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CHARACTERIZATION OF ABIOTIC STRESS GENES FROM DIFFERENT SPECIES OF *EUCALYPTUS*

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Abstract

The stresses causing dehydration damage to the plant cell like cold, drought, and high salinity are the most frequent environmental stresses that influence plant growth, development and restraining productivity in cultivated areas world-wide. Many drought, salinity and cold inducible genes causing tolerance to environmental stresses in many plants include *Dehydrin1 (DHN1), Dehydrin2 (DHN2), Dehydrin10 (DHN10), putative phosphate transporter (Ecpt2), choline monooxygenase (CMO)* and *DREB/CBF1c* genes. Gene specific primer pairs were designed for each gene using DNAStar software. These genes were amplified from different species of eucalyptus such as *Eucalyptus camaldulensis, E. globulus, E. tereticornis* and *E. gunii* through PCR. *Dehydrin2* gene of *E. camaldulensis* and *dehydrin10* gene of *E. globulus* were cloned using the TA Cloning® Kit with pCR®2.1 vector and sequenced. The *Dehydrin* genes sequences were submitted to GeneBank: *Eucalyptus globulus dehydrin10* gene (Accession No. HG915712) and *E. camaldulensis dehydrin 2* gene (Accession No. HG813113). The amino acid sequence of *Dehydrin10* from *E. globulus* showed 97% homology to *E. globulus DHN10* (JN052210) and *Dehydrin2* from *E. camaldulensis* presented 94% homology to *E. globulus DHN2* (JN052209). These genes can be employed in generating drought resistant crop plants.

Key words: Drought, Cold, Salinity tolerance, *Dehydrin* gene, *Eucalyptus*.

Introduction

The genus Eucalyptus belongs to the Myrtaceae family and is the second most important forestry plant that consists of approximately 734 species and 3 hybrids growing in different regions. *Eucalyptus* is native to Australia and introduced in Asia, Africa, America and South Europe with minimum 12 million of hectares planted (Carbonnier *et al.,* 2004). It is a commercially important woody tree for paper pulp as it is major source of cellulose, timber and in furniture industries.

Several species of the *Eucalyptus* genus are used for medicinal values in the Chinese folk medicine. The leaves of the *Eucalyptus* species are traditionally used as anti-inflammatory, antipyretic remedies, analgesic for symptoms of the respiratory infections, such as cold and sinus congestion (Sen-Sung *et al*., 2009). It is vital source of essential oil production, which has the medicinal and pharmaceutical importance (Hamed *et al.,* 2012).

Plants exposed to environmental stress causes is an adverse effect on plants that prevents the normal functions of a biological system. The plant stresses involve, abiotic stresses like drought, cold, heat, salinity, chemicals and pollutant, wind, radiations, nutrient deprivation and biotic stresses like insects, pathogen, herbivores, rodents (Jones & Jones, 1989). The drought and salinity are broadly occurrence in various regions and gradually raise day by day (Burke *et al.,* 2006). The abiotic stresses result on negative effects in the plant growth development and quality of their products. (Chinnusamy *et al.,* 2004).

The plant species respond to these circumstances by the gathering of physiological, morphological, molecular and the changes in biochemical reactions that support plants for reproduction and survival (Fernández *et al*., 2006). When the plant exposed to abiotic related stresses, a variety of the genes with various tasks are suppressed and induced. These proteins might be characterized into two groups. The first group comprise functional proteins viz. antifreeze proteins, late embryogenesis abundant (LEA) proteins, molecular chaperones, the key enzymes for biosynthesis of osmolytes like sugar and sugar alcohols, proline, betaines, water channel proteins, detoxification enzymes and membrane transporters which are rightly linked with the protection of plants from harsh effects of abiotic stress. The second group included proteins that are regulatory in the nature and further regulate stress-responsive gene expression and signal transduction. These include several transcription factors, protein kinases, enzymes involved in metabolism, and other signaling molecules. Therefore, it is important to study and discover new abiotic stress genes to promote stress tolerance in *Eucalyptus* or in others plant species via genetic transformation (Silva *et al.*, 2013).

The gene *Dehydrins* identified to the group 2 LEA proteins (i.e. Late embryogenesis abundant) accumulate in responses of both low temperature and dehydration (Close, 1997). Usually abiotic stresses induced by Dehydrins subgroup of LEA protein family which include cell desiccation and it is widely studied group. It contain the lysine-rich K-segment, 15-amino acid motif (EKKGIMDKIKEKLPG), (Ramanjulu & Bartels, 2002; Bartels & Salamini, 2001). The proteins related to dehydrin present in numerous woody plant species like *Eucalyptus*, birch, pistachio, *Rhododendron*, blueberry and peach during autumn, attaining maximum level during winter concurring maximum freezing resistance (Fernandez *et al.,* 2012).

Eucalyptus species are used for a variety of amenity and wood and tolerant for water logging, salinity, cold and drought. The origin and variation of various traits of eucalyptus is large, so collection of the stock is significant when planting. It is grown widely; so much of its silvicultural and pest information is recognized (Potts & Dungey, 2004). In the current study isolation, identification and characterization of stress related genes from four *Eucalyptus* species: *Eucalyptus camaldulensis, E. globulus, E. gunni, E. teriticornis* was carried out using different bioinformatics tools.

Materials and Methods

Collection of plant material: The young leaves of four different *Eucalyptus* species (*E. camaldulensis, E. globulus, E. gunni* and *E. teriticonis*) were collected from different areas of Lahore, Pakistan.

DNA extraction and primer designing: Genomic DNA was isolated from young leaf tissues of *Eucalyptus* species by using CTAB method (hexadecyltrimethyl ammonium bromide) (Doyle & Doyle, 1990; Stewart and JrVia, 1993) with some modifications. One g of leaves were chopped in liquid nitrogen to a fine powder and collected in 2 ml eppendorf. Preheated Extraction buffer (20 mM EDTA, 2%CTAB, 100 mM Tris–HCl, NaCl and 2% β–mercaptoethanol) was added to this eppendorf tube and incubated at 65°C for 30 min. mixed with chloroform–octanol (24: 1 v/v) and centrifuged at 7000 rpm for 10 min. The aqueous layer was separated, mixed with an equal volume of chilled isopropanol and centrifuged, later on pellet was washed with 70% ethanol and dried. Then stored in TE buffer pH 8.0 (Tris–HCl 10mM; EDTA 1mM) to dissolve DNA pellet. The DNA concentration was determined by UV-spectrophotometer (at 260 nm). The forward and reverse primers were designed (Table 1) to amplify full length *dehydrin 1 gene, dehydrin 2 gene, dehydrin 10* gene, *choline monooxygenase* gene, *Ecpt* 2 *(putative phosphate transporter*) gene, and *DREB/CBF1c* genes (Tables 1, 2).

Table 1. *Eucalyptus* gene specific primers.

DHN 1Forwad	GAG CGT GAT TTC TTG GTC CTT AGT
DHN1Reverse	ACA AAT ACG TAC CCC CGC TTA GAT
DHN2 Forward	GCC ACC CAG CTT ATT CCT CAG
DHN2 Reverse	AAA GGC GGT TCT CCC CCT CAG T
DHN10Forward	CCC AGG GCA AAC CAG AAT CAA ATC
DHN10Reverse	GCA AGA ACC AAA CGC ACC CTC AGA
CMO Forward	GTA CCG GAA GCA ACC AAA AAC C
CMO Reverse	TCC TCA CCG AAC AAG ATA CC
CBF1 Forward	CCA GCA GTC CAG CAC GAG CAC ATT
CBF1 Reverse	TAA CGG CAC GCC CTA AAA GAT TGG
EcPT2 Forward	ATG GCT AAA GAA AAT CTT GGG GTG C
EcPT2 Reverse	CTA ACC ACC AAA TGG TCC CAA TG

PCR amplification: The PCR reactions were performed in 25 µl volume with reaction mixture containing 2 µl of 20ng μL^{-1} genomic DNA, 2.5µl 10x PCR buffer, 1 µl $MgCl_2$(50 mM), 2 µl of 2.5mM dNTPs, 0.6 µl each primers(10 mM) and 0.2µl Taq polymerase (Thermo Scientific Gene JET PCR Purification Kit CAT. # 00129583). The PCR amplifications were initiated by 95°C for 5 min followed by 35 cycles consisting of denaturation at 95°C for 30 s; annealing at 58°C for 45s, extension at 72°C for 1:30 min; and a final extension of 7 min at 72°C (Primus 96 Programmable thermal cyclers). The PCR products were visualized on 0.8% agarose gel with molecular DNA marker (Fermentas life science) stained in ethidium bromide. The amplified PCR products were cloned in InsTA clone™ PCR Product Cloning Kit (CAT # 1275240) from Invitrogen Life Technologies.

Cloning of PCR product and sequence analysis: The PCR product was ligated in T/A cloning vector and transferred into *E.coli* (DH5α strain). The white colonies were used to isolate plasmid and screened for the presence of insert by double restriction (Fig. 3). The positive clones were sequenced (Macrogen Company). The consensus sequence was generated from plus and minus strand sequence by using DNASTAR seqman software. The final sequence was submitted to EMBL database (http://www.ebi.ac.uk/ena). The gene sequences were run on BLAST n search of NCBI Gene Bank and selected sequences were used from results of BLAST n to generate phylogenetic tree.

Results and Discussion

We have characterized Dehydrins gene *dehydrin 1, dehydrin 2, and dehydrin 10* gene, *putative phosphate transporter* gene, *DREB/CBF1c* and *choline monooxygenase* gene from different *Eucalyptus* species such as *E. camaldulensis, E. globulus, E. tereticornis* and *E. gunii.* Dehydrin gene is responsible for abiotic stress resistance in plants. It is involved in drought, cold and salinity tolerance. The *dehydrin1* gene fragment of approximately 959 bp was amplified through PCR from *Eucalyptus camaldulensis*, with the designed forward and reverse primers.

Table 2. Stress Genes of *Eucalyptus*.

Sr. No.	Gene	Accession No.	Gene size	Product size	Primer range	Tem (°C)
1.	*Dehydrin 1* (DHN 1)	JN052208	1575 bp	959 bp	F: 5′ 523-546 3′ R: 3′ 1482-1505 5′	63.5
2.	*Dehydrin 2* (DHN 20)	JN052209	2130 bp	1171 bp	F:5′ 783-803 3′ R:3′ 1958-1980 5′	63.3
3.	*Dehydrin 10* (DHN 10)	JN052210	730 bp	525 bp	F:5′ 118-141 3′ R:3′ 643-666 5′	65.1
4.	*Putative phosphate transporter* (Ecpt 2)	AB242817	1865 bp	1843 bp	F:5′ 1 - 25 3′ R:3′ 1853-1865 5′	63
5.	DREB/CBF1c (CBF)	EU794855	1146 bp	868 bp	F:5′ 46 -69 3′ R:3′ 914-937 5′	65,3
6.	*Choline monooxygenase Gene* (CMO)	JN616285	4036 bp	3925 bp	F:5′ 37 -58 3′ F:3′ 3962-3981 5′	58.4

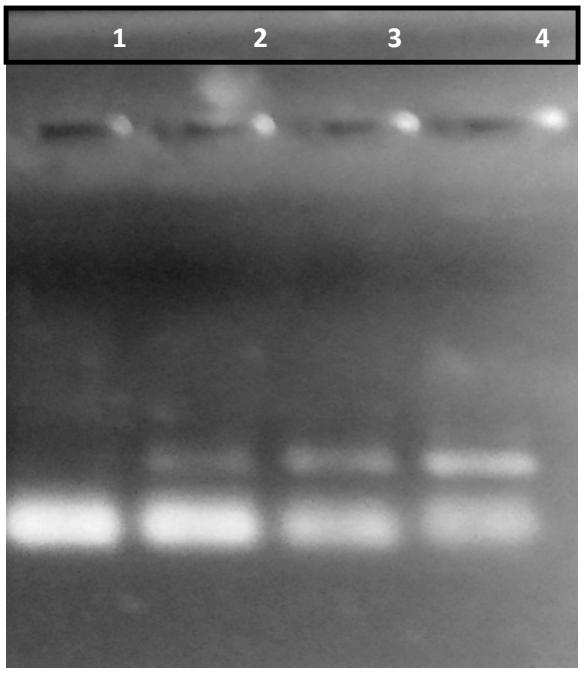

Fig. 1. The *dehydrin10* gene fragment of approximately 525 bp was amplified through PCR from *Eucalyptus gunii* (lane 1), *Eucalyptus tereticornis* (lane 2), *Eucalylptus globulus* (lane 3), *Eucalyptus camaldulensis* (lane 4) with the gene specific primers.

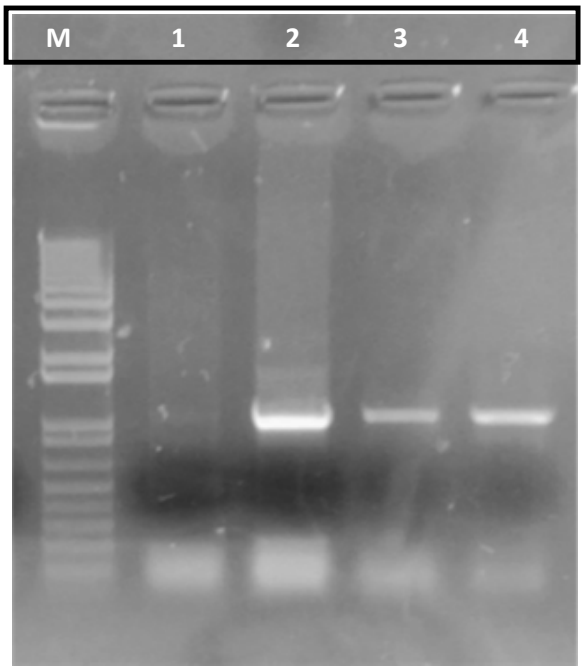

Fig. 2. The *dehydrin2* gene fragment of approximately 2.1 kb was amplified through PCR from *Eucalyptus gunii* (lane 1), *Eucalyptus tereticornis* (lane 2), *Eucalylptus globulus* (lane 3), *Eucalyptus camaldulensis* (lane 4) with the gene specific primers.

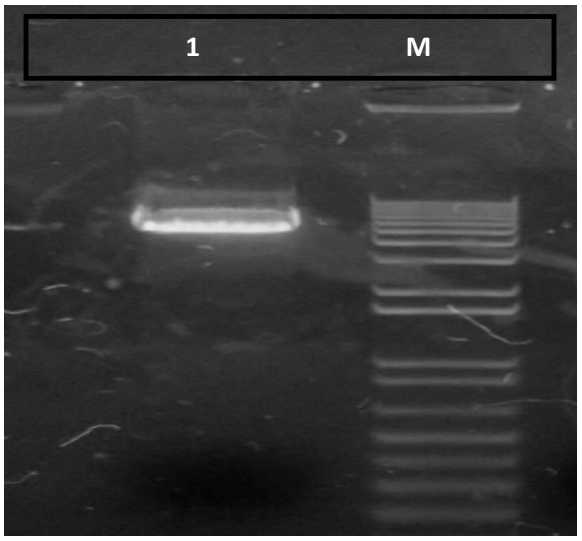

Fig. 3. Single Restriction of T/A Cloned *dehydrin 2* gene from *E. globulus* with *Hind III* and EcoR1 (lane 1), DNA ladder (lane M).

The gene *dehydrin 10* of *Eucalyptus globulus* was amplified via polymerase chain reaction (Fig. 1), then cloned inside the cloning vector pCR 2.1 and transferred into *E.coli* DH5α strain. The sequenced gene was consists of 549 nucleotides encoding a 98 residues. The protein consisted of two functional domains, with a calculated molecular mass of 10.94 kDa and pI value of 6.45, it belong to KS group of dehydrin. Similarly Fernandez *et al.* (2012) isolated Eugl DHN10 (Genbank Accession: JN052210) having an open reading frame of intron less 294 bases, 98 amino acids with calculated pI value of 6.87 and deduced molecular mass of approximately 11 kDa, this Dehydrin belongs to the KS group.

The *dehydrin 2* gene of *Eucalyptus camaldulensis* was amplified through PCR (Fig. 2), then cloned inside into pCR 2.1 cloning vector and transferred in *E. coli* DH5α strain. The sequenced DNA segment has 1051 bp containing one intron and a coding sequence of 777 nucleotide bases that consists of 259 amino acids, molecular mass of 29.47 kDa and pI value of 5.20, it belong to SK2 group of dehydrin. These results are strongly agreed with the study of Fernandez *et al.* (2012) isolated *EuglDHN2* (JN052209) is 2.130 bp long; A coding sequence consisting of 777 nucleotide bases and is composed of about 259 nucleotide bases. It is about 130 base pair in length, has an intron of about 113 bp in length and a coding sequence consisting of about 774 bases which code for 258 amino acids with a 30kDa molecular mass, and an isoelectric point calculated to be 4.82, it belongs to SK2 group.

Sequencing of *dehydrin 2* & *dehydrin 10* genes: Recombinant plasmid DNA were prepared from the confirmed positive screened clones of *dehydrin* genes and sequenced using M13 forward and reverse primers. The sequences of these *Dehydrin* genes were submitted to GeneBank under accession no. HG915712 and *E. camaldulensis dehydrin 2* gene under accession no. HG813113. *E. globulus dehydrin 10* gene shows 99% homology with *E. globulus dehydrin 10*, and *E. camaldulensis dehydrin2* gene shows 94% homology with *E. globulus dehydrin 2* gene. The amino acid sequence of Dehydrin 2 *E. camaldulensis* and Dehydrin 10 *E. globulus* were compared with previously sequenced Dehydrin and phylogenetic relationship among them were determined.

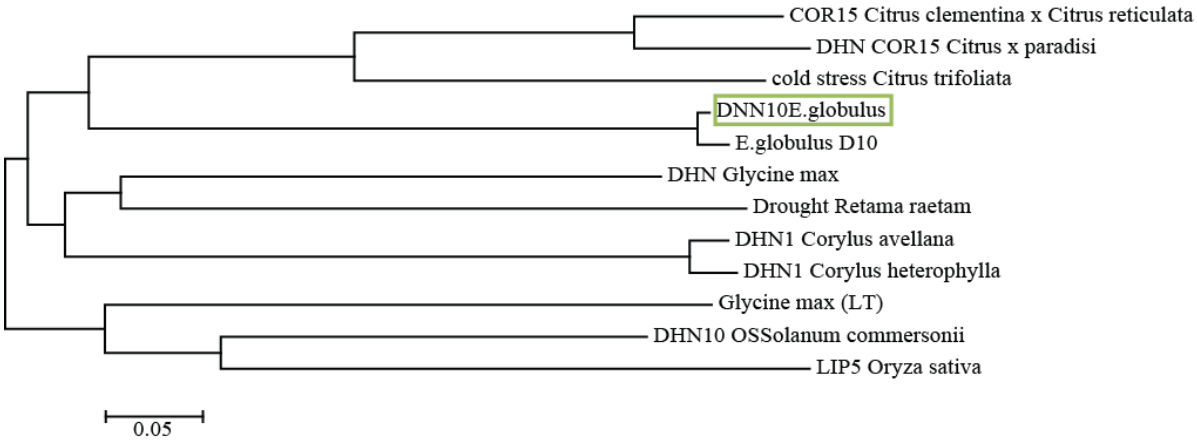

Fig. 4. Phylogenetic relationship among Dehydrin proteins from Dehydrin 10 *E. globulus* protein. The tree was generated by ClustalW and neighbor-joining algorithms using MEGA 2.1 software.

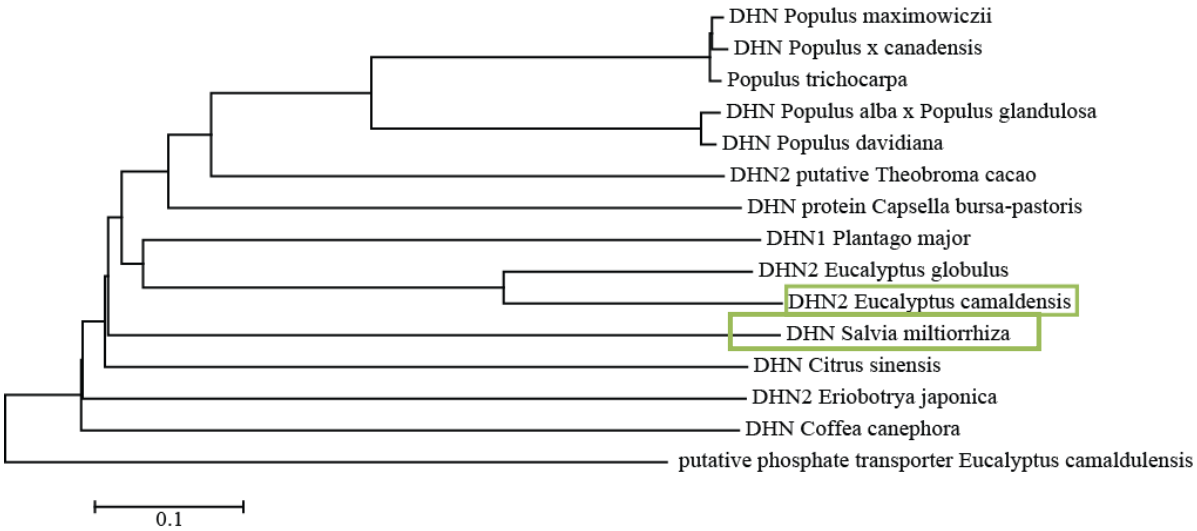

Fig. 5. Phylogenetic relationship among Dehydrin proteins with *E. camaldulensis* Dehydrin 2. The tree was generated by ClustalW and neighbor-joining algorithms using MEGA 2.1 software.

The Dehydrin 10 *Eucalyptus globulus* (Fig. 4) presented 98% homology with *E. globulus* Dehydrin10 (AER27689.1), reported by Fernandez *et al.,* 2011 from Chile region and this outgroup shows 64% homology with cold stress protein *citrus trifoliate* (gb|AAA99963.1|), 64% low temperature inducible SLT166 *Glycine max* (AB070349.1), 58% with LIPS *Oryza sativa* (BAA24979.1), 55% and 54% with Drought induced protein in *Retama raetam* and dehydrin 1 *Corylusavellana*,*-paradise*. The gene KS-type dehydrin (K-segment containing lysine) EUGLDHN10 is present as a multicopy gene which is alike to the core 15 gene in *Citrus* genome (Porat *et al*., 2002).

The *Eucalyptus camaldulensis* Dehydrin 2 (Fig. 5) show 94% homology with *E. globulus* Dehydrin 2 reported by Fernandez *et al*., 2011 from Chile region, and this outgroup show 61% homology with Dehydrin1 *plantago major*, 62% homology Dehydrin *salvia miltiorrliza* and Dehydrin protein *capsella burea-pastoris,* 63% with dehydrin *Populus alba x Populus glandulosa*, 61% with *Populus maximowiczii* and *Populus davidiana*, 46% with *coffea canephora*. A distinct gene present in the genome of *E. globulus* encodes EuglDHN10 which is also seen in the gene ccDH2DHN in the *C. canephora* genome (Hinniger *et al*., 2006) and the gene csDHN in *Citrus sinensis* (Porat *et al*., 2004).

The absence of glycine-rich repeat and acidic nature of EuglDHN2 recommend that it belongs to acidic dehydrin subfamily (Danyluk *et al.,* 1998). It had been reported in wheat that it preferentially associated in the sensitive vascular transition area with plasma membrane where cold-induced dehydration is expected to be most severe. Danyluk *et al.* (1998) reported that this acidic Dehydrin may play an imported role under dehydrative conditions in preventing destabilization of plasma membrane. Moreover, the hydrophilic nature of EuglDHN1 and EuglDHN2 polypeptides is well suitable to stabilize membranes and replace water during dehydration through polar interactions.

E. globulus Dehydrin10 (Fig. 6) presents 97% similarity with a Eugl DHN10 (JN052210) and 62% similarity with a Dehydrin10 from the *Solanum commersonii* (ACJ26759).

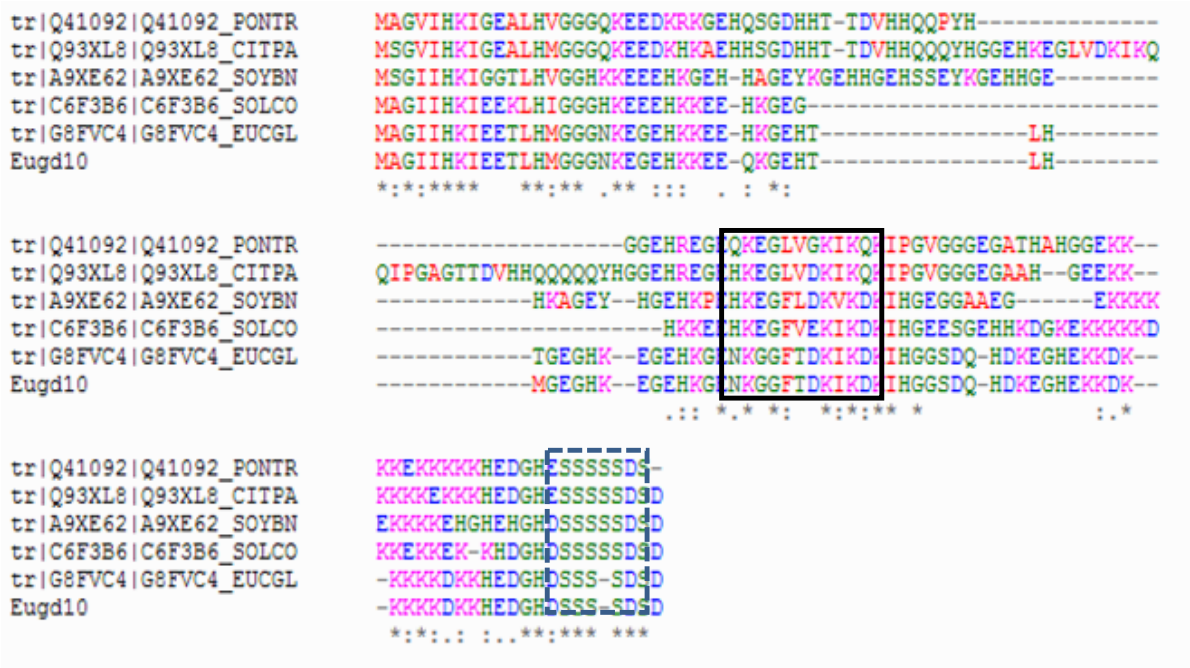

Fig. 6. The optimized alignment of *E. globulus* Dehydrins with Dehydrins homologues. This amino acid alignment was made using the Clustal W program and then optimized further manually. The shaded block shows identical amino acids. The rectangles with broken lines shows the S segments and the solid dark rectangles demarcate the K segments.

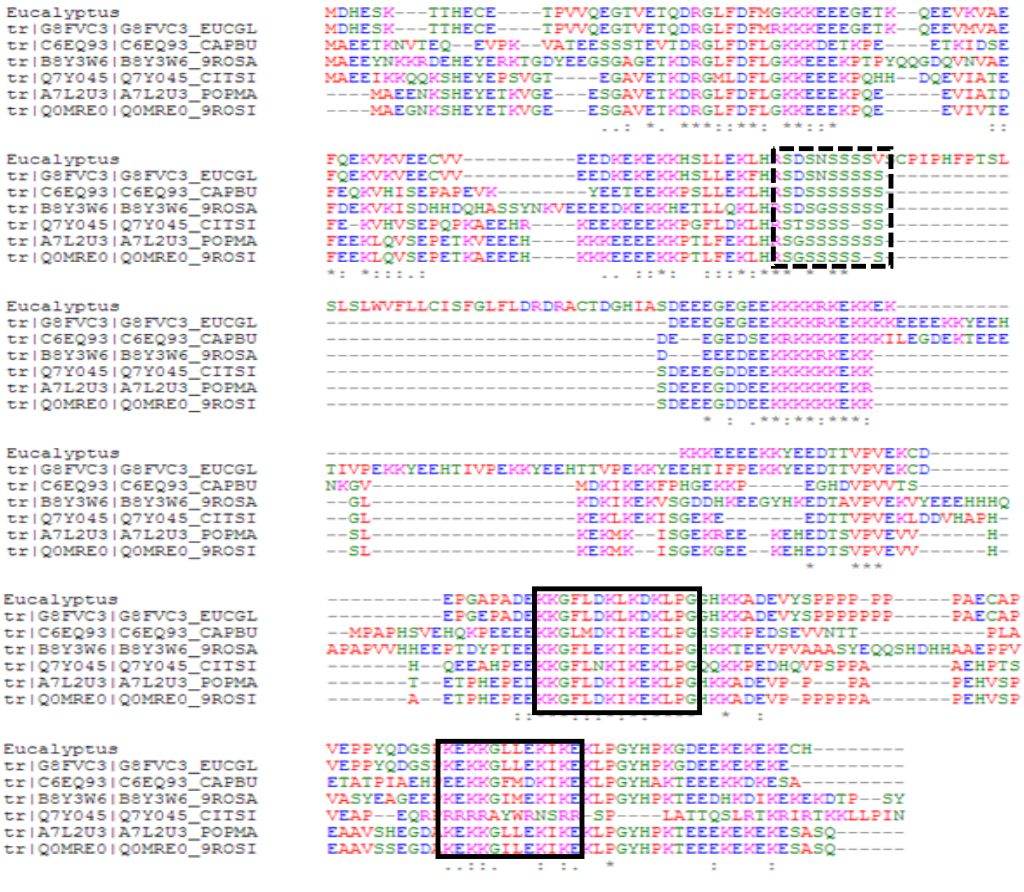

Fig. 7. The optimized alignment of *E. globulus dehydrin* with DHNs homologues. This amino acid alignment was generated using the software and then optimized further manually. The Shaded blocks show identical amino acids.

In the present study, analysis of EuglDHN10 protein showed that the Histidine residue composes 12.2% of the total content of amino acids. Also, the EuglDHN10 residues had a high level of transcript in the tissues of stem. This protein EuglDHN10 might be involved in the long distance transport mediated by the phloem which has been reported in castor bean DHN (Kruger *et al*., 2002). *Solanum sogarandinum* and *Solanum tuberosum* report the same phenomenon which provides support to the supposed role of EuglDHN10 in transport of iron, and might also be involved in the mechanism providing protection against oxidative stress (Rorat *et al*., 2004) in cold. *E. camaldulensis* Dehydrin2 shows (Fig. 7) 94% similarity with EuglDHN2 (JN052209) and shows 53 and 51% similarity with two poplar DHNs, *Populus alba* × *Populus tremula var. glandulosa* dehydrin (ABH11546) and *Populus maximowiczii* dehydrin (ABS12346) respectively.

EuglDHN1 and EuglDHN2 are hydrophilic polypeptides which substitute water and also contribute towards stabilization of membranes by forming polar interactions during the process of dehydration. The KS-type DHNs have a physiological role of the iron binding quality of a castor bean (*Ricinus communis*), DHN (Kruger *et al*., 2002) along with *Citrus unshiu* DHN which is rich in His residues (Hara *et al.,* 2005). His residues do not much protein, and they build up only 2% of amino acids (Ueda *et al*., 2003). The *dehydrin 10 Eucalyptus globulus* gene consist of 98 amino acids (Fig. 8), has a change in position number 28 and 36, Gln (Q) with Histidine (H) and Met (M) with Thr (T) from *E. globulus* DHN10 (AER27689).

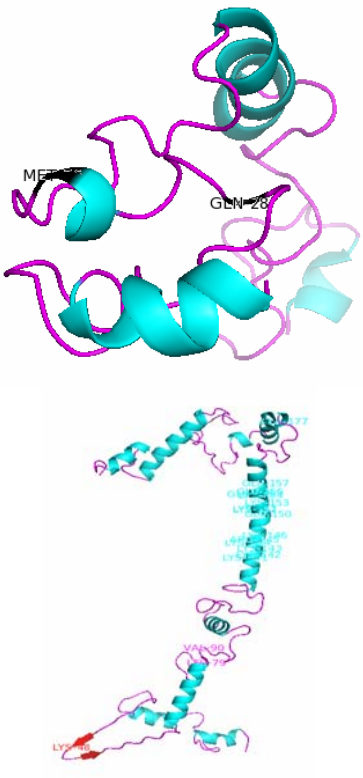

Fig. 8. Predicted 3D structure of Dehydrin 10 *Eucalyptus globulus* and Dehydrin2 *Eucalyptus camaldulensis* using SCARTCH.

The *dehydrin 2 E. camaldulensis* gene consists of 259 amino acids, (Fig. 8) change in position 48 Lys(K) with Met (M), 79 Leu (L) with Phe (f), 90 Val (V) with Ser (S), from 141 to 146 KKKKKK with EHTTVP, from 150 to 157 EKKKKEEE with YEEHTIFP, at position 177Ala (A) with Glu (E) from dehydrin *E. globulus.* It had been suggested that Dehydrin are highly evolved proteins, through conformational modifications in their K segment, might stabilize cellular membranes beneath stress conditions (Koag *et al.,* 2003) and selected to sustain maximum configurational flexibility to resist unspecific aggregation and collapse (Mouillon *et al.,* 2006; Koag *et al.,* 2009).

Conclusion and Future prospects

Abiotic stress signaling is an important area with respect to increase in plant productivity under adverse conditions. The products of these genes may be characterized for their stress tolerance levels and potent could be further used for transgenesis of stress tolerance in economically important crops.

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