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Molecular cloning and characterization of salt inducible dehydrin gene from the C4 plant *Pennisetum glaucum*

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ABSTRACT

Dehydrins (DHNs) or group 2 LEA (late embryogenesis abundant) proteins play a protective role in plants under different abiotic stress conditions like drought, salinity, cold and heat stress. DHNs are expressed in late embryogenesis and accumulate in vegetative tissues in response to desiccation stress in all photosynthetic organisms. Here we report the cloning and characterization of a *PgDHN* gene from the C4 plant *Pennisetum glaucum*. The PgDHN cDNA encoded for a polypeptide of 133 amino acids with an estimated molecular weight of 13.87 kDa and isoelectric point of 6.81. The protein sequence analysis of PgDHN classified it into the YnSKn subgroup of dehydrins. Phylogenetic analysis revealed that PgDHN is evolutionarily related to a *Setaria italica* DHN. In silico sequence analysis of the PgDHN promoter identified a distinct set of *cis*-elements and transcription factor binding sites. *PgDHN* mRNA accumulated in leaves of *P. glaucum* upon treatment with NaCl stress. Recombinant PgDHN transformed *E. coli* cells showed improved tolerance and exhibited better growth rate under high salt concentration (750 mM) and heat stress in comparison to their respective controls. Heterologous expression of PgDHN in transgenic yeast showed increased tolerance to multiple abiotic stresses. This study provides a possible role of PgDHN in stress adaptation and stress tolerance in pearl millet.

1. Introduction

Plants are subjected to multiple abiotic stresses like temperature, salinity, cold, and drought at different stages of their development due to their sessile nature. These stresses are responsible for huge crop losses worldwide, both quantitatively and qualitatively (Oerke, 2006). It has been estimated that the average yields for most major crops are curtailed by more than 50% due to the abiotic stresses (Bray et al., 2000). The situation is going to exacerbate, with salinization alone expected to reduce the cultivable land by 50% by the year 2050 (Wang et al., 2003). In addition, the earth's average temperature is anticipated to rise by 1.5–5.88 °C in the 21st century, severely hampering crop productivity (Ruan et al., 2012). The negatively impacting climate conditions coupled with an unprecedented increase in global food demand due to staggering population growth have made it imperative to develop stress-tolerant crop varieties.

Different abiotic stress factors usually are interconnected and cause similar damage at the cellular and molecular level, which result in various morphological and physiological abnormalities of plants (Wang et al., 2000, 2003). Salinity and drought stress are perceived as signals of water deficiency by plants. The presence of high salt concentrations in soil reduces its water potential, thereby limiting water availability (Hasegawa et al., 2000) and causing osmotic stress (Evelin et al., 2009). Furthermore, the problem of the high NaCl concentration outside plant cells in solution also cause ion toxicity and nutrient imbalance, which are debilitating for plants (Evelin et al., 2009). Various abiotic stresses including drought, salinity, cold, heat and chemical pollution culminate into oxidative stress and trigger defence mechanisms in plants (Gill and Tuteja, 2010). Plants express a number of different set of genes to combat these stresses. These include genes involved in signalling pathways like MAP kinases, SOS kinase, transcription factors such as the CBF/DREB and ABF/ABAE families (Li et al., 2013), genes encoding enzymes for the biosynthesis of osmoprotectants like proline and sucrose, enzymes for scavenging reactive oxygen intermediates, heat shock proteins (HSPs), late embryogenesis-abundant (LEA) proteins (Mahajan and Tuteja, 2005), enzymes modifying membrane lipid saturation, proteins required

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for ion homeostasis (Zhang et al., 2000), and small RNAs (Atkinson and Urwin, 2012).

LEA proteins are profusely expressed during the desiccation phase of seed maturation and also accumulate in the vegetative tissues of different plant species in response to ABA, drought, salinity, cold and freezing stress (Hanin et al., 2011; Reddy et al., 2012). LEA proteins are categorized into various structural groups. One of the groups, named LEA II, containing the dehydrins, is present in all photosynthetic organisms (Puhakainen et al., 2004). Dehydrins are classified as proteins possessing 1–11 copies of a lysine rich conserved K-segment (EKKG IME/DKIKEKLPG) near their C terminus (Hanin et al., 2011). The other two conserved sequences of amino acids found in DHNs are the tyrosine-rich Y-segment (consensus (V/T) D (E/Q) YGNP) and a serine-rich S-segment (Hanin et al., 2011). The Y-segment is found in 1–3 copies near the N-terminus, whereas the S-segment is a phosphorylatable patch of 4–10 serine residues, part of a conserved sequence LHRSGS4-10(E/D)3 (Hanin et al., 2011). Recent studies have shown that desiccation and salt are the leading stresses resulting in higher expression of YnSKn type dehydrins. Whereas the Kn, SKn, and KnS proteins are mainly upregulated by cold stress, although a few are upregulated by desiccation and salt too (Graether and Boddington, 2014). In vitro studies and localization experiments have suggested the involvement of dehydrins in diverse functions, including membrane stabilization, cryoprotection of enzymes, and protection from reactive oxygen species (Graether and Boddington, 2014). The expression pattern of dehydrins in different species indicates that the Y-segment plays a more important role in protection from drought and salt stress as compared to cold stress. The reason behind this could be that the Y-segment is not involved in membrane binding and since the cold stress is predominantly more damaging to the membranes (Steponkus, 1984). Therefore, the role of Y-segment could be to provide more resistance to desiccation and salt stress than to cold (Graether and Boddington, 2014).

Transgenic plants overexpressing DHNs have shown enhanced tolerance to a variety of abiotic stresses. Recently, it has been reported that transgenic rice plants overexpressing *OsRab16A* gene (belonging to group II Lea/dehydrin family) performed better than the control plants when subjected to salinity stress (Ganguly et al., 2012). Transgenic *Arabidopsis* plants overexpressing *RcDHN5* (a dehydrin from *Rhododendron catawbiense*) showed enhanced tolerance to freezing stress (Peng et al., 2008). Similarly, the expression of *DHN24* from *Solanum sogarandinum* showed improved tolerance to cold stress in transgenic cucumber seedlings (Yin et al., 2006). DHNs have also been implicated in conferring resistance to salt, osmotic and drought stress in plant species including *Arabidopsis*, tobacco and rice (Brini et al., 2007; RoyChoudhury et al., 2007; Cheng et al., 2002). Furthermore, it has been shown that the expression of dehydrins *BjDHN2* and *BjDHN3* from *Brassica juncea* provide resistance to heavy metal (Cd^{2+} and Zn^{2+}) stress in transgenic tobacco plants (Xu et al., 2008). The above studies establish the role of dehydrins in combating various abiotic stress conditions.

Pennisetum glaucum (*P. glaucum*), is a C4 plant, commonly known as 'pearl millet'. It is the most widely cultivated type of millet. It is a stress tolerant crop and is well adapted to fields with limited soil fertility, drought, and heat stress conditions. Therefore, owing to its stress resistant nature, *P. glaucum* is contemplated to be equipped with better defence mechanisms to combat different abiotic stresses. In the present study, we have isolated a dehydrin gene from *P. glaucum*, referred to as *PgDHN*. The expression of *PgDHN* is upregulated in response to drought, salinity, heat stress, and cold stress. Its role in rendering tolerance to salinity and heat stress is further indicated by its expression analysis in *Escherichia coli* and yeast cells. These results indicate that *PgDHN* plays a protective role under various abiotic stress conditions and could be used as a tool to improve the abiotic stress tolerance of crop plants along with other stress responsive genes.

2. Materials and methods

2.1. Plant material and stress treatments

P. glaucum seeds were surface-sterilized and grown in vermiculite-containing pots; control conditions included a 14/10-h light/dark cycle under greenhouse conditions. For each type of treatment, two sets of plants were grown, where one set was treated as control (plants were irrigated with water) and the other set was used for various types of stress treatments. For transcript analysis, 14 days old seedlings were exposed to different stress treatments for a variable time duration. Dehydration stress was administered by withholding water from plants. For cold treatment, plants were incubated at 4 °C. For heat stress, seedlings were kept at 45 °C in an incubator. Salt stress was administered via a hydroponic system, wherein seedlings were dipped in a tray containing 250 mM salt solution. After treatment, the seedlings were harvested from both stress and control samples and stored in −80 °C until RNA isolation.

2.2. Cloning of Pennisetum DHN cDNA

An EST clone from the stress responsive EST database (Mishra et al., 2007) that showed maximum homology to the dehydrin gene (GenBank ACC. CD725588) was used as probe for screening the *Pennisetum* salt stress cDNA library using the plaque hybridization method (Reddy et al., 2015). Plaques showing positive signals were purified to homogeneity following two rounds of screening. The positive recombinant cDNA inserts were prepared for sequencing (Reddy et al., 2008b) and submitted to GenBank (GenBank ACC. AY823548).

2.3. PgDHN promoter isolation by using genome walking method

The 5′ flanking genomic sequence region of PgDHN cDNA sequence (GenBank ACC. KM575846) was cloned using the PCR-based directional genome walking method (Reddy et al., 2008a). Two rounds of successive PCR amplifications were done by using walker primers and their corresponding nested primers (DHN1: 5′-CAAGACTGACGGCCTCCTT-3′ and DHN2: 5′ CCAACCAAGCCAACGAGTAC 3′) for the amplification of the flanking genomic DNA fragment (Reddy et al., 2008a). Genomic fragment was cloned into Topo-TA vector (Invitrogen) and completely sequenced at the Macrogen commercial facility. The promoter sequences were screened for putative *cis*-acting elements using PlantPAN (Chang et al., 2008), PLACE (Higo et al., 1999) and PlantCARE (Lescot et al., 2002) databases as well as motifs taken from the literature.

2.4. Protein sequence and phylogenetic analyses

To find out the sequence conservation and functional homology of *DHN* gene in various plant species selected, multiple sequence alignment of selected candidate genes was carried out using ClustalX (version 2.0.8) (http://www.clustal.org/download/2.0.8). To infer the evolutionary history of selected *DHN* genes, an un-rooted phylogenetic tree was constructed employing MEGA version 5.0 using default parameters. The tree was generated by neighbour-joining (NJ) algorithm with p-distance method and gapped pairwise deletion. To test the phylogeny, a bootstrap statistical analysis was performed with 1000 replicates.

2.5. RNA isolation, cDNA synthesis and qRT-PCR analysis

Total RNA was isolated from *Pennisetum* seedlings exposed to different abiotic stress conditions and their corresponding controls using the TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany). First-strand cDNA was synthesized from each RNA sample using first strand cDNA synthesis kit (Invitrogen GmbH, Karlsruhe, Germany) and used for quantitative PCR amplification using specific oligonucleotide primers (5'AGGAGGAAGAAAGGCATCAAG-3′ and 5'TCCTGGATCTTGTCCATG

AGT-3′ for *PgDHN* and 5′-CAAAGGTGGGTGTAGCAAGC-3′ and 5′-CCGA AGGTGTCTGATACT GTGG-3′ for *ß-tubulin* as a housekeeping reference gene). Quantitative real-time PCR was performed in an optical 96-well plate with an iCycler (BioRad, USA) using KAPA SYBR FAST Master Mix (2X) Universal SYBR® Green (Kapa Biosystems, Wilmington). The PCR reactions of all samples were done by following a standard thermal profile: 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplicon dissociation curves were recorded after cycle 45 by heating from 60 to 95 °C with a ramp speed of 1.9 °C min^{-1}. The experiments were independently repeated three times, and the data from these experiments were averaged. The relative change in expression levels of PgDHN transcripts in response to different abiotic stresses was estimated using REST software (Pfaffl et al., 2002) using *ß-tubulin* as the reference gene.

2.6. Cloning, expression and purification of recombinant PgDHN in E. coli

The PgDHN cDNA sequence was PCR amplified using 150 ng of each forward (5′ ACTACATATGATGGAGCACCAGGGGCAGCACG-3′) and reverse (5′-TAATGCGGCCGCTTAGTGCTGCCCGGGAAGCTT-3′) primers with NdeI in forward and NotI site in reverse primer in a 50 μl reaction volume containing 200 μM dNTPs, 1 U Taq DNA polymerase, 1X Taq buffer and 10 ng of PgDHN template. PCR conditions were 94 °C for 5 min; 94 °C for 1 min; 58 °C for 1 min and 72 °C for 1 min for 30 cycles, final extension for 72 °C for 10 min. PCR amplified DNA fragment was digested and ligated into NdeI and NotI sites of pET-28a vector. The PgDHN-pET28a construct was transformed into *E. coli* BL21 (DE3) cells. The expression and purification was done as described earlier (Singh et al., 2012; Reddy et al., 2014).

2.7. Stress tolerance competence of E. coli cells transformed with PgDHN

Stress tolerance assay of *E. coli* cells was performed as described previously (Singh et al., 2012; Reddy et al., 2014). Bacterial cells (*E. coli* BL21 (DE3)) containing pET28a (vector alone) or pET28a-PgDHN plasmids were overgrown in LB broth containing 50 μg/ml kanamycin. One percent inoculums were added from both the tubes to fresh sterile LB broth containing 50 μg/ml kanamycin. The absorbance was monitored at 600 nm; IPTG (1 mM) was added after the OD at 600 nm of the cultures reached 0.2. The bacterial cultures were then administered with different stress conditions i.e. NaCl (0–750 mM) for salinity stress and temperature (37–55 °C) for heat stress. The culture growth was further analysed for a period of 12 h for each stress, individually. Each experiment was performed in three sets and mean values for each stress were calculated.

2.8. Cloning of PgDHN cDNA into yeast expression vector and abiotic stress tolerance studies in yeast

The coding region of *PgDHN* gene was released from pCR8/GW/TOPO TA Vector plasmid with EcoRI and cloned into *Pichia pastoris* expression pPICZ A vector (Invitrogen, USA) at the EcoRI site. The clone with proper orientation was selected for transformation in *P. pastoris* X-33 strain by electroporation according to the manufacturer's instructions (Invitrogen); empty pPICZ A plasmid was also used for *P. pastoris* X-33 electroporation for vector control clones. Electroporated cells were plated on YPDS plates containing 200 mg/l zeocin antibiotic. After incubation of 3 days at 30 °C, several colonies were obtained, these colonies were further patched on YPD selection media (zeocin 200 mg/l) and maintained. Two vector control and two PgDHN clones were inoculated to YPD broth and allowed to grow for 24 h at 30 °C. The OD600 of the cultures were measured and inoculated to 5 ml of BMGY broth to an OD of 0.6 then grown at 30 °C for 24 h with shaking at 200 rpm. Next day the cultures were centrifuged at 4000 rpm for 10 min then the supernatant was discarded, cell pellet was suspended in 5 ml BMMY (methanol induction media) broth and allowed to grow for 24 h at 30 °C. Methanol induced cultures derived from vector control clones and PgDHN clones were inoculated freshly to YPD media to an OD600 of 0.4 with different abiotic stress treatments such as 2.5 M NaCl, 15% poly ethylene glycol (PEG 6000) and heat stress treatment at 40 °C. After 24 h of stress treatments, the OD600 of the cultures were plotted. Simultaneously the cultures were subjected to 1/10 serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) then 3 ul from each dilution of treated (vector control and PgDHN recombinant yeast cells) and their corresponding untreated cultures were spotted on YPD agar and incubated at 30 °C for 3 days.

3. Results and discussion

3.1. Cloning, sequence and phylogenetic analysis of the PgDHN gene

The DHN encoding full-length cDNA sequence was isolated by screening a *P. glaucum* cDNA library using PgDHN EST (CD725588) as a radioactive probe. The full-length cDNA sequence of *PgDHN* gene comprised a 99 bp 5′ untranslated region (UTR), open reading frame (ORF) of 402 bp and a 3′ UTR of 115 bp (Fig. 1; GenBank ACC. AY823548). Analysis of the protein sequence indicated that PgDHN contained 133 amino acids with a calculated isoelectric point of 6.81 and a molecular mass of 13.87 kDa. Further analysis showed that the PgDHN protein contained the characteristic domains of the dehydrin family with serine, tyrosine and lysine rich segments (Fig. 2). The dehydrins can be classified into five different structural subgroups: Kn, SKn, KnS, YnKn and YnSKn, depending on the presence of the K-, S- and Y-segments. The sequence analysis of the PgDHN isolated in this study shows that it belongs to the YnSKn subgroup with one Y segment, one S segment and two K segments (Fig. 2).

The result of BLASTX showed that it was highly homologous to a DHN3-like gene of *Setaria italica* (ACC. KJ000690), DHN1 from *Aegilops tauschii* (ACC. EMT30992), DHN1 from *Hordeum vulgare* (ACC. P12951), and DHN3 from *Brachypodium distachyon* (ACC. XP_003574997). The secondary structure profile predicted by using PSIPRED indicated the presence of 3 beta strands and 2 helices (data not shown). The PgDHN protein sequence alignment showed 80% similarity with dehydrin DHN3-like protein of *S. italica* (Figs. 2 and 3). Since both *P. glaucum* and *S. italica* are C4 plants and members of the same subfamily Panicoideae (in the Poaceae family), this sequence homology is expected. Further, the PgDHN was closely related to *Sorghum bicolor* and *Zea mays*, which are also C4 plants (Fig. 3). *H. vulgare*, *Triticum durum*, and *Lophopyrum elongatum* were clustered into a separate clade as they belong to C3 type plants (Fig. 3). Moreover, the PgDHN was clustered together with all the monocots and the dicots comprising of *Arabidopsis thaliana*, *Vitis rotundifolia*, and *Cochliobolus sativus* formed a separate group (data not shown). The branching pattern of the phylogenetic tree suggests that the dehydrin gene diverged at an early stage in evolution before the dicots and monocots formation.

3.2. In silico promoter analysis of PgDHN gene

In silico sequence analysis of 0.817 kb (GeneBank ACC. KM575846) of the PgDHN gene promoter identified putative *cis*-acting regulatory elements like GTAC-motifs (−220, +/− strand; −285, +/− strand; −291, +/− strand; −477, +/− strand) for anoxic stress responsive expression, Sp1 (−433, − strand; −464, − strand) and G-box motifs for light responsive expression, MBS-motif (−86, − strand) for drought-inducibility, LTR element (−244, + strand) for low temperature responsive expression and ARE element for anaerobic induction in addition to core sequences of ABRE elements (−223, − strand; −283, + strand; −284, + strand; −286, − strand; −287, − strand; −288, − strand; −290, − strand) for ABA responsive expression (Fig. 1). Additionally, several *cis*-acting motifs related to root and endosperm specific elements motif I (−286, + strand) and Skn-1 motif (−551, + strand; −807, + strand) conferring endosperm-

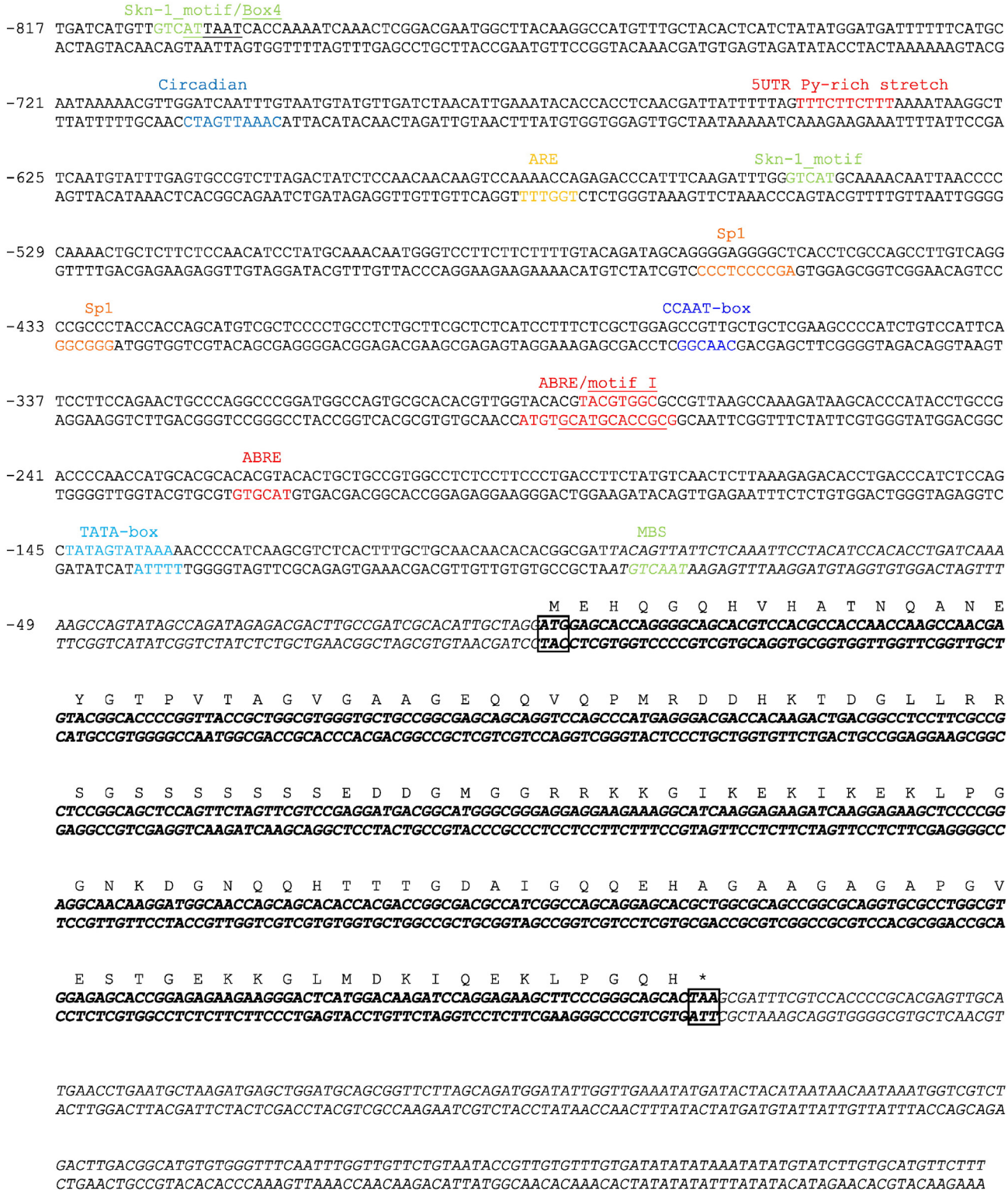

Fig. 1. The nucleotide sequence, amino acid sequence and promoter sequence of the *PgDHN gene*. The deduced amino acid sequence is shown on top of the nucleotide sequence (single letter code). The coding region of the *PgDHN* gene is shown with bold italics, whereas the 5′ and 3′ UTR regions are italicized. The translation initiation and termination regions are shown in boxes. Various putative *cis*-regulatory elements have been marked in the upstream region of the gene in different colours.

specific gene expression were also enriched. This suggests that the *DHN* gene is not only expressed during salt stress, but also expressed during other environmental stresses to protect the plants. However, the presence of different *cis*-regulatory elements and their concerted action in association with their corresponding transcription factors may regulate the expression of PgDHN transcript under various environmental conditions and also in the plant development.

3.3. Transcript analysis of PgDHN in response to different abiotic stress conditions

Fifteen day old *Pennisetum* plants were subjected to various abiotic stress treatments to map the transcription profile of the *PgDHN* gene. *PgDHN* transcript levels varied under different abiotic stress conditions. Exposure to drought stress hiked the levels of

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Pg     1 MEHQG--QHVH-ATNQANEYGTPVTA----------GVGAAGE-------------QQVQPMRDDHKTDGLLRRSGSSSSSS-EDDG  61
Si     1 MEHQG--QHGH-ATNQANEYGNPVAA----------GHGATGVG---------AAGDQVQPMRDDHKTDGLLRRSGSSSSSS-EDDG  65
Hv     1 MEYQG--QHGH-ATDKVEEYGQPVAG---------HGGFTGGPTG--THGAAGVGGAQLQATRDGHKTDGVLRRSGSSSSSS-EDDG  73
Zm     1 MEYGQQGQHGHGATGHVDQYGNPVGGVEHGTGGMRHGTGTGGMGQLGEHGGAGMGGGQFQPAREEHKTGGILHRSGSSSSSS-EDDG  87
Ta     1 MEYQGHQQHGQ-ATNRVDEYGNPVAG---------HGVGTG----------AAAGGHFQPSGEEHKAGGILQRSGSSSSSSSEDDG  67
Sb     1 MEYGQQGQHGHGTTGRVDQYGNPVG---AGTGGM--GTGTGGMGQLGEHGGAGMGGGQFQPAREEHKTGGILHRSGSSSSSS-EDDG  82
          **     ** . .* .  .** **           *  .                . *   . **. *.*.********** ****

Pg    62 MGGRRKKGIKEKIKEKLPGG-NKDGN-QQHTTT----------GDAIGQQEHAGAAGAGAPGVESTGEKKGLMDKIQEKLPG-QH 133
Si    66 MGGRRKKGIKEKIKEKLPGG-NKDSTGQQHTTT----------GGAVGQQGHAGATGTGAHGTEGTGEKKGLMDKIKEKLPG-QH 138
Hv    74 VGGRRKKGMKEKIKEKLPGGAHKDAAGQQQQTA----------MAGEY-------AGTHGTEATGEKKGVMDKIKEKLPGGQH 139
Zm    88 MGGRRKKGIKEKIKEKLPGG-HKDDQHATATTGGAYGQQG---HTGSAYGQQGHTGG--AYATGTEGTGEKKGIMDKIKEKLPG-QH 167
Ta    68 MGGRRKKGIKDKIKEKLPGG-HGDQQQTTDNTYGQQGHTAGMAGTGGTYGQPGHTGMAGTGTHGTDGTGEKKGVMDKIKEKLPG-QH 152
Sb    83 MGGRRKKGIKEKIKEKLPGG-HKDNQHATATGG----------GAAYGQQEHTG---AGTYGTEGTGEKKGIMDKIKEKLPG-QH 152
          .*******.*.********* . *                    *.        . . * . ******.****.***** **
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Fig. 2. Multiple sequence alignment of PgDHN with various plant species dehydrins showing their characteristic domains and conserved residues (red – Y segment, blue – S rich segment, green – two K rich segments, Pg – *Pennisetum glaucum*, Si – *Setaria italica*, Hv – *Hordeum vulgare*, Zm – *Zea mays*, Ta – *Triticum aestivum*, Sb – *Sorghum bicolor*).

PgDHN mRNA nearly 2.5 times after 12 h (Fig. 4A) and was maintained up to 48 h. The transcript level peaked after 72 h with an enhancement of 3.5 fold (Fig. 4A). The NaCl treatment had the maximum effect on the mRNA expression with more than a 3 fold hike after 4 h of treatment (Fig. 4B); the expression of *PgDHN* reached to a maximum of 13 fold after a time span of 12 h (Fig. 4B). The salinity stress had a lasting effect and was measured for 36 h (Fig. 4B). In addition, there was no dramatic change in the expression level of *PgDHN* in response to heat stress (Fig. 4C). Cold stress marginally reduced the expression of *PgDHN* as compared to the controls (Fig. 4D).

Different stress conditions had different effects on the transcript level of *PgDHN*, the expression of the gene being significantly up-regulated in salt and drought stress conditions. RAB proteins belong to group II LEA protein, also known as dehydrins. The RAB/LEA/DHN polypeptides are thought to play a role in tolerance to water stress. These groups of protein are known to be differentially up-regulated at the transcriptional level under environmental stresses such as water deficit,

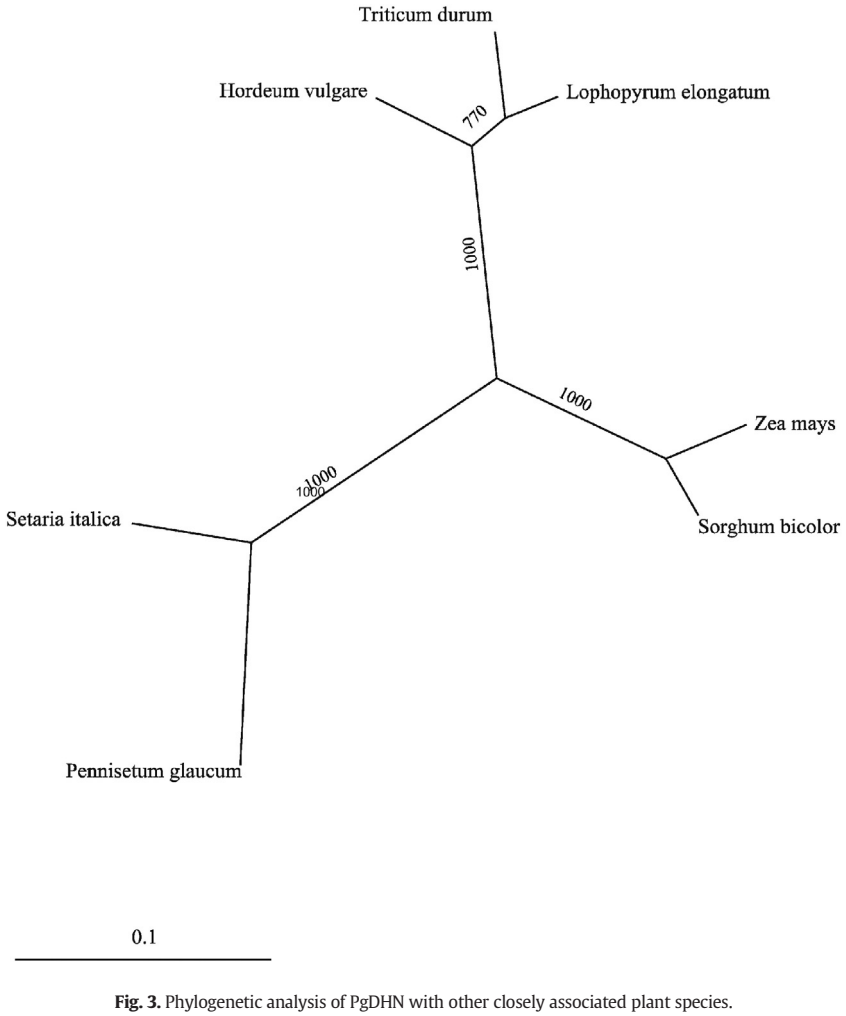

Fig. 3. Phylogenetic analysis of PgDHN with other closely associated plant species.

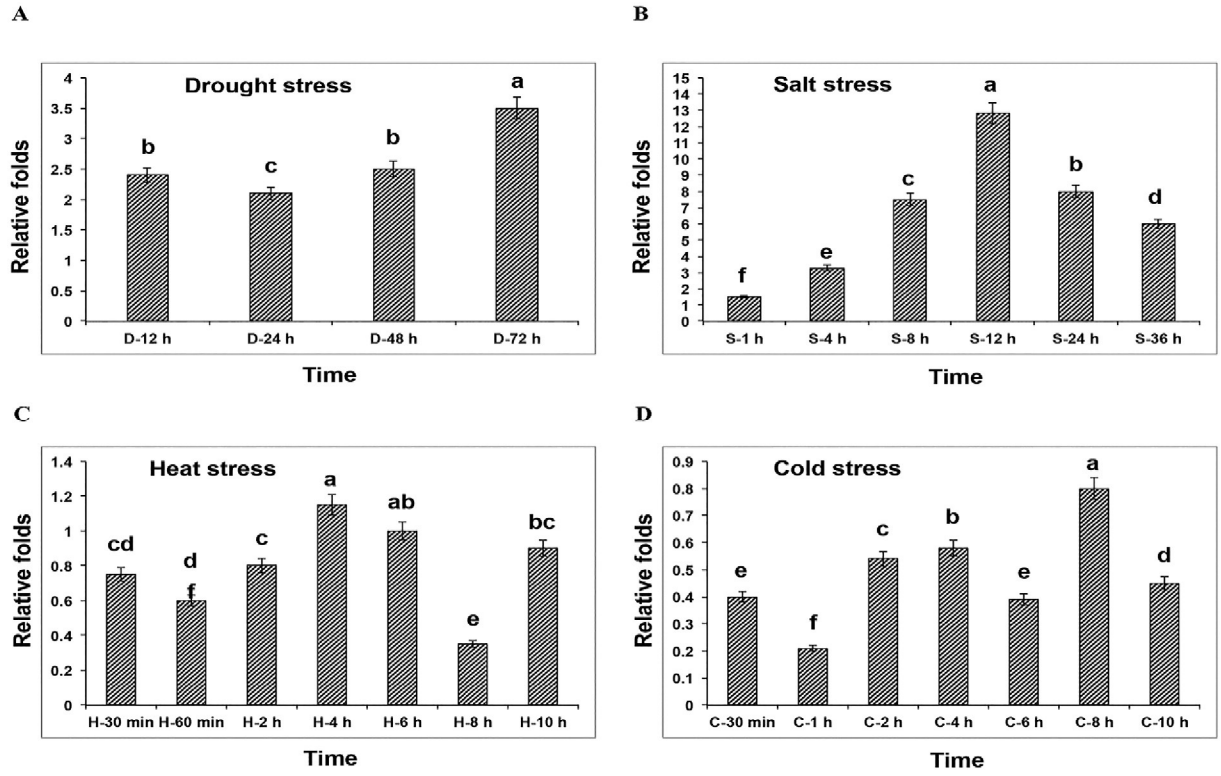

Fig. 4. qRT-PCR analysis of PgDHN transcript under different abiotic stress conditions. Relative expression of PgDHN transcript during drought stress (A), salt (B), heat (C) and cold stress (D) conditions in comparison to its control as revealed by quantitative RT-PCR analysis. Values represent the relative expression fold changes obtained after normalizing against the control value. The X-axis represents the duration of the stress treatment and the Y-axis represents the relative fold-increase in expression as compared to respective controls. All samples were analysed in triplicate, in three independent experiments. Bars with different letters indicate a significant difference ($P < 0.05$) between different time points in the different abiotic stress treatments.

salinity, and low temperature and in response to ABA (Qian et al., 2011). The gene *Rab16A* has been reported to be ABA-inducible and highly expressed during salinity stress and dehydration (Mundy and Chua, 1988; Park et al., 2006). Moreover, it has also been reported that the salt-tolerant rice varieties have constitutive and higher levels of *Rab* gene expression compared with the salt-sensitive varieties (RoyChoudhury et al., 2008). The accumulation of dehydrin proteins has also been reported in cold stress conditions (Hanin et al., 2011; Houde et al., 2004). However, in the present case, there was no significant change in the expression level of *PgDHN* in response to temperature stress conditions.

3.4. PgDHN protein expression in E. coli

The PgDHN protein was purified near to homogeneity using the heterologous expression system of *E. coli*. The cDNA of *PgDHN* gene was PCR amplified and cloned in pET28a expression vector at NdeI and NotI sites under the regulation of T7 promoter. The recombinant vector PgDHN-pET28a was transformed into *E. coli* BL21 (DE3) cells. IPTG was used for induction and the expression of the PgDHN recombinant protein. The recombinant PgDHN protein was purified from clarified lysate using Ni-NTA column chromatography. The purified fractions along with the uninduced and induced fractions were run on an SDS-PAGE. The purified fractions appeared as single band near to 14 kD (Fig. 5A).

3.5. PgDHN confers abiotic stress tolerance in E. coli

To evaluate whether recombinant PgDHN protein confers stress tolerance, *E. coli* cells overexpressing recombinant PgDHN were exposed to heat stress (37–55 °C) and their growth rates were monitored and compared with control *E. coli* cells. At heat stress (45 °C) the pET28a-PgDHN-containing *E. coli* cells showed considerably higher growth rates than the corresponding control *E. coli* cells (Fig. 5B). To study the role of recombinant PgDHN protein in tolerance to salt stress, *E. coli* cultures with and without expression of recombinant PgDHN were exposed to different salt concentrations (0–750 mM NaCl) for 12 h during which their growth was monitored. *E. coli* cells that overexpressed recombinant PgDHN showed a growth advantage under salt stress compared to the empty-vector control *E. coli* cells (Fig. 5C). The *E. coli* cells overexpressing PgDHN showed significantly less reduction in growth rate even at higher concentrations of NaCl as compared to the cells harbouring the empty vector. Few recent studies have reported enhanced growth of *E. coli* cells by overexpression of plant stress-tolerant functional genes (Liu and Zheng, 2005; Reddy et al., 2010, 2011, 2012, Singh et al., 2012). A pea DNA helicase (PDH 45), which has been shown to confer salinity stress tolerance in tobacco plants (Sanan-Mishra et al., 2005) provides resistance to salt stress in *E. coli* too (Tajrishi et al., 2011). The finding indicates that the high salinity stress evokes similar cellular response, which is conserved across prokaryotes and plant kingdom (Tajrishi et al., 2011). Additionally group II LEA proteins have been shown to be stress responsive, being highly up-regulated under salt stress conditions, which shows that they play a protective role in salinity stress tolerance (Battaglia et al., 2008).

3.6. PgDHN protein expression in yeast confers enhanced abiotic stress tolerance

To further confirm the stress tolerance properties of PgDHN in a eukaryotic system, PgDHN was expressed in *P. pastoris*. The stress

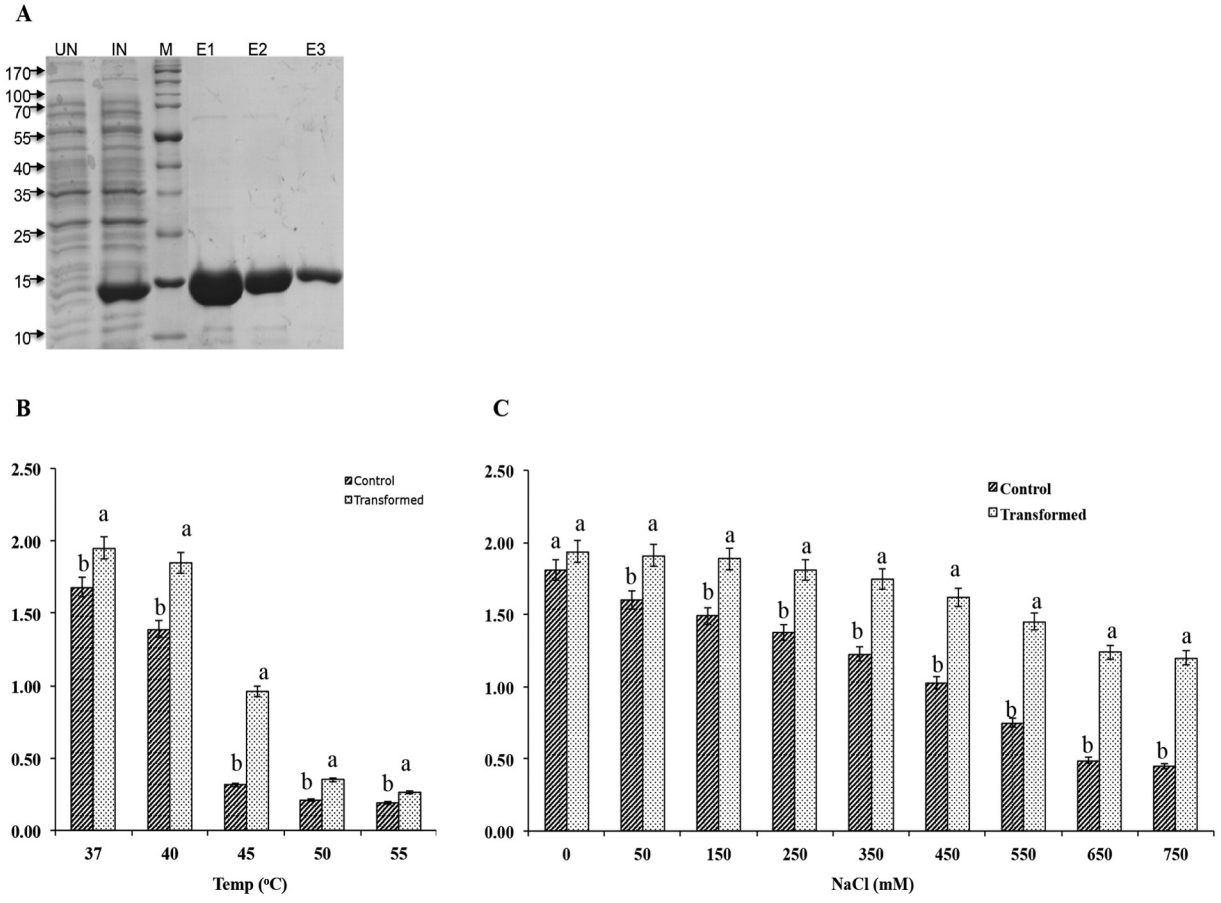

Fig. 5. Recombinant PgDHN protein charecterization in *E. coli*. (A) Expression and purification of PgDHN protein, Lanes, UN uninduced, IN induced, M molecular weight marker, E1, E2 and E3 are different elution fractions. Relative growth analysis of *E. coli* cells over expressing recombinant PgDHN protein with the controls under heat (B) and salt stress (C) conditions. The X-axis represents the range of temperatures/NaCl concentrations at which the *E. coli* cultures were grown and the Y-axis represents the OD at 600 nm. The data shows mean ± SD of three replicates. All the comparisons between control and transformed *E. coli* in different temperature and NaCl concentrations were significantly different at $P < 0.005$ and $P < 0.001$ respectively. Bars with different letters indicate significant difference between the control and transformed *E. coli* cells.

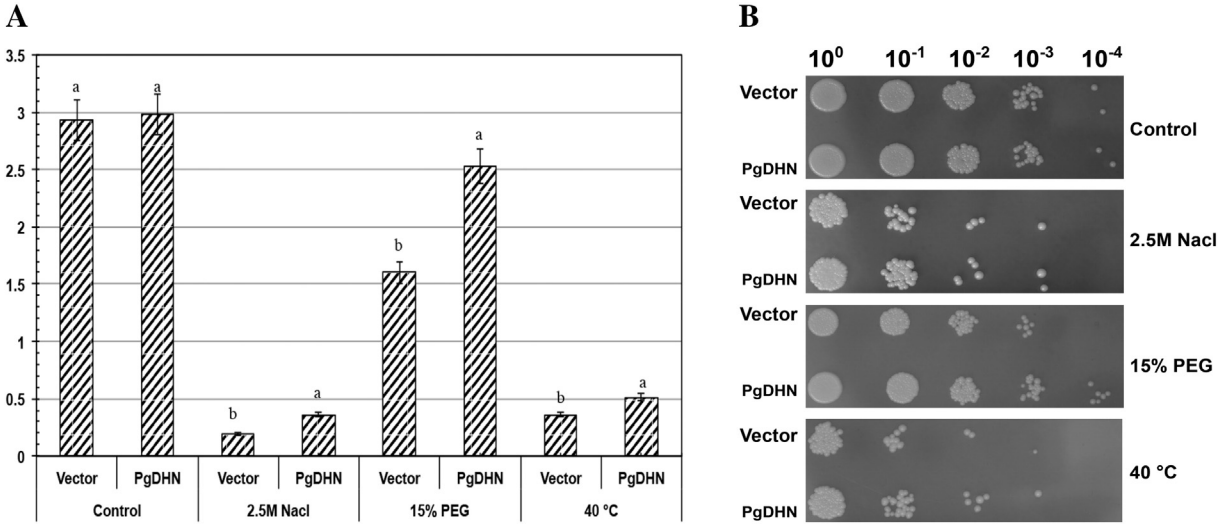

Fig. 6. Role of PgDHN in abiotic stress tolerance of yeast. A). The OD 600 of stress treated cultures (2.5 M NaCl, 15% PEG 6000 and heat stress treatment at 40 °C) of both vector control clone and PgDHN clone were plotted. Simultaneously OD 600 of untreated cultures of both vector control clone and PgDHN clone were also plotted. The data shows mean ± SD of three replicates. All the comparisons between vector and PgDHN under different abiotic stress treatments were significantly different at $P < 0.05$. Bars with different letters indicate significant difference between the vector and PgDHN in different stress treatments at $P < 0.05$. B). For the spot assay, the different abiotic stress treatments including salt, osmotic (PEG) and heat stress (40 °C), yeast cells were incubated in 2.5 M NaCl, 15% PEG and 40 °C for 24 h respectively. Non-stress yeast cells were incubated at 30 °C for 24 h. Serial dilutions were subjected for PgDHN recombinant and control yeast cells to 1/10 serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) then 3ul from each dilution was spotted on YPD agar and incubated at 30 °C for 3 days. Simultaneously untreated cultures of both vector control and PgDHN recombinant cells were diluted and spotted.

response was investigated on the basis of a spotting assay and the growth pattern of stress treated cultures determined by measuring the OD600 value. Under controlled conditions, both the PgDHN and the empty vector transformed yeast (control) cells showed similar growth characteristics (Fig. 6A and B). However, when subjected to various abiotic stress conditions (2.5 M NaCl, 15% PEG and 40 °C) the PgDHN transformed cells had a significant growth advantage over the control cells (Fig. 6A and B). Exposure to 2.5 M NaCl and 40 °C heat stress drastically reduced the growth of both PgDHN transformed and controlled cell culture (Fig. 6A and B). However, the PgDHN transformed cells were more tolerant to these stress conditions and exhibited better growth than the control cells as evidenced by the bigger size and higher number of colonies (Fig. 6B) and the OD600 values (Fig. 6A). PgDHN transformed *P. pastoris* culture showed noticeably enhanced growth compared to control cell culture in the presence of 15% PEG (Fig. 6A and B). The numbers of colonies of PgDHN transformed cell culture were significantly higher and bigger in size than the control cells when measured by the spot assay (Fig. 6B). The OD600 value of PgDHN transformed yeast culture was ~1.5 times of the control one when measured after 24 h of stress treatment (Fig. 6A). These results clearly show that PgDHN is functional in *P. pastoris* and enhances the tolerance and viability of cells in the presence of NaCl, PEG and heat stress. Dehydrins have also been shown to confer resistance in yeast cells to various abiotic stresses including drought, salt, oxidative, metal (Zn), freezing and thawing (Kim et al., 2013). Moreover expression of dehydrin enhances the fermentation capacity of yeast cells (Kim et al., 2013). The expression of *CaDHN* gene from Arctic *Cerastium arcticum* enhances the tolerance in *Saccharomyces cerevisiae* against abiotic stresses including salinity, H_2O_2, MD, and t-BOOH by improving redox homeostasis (Kim et al., 2013). A wheat dehydrin like gene, wzy2, confers tolerance to drought and salinity stress in *P. pastoris* (Zhu et al., 2012). Similarly, yeast complementation assay of an *A. thaliana* late embryogenesis abundant (LEA)-like protein shows that it plays a role in oxidative stress tolerance (Mowla et al., 2006). Dehydrin like proteins have also been reported in the fungus *Aspergillus fumigatus* (Hoi et al., 2011) and *Alternaria brassicicola* (Pochon et al., 2013) and are involved in a stress response. The functions of many stress responsive genes including CAP2 (Shukla et al., 2009), DREB2B (Li et al., 2014) have been studied and validated by using yeast as an expression system. These reports indicate that yeast is a good model system for studying and functional characterization of various abiotic stress responsive genes including dehydrins.

4. Conclusions

We isolated a full-length cDNA encoding a transcript of the dehydrin gene, a subgroup of the Lea gene family, from the stress adaptive *P. glaucum* plant. The sequence analysis of the full length protein revealed the presence of all the conserved motifs characteristic of dehydrins. Transcript profiles in leaves under different abiotic stress conditions showed that PgDHN is upregulated particularly in salinity stress. *E. coli* cells expressing recombinant PgDHN exhibited higher growth rates as compared to the control cells (without PgDHN) when exposed to heat and salinity stress. PgDHN was shown to confer salt, 15% PEG and heat stress tolerance in transgenic yeast cells. These findings suggest that PgDHN plays a crucial role in stress adaptation and provides resistance against different abiotic stresses in *P. glaucum*. Therefore, PgDHN can be used to develop transgenic crop plants to combat multiple abiotic stress conditions.

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