Molecular Biology Reports
https://doi.org/10.1007/s11033-019-05039-4

ORIGINAL ARTICLE

Isolation and functional characterization of three abiotic stress-inducible (*Apx, Dhn* and *Hsc70*) promoters from pearl millet (*Pennisetum glaucum* L.)

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Received: 4 May 2019 / Accepted: 20 August 2019

Abstract

Pearl millet is a C_4 cereal crop that grows in arid and semi-arid climatic conditions with the remarkable abiotic stress tolerance. It contributed to the understanding of stress tolerance not only at the physiological level but also at the genetic level. In the present study, we functionally cloned and characterized three abiotic stress-inducible promoters namely cytoplasmic *Apx1* (*Ascorbate peroxidase*), *Dhn* (*Dehydrin*), and *Hsc70* (*Heat shock cognate*) from pearl millet. Sequence analysis revealed that all three promoters have several *cis*-acting elements specific for temporal and spatial expression. *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* were fused with *uid*A gene in Gateway-based plant transformation pMDC164 vector and transferred into tobacco through leaf-disc method. While *PgApx pro* and *PgDhn pro* were active in seedling stages, *PgHsc70 pro* was active in stem and root tissues of the T_2 transgenic tobacco plants under control conditions. Higher activity was observed under high temperature and drought, and less in salt and cold stress conditions. Further, all three promoters displayed higher *GUS* gene expression in the stem, moderate expression in roots, and less expression in leaves under similar conditions. While RT-qPCR data showed that *PgApx pro* and *PgDhn pro* were expressed highly in high temperature, salt and drought, *PgHsc70 pro* was fairly expressed during high temperature stress only. Histochemical and RT-qPCR assays showed that all three promoters are inducible under abiotic stress conditions. Thus, these promoters appear to be immediate candidates for developing abiotic stress tolerant crops as these promoter-driven transgenics confer high degree of tolerance in comparison with the wild-type (WT) plants.

Keywords Abiotic stress-inducible promoters · Pearl millet · *Cis*-acting elements · *PgApx pro* · *PgDhn pro* · *PgHsc70 pro*

Abbreviations

Pg	*Pennisitum glaucum*
Apx	Ascorbate peroxidase
Dhn	Dehydrin
Hsc70	Heat shock cognate 70
GE	Genetic engineering
SOD	Superoxide dismutase
BAP	6-benzylaminopurine
NAA	Napthaleneacetic acid
GUS	ß-glucuronidase
MS	Murashige and Skoog

Introduction

Abiotic stresses such as salt, drought and extreme temperatures affect plant growth and result in the loss of nearly 50% of world's agricultural productivity [1]. They modulate biochemical, physiological, and molecular activities from seedling stage till flowering. When the plants are subjected to different abiotic stresses, gene expressions are either up- or down-regulated leading to increased or decreased levels of metabolites and proteins, of which some may be conferring certain degrees of tolerance against stresses [2]. But, plants exhibit several intricate mechanisms to withstand such

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11033-019-05039-4) contains supplementary material, which is available to authorized users.

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Published online: 29 August 2019

adverse stress conditions [3, 4]. At the molecular level, several signal cascades are modulated leading to altered accumulation of osmoprotectants. Large number of genes that are induced by various abiotic stresses have been identified using multi-disciplinary approaches such as subtractive cDNA libraries [5], microarray [6] and RNA sequencing by NGS [7, 8]. The identified genes are broadly classified into two groups [9]. The first group of proteins function directly against abiotic stress and are named as heat shock proteins (Hsps), late embryogenesis abundant (LEA) proteins, osmotins, antifreeze proteins, aquaporins, sugar and proline transporters [10]. The second group is involved in the signal transduction pathways and is modulated mainly by transcription factors [9]. Therefore, understanding the molecular and functional mechanisms behind the regulation of the identified genes/promoters against abiotic stress is vital. To dissect out the mechanisms, several candidate genes were characterized. But, very few reports on promoters like *rd29a* [11], *PR10* [12], and *SEOF1* [13] are available. Rice *HsfB2c* and *PM19* are highly heat-inducible and effective as heat-inducible promoters for plant genetic engineering [14]. The *PR10* promoter from *Erianthus* though highly constitutive, was quickly induced upon wounding as well as with ABA and methyl jasmonate hormones [15]. The *OsABA2 pro* drives a low constitutive expression under normal conditions, but high expression in response to ABA, salt and drought stresses. Promoter from the maize *Type-II* H^+*-pyrophosphatase* gene showed higher expression when compared to constitutive *35S pro* under drought and salinity stresses [16]. Similarly, *AtUSP* has been found more active in response to phytohormones as well as multiple abiotic stresses. This promoter is used as a stress-inducible promoter to develop crops tolerant to multiple stresses [17]. Transgenics with stress-inducible promoters confer better resistance to abiotic stresses than the constitutive one [18–20]. Hence, isolation and validation of promoters from drought tolerant crop like pearl millet may have an advantage over others due to its inherent stress tolerance nature.

In the present study, three abiotic stress-inducible promoters were selected from group I proteins. Cytoplasmic ascorbate peroxidase1 (here after called as APX) belongs to class I peroxidase seen extensively in photosynthetic algae and all higher plant species. APX is a potential candidate in eliminating H_2O_2 and other reactive oxygen species (ROS). Simultaneous overexpression of SOD and APX has been shown to enable the plants to withstand severe saline conditions [21]. Increased levels of *APX* from *Pisum sativum* in transgenic tomato plants play a significant role in ameliorating oxidative stress activated upon chilling and salt stresses [22]. Wang et al. [23] demonstrated that transgenic tomato overexpressing *APX* exhibit elevated tolerance to drought, UV-B as well as heat stresses. Yabuta et al. [24] reported that overexpression of tobacco with *APX* results in high tolerance to methyl viologen and chilling stresses [24]. *OsAPX* in rice allows more spikelet fertility under cold stress [25]. *AtAPX* in tobacco enhances the tolerance to heat stress [26]. Similarly, Sun et al. [27] demonstrated enhanced tolerance to osmotic stress in tobacco by incorporating APX gene. This shows that *APX* indeed has a key role to play in abiotic stress tolerance and may be the suitable candidate for the development of transgenic plants.

Recent studies show that exposure of plants to desiccation and salt stress lead to higher expression of dehydrins (YnSKn-type). Kn, SKn, and KnS proteins are mostly regulated during cold stress, though some of them are highly expressed under desiccation and salt stresses [28]. Expression of *Dhn* genes under different abiotic stresses reveal higher expression in salt stress, high temperature and drought stresses [29, 30]. From in vitro as well as localization experiments, it is noticed that Dhns play prominent functions such as stabilization of cellular membrane, protection of enzymes under low temperature and from ROS [28]. Transgenic plants overexpressing different *Dhn* members display enhanced tolerance to different abiotic stresses [31, 32]. *Dhns* are implicated in conferring resistance to multiple abiotic stresses in different plants [33–35]. These studies demonstrate the vital roles that *Dhns* play in tackling varied abiotic stresses.

To protect plants from heat stress, a group of specialized proteins take part and are called heat shock proteins (Hsps) [36–40]. Most abundant Hsps in the eukaryotic cells are Hsp70 class which are known to play a chaperonic function for newly synthesized proteins for preventing their accumulations and guide proper folding and also aid in translocation in an ATP-dependent way [36, 41]. Four subgroups of Hsp70 are known, and also each of their subcellular location in compartments like mitochondria, chloroplasts, endoplasmic reticulum (ER) and plastids [36]. Cytosolic Hsp70 s are again categorized into two types: one is heat-inducible and the second heat-shock cognate Hsp70 or Hsc70, upregulated under control conditions. Under high temperature stress, Hsc70 cognates are essential in protein homeostasis control, sorting of proteins by interacting with mitochondrial as well as chloroplast protein import complexes, and eventually make a way to the ubiquitin-mediated proteasomal degradation pathway [42–44]. Further, overexpression and knockout gene studies show that Hsp70 plays a protective role during dehydration stress in tobacco, soybean and citrus [45]. Due to the important roles being played by *Apx, Dhn* and *Hsc70* genes during abiotic stress tolerance, it is presumed that their promoter regions might have a crucial function in imparting tolerance to abiotic stresses. To validate this concept, upstream regions of the three genes were cloned and transferred into tobacco for functional validation.

Materials and methods

Cloning and *cis*-motif analysis of the *PgHsc70, PgDhn* and *PgApx* promoters

Genomic DNA was extracted from *Pennisetum glaucum* leaves using the DNeasy plant mini kit (Qiagen, Germany). Upstream regions of the *PgApx*, *PgDhn* and *PgHsc70* genes were PCR amplified [46] using respective promoter specific primers and cloned separately into pCR™8/GW/TOPO® TA vector (Invitrogen). The recombinant plasmids of *PgHsc70*, *PgDhn pro* and *PgApx pro* were sequenced at Macrogen commercial facility, South Korea. Putative *cis*-acting elements of three promoter sequences were analyzed in silico by employing databases like PlantCARE [47] and PlantPAN [48]. Putative *cis*-acting elements and their positions were looked in both forward as well as reverse strands of the three promoter regions.

Cloning into the pMDC164 plant transformation vector

The recombinant plasmids containing *PgApx pro, PgDhn pro* and *PgHsc70 pro* were cloned with *uid*A reporter gene in the binary vector pMDC164 using Gateway cloning protocol. The fusion constructs *PgApx pro-uid*A-*nosT*, *PgDhn pro-uid*A-*nosT* and *PgHsc70 pro-uid*A-*nosT* in pMDC164 were further mobilized to *Agrobacterium tumefaciens* (EHA105) by electroporation and recombinant clones were verified further by colony PCR and sequencing.

Development of transgenic tobacco plants

Agrobacterium culture containing *PgApx pro-uid*A-*NosT, PgDhn pro-uid*A-*NosT* and *PgHsc70 pro-uid*A-*NosT* constructs were transferred individually to tobacco [*Nicotiana tabacum* (L.) variety Xanthi] through leaf-disc method [49]. The leaf-discs were immersed for 10 min in the *Agrobacterial* suspension containing 100 µM acetosyringone. Leaf-discs were dried between folds of sterile filter paper and placed on Murashige and Skoog's (MS) medium fortified with 2 mg/l 6-benzylaminopurine (BAP), 0.1 mg/l naphthaleneacetic acid (NAA), 250 mg/l cefotaxime, and 50 mg/l hygromycin. Co-cultivated plates were maintained in continuous dark at 25 ± 2 °C for 14 days; and resistant calli sub-cultured again for 14 days in the same medium. Calli resistant to hygromycin, which still survived after two laps of selection were then shifted to regeneration medium (MS medium containing 3 mg/l, BAP, 0.5 mg/l NAA, and 50 mg/l hygromycin). These plates were incubated in dark for 6 days, and then transferred to light. Shoots were rooted on MS basal medium containing 50 mg/l hygromycin. Transgenic tobacco plants generated with *PgApx pro, PgDhn pro* and *PgHsc70 pro* were screened preliminarily on a medium containing 50 mg/l hygromycin and established in the containment greenhouse and used for molecular characterization and maintained until flowering and seed set.

Table 1 Primers used for promoters cloning into pCR8/GW/TOPO TA vector and transgenic confirmation of transgenic tobacco plants

S.No	Primer	Sequence (5′–3′)
1	Apx_PC_F	CGACGGCGTAGTATTTCCAT
2	Apx_PC_R	GGCTGCGGGCGGAGAAGCTTTCG
3	Dhn_PC_F	TGTTGTCATTAATCACCAAAATCAA
4	Dhn_PC_R	CCTAGCAATGTGCGATCGGCAAGT
5	Hsc70_PC_F	CCCGATGTTGATAAACTTAAGATAG
6	Hsc70_PC_R	GGGAATCTGCTTCCTCCTAGTC
1	Trans-APX_F	ACGGGAGATTGGATGGATCAGG
3	Trans-Dhn P_F	ACAAGTCCAAAACCAGAGACCCA
5	Trans-Hsc70_F	GCTCAACAAGGCTGACGATGAT
7	T-GUS_R	GATACGTACACTTTTCCCGGCA
8	HygF	TTGACATTGGGGAGTTTAGCGA
9	HygR	GTTTCCACTATCGGCGAGTACT

Molecular analysis of the transgenic tobacco plants

In order to confirm the presence of transgenes, putative T_0 transgenic tobacco seedlings were screened preliminarily by hygromycin selection medium. Genomic DNA was extracted from the hygromycin-resistant plants with DNeasy plant mini kit (Qiagen, Germany). Primers used for amplification of *uid*A gene fragment along with the respective promoter specific sequences are shown in Table 1. PCR amplification of the transgene was performed in a reaction volume of 25 µl containing 2.5 µl buffer, 0.5 µl 10 mM dNTPs, 500 ng of the tobacco genomic DNA, 0.5 µl each of forward and reverse primers (10 µM) and 0.2 µl Taq DNA polymerase (2.5U). PCR conditions used were 95 °C for 3 min; 94 °C for 60 s, 60 °C for 60 s; 72 °C for 60 s; 32 cycles in all, at 72 °C for 10 min. DNA extracted from the untransformed plants was taken as a negative control and the corresponding recombinant pMDC164 plasmid was used as a positive control.

Generation of T_2 transgenics

Around 100 transgenic T_1 tobacco seeds harboring three promoters (*PgApx pro, PgDhn pro* and *PgHsc70 pro*)-*uid*A gene constructs were separately surface sterilized and plated on MS basal medium containing sucrose (3%) and hygromycin (50 mg/l) for selection. T_1 transgenic tobacco seeds were germinated in accordance with Mendelian ratio on the selection medium within 7–10 days.

The germinated plants were transferred to jiffy cups and leaf tissue was collected from all the plants separately and used for genomic DNA isolation. PCR amplification was carried out with two sets of primers which includes specific promoter F–*uid*A gene R and Hyg F–Hyg R (Table 1). The plants were confirmed by comparing with both sets of primers and the PCR positive transgenics transferred to glasshouse and seeds harvested. The harvested seeds (T_2 transformants) were kept for germination on MS basal medium supplemented with 50 mg/l hygromycin. Plants that survived were then subjected to PCR analysis. PCR-positive plants in triplicates were subjected to GUS histochemical assay and RT-qPCR for *uid*A gene expression.

Abiotic stress treatments

Experiments involving primary abiotic stresses (heat, cold, salt and drought) were performed on 30-day-old plants grown in the glasshouse after shifting from jiffy cups. These plants were incubated for 4 h at 45 °C and 4 °C for high temperature and cold stresses respectively. Dehydration stress was imposed by withholding water supply for 5 days and 250 mM NaCl solution was applied for 48 h. Leaf, stem and root tissues were collected from respective control as well as stress imposed plants immediately after stress imposition. For dehydration stress: after withholding water supply for 5 days and after 48 h of 250 mM NaCl solution imposition for salt stress to study the GUS histochemical activity as well as for RNA extraction.

Histochemical GUS assay

Abiotic stress treated and control tissue samples were vacuum infiltrated for 10 min in the GUS staining solution containing 1 mM 5-bromo-4-chloro-3-indo-lyl-β-D-glucuronide (X-gluc) and 50 mM sodium phosphate buffer as described by Jefferson et al. [50] with slight modifications, and then incubated overnight at 37 °C. Their action was stopped and chlorophyll destained by adding 75% ethanol. Sections of stained tissue samples were taken, cut, and dehydrated by passing through graded ethanol series (70–95% v/v). For studying abiotic stress-induced expression pattern of these promoters, T_2 seeds were kept for germination on MS medium containing 50 mg/l hygromycin and PCR-confirmed plantlets were subjected for the GUS histochemical studies. Seedling, leaf, stem, root and floral tissues of the three constructs were immersed in the GUS staining solution.

RNA isolation and RT-qPCR analysis

Total RNA was extracted from the plants exposed to different abiotic stress treatments using 100 mg of tissue and RNeasy Plant Mini kit (Qiagen, Germany). NanoVue plus spectrophotometer (GE health care, USA) was used to check the quality and quantity of RNA and the ratio of the absorbance measured at 260 and 280 nm (260/280) of the samples ranged from 1.8 to 2.0. Integrity of the RNA was further verified by denaturing 1.4% agarose gel and bioanalyzer. The RNA extracted was diluted to 30 ng/µl concentrations for their direct use in RT-qPCR. RT-qPCR reactions were set and carried out in the optical plates with 96 wells on a Realplex (Eppendorf, Germany). Reactions were performed with 1 µl of RNA (30 ng), 400 nM of primer each, 2X one step SYBR RT-PCR buffer 5 µl (Takara, Japan) and 0.5 µl of Prime Script One Step Enzyme Mix 2 (Takara, Japan) and the volume was made to 10 µl with RNase-free H_2O. The RT-qPCR reactions were carried out by following standard thermal profile: 42 °C for 5 min and 95 °C for 10 s (reverse transcription) and then 40 cycles of 15 s at 95 °C, 15 s at 62 °C with fluorescent signal recording and 15 s at 72 °C. After 40th cycle, amplicon dissociation curves were measured by heating at 58 to 95 °C with fluorescence measured within 20 min. All RT-qPCR data were obtained from three biological replicates and with three technical replicates. Normalized expression of the *uid*A gene was calculated with qbase + software with reference genes *NtUBC* and *NtEF-1α*. The relative expression of *uid*A gene in reflex to various abiotic stress treatments was calculated using qbase + software by normalizing with corresponding control samples as well as with *NtUBC* and *NtEF-1α* reference genes [51].

Statistical analysis

After 7–10 days of seed germination of *PgApx pro, PgDhn pro* and *PgHsc70 pro*, the number of sensitive and resistant seedlings were counted and checked for the Mendelian inheritance with Chi square (χ^2) test. χ^2 values were calculated. Results were expressed as the mean ± SE from at least three experiments. For all the stress experiments (high temperature, cold, salt and drought), relative expression data were analysed with CoStat version 6.204 (Cohort Software, Monterey, CA, USA). One-way ANOVA was performed to check for expression difference among the transgenic events and wild type plants. The means were compared using the Tukey–Kramer test as well as LSD (at P = 5% significance).

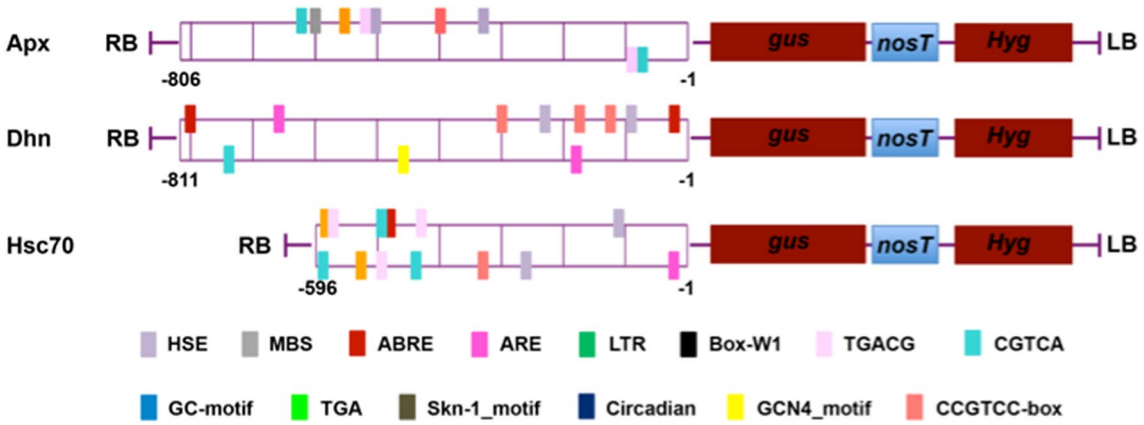

Fig. 1 Sequence analysis and cloning of *PgApx*, *PgDhn* and *PgHsc70* promoters into plant transformation pMDC164 vector. Positions of *cis*-acting elements which are present in the promoter regions of three pearl millet abiotic stress inducible genes. Promoter analysis was performed with PlantCARE and PlantPan databases. *PgApx pro, PgDhn pro* and *PgHsc70 pro* were cloned into pMDC164 vector through gateway cloning. *Gus uid*A gene, *nosT* nopaline synthase terminator, *Hyg* Hygromycin, *RB* right border, *LB* left border

Results

PgApx pro, PgDhn pro and *PgHsc70 pro* cloning and *cis*-motif analysis

Three pearl millet abiotic stress-inducible genes (*PgApx, PgDhn* and *PgHsc70*) were identified in our previous studies. All three promoters were cloned in pCR™8/GW/TOPO® TA vector and sequenced. In silico promoter analysis by PlantCARE and PlantPan software revealed that all three promoters are a rich source for the abiotic stress-inducible *cis*-motifs as well as tissue specific motifs. CAAT-box, G-box and Sp1 (light-responsive elements) are the most commonly present in all the three promoters. A-box, CCG TCC-box (meristem specific element), CGTCA-motif and TGACG-motif (MeJA-responsiveness) were present in *PgApx pro* and *PgHsc70 pro*. Skn-1_motif (endosperm) was noticed in *PgApx* and *PgDhn pro*. CCAAT-box (MYBHv1 binding site) and MBS (drought-inducibility) were present in *PgDhn* and *PgHsc70 pro*. GC-motif (anoxic specific), LTRE (low-temperature responsive element), TC-rich repeats (defense and stress responsiveness), TCA-elements (salicylic acid-responsiveness) were noticed in *PgApx pro*. ABRE (abscisic acid-response elements), ARE (anaerobic response elements), circadian (circadian control) and motif I (specific to root) were present in *PgDhn pro*. Box-W1 (fungal elicitor), CAT-box (meristem expression), HSE (heat stress-responsiveness) and TGA (auxin-responsive) were noticed in *PgHsc70 pro* (Fig. 1 and Table 2).

Cloning and generation of the transgenic tobacco plants

Recombinant *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* plasmids were subsequently cloned into pMDC164 vector upstream to the *uid*A gene individually through Gateway cloning (Fig. 1). The fusion constructs containing *PgApx pro-UidA-nosT*, *PgDhn pro-uidA-nosT* and *PgHsc70 pro-uidA-nosT* in pMDC164 were transferred into *Agrobacterium tumefaciens* (EHA105) by electroporation and verified by colony PCR using promoter specific forward and gene specific reverse primers which gave amplicon sizes of 806 bp, 811 bp and 596 bp to *PgApx pro, PgDhn pro* and *PgHsc70 pro* respectively. The *Agrobacterium* harbouring *PgApx pro-uidA-nosT*, *PgDhn pro-uidA-nosT* and *PgHsc70 pro-uidA-nosT* were used to co-cultivate with tobacco leaf-discs. The sterile leaf explants were infected and whole plants were regenerated on MS medium supplemented with plant growth regulators and 50 mg/l hygromycin according to Horsch et al. (1989) with minor modifications (Fig. 2). A total of 35, 32 and 32 hygromycin-resistant putative transgenic tobacco plants were recovered for *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* respectively in the T_0 generation and transferred to the soil for seed setting.

Molecular analysis, development of T_2 generation and statistical analysis

Genomic DNA isolated from the putative transgenic tobacco plants was used for confirmation of the transgenic plants. Two different sets of primers were used to confirm the transgene integration: promoter specific F–*uid*A gene R, Hyg F–Hyg R. In all, 24, 27 and 18 plants were PCR positive for *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* respectively. The

Table 2 *Cis*-acting elements in the promoter regions of *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* regions identified using PlantCare and PlantPan

Motif	No. of *cis*-acting elements			Function
	Apx pro	*Dhn pro*	*Hsc70 pro*	
A-box	1	–	1	*Cis*-acting regulatory element
CAAT-box	1	1	1	Common *cis*-acting element in promoter and enhancer regions
CATT-motif	1	–	–	Part of a light responsive element
CCGTCC-box	1	–	1	*Cis*-acting regulatory element related to meristem specific activation
CGTCA-motif	1	–	1	*Cis*-acting regulatory element involved in the MeJA-responsiveness
G-box	1	2	1	*Cis*-acting regulatory element involved in light responsiveness
GC-motif	1	–	–	Enhancer-like element involved in anoxic specific inducibility
GTGGC-motif	1	–	–	Part of a light responsive element
LTR	1	–	–	*Cis*-acting element involved in low-temperature responsiveness
Skn-1_motif	1	1	–	*Cis*-acting regulatory element required for endosperm expression
Sp1	1	1	1	Light responsive element
TATA-box	1	1	–	Core promoter element around -30 of transcription start
TC-rich repeats	1	–	–	*Cis*-acting element involved in defense and stress responsiveness
TCA-element	1	–	–	*Cis*-acting element involved in salicylic acid responsiveness
TGACG-motif	1	–	1	*Cis*-acting regulatory element involved in the MeJA-responsiveness
rbcS-CMA7a	1	–	–	Part of a light responsive element
5UTR Py-rich stretch	–	1	–	*Cis*-acting element conferring high transcription levels
ABRE	–	1	–	*Cis*-acting element involved in the abscisic acid responsiveness
ARE	–	1	–	*Cis*-acting regulatory element essential for the anaerobic induction
Box 4	–	1	–	Part of a conserved DNA module involved in light responsiveness
Box II -like sequence	–	1	–	*Cis*-acting regulatory element
CCAAT-box	–	1	1	MYBHv1 binding site
GAG-motif	–	1	1	Part of a light responsive element
GT1-motif	–	1	–	Light responsive element
I-box	–	1	–	Part of a light responsive element
LAMP-element	–	1	–	Part of a light responsive element
MBS	–	1	1	MYB binding site involved in drought-inducibility
box E	–	1	–	–
Circadian	–	1	–	*Cis*-acting regulatory element involved in circadian control
motif I	–	1	–	*Cis*-acting regulatory element root specific
Box-W1	–	–	1	fungal elicitor responsive element
CAT-box	–	–	1	*Cis*-acting regulatory element related to meristem expression
HSE	–	–	1	*Cis*-acting element involved in heat stress responsiveness
TGA-element	–	–	1	Auxin-responsive element
W box	–	–	1	–

list of all the primers used are given in Table 1. PCR-positive plants were successfully transferred to the glasshouse. The seeds of these plants were used for generating T_2 plants and also for analysis of the Mendelian segregation ratio of the transgene. All the hygromycin-resistant T_1 tobacco seedlings were analyzed for gene amplification using PCR and PCR confirmed plants were raised again and T_2 seeds collected after maturity. T_2 seeds of *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* containing transformants were germinated on MS basal medium supplemented with 50 mg/l hygromycin. These plants showed 100% seed germination and the events were confirmed by PCR and transferred to pots. Three T_2 transgenic events for each promoter were selected for further analysis. Using the Chi square (χ^2) test, goodness of fit of segregation ratio observed for the transgene was tested against the Mendelian segregation ratio (3:1). Consistency was noticed in the segregation ratio of all of the progeny with the presence of a T-DNA insertion locus, whereas the progeny of the primary transgenics (T_1 generation) showed significantly less transgene events than expected from a monogenic segregation. Deviation from monogenic segregation (as shown by χ^2 test) was not significant among

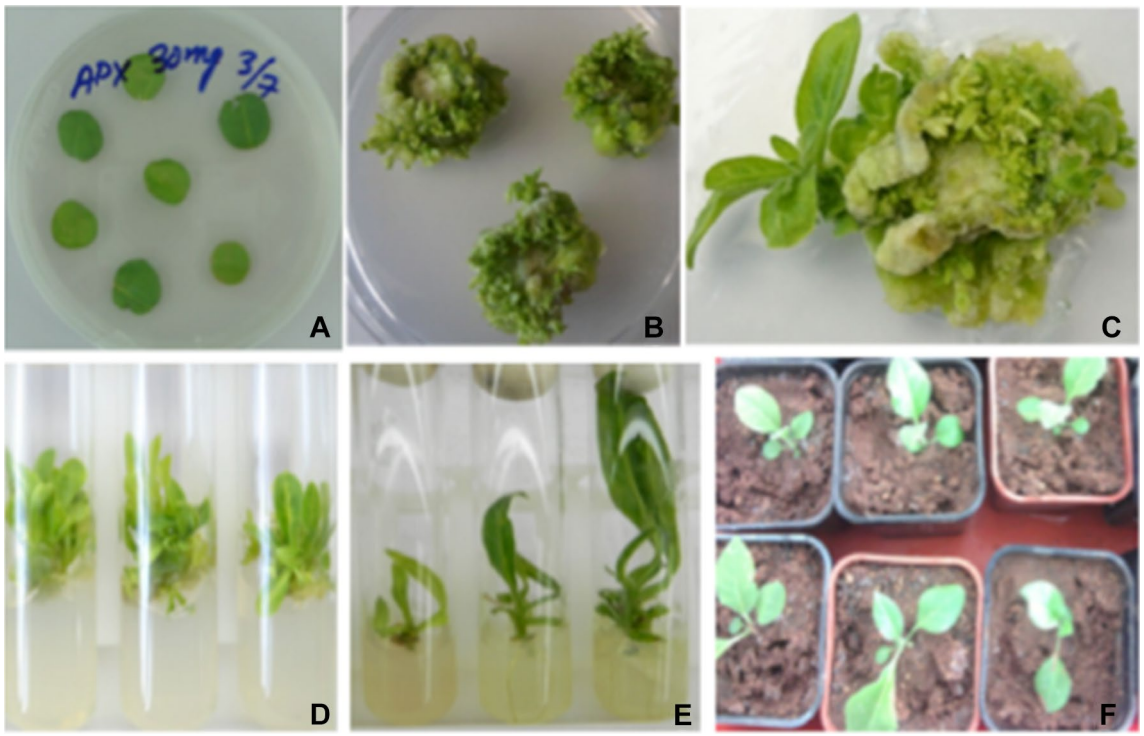

Fig. 2 Tobacco genetic transformation mediated by *Agrobacterium* by Leaf-disc method. Leaf-discs from the leaf explants after co-cultivation with *Agrobacterium* culture harboring the binary plasmids carrying the *PgApx pro, PgDhn pro* and *PgHsc70 pro* with *uid*A gene; **b** and **c** multiple shoot induction and regeneration on MS4C medium; **d** Multiple shoots sub-culturing on MS4C medium for shoot elongation; **e** Root induction of individual shoots on MS medium; **f** Healthy tobacco plants shifted to pots having sand:soil mixture and maintained in P2 greenhouse

Table 3 Segregation and seed germination efficiency of *PgApx pro, PgDhn pro* and *PgHsc70 pro* constructs

S. No	Construct	Event	Total seeds	Total no. of seeds germinated	Total no. of seeds non germinated	Segregation ratio	χ^2 value as to expected ratio of 3:1	Likelihood (P) according to χ^2 test
1.	1	19	769	586	183	3.2:1	0.59	< 0.05
2.	1	25	650	480	170	2.8:1	0.46	< 0.05
3.	4	6	606	471	135	3.4:1	2.39	< 0.05
4.	4	25	662	500	162	3.0:1	0.09	< 0.05
5.	4	23	730	551	179	3.0:1	0.08	< 0.05
6.	5	40	730	540	190	2.8:1	0.41	< 0.05

* χ^2 value at 0.05% probability at 1 df is 3.84. Calculated values below 3.84 were non-significant, and the samples fit for 3:1 segregation ratio. Deviation from monogenic segregation (as shown by χ^2 test) was not significant among any of the progeny

any of the progenies tested (Table 3 and Supplementary Table 2). Data are shown as mean ± standard error (SE) in Table 3. Statistical significance was determined by one-way ANOVA, and means were compared with Tukey–Kramer test and $p < 0.05$ was set as statistical significance.

Tissue specific induction

Different tissues (seedling, leaf, stem and root) of tobacco transgenic T_2 plants grown under controlled (well-watered) conditions were immersed overnight in GUS staining solution to check for the tissue specific expression of *uid*A driven by three abiotic stress-inducible promoters. The GUS histochemical assay results showed that *PgApx pro* and *PgDhn pro* were active in the whole seedling, while *PgHsc70 pro* in roots of seedling (Fig. 3a). *PgDhn pro* and *PgHsc70 pro* exhibited the highest expression in stem and root tissues compared to the leaf. Similar expression pattern was noticed even in *PgApx pro* tissues but with low expression in comparison with *PgDhn pro* and *PgHsc70 pro* (Fig. 3b).

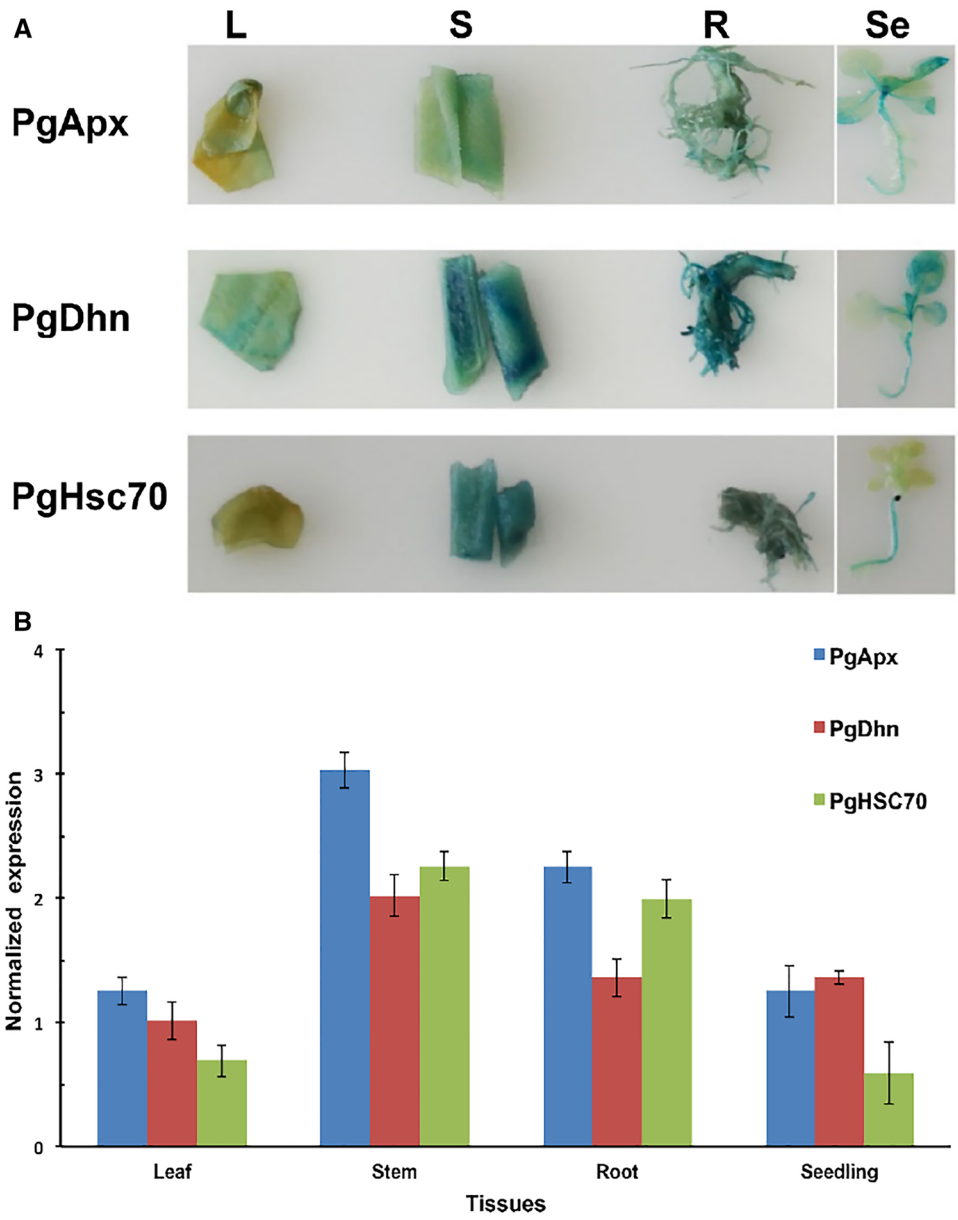

Fig. 3 Tissue specific expression of the three pearl millet promoters. **a** Histochemical assay for *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* in transgenic tobacco plants under control conditions in leaf, stem and root tissues. **b** Normalized expression of *uid*A gene controlled by *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* in transgenic tobacco plants under control conditions in leaf, stem and root tissues. Values represent the normalized expression values after normalizing with the reference genes. Samples were analyzed in triplicates in three independent experiments. Different tissues are represented on the X-axis, whereas, normalized expression of *uid*A gene represented on the Y-axis. Standard error bars are shown. *L* Leaf, *S* Stem, *R* Root and *Se* Seedling

Compared to the mature leaf, stem and root tissues, seedlings exhibited very low expression indicating the regulation is developmental-stage specific.

Abiotic stress inducible promoters

Expression of the *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* were confirmed by GUS staining in the leaf, stem and root tissues of the T_2 transgenic tobacco plants under different abiotic stress conditions. *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* under non-stress conditions exhibited significantly lower or no GUS activity in comparison with stress-imposed tissues. The GUS expression staining pattern under different abiotic stresses is given below.

High temperature stress

In order to check the regulation of *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* under high temperature stress, stress-imposed tissues were collected and subjected to GUS staining. *PgApx pro* and *PgDhn pro* exhibited the highest expression in stem and root tissues in comparison with leaf. Surprisingly, *PgHsc70 pro* showed less expression in comparison with *PgApx pro* and *PgDhn pro*, but still was fairly regulated under high temperature stress (Fig. 4).

Cold stress

In order to check the activity of these three promoters under cold shock, transgenics harboring these promoters

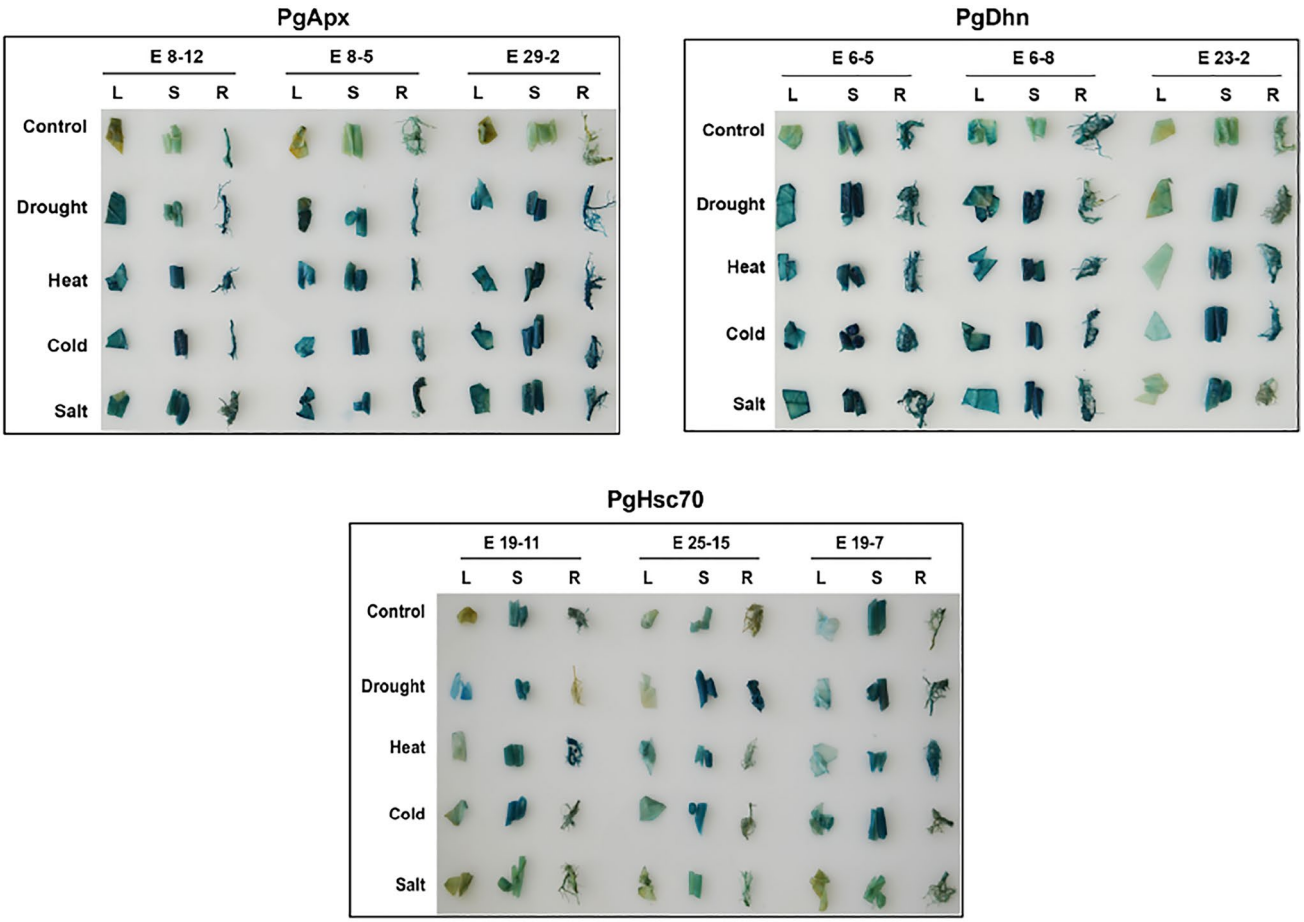

Fig. 4 Histochemical assay for GUS expression analysis in transformed tobacco tissues under different abiotic stress conditions. Transgenic tobacco leaves, stem and roots which were treated with different abiotic stress conditions and stained by GUS staining solution for studying their GUS activity, localization and intensity upon treatment with salt, heat, cold and drought stresses and in control conditions. *L* Leaf, *S* Stem and *R* Root

were subjected to 4 °C for 4 h. Amongst all the stresses, promoter-induced activity was less under cold stress conditions. This is attributed to the lack of cold-specific *cis*-acting elements upstream of the gene sequence. *PgApx pro* and *PgDhn pro* showed higher expression patterns in stem than root and least expression in leaf. In *PgHsc70 pro*-containing transgenics, least inducibility was recorded in comparison with other two promoters, with the highest intensity in stem, followed by root and negligible expression in leaf tissues (Fig. 4).

Salt stress

Upon exposure to 250 mM NaCl stress, *PgApx pro* transgenic tobacco plants showed high GUS activity followed by *PgDhn pro* and then *PgHsc70 pro*. *PgApx pro* showed the highest expression in root, and stem but less expression in the leaf tissues. *PgDhn pro* showed similar expression pattern but with less intensity when compared to *PgApx pro*. *PgHsc70 pro* showed the least expression in all the leaf, stem and root tissues in comparison with *PgApx pro* and *PgDhn pro* and negligible expression or no expression in leaf tissues of *PgHsc70 pro* (Fig. 4).

Drought stress

High GUS activity was noticed in *PgDhn pro* transgenic stems and roots under drought stress, followed by leaf. Similar observations were recorded for *PgApx pro* but with less intensity in comparison with *PgDhn pro*. GUS induction of *PgHsc70 pro* was less in comparison with *PgApx pro* and *PgDhn pro*, and least in all the leaf, stem and root, especially in leaf tissues (Fig. 4).

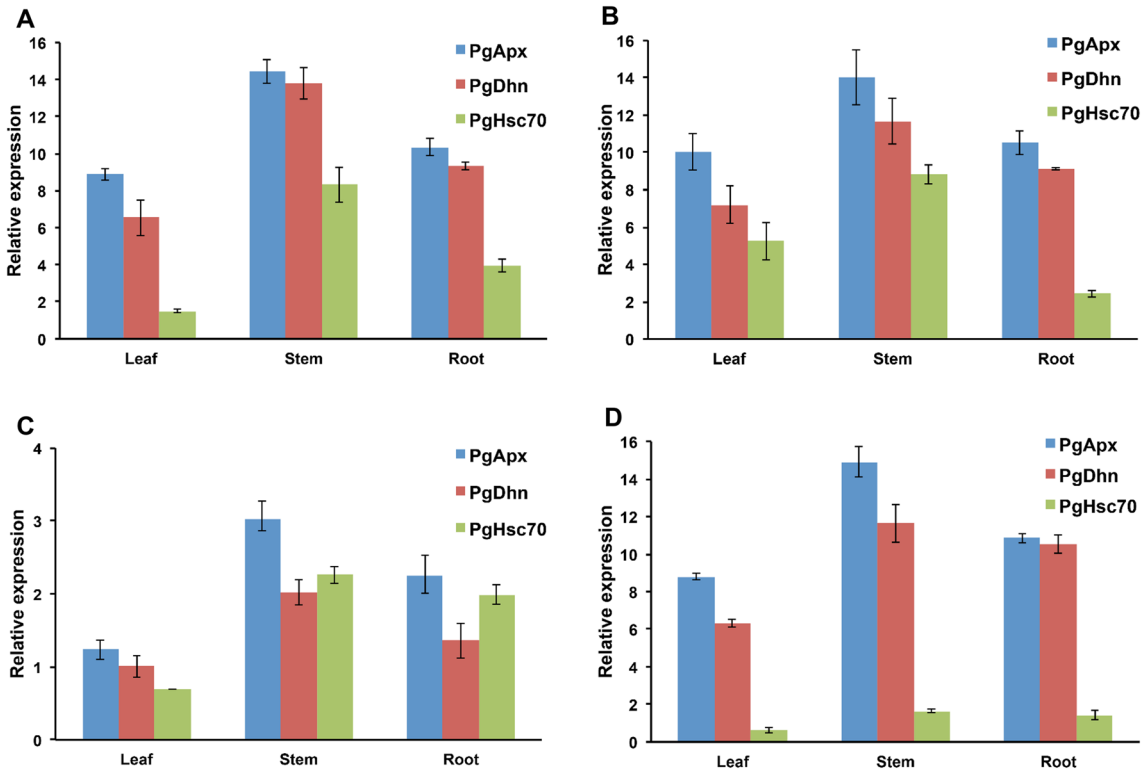

Fig. 5 Relative expression analysis of *uid*A transcript in leaf, stem and root tissues of *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* transgenic tobacco plants in various abiotic stress conditions i.e., **a** drought, **b** heat, **c** cold and **d** salt stresses. Values represent the expression folds obtained after normalizing against the reference genes as well as corresponding control samples. All samples were analyzed in triplicate for each experiment. Different abiotic stress treatments are represented on the X-axis, and relative expression of *uid*A gene expression are represented on the Y-axis. Standard error bars are shown

Quantification of *GUS* gene activity as measured by RT-qPCR under abiotic stresses

Quantitative RT-PCR was performed to understand the *uid*A gene expression in leaf, stem and root tissues of the transgenic tobacco plants under different abiotic stress conditions. In comparison with the untreated tissues, higher accumulation of *uid*A transcripts was observed in different abiotic stress-exposed plants (Fig. 5). These findings are completely in accordance with the histochemical assays. For normalized expression studies, equal expression was recorded in the seedlings of *PgApx pro* and *PgDhn pro*. Comparatively, *PgApx pro* and *PgHsc70 pro* showed slightly higher expression in stem and root tissues than leaf. Expression was less in the leaf, stem and root tissues of *PgDhn pro*. Under high temperature stress, expression of *uid*A was intense in the stem and root tissues but less in both *PgApx pro* and *PgDhn pro*. Comparatively, *PgHsc70 pro* driven *uid*A displayed lower expression in the leaf, stem and root tissues than *PgApx pro* and *PgDhn pro*. Under cold stress, *PgApx pro* and *PgDhn pro* showed similar expression patterns in stem and root but mild expression in the corresponding leaf tissues. *PgHsc70 pro* in stem and root tissues showed mild expression (which is lower than *PgApx pro* and *PgDhn pro* tissues) with no or very limited expression in the leaf tissues (Fig. 5). Under salt stress, *PgApx pro* recorded higher expression in stem than root tissues and the least in the leaf tissues. *PgDhn pro* also displayed the same expression pattern across different tissues but with lower activity in comparison with *PgApx pro*. Mild expression of *PgHsc70 pro* was noticed in the stem and root but not in the leaf tissue. In case of drought stress, *PgDhn pro* showed the highest expression in the stem and root tissues followed by leaf. Intense expression levels of *PgApx pro* were observed in the stem and root tissues followed by leaf but lesser expression compared to the *PgDhn pro*. Contrarily, *PgHsc70 pro* showcased mild expression in the stem and root but least expression in the leaf tissues (Fig. 5). Comparison of expression levels between transgenic and wild-type plants under stress and normal conditions showed that there was a statistically significant difference between stressed and non-stressed plants as well as plants containing *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* ($p < 0.05$). Tukey–Kramer test distinguished mean expression values of both transgenic plants under normal and transgenic plants under stress conditions (Supplementary Table 2).

Discussion

If transgenes are expressed under the influence of constitutive promoters, an additional metabolic load on the plant systems is usually observed. Use of constitutive promoters may even, in some cases, result in undesirable phenotypes and/or low yield. Plants need to divert their useful resources for growth in normal as well as under stressed conditions, hence, usage of stress-inducible promoters provide ideal conditions for transgene modulation and better performance of the transgenics. Pearl millet is a stress tolerant crop and grows in the arid regions. Stress tolerance mechanisms associated with water deficit conditions were studied earlier in millets including pearl millet [52–54]. Based on high vapour pressure deficit and other studies related to drought, it has been inferred that these plants perform well under harsh conditions [54]. It is predicted that there are good chances of this crop plant containing stress-inducible genes and promoter sequences that can be exploited for generating drought-tolerant crops. They may confer superior stress tolerance and therefore, validating such promoters under stress conditions is of considerable interest. Hence, studies on the abiotic stress-inducible genes or promoters would be a logical move which would help in the crop improvement in both forward, and reverse genetics as well as in genetic engineering approaches. Present study involves cloning and functional characterization of three pearl millet abiotic stress-inducible promoters namely *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* and their expression in the tobacco model system. The respective genes were selected based on the earlier studies which revealed that they play pivotal roles and contribute to the abiotic stress tolerance [5, 29, 36, 55]. In silico analysis of *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* with PlantCare and PlantPan showed multiple *cis*-acting elements. This indicates that these promoters modulate transcription in a stress-inducible and tissue specific manner, but that does not ensure efficacy when put into a transgene. Hence, it is of utmost importance to check the efficacy of these promoters in transgenic tobacco by fusing them with a *uid*A reporter gene. If the promoters are to be employed for developing a transgenic crop plant, they ought to be analyzed first functionally. Accordingly, transgenic tobacco plants were developed using three promoters (Fig. 2). Also, *uid*A gene expression was analyzed under control (devoid of stress) (Fig. 3) as well as different abiotic stress conditions like high temperature, cold, salt and drought stresses in the glasshouse.

Expression of the *uid*A gene with *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* was confirmed by GUS histochemical staining in leaf, stem and root tissues of the T_2 transgenic tobacco plants. *PgApx pro* and *PgDhn pro* showed high expression compared to *PgHsc70 pro* in high temperature and cold stress conditions. *PgApx pro* showed high expression in salt stress conditions, followed by *PgDhn pro* and the lowest in *PgHsc70 pro*. The results indicate that *PgApx pro* could be considered as a better choice for using it while developing transgenics. On the other hand, *PgDhn pro* unveiled high expression under drought stress conditions followed by *PgApx pro* and comparatively least activity in *PgHsc70 pro*. Heat stress as well as other abiotic stresses cause severe damage to the plant cells and ultimately final productivity of crop [56]. When the three promoters in the transgenic tobacco plants were subjected to high temperature stress (40 °C), all the three displayed high expressions (Figs. 4, 5) which indicate that these promoters are associated with abiotic stress conditions. *PgHsc70 pro* and *PgApx pro* consist of HSE *cis*-acting elements and likely that this element is responsible for the promoter activation under high temperature stress [57]. In contrast, *PgDhn pro* does not possess the HSE *cis*-element but still activated under high temperature stress which was also demonstrated in different plant species. Sato et al. [58] noticed that the HSE in the rice *Apx* promoter helps in the heat shock induction of the *Apx* gene. Also, *Dhn1* and *Dhn2* genes from grapevine were reported to be induced under high temperature stress. Yang et al. [59] noted that the presence of specific *cis*-acting elements within the upstream regions of each gene was positively correlated with its expression. Our studies infer that both the *PgDhn pro* and *PgApx pro* are activated under high temperature stress conditions. From earlier studies, it is evident that ROS-mediated stress tolerant mechanisms play a significant role in shielding crops against extreme temperature stresses [60]. The expression of rice *OsApxa pro* protects rice seedlings against chilling injury as demonstrated by Sato et al. [58]. *PgApx pro* transgenic tobacco plants recorded the highest expression when exposed to cold stress (4 °C) conditions in the present study. *PgDhn pro* and *PgHsc70 pro* under cold stress exhibited higher expressions of the *uid*A gene that might have been controlled by *cis*-elements. Else, there might be some unknown *cis*-acting elements within the promoter region that have not yet been identified or their functions not known. Higher expression noticed in the *PgApx pro* transgenic plants could be because of increased Apx activity with the elevation in the level of stress as was also noticed earlier in diverse plant species [21, 61, 62]. Halder et al. [63] demonstrated that dehydrin (*SbDhn2*) driven under the influence of *CaMV35 s pro* confers tolerance to oxidative damage caused by different abiotic stresses. These plants revealed protection against hydroxyl radical and it is possible that such a mechanism also operates in transgenic tobacco plants containing *PgDhn pro* to confer resistance against salt stress. Least or no expression was recorded in the transgenics transformed with *PgHsc70 pro* possibly because of the absence of the specific elements in its promoter sequence.

Transgenic tobacco plants containing the three promoters showed higher expression of *uid*A gene against drought stress. In spite of the absence of any drought specific elements, *PgApx pro* transgenic tobacco plants demonstrated higher expression of the GUS. This might be because of the presence of GC motif and TC-rich repeats (Figs. 1, 4, 5). Evidence was presented earlier from similar studies, where transgenic plants overexpressing *Apx* gene under the influence of *SWAP2* pro conferred drought stress tolerance. This could be because of the presence of other abiotic stress-related *cis*-acting elements [64]. Both *PgDhn pro* and *PgHsc70 pro* plants displayed resistance to drought, due to the presence of drought specific *cis*-acting elements like CCAAT-box and MBS. Our results corroborate the studies on *dehydrin* promoter region (*PpDhn2*) of peach that conferred tolerance to dehydration stress [65].

This is the first study on the isolation and functional characterization of abiotic stress-inducible promoters from a naturally drought-adapted crop plant like pearl millet. Comparative functional validation of these three stress-inducible promoters provided vital information like the specificity and inducibility of stress-responsive genes. Interestingly, in transgenic tobacco plants, *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* are induced differentially under high temperature, cold, salt and drought stress conditions. The results indicate that these promoters can be employed effectively under varied abiotic stress conditions and such transgenics may exhibit better productivity under stress. Present results also pave the way for a detailed study of these promoter region analysis, because these could be potential candidate promoters for developing abiotic stress tolerant crop plants through genetic engineering approaches, or at least to understand their role in the adaptation to water/high temperature stress in pearl millet. These promoters drive low constitutive transgene expression in normal conditions, but high activity under high temperature, cold, salt and drought stresses. Hence, usage of *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* promoters would avoid many harmful effects posed during the overexpression of target genes under the influence of constitutive promoters in transgenics.

Conclusions

Genetic engineering technology is a robust tool to validate, understand and alter the reflexes of plants to different stress conditions. There is a need to dissect out the genetic elements viz., genes and functionality of the promoters especially in agronomically superior crops and the phenotype they lead to under abiotic stress conditions since their manipulation may aid to widen our knowledge of crop tolerance to such stresses. To our knowledge, the *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* are the first stress-inducible promoters being documented especially from pearl millet. From the experiments, it is concluded that the low constitutive expression under normal conditions and specific inducible expression patterns of *PgApx pro*, *PgDhn pro* and *PgHsc70 pro* in seedlings, leaf, stem and root tissues on exposure to abiotic stresses make them crucial candidate promoters for understanding abiotic stress responses. These promoters appeared highly inducible under different abiotic stress conditions. This would enable these promoters to be valuable tools for the targeted expression of the downstream genes like *uid*A (present study) for engineering abiotic stress protection to the crop plants. Research in the areas of stress biology in the future would highlight the need for stress-inducible expression of the transgenes. Further, transgenes in combination with the proper promoter regions involving transcription factors will be a new genetic alteration tool for steering the regulation of varied stress-responsive genes.

Acknowledgements PSR acknowledges the Department of Science and Technology, New Delhi, Government of India for the research grant and award through INSPIRE Faculty (Award No. IFA11-LSPA-06). This work was undertaken as part of the CGIAR Research Program on GLDC.

Author contributions PSR and VV designed the experiments, KD, PSR, PBM and PS executed the study, PSR, KD and PBK analyzed data. PSR, KKS, PBK and KD wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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