

Metabolic engineering of SK2-type of dehydrin1 (*DHN1*) gene isolated from *Sorghum bicolor* enhances tolerance to water-deficit and NaCl stresses in transgenic tobacco

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Abstract

A stress-inducible dehydrin (*DHN1*) gene was isolated from *S. bicolor* and its role in water-deficit and NaCl stresses was studied in transgenic tobacco. The cDNA sequence of *SbDHN1* gene was 638bp in length and the ORF encoded a predicted polypeptide of 153 amino acid residues. Multiple alignment of amino acid sequence of *SbDHN1* with *DHN* from other plant species revealed the presence of two K-segments and an S-segment, revealing it to be an SK2 type of dehydrin. Induction of *SbDHN1* under water-deficit, salinity, high and low temperature stresses indicated its role in various abiotic stresses. Assays carried out using transformed *E.coli* cells expressing *SbDHN1* showed enhanced tolerance to multiple abiotic stresses. Transgenic tobacco plants expressing *SbDHN1* gene were generated. GUS histochemical assay and PCR analysis indicated the transgenic nature of the putative T₀ transgenics. Molecular characterization of the T₁ and T₂ transgenics using PCR, RT-PCR and Southern analysis confirmed the stable integration, expression and carrying forward of the transgene. When subjected to water-deficit (-1.0 MPa) and salt (300 mM NaCl) stresses, the transgenics exhibited higher germination rate as well as shoot and root lengths. Also the leaf discs of the transgenics retained more leaf water content (10.12%) and chlorophyll content (22.5%) in comparison to their untransformed controls. Water-deficit stress tolerance of the T₂ transgenics was revealed by maintenance of higher relative water content, membrane stability and superoxide dismutase activity. Thus, these results indicate the role of *SbDHN1* gene in imparting tolerance to water-deficit and NaCl stresses.

Keywords: Abiotic stress tolerance, Dehydrin, Sorghum, Tobacco, Transgenic plants.

Abbreviations: GUS-β glucuronidase, MDA-Malondialdehyde, MSI-Membrane stability index, RWC- Relative water content, SOD: Superoxide dismutase.

Introduction

Abiotic stresses such as drought, salinity and high temperature are the major constraints in agriculture that adversely affect crop growth and yield. One of the important molecular responses to these stresses is the induction of various types of genes and gene products. Dehydrins, a class of group 2 LEA or LEA-D11 proteins are ubiquitous in plants. They are highly hydrophilic and thermo stable proteins composed largely of the amino acids glycine, alanine and glutamine and lack cysteine and tryptophan. Due to the presence of charged and polar amino acids, they exhibit highly flexible structures (Close 1996; Wang et al., 2014). Genes encoding these proteins are expressed during late embryogenesis, as well as in vegetative tissues subjected to drought, low temperature and high salt conditions (Xu et al., 2008; Yang et al., 2012). It is well established that dehydrins function to protect the cells from damage caused by stress-induced dehydration or high temperature conditions by playing a role in membrane stabilization and as chaperones to prevent the aggregation of proteins (Eriksson and Harryson 2011; Yang et al., 2012). Dehydrins are structurally characterized by the presence of several conserved domains known as K, S and Y-segments. The K-segment is located at

the C-terminal end of the protein and has the ability to form amphipathic α helix-like structure that may play a role in its interaction with membranes and proteins (Close 1996; Yang et al., 2012). The S-segment consists of a track of serine residues that can be modified through phosphorylation and may function in the regulation of protein conformation and ion binding activity (Jensen et al., 1996; Alseikh et al., 2005). Based on these three segments, dehydrins are classified as SKn, YnSKn, YnKn, KnS, etc. Different classes of dehydrins respond to different kinds of cellular dehydration. For instance, the YnSK2 type responds to ABA and dehydration but not to low temperature, Kn and Y2Kn types are activated by low temperature primarily while the SKn type is induced by low temperature, salinity, drought and wounding, and the KnS type responds to drought and low temperature (Close 1996; Kalemba 2014). Transgenic plants expressing dehydrin family genes have been shown to impart tolerance to abiotic stresses such as low temperature (Ochoa-Alfaro et al., 2012), osmotic stress (Wang et al., 2011) and high salinity (Shekhawat et al., 2011). Although *DHN1* genes isolated from various crop species have been shown to play a role in multiple stresses (Bae et al., 2009; Kalemba 2014), clear

evidence supporting its physiological mechanism in stress tolerance is still lacking. Sorghum is one of the most drought tolerant crop species and is an important model system for studying physiological and molecular mechanisms underlying drought tolerance (Ludlow and Muchow 1990). Therefore, the present investigation was aimed at isolation, cloning and characterization of *DHNI* gene from *S.bicolor* and to elucidate its role in water-deficit and NaCl stress tolerance.

Results

Isolation, cloning and characterization of *SbDHNI*

In the present study, water-deficit stress inducible gene *SbDHNI* was isolated, cloned and characterized. Amplification of *SbDHNI* gene from genomic and cDNA templates resulted in 770 bp and 638 bp products respectively (Fig. 1A). The difference in the molecular weights of the amplified products indicated the presence of 132 bp of intron. BLASTn search of genomic and cDNA sequences revealed homology with the *DHNI* sequences of *TaDHN* (90%), *ZmDHN* (87%) and *SiDHN* (87%) available in the database. Sequence analysis revealed that the cDNA of *SbDHNI* was 638bp in length. ORF finder indicated that the coding region was 459bp in length with its initiation codon at the nucleotide position of 22bp and the termination codon at the position of 480bp. It also revealed the presence of 21bp of 5' and 158bp of 3' untranslated regions. The genomic and cDNA sequences of *SbDHNI* were registered in the NCBI Gene Bank with the accession numbers GU137311 and HM243499 respectively. The open reading frame (ORF) encoded a predicted polypeptide of 153 amino acid residues with a molecular mass of 15.7 kDa and pI of 8.81. The high percentage of charged and polar amino acids such as Glu (7.2%), Lys (8.6%) and serine (5.3%) revealed the hydrophilic nature of *SbDHNI* protein. Blastp analysis of the deduced *SbDHNI* polypeptide revealed significant sequence similarity with *ZmDHN* (84%), *SiDHN* (74%), *OsDHN* (67%), *HvDHN*(60%) and *TaDHN* (60%). Multiple alignment of these sequences using Clustal W program revealed the presence of conserved regions corresponding to two lysine-rich K-segments and one 7-serine rich S-segment, characteristic feature of SK2-type of dehydrins (Fig. 1B). Semi-quantitative RT-PCR analysis showed the induction of *SbDHNI* gene under different abiotic stresses. High level of expression was observed in low temperature and salt stresses followed by water deficit and high temperature stress. No such amplification was observed in the control (Fig. 1C).

E.coli cells harbouring *SbDHNI* gene showed the induction of 15.7kDa fusion protein upon addition of 1mM IPTG. The concentration of the *SbDHNI* protein increased with an increase in the time period from 0.5 to 4h (Fig. 2A). Abiotic stress tolerance assays using varying concentrations of stress-inducing agents such as mannitol, NaCl and methyl viologen indicated that the growth of the *E.coli* cells harbouring pQE30UA vector only and pQE30UA *SbDHNI* was inhibited. The impact of growth inhibition was less in pQE30UA *SbDHNI* when compared to the *E.coli* cells containing only vector (Fig. 2B-D).

Generation of *SbDHNI* tobacco transgenics and their characterization

The plant expression vector pCAMBIA1303*SbDHNI* was constructed (Fig. 3A) and was used for *Agrobacterium*

mediated transformation of tobacco. Twenty putative T₀ tobacco transgenics with *SbDHNI* gene were generated. The blue colouration in the leaf discs upon histochemical assay suggested the presence and expression of the *GUS* gene (Fig. 3B). PCR amplification of the genomic DNA isolated from putative T₀ transgenics with *hpt* and *SbDHNI* gene specific primers (P3 and P4) resulted in 800 bp and 459 bp products respectively (Fig. 3C and D). No such amplification was found in untransformed control. Of the 20 T₀ transgenics, 16 were found to be PCR positive. Among them, three T₀ transgenics (D7, D17 and D19) were selected for further evaluation. Based on the germination percentage on hygromycin (50 mg/L), it was observed that the progeny of all the 3 selected lines tested showed 3:1 Mendelian segregation pattern (table 1). T₁ transgenics of these 3 lines were also found to be positive by PCR analysis using *hpt* and *SbDHNI* gene specific primers (Fig. 3E and F). PCR and RT-PCR analysis of selected 3 T₂ transgenic lines using *hpt* and *SbDHNI* gene specific primers confirmed the carrying forward of the transgenes (Fig.4A-D). Southern blot analysis in these transgenic lines further confirmed the stable integration of *SbDHNI* (Fig. 4E). Semi-quantitative RT-PCR analysis of these 3 (D7, D17 and D19) transgenic lines using *SbDHNI* gene specific primers revealed that the transgenic line D19 had high level of expression compared to D7 and D17 (Fig. 4F). Seed germination of *SbDHNI* transgenics (T₁) in the presence of high concentration of mannitol (-1.0MPa) and NaCl (300mM) revealed its tolerance to water-deficit and salt stresses (Fig. 5). Also the root and shoot lengths of the transgenics were higher when compared to untransformed control (table 2). The loss of chlorophyll in the transgenics subjected to salt stress was also evident. Leaf disc assay for water-deficit stress tolerance revealed less reduction in the leaf water content (9.8-16%) of the transgenic lines when compared to the untransformed control (20.9%) (table 3) in the presence of mannitol (-1.0MPa). When subjected to salt stress, the transgenic lines showed relatively less reduction in total chlorophyll content (45.13%) when compared to untransformed control (48.9%) (table 3). Leaf discs of transgenics and the untransformed control floated on water showed no significant differences.

Evaluation of *SbDHNI* transgenics for water-deficit stress tolerance in pot culture

Withholding water for a period of 7 days led to a decrease in soil moisture content. The soil moisture content in the control pots was 17% while in the stressed pots, it was 7% (table 4). This reduction in soil moisture content resulted in leaf wilting symptoms in the stressed plants. Appearance of leaf wilting symptoms was delayed in the transgenic plants when compared to the untransformed control plants. The percent decrease in RWC in the transgenics under water-deficit stress was lower when compared to the untransformed control (Fig. 6A). The transgenics maintained relatively better membrane stability as indicated by their lower MDA content (15.3-17.4%) when compared to the untransformed control (41.3%) (Fig. 6 B). Also the reduction in MSI of the transgenics was lower (4.9-8.5%) when compared to the untransformed control (21.7%) (Fig. 6C). Among the transgenic lines, D17 had the highest MSI followed by D19 and D7 (Fig. 6C). The SOD activity was significantly higher under water-deficit stress in both the transgenic lines and the untransformed control. There was a 2 fold increase in SOD activity in the transgenic lines when compared to the untransformed control under stress conditions (Fig. 6D). Significant variations were

Table 1. Segregation analysis in T₀ seeds of *SbDHN1* tobacco transgenics.

| Transgenic (T ₀) lines | Number of seeds tested | Number of seeds germinated | Number of seeds not germinated | Segregation ratio | χ^2 value |
|------------------------------------|------------------------|----------------------------|--------------------------------|-------------------|----------------|
| D7 | 83 | 62 | 21 | 3:1 | 0.148 |
| D17 | 118 | 84 | 34 | 3:1 | 0.914 |
| D19 | 108 | 78 | 30 | 3:1 | 0.444 |

*Tabular χ^2 values for 1 d.f = 3.841 at 5% level of significance

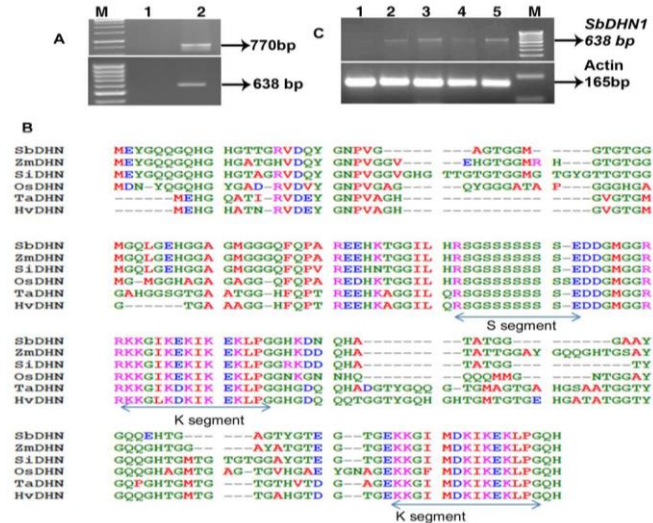


Fig 1. (A) PCR amplification of *SbDHN1* gene from genomic DNA and cDNA of *Sorghum bicolor*. Lane M- 1Kb DNA molecular weight marker; Lane 1- control seedlings; Lane 2- water-deficit stressed seedlings (B) Comparison of deduced amino acid sequence of *sorghum bicolor* (*SbDHN1*) with *DHN* sequences of other plant species. The domain regions i.e one S segment- polyserine rich region, and two K segments - lysine rich element 1 and 2 which are characteristic features of SK2 type of dehydrins are indicated with arrow marks (C) Expression analysis of *SbDHN1* gene in response to various stress treatments using semi-quantitative RT-PCR. Lane 1- control seedlings; Lane 2- Water-deficit; Lane 3- Salt (NaCl); Lane 4- High temperature; Lane 5- Low temperature. Actin served as an internal control.

Table 2. Shoot and root growth in *SbDHN1* tobacco transgenic (T₁) lines and untransformed control (UC) subjected to water-deficit (-1.0 MPa mannitol) and salt (300mM NaCl) stress treatments. Means \pm SE, n=3. LSD at 5% level of significance.

| | Water-deficit stress | | Salt stress | |
|-----|------------------------------|------------------------------|-------------------------------|------------------------------|
| | Shoot length(cm) | Root length(cm) | Shoot length(cm) | Root length(cm) |
| UC | 0.07 ^c \pm 0.02 | 0.40 ^d \pm 0.10 | 0.0 ^c \pm 0.0 | 0.0 ^d \pm 0.0 |
| D7 | 0.30 ^b \pm 0.01 | 2.67 ^c \pm 0.09 | 0.31 ^b \pm 0.03 | 2.60 ^c \pm 0.12 |
| D17 | 0.39 ^a \pm 0.01 | 3.30 ^b \pm 0.17 | 0.36 ^{ab} \pm 0.02 | 2.83 ^b \pm 0.07 |
| D19 | 0.43 ^a \pm 0.03 | 3.80 ^a \pm 0.23 | 0.41 ^a \pm 0.01 | 3.97 ^a \pm 0.12 |

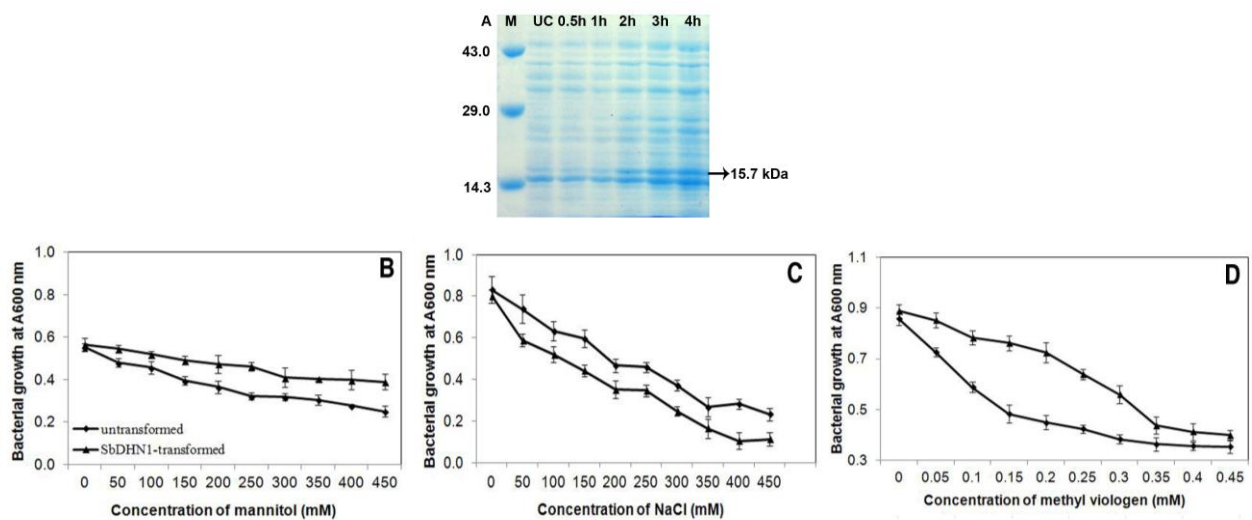


Fig 2. (A) Time course expression analysis of *SbDHN1* protein induced with 1mM IPTG in transformed *E.coli* cells. M: medium range protein marker, UC: un-induced control. Abiotic stress tolerance assays in *E.coli* cells transformed with *SbDHN1* using different concentrations of (B) mannitol (C) NaCl and (D) methyl viologen.

Table 3. Leaf disc assays in transgenic (T_1) lines and untransformed control (UC) subjected to water-deficit (-1.0 MPa mannitol) and salt (300mM NaCl) stress treatments.

| Lines | Water-deficit stress | | Salt stress | |
|-----------------------|------------------------|-------------------|-------------------------------|------------------|
| | Leaf water content (%) | | Chlorophyll content (mg/g DW) | |
| | Control | Stress | Control | Stress |
| UC | 90.9 ^b | 71.9 ^c | 9.4 ^b | 4.8 ^b |
| D7 | 92.2 ^{ab} | 77.5 ^b | 11.1 ^a | 6.1 ^a |
| D17 | 93.8 ^a | 80.4 ^a | 11.8 ^a | 6.2 ^a |
| D19 | 91 ^b | 82.1 ^a | 11 ^a | 6.3 ^a |
| LSD _{0.05} : | | | | |
| Genotype (G) | | 0.725 | | 0.449 |
| Treatment (T) | | 0.513 | | 0.318 |
| G X T | | 1.025 | | NS |

LSD: Least significant difference

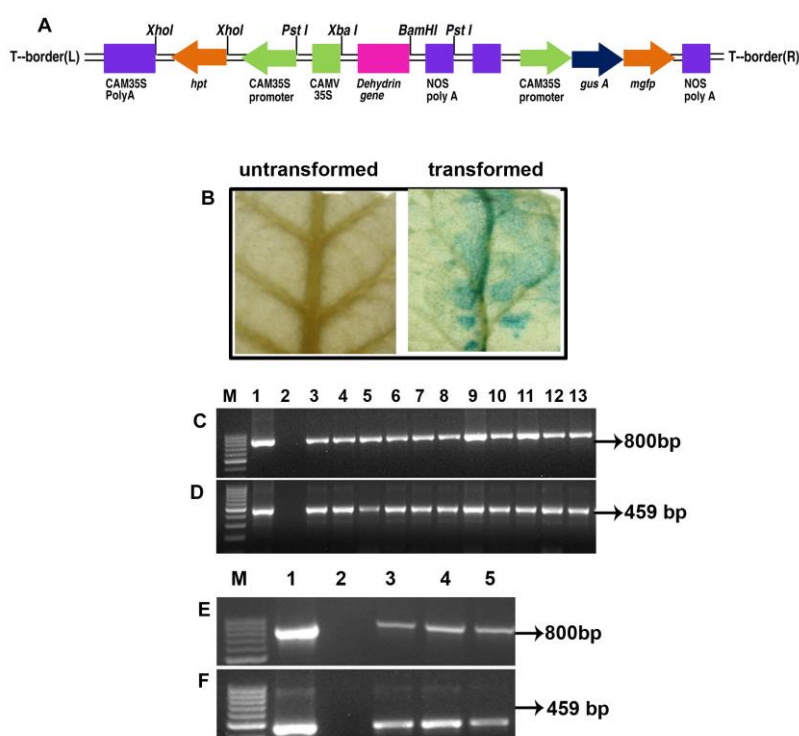


Fig 3. (A) Recombinant vector map of *pCambia 1303 SbdDHN1* T-DNA fragment (7453bp) (B) GUS expression in the infected leaf disc of tobacco. (C and D) Molecular characterization of *SbdDHN1* tobacco transgenic plants through PCR analysis in T_0 transgenics using *hpt* and *SbdDHN1* gene specific primer pairs respectively. Lane M-1kb DNA ladder; Lane 1- positive control; Lane 2- untransformed control; Lane 3-13 T_0 transgenics. (E and F) PCR analysis in three selected T_1 transgenics using *hpt* and *SbdDHN1* gene specific primer pairs respectively. Lane M-1kb DNA ladder; Lane 1- positive control; Lane 2- untransformed control; Lane 3- 5 transgenic lines D7, D17 and D19 respectively.

observed between the genotypes, treatments and their interaction for all the parameters analyzed. On the whole, analysis of various physiological parameters indicated that transgenic lines performed better than untransformed plants under water-deficit stress conditions. Among the three transgenic lines, D19 performed better followed by D17 and D7 in terms of most of the physiological parameters assessed.

Discussion

In the present investigation, a stress-inducible dehydrin gene (*SbdDHN1*) was cloned and characterized from the stressed

seedlings of *S. bicolor* to elucidate its role in water-deficit and NaCl stress tolerance. *SbdDHN1* gene isolated was 638bp in size and showed high sequence homology with *DHN* genes from other plant species. Although the sequence was similar in other crop species, reports indicating its role in abiotic stress tolerance are unavailable. Sequence analysis revealed the presence of two lysine rich K segments and one serine rich S segment, revealing it to be an SK2 type of dehydrin. Classification of dehydrins based on these conserved segments has been earlier reported by Close 1996. The hydrophilic nature of *SbdDHN1* was indicated by the presence of high percentage of charged and polar amino acids such as

Table 4. Soil moisture percent in the control and stressed pots of untransformed control (UC) and the transgenic lines (D7, D17 and D19) subjected to water-deficit stress treatment.

LSD_{0.05}: G = NS, T = 0.862, GxT = NS

| | Soil moisture (%) | |
|-----|-------------------------|------------------------|
| | Control | Stress |
| UC | 17.7 ^a ± 0.4 | 6.7 ± 0.3 ^b |
| D7 | 17.3 ^a ± 0.9 | 7.0 ± 0.6 ^b |
| D17 | 17.1 ^a ± 0.3 | 7.7 ± 0.9 ^b |
| D19 | 16.7 ^a ± 0.3 | 6.0 ± 0.7 ^b |

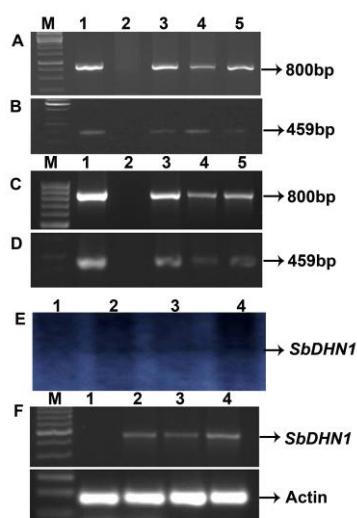


Fig 4. Molecular characterization of *SbDHN1* tobacco T₂ transgenic plants using PCR and RT-PCR analysis. (A and C) using *hpt* primers (B and D) *SbDHN1* primers. (Lane M-100bp ladder; Lane 1- positive control; Lane 2- untransformed control; Lane 3-5 transgenic lines D7, D17 and D19 respectively. (E) Southern blot analysis Lane 1- untransformed control Lane 2-4 transgenic lines D7, D17 and D19 respectively (F) Semiquantitative RT-PCR analysis Lane 1- untransformed control Lane 2-4 transgenic lines D7, D17 and D19 respectively.

Glu, Lys and serine. These amino acids are known to play an important role in maintaining highly extended conformations (Ingram and Bartels 1996). The induction of *SbDHN1* under water-deficit, salt, low and high temperature stresses suggested its role in tolerance to multiple abiotic stresses. The role of *DHN1* gene in multiple stress tolerance was reported earlier in different crop species by Close 1996; Kalemba 2014. In the present study, the induction of *SbDHN1* under various abiotic stresses is in consistency with the role of SK2 type of dehydrins in multiple stress tolerance as was also reviewed earlier in wheat (Wang et al., 2014). SDS-PAGE analysis revealed *SbDHN1* protein to be 15.7kDa in molecular weight whose expression was found to increase gradually with an increase in time period. The ability of *E.coli* cells expressing *SbDHN1* gene to tolerate higher concentrations of mannitol, NaCl and methyl viologen further revealed its role in water-deficit, salinity and oxidative stresses. Yadav et al., 2012 also reported the tolerance of *E.coli* cells expressing the *SbSI-1* gene to 500mM NaCl and 600mM mannitol. Tobacco transgenics harbouring pCAMBIA1303:*SbDHN1* were generated by *Agrobacterium* mediated transformation. Blue coloration in the leaf discs of

putative *SbDHN1* tobacco transgenics upon histochemical assay suggested the presence and expression of the GUS gene. PCR analysis of the putative T₀ transgenics with *hpt* and *SbDHN1* gene specific primers confirmed the integration of the transgenes respectively. Of the 20 T₀ transgenics generated, 16 were found to be positive by PCR analysis. Among them, 3 transgenics (D7, D17 and D19) selected showed 3:1 Mendelian segregation ratio and were carried for further studies. The Mendelian segregation ratio of 3:1 in these transgenics indicated single gene insertion. The amplification of 638bp fragment in the T₁ lines of D7, D17 and D19 revealed the carrying forward of the transgene. These selected transgenics were carried forward to T₂ generation. PCR and RT-PCR analysis of the T₂ transgenics further revealed the stable integration of *SbDHN1* transgene. Further, the transcript levels of *SbDHN1* were found to be higher in the transgenic line D19 when compared to D7 and D17 as revealed by semi-quantitative RT-PCR analysis. When subjected to water-deficit and salt stresses, the *SbDHN1* transgenics germinated on high concentrations of mannitol and NaCl. The root and shoot lengths of the transgenics were found to be higher when compared to untransformed control. Increased root and shoot lengths in dehydrin transgenics were also reported previously by Yang et al., 2014. The transgenic seedlings germinated on NaCl showed pale green color in contrast to the thick green colour of the transgenics germinated on mannitol medium. This could be due to the bleaching of chlorophyll by NaCl under salt stress (Jami et al., 2008). Leaf-disc assay confirmed water-deficit stress tolerance of the transgenic lines as revealed by the maintenance of better leaf water content (13.36%) in the transgenics over the untransformed control. While in case of salt stress, the leaf discs of the transgenic lines showed less reduction in chlorophyll content when compared to the untransformed control. These typical stress specific responses are in conformity with the already proven effects of stress (Xiong et al., 2002).

Evaluation of the *SbDHN1* transgenics (T₂) in pot culture for water-deficit stress tolerance revealed that the transgenic lines performed better than the untransformed control. The transgenic lines were found to be more tolerant to water-deficit stress as indicated by their better maintenance of RWC and MSI. Also the SOD activity was higher in the transgenics when compared to untransformed control. High SOD activities may detoxify excessive ROS contributing to high membrane stability that would ultimately result in maintaining high relative water content. Among the transgenics, D19 performed better when compared to D17 and D7. The differences in the performance of the transgenics may be due to the differences in the expression levels of the transgene.

Materials and Methods

Plant material and stress treatment

Seeds of *Sorghum bicolor* cv. SPV 462 obtained from ICAR-Indian Institute of Millets Research, Hyderabad, Telangana, India were used in the present study. Surface sterilized seeds were germinated in sterile wet cotton bottles and maintained under controlled conditions at 25±2° C and 70% RH. Fifteen days old seedlings were exposed to water-deficit stress treatment, i.e. through controlled dehydration in the dessicator for 48h. The stressed seedlings were then used for the isolation of genomic DNA and total RNA.

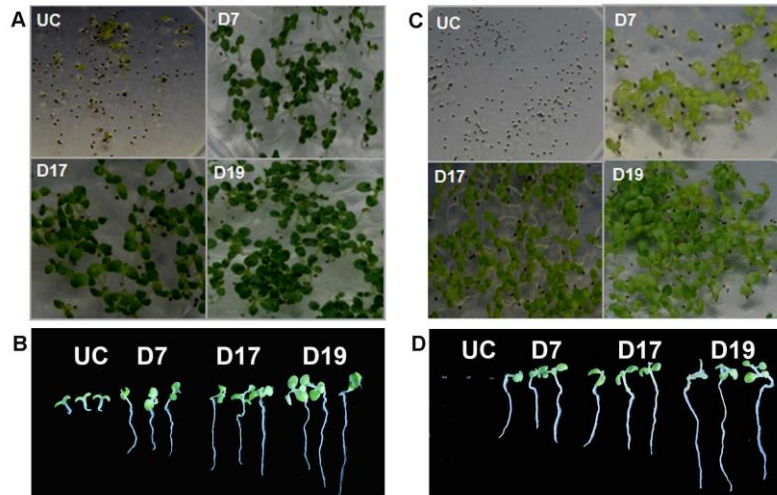


Fig 5. Seed germination of untransformed control (UC) and T_1 tobacco *SbdHN1*-transgenics (D7, D17 and D19) on MS media supplemented with (A) mannitol (-1.0 MPa) (C) NaCl (300mM). Shoot and root lengths of seedling germinated on (B) mannitol (-1.0 MPa) (D) NaCl (300mM).

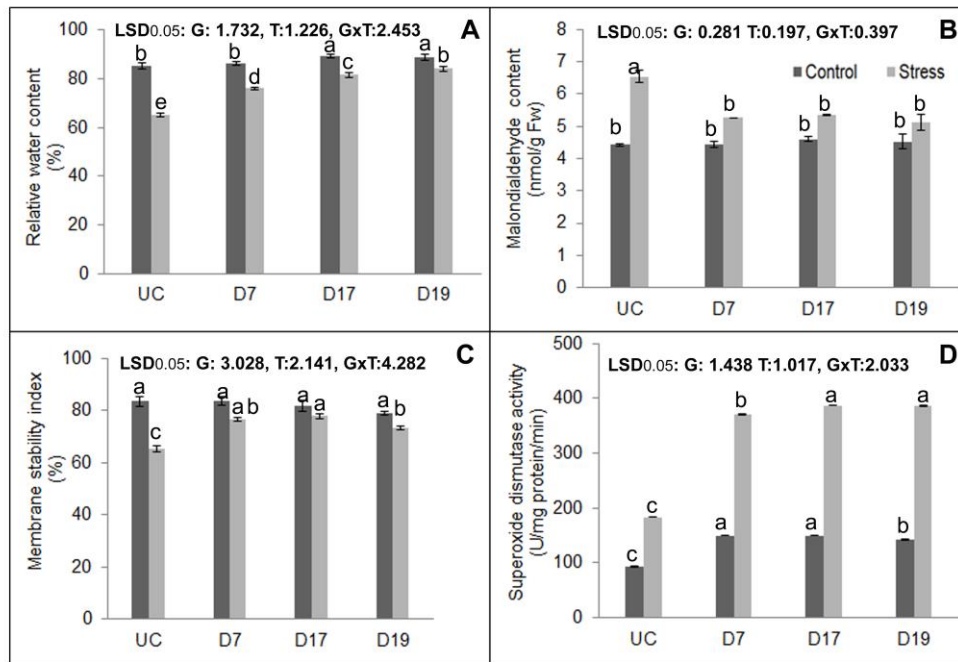


Fig 6. Effect of water-deficit stress on (A) relative water content (B) malondialdehyde content (C) membrane stability index (D) superoxide dismutase activity of *SbdHN1* (T_2) tobacco transgenic plants D7, D17 and D19 and untransformed control (UC) under well-watered and water-deficit stress conditions. (LSD_{0.05}: Least significance difference at 5%; G=genotypes, T=treatments GxT= Genotypes x Treatment interaction).

Cloning and sequence analysis of *SbdHN1*

Genomic DNA was isolated from the stressed seedlings using CTAB method (Doyle and Doyle 1990) and the total RNA was isolated using TRI reagent (Sigma, USA) according to the manufacturer's instructions. Five μ g of total RNA was used to synthesize first-strand cDNA using Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). A pair of gene specific primers P1: 5'-ACCAAATTAAAGCCGTGCAG 3' and P2: 5' TCTCACTCGCAAGTCACACC 3'

corresponding to the full length gene sequence including UTR regions was designed based on the *Sorghum bicolor* *DHN1* sequence (Accession no. U11696) available in the NCBI website. The genomic and cDNA sequences of *SbdHN1* gene were amplified using genomic DNA and first strand cDNA as templates in individual reactions according to the following conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 45s, 55°C for 45s and 72°C for 45s and final extension at 72°C for 5 min. PCR was performed in *Gene AmpR* PCR system 9700 (*Applied Biosystems*, Foster city, CA, USA). The resultant PCR

products were purified with QIA quick gel extraction kit (Qiagen, Germany), cloned into pTZ57R/T vector (Fermentas, Germany) and designated as pTZ57R-5*SbDHN1*. The *SbDHN1* gene plasmid was sequenced at Xcelaris Ltd (Ahmedabad, India). The sequence similarity search of the *SbDHN1* gene was performed using BLASTn and BLASTp tools available at NCBI website (<http://www.ncbi.nlm.nih.gov>). Nucleotide translations (DNA/RNA to protein) and the *SbDHN1* protein properties were deduced using tools available at Expasy website (<http://www.expasy.ch>). To identify the conserved domains in the *SbDHN1* protein sequence, the full length amino acid sequences from related crop species i.e *Zea mays*, *Setaria italic*, *Oryza sativa*, *Hordeum vulgare* and *Triticum aestivum* were aligned using CLUSTAL W program available at European Bioinformatics Institute (<http://www.ebi.ac.uk>).

Semi-quantitative RT-PCR analysis

Total RNA was isolated using TRIZOL reagent (Sigma, USA) from control and stressed seedlings exposed to water-deficit (controlled dehydration), salinity (250 mM NaCl), low temperature (4°C) and high temperature (45°C) stresses for 24 h. For semiquantitative RT-PCR analysis, the concentration of RNA was accurately quantified by spectrophotometric measurements. Five micrograms of RNA was used for the cDNA synthesis. Control reactions with actin primers were performed to ensure that equal amounts of RNA were used in each set of reaction. Second strand cDNA synthesis was carried out by using gene specific primers P1 and P2. The experiment was repeated thrice.

Expression of *SbDHN1* protein in *E. coli* cells

The isolated *SbDHN1* gene was cloned into bacterial expression vector pQE30UA using UA cloning kit (Qiagen, Germany) and transformed into *E. coli* host strain M15 [pREP4]. For carrying out time course expression analysis of *SbDHN1* protein, overnight grown cultures were inoculated into fresh LB medium (10ml) containing ampicillin (100 mg/ml) and kanamycin (25mg/ml) and cultured at 37°C until the absorbance at 600 nm reached a value of 0.6. *SbDHN1* protein was then induced by the addition of 1mM isopropyl-d-thiogalactoside (IPTG) and the cultures were collected at various time intervals i.e 0.5, 1, 2, 3 and 4h. Cultures were lysed by sonication (6 cycles of 10s pulse followed by a gap of 10s). The lysed cell extracts were resolved on 10% SDS-PAGE.

Abiotic stress tolerance assays in transformed *E.coli* cells

Overnight grown cultures of pQE30UA (only vector) and pQE30UA *SbDHN1* plasmids were cultured at 37°C in the LB medium containing ampicillin (100 mg/ml) and kanamycin (25 mg/ml). When the absorbance at 600 nm reached a value of 0.6, 1mM IPTG and varying concentrations of mannitol (50, 100, 150, 200, 250, 300, 400 and 450 mM), NaCl (50, 100, 150, 200, 250, 300, 350, 400 and 450 mM), and methyl viologen (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4 and 0.45 mM) were added simultaneously in independent experiments and the cultures were inoculated at 37 °C for 12 h in a shaking incubator and the cell growth was monitored by measuring the absorbance at 600nm and the growth of the cells was determined graphically. There were three replicates for each treatment.

Construction of plant expression vector and *Agrobacterium* mediated transformation into tobacco

The plasmid pTZ57R-*SbDHN1* was digested with *Xba*I and *Bam*HI, and cloned into the corresponding sites of pRT100 for mobilization of promoter and terminator. The gene cassette was then sub-cloned into plant expression vector pCAMBIA1303 and the construct pCAMBIA1303 35S:*SbDHN1* was mobilized into *A. tumefaciens* strain LBA4404 by freeze-thaw method. The transgenic tobacco plants were generated using pCAMBIA1303 35S:*SbDHN1*, according to the standard leaf disc transformation procedure of Horsch et al., (1985). Putative transgenic plants regenerated directly from edges of leaf discs growing on hygromycin selection medium were transferred to jam bottles containing MS basal medium supplemented with hygromycin (50mg/L). The rooted plantlets were transferred to cocopeat for hardening and then finally to pots maintained in the transgenic glass house. The plants were allowed to grow to maturity and T₀ seed was collected.

Histochemical GUS assay

Transient histochemical GUS staining activity (Jefferson et al., 1987) was carried out in the leaf discs of putative T₀ tobacco transgenics. Leaves from control and transgenic plants were incubated in a solution containing 50 mM sodium phosphate buffer pH 7.0, 0.1% TritonX-100, 10 mM EDTA and 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-d-glucuronide), vacuum infiltrated for 10 min and then incubated overnight at 37°C in the dark. The tissues were then incubated in 80% ethanol for 4 h and then rinsed to remove chlorophyll.

PCR, RT-PCR and Semiquantitative RT-PCR analysis

Genomic DNA and total RNA were isolated from putative transgenics and untransformed control plants. PCR analysis was carried out in all the twenty T₀ transgenics generated and 3 selected (D7, D17 and D19) T₁ lines and their T₂ progeny. RT-PCR analysis was carried out in 3 selected T₂ transgenic plants. PCR and RT-PCR analysis were carried out using *hpt* and a pair of internal *SbDHN1* gene specific primers (P3: ATGGAGTACGGTCAGCAGGGA and P4: TTAGTGCTGTCCGGGCAGCTT) spanning the complete coding region of the gene using the following PCR conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 45 s and final extension at 72°C for 5 min. The plasmid DNA of pCAMBIA1303*SbDHN1* and genomic DNA from the untransformed plants were used as positive and negative controls respectively. Semiquantitative RT-PCR analysis was also carried out in the selected three (D7, D17 and D19) transgenic lines (T₂) using the *SbDHN1* gene specific primers P3 and P4. Actin was used as internal control.

Southern blot analysis

Southern blot analysis was carried out using 10 µg of DNA according to Sambrook and Russel (2001). Genomic DNA of three selected PCR positive T₂ transgenics along with untransformed control were digested with *Xba*I and *Bam*HI. The digested genomic DNA was separated on 0.8 % agarose gel and blotted onto positively charged nylon membrane (*Hybond-N*, Amersham, Buckinghamshire, UK) by capillary method. The coding sequence of *SbDHN1* gene was used as

probe and labeled with α -³²P-dCTP by random priming kit, as per the manufacturer's guidelines (Takara, Japan). Hybridization was carried out for 18–24 h at 42°C. After post hybridization washes, the membrane was exposed to X-ray film (Kodak) at -70°C, and finally developed to detect the signals.

Segregation analysis

The inheritance of the transgene (*hpt*) in the T₁ lines was determined by evaluating the potential of germination on half-strength MS basal medium supplemented with 50 mg/L hygromycin in three selected T₀ transgenics (D7, D17 and D19). Germination percentage was scored after 20 days.

Seed germination assay for water-deficit and salt stress tolerance

The tolerance of the tobacco *SbDHN1* transgenics (T₁) to water-deficit (-1.0 MPa mannitol) and salt (300 mM NaCl) stresses was tested. The seeds of 3 selected (D7, D17 and D19) transgenic lines (T₁) along with the untransformed control were surface sterilized and inoculated onto half-strength MS basal medium supplemented with mannitol (-1.0 MPa) and NaCl (300 mM) in independent experiments and maintained at 25±2°C. After culturing for 20 days, visual observations on germination as well as the shoot and root lengths were recorded. The surviving seedlings were transferred to pots and taken to maturity to collect the seeds. There were three replications for each treatment.

Leaf disc assay for water-deficit and salt stress tolerance

When the T₁ plants were eight weeks old, leaf segments were cut from youngest fully expanded leaves and used for leaf disc assay. Untransformed control plants were also maintained. For water-deficit (-1.0 MPa mannitol) and salt (300 mM NaCl) stress tolerance assays, leaf segments were floated on solutions of mannitol and NaCl for 48h respectively. Leaf water content was determined in the leaf discs subjected to water-deficit stress. The fresh weight of leaf segments was recorded before and after floating on various solutions. At the end of the experiment dry weight was recorded. Leaf water content was calculated as

$$LWC = [(freshweight - dryweight) / freshweight] \times 100$$

Chlorophyll contents were determined according to the method described by Arnon (1949) in the salt stressed leaf discs. The leaf segments floated on water served as control. There were three replications for each treatment.

Evaluation of *SbDHN1* transgenics (T₂) for water-deficit stress tolerance in pot culture

Eight weeks old T₂ transgenics along with the untransformed control plants growing in the pots in the transgenic glass house were used. There were two treatments; well-watered control plants and water-deficit stressed plants. There were three replicates for each treatment. The plants were subjected to water-deficit stress by withholding watering for 7 days. At the end of the stress period, soil moisture content was recorded gravimetrically in both control and stressed pots. Also various physiological parameters such as relative water content (RWC), lipid peroxidation (LP), membrane stability index (MSI) and *in vitro* assay of superoxide dismutase (SOD) were assessed both in the transgenic lines and the

untransformed control plants. Relative water content (RWC) was determined in the youngest fully expanded leaves according to Bars and Weatherly (1962). RWC was calculated using the following equation:

$$RWC(\%) = [(FW - DW) / (TW - DW)] \times 100,$$

Where FW is fresh weight of the leaf tissue taken, TW is the turgid weight after rehydration and DW is the dry weight after oven drying. Lipid Peroxidation (LP) was measured in terms of malondialdehyde content (MDA), a product of lipid peroxidation according to Heath and Packer (1968). The absorbance of the supernatant was recorded at 532 nm and the non-specific absorption at 600 nm was subtracted. The MDA content was calculated using its absorption coefficient of 155 mM⁻¹cm⁻¹. Membrane stability index (MSI) was determined according to Premachandra et al., (1991) as modified by Sairam (1994). MSI was recorded using the electric conductivity meter and calculated using the following formula:

$$MSI = [1 - (C1 / C2)] \times 100,$$

Where, C1 and C2 are the electric conductivities recorded at 40°C and 100°C respectively. Superoxide dismutase activity (SOD) was carried out by recording the enzyme induced decrease in absorbance of formazone formed by nitro-blue tetrazolium chloride with superoxide radicals and the decrease in absorbance was recorded at 560 nm (Dhindsa et al., 1981). One unit of SOD activity was defined as the quantity of enzyme required to cause 50% inhibition and expressed as enzyme units/mg protein/min.

Statistical analysis

The standard errors, LSD and chi-square (χ^2) values were calculated according to the standard statistical procedures (Snedecor and Cochran 1980).

Conclusion

In summary, the *SbDHN1* gene isolated in the present study revealed its role in imparting tolerance to water-deficit and NaCl stresses. Therefore, this gene can be used for developing transgenic crop plants with multiple stress tolerance.

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