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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