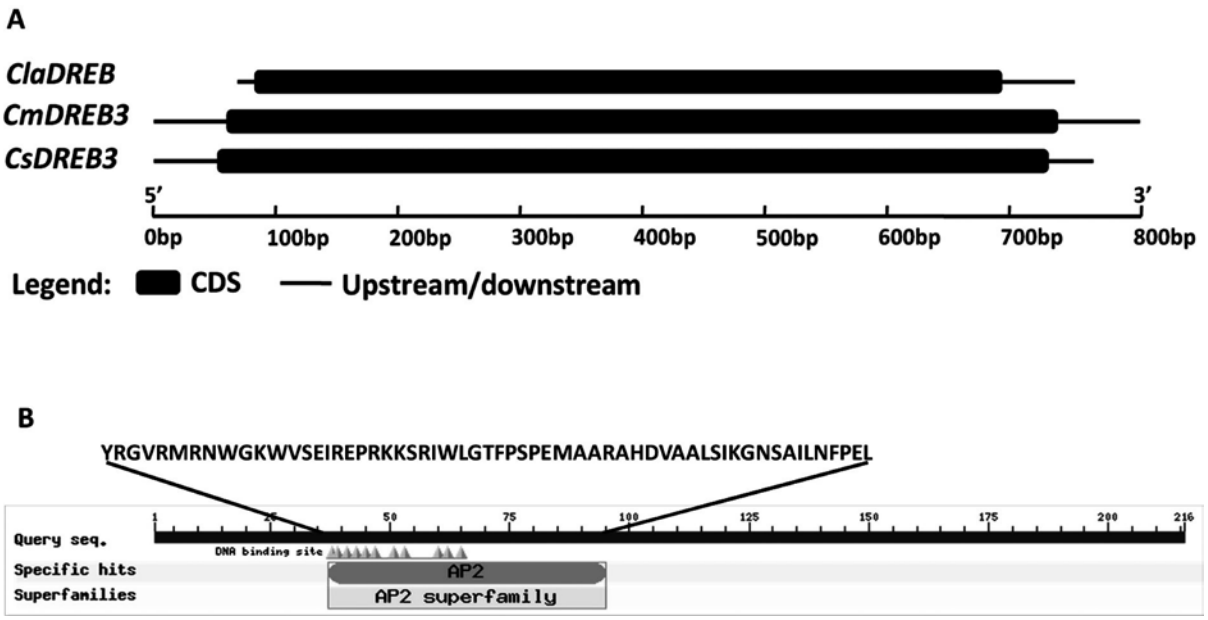

Fig. 2. Structural analysis of *ClaDREB*-like gene and protein. A, represents the structural organization of *ClaDREB*-like gene and comparison with *CsDREB3* and *CmDREB3* gene. B, represents the AP2/ERF domain present in *ClaDREB*-like protein with consensus sequence.

The characteristics features of *ClaDREB*-like gene, *i.e*., 3 Beta sheet and a antiparallel alpha helix with two functional conserved amino acids, valine and glutamine, at 14 and 19 positions, respectively in Beta sheet was found similar to reported findings (Sakuma *et al*., 9; Lata *et al*., 7).

For evaluating the evolutionary relationships between *ClaDREB*-like protein of watermelon and DREB proteins of other plants, a Neighbor-Joining, unrooted phylogenetic tree with 1,000 bootstrap replicates was generated. The phylogenetic analysis grouped them into three major distinct clusters. Predictably, *ClaDREB*-like protein clustered with other cucurbit DREB protein (Fig. 3).

Question arose that whether *ClaDREB*-like gene contains same DREB responsive *cis*-regulatory elements as described in other plants. To get insight into this query, a 1.5 kb upstream region as a putative promoter of *ClaDREB*-like gene was identified in cucurbit genomic database. The putative promoter was subjected to search for *cis*-regulatory elements using PlantCare. The analysis revealed many drought responsive *cis*-regulatory consensus sequences (*TAACTG*, *CANNTG*, *WAACAA*, *CNGTTR*, *YAACKG*, *ACTGT*, *ACGT* and *CATGTG*) in the putative promoter region. The presence of such elements in the promoter indicates its strong possibility in drought-inducibility. The similar drought responsive elements were described in *Arabidopsis thaliana* (Abe *et al*., 1; Simpson *et al*., 11) and rice (Oh *et al*., 8).

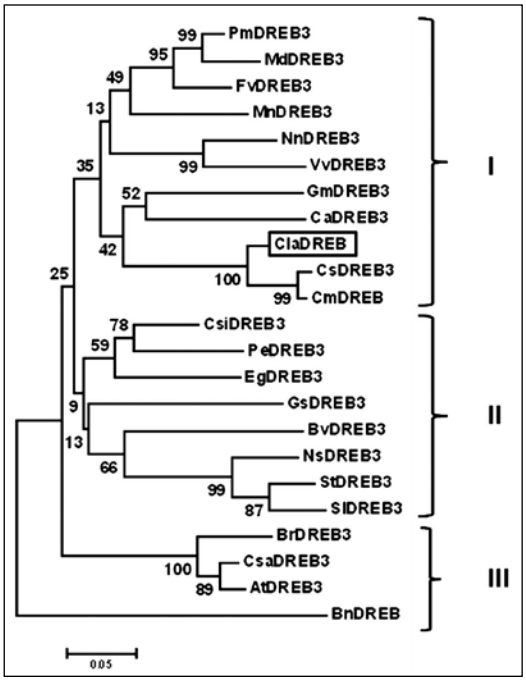

Fig. 3. Phylogenetic tree analysis among ClaDREB-like and DREB3 protein of other plants. For tree construct the Neighbor-Joining method with bootstrap consensus tree inferred 1000 replicates was taken to represent the evolutionary history of the *DREB* genes. The value on braches indicates percent confidence of bootstrap. The ClaDREB-like protein is indicated in rectangular box.

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