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Functional characterization of the promoter of pearl millet heat shock protein 10 (PgHsp10) in response to abiotic stresses in transgenic tobacco plants

Divya Kummari, Pooja Bhatnagar-Mathur, Kiran K. Sharma, Vincent Vadez, Sudhakar Reddy Palakolanu *

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad 502 324, India

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ABSTRACT

In the present study, the promoter region of the pearl millet *heat shock protein 10* (*PgHsp10*) gene was cloned and characterized. The *PgHsp10* promoter (*PgHsp10pro*) sequence region has all the *cis*-motifs required for tissue and abiotic stress inducibility. The complete *PgHsp10pro* (*PgHsp10PC*) region and a series of 5′ truncations of *PgHsp10* (*PgHsp10D1* and *PgHsp10D2*) and an antisense form of *PgHsp10pro* (*PgHsp10AS*) were cloned into a plant expression vector (pMDC164) through gateway cloning. All four constructs were separately transformed into tobacco through *Agrobacterium-mediated* genetic transformation, and PCR-confirmed transgenic plants progressed to T_1 and T_2 generations. The T_2 transgenic tobacco plants comprising all *PgHsp10pro* fragments were used for GUS histochemical and qRT-PCR assays in different tissues under control and abiotic stresses. The *PgHsp10PC pro* expression was specific to stem and seedlings under control conditions. Under different abiotic stresses, particularly heat stress, *PgHsp10PCpro* had relatively higher activity than *PgHsp10D1pro*, *PgHsp10D2pro* and *PgHsp10ASpro*. *PgHsp10pro* from a stress resilient crop like pearl millet responds positively to a range of abiotic stresses, in particular heat, when expressed in heterologous plant systems such as tobacco. Hence, *PgHsp10pro* appears to be a potential promoter candidate for developing heat and drought stress-tolerant crop plants.

1. Introduction

Understanding the role of heat shock proteins (Hsps) in plants has enhanced agricultural productivity and unraveled the molecular mechanism underlying protein folding, misfolding and aggregation. Upon encountering the negative effects of protein misfolding and aggregation, cells are perceived to develop a system known as protein quality control (PQC) for maintaining homeostasis [1]. This is done with the aid of the proteasome and chaperonic system along with molecular chaperones and Hsps. Under stress conditions, by default Hsps act as molecular chaperons, whereas under nonstress conditions, their function could be different [2–6]. In fact, plant adaptation to abiotic stresses, including heat stress, was shown to be strongly dependent on Hsps, as exemplified by the diversification of their families. Based on the molecular weight, Hsps were classified into five subfamilies comprising small Hsp (sHsp), chaperonins (Hsp10 and Hsp60), Hsp70, Hsp90, and Hsp100 [3,7–9]. While Hsps are known to play a role in maintaining cellular homeostasis, some Hsps (e.g., sHsp and Hsp70) stabilize protein folding, prevent aggregation and maintain the non-native protein in a competent state for subsequent refolding with the help of some Hsps such as Hsp60, Hsp70 and Hsp90 [7,10]. The denatured or misfolded proteins form aggregates that are further resolubilized by Hsp100/Clp followed by refolding or degradation by proteases [11]. Some Hsps/chaperones (e.g., Hsp70, Hsp90) accompanying the signal transduction and activation of specific transcription factors (TFs) lead to the synthesis of other members of Hsps/chaperones [7,12].

The chaperonin (Cpn) family comprises important molecular chaperones that can be found in prokaryotes and eukaryotes [13]. The Cpn family has been categorized into two types: group I chaperonions (Hsp60) found in bacteria, mitochondria and chloroplasts, and group II chaperonions such as thermosomes and TRiC found in archaea and the eukaryotic cytosol [14]. Hsp10 family proteins functionally cooperate with Hsp60 chaperonions in an ATP-dependent manner and form the lid of the protein-folding cage and assist in precise protein folding, including that of larger proteins [14]. Hsp10 (Cpn10) belongs to the multigene family where high sequence conservation is observed between the family members and other plant homologs [15]. To date, very few expression and functional studies have been reported to unravel the role of *Hsp10* genes under different abiotic stresses. *Hsp10* genes are known to be expressed under different abiotic stress conditions [15–17]. Tan

* Corresponding author at: Cell, Molecular Biology & Genetic Engineering Group, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru-502 324, Hyderabad, Telangana, India.
E-mail address: p.sudhakarreddy@cgiar.org (S.R. Palakolanu).

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Table 1
Primers used for cloning the fragments of *PgHsp10* promoter into the pCR8/GW/TOPO TA vector.

S. No.	Primer	Sequence (5′ - 3′)	Product size (bp)
1	Hsp10_PC_F	AGTTCCAGGCCGAAGTGAAT	896
2	Hsp10_PC_R	CGCCGCCTTCTCTTCTTCTTCTT	
3	Hsp10_PD1_F	AGCGAACTACGCGTGTACCT	561
4	Hsp10_PC_R	CGCCGCCTTCTCTTCTTCTTCTT	
5	Hsp10_PD2_F	CATGGCTCAATACCTGAACATTT	319
6	Hsp10_PC_R	CGCCGCCTTCTCTTCTTCTTCTT	
7	Hsp10_PC_AS_F	CGCCGCCTTCTCTTCTTCTTCTTG	896
8	Hsp10_PC_AS_R	ATTAGAGTTCCAGGCCGAAGTG	

et al. (17) observed that both *GdHsp10* and *GdHsp60* were induced under heat and cold stresses and that two genes were expressed from high to low in *G. daurica* eggs over time. In sea cucumber (*A. japonicas*), *Hsp10* and *Hsp60* transcript expression was highly upregulated with time and temperature, suggesting their role in heat stress response. Further studies of Hsp60 and the cochaperone in *E. coli* and yeast demonstrate their tolerance against osmotic and salt stresses. A recent study in pearl millet showed that *PgHsp10* was expressed at high levels under heat stress compared to other abiotic stresses. Furthermore, *E. coli* cells overexpressing PgHsp10 show better tolerance to heat and salt stresses [15].

Pearl millet (*Pennisetum glaucum*) is one of the few resilient crops that can adapt to climate change scenarios, particularly under increasing adverse environmental conditions with a limited need for fertilizer supplements. Therefore, owing to its abiotic stress-resistant nature, pearl millet is anticipated to be equipped with better tolerance mechanisms than other cereals to combat different abiotic stresses. Hence, characterization of Hsp10 from naturally drought-tolerant crops such as pearl millet would help in understanding the tolerance mechanism and development of abiotic stress-tolerant plants. In the present study, we cloned and characterized the *Hsp10* promoter (*Hsp10pro*) region from pearl millet. The activity of *PgHsp10pro* along with its truncations and antisense promoter regions was functionally characterized in transgenic tobacco plants.

2. Materials and methods

2.1. Cloning of PgHsp10pro regions

The upstream region of the *PgHsp10* gene comprising of the promoter was cloned from the pearl millet (variety PRLT2) genomic DNA according to [15] using promoter-specific primers listed in Table 1. Full-length sense, antisense and truncated *PgHsp10pro* sequences were PCR amplified by the polymerase chain reaction (PCR) using respective forward and reverse primers with proofreading DNA polymerase (*Pfu*, Thermo Scientific) with 200 μM of dNTPs and 300 ng of genomic DNA. PCR was performed as follows: 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min for 32 cycles with a final extension of 72 °C for 10 min. The amplified PCR products were cloned into the pCR™8/GW/TOPO® TA vector (Invitrogen) and sequenced. *Cis*-elements present in the *PgHsp10pro* regions were analyzed using literature and the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [18].

2.2. Cloning, development of the transgenic tobacco plants and abiotic stress treatments

pCR™8/GW/TOPO® TA recombinant plasmids harboring *PgHsp10PCpro*, *PgHsp10ASpro*, *PgHsp10D1pro* and *PgHsp10D2pro* were

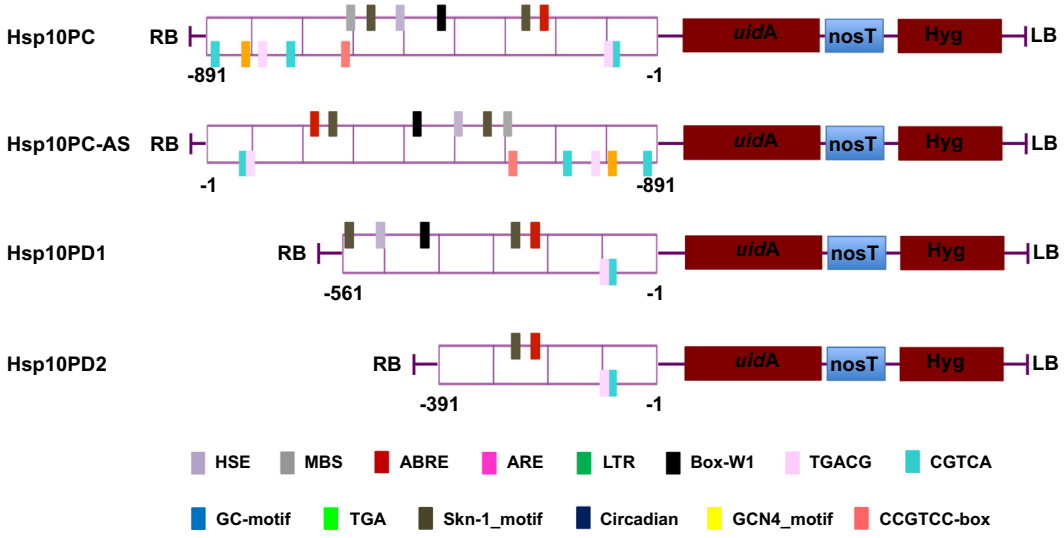

Fig. 1. Position of *cis*-acting elements present in the *Hsp10pro* region. The analysis was performed using the PlantCARE database. Gus- *uid*A gene, *nos*T - Nopaline synthase terminator, *Hyg*-Hygromycin, RB-Right border, LB-Left border.

cloned separately with the *uid*A gene in the plant binary vector pMDC164 using gateway cloning. The resulting recombinant clones containing PgHsp10PC-uidA-NosT, PgHsp10AS-uidA-NosT, PgHsp10D1-uidA-NosT and PgHsp10D2-uidA-NosT in pMDC164 vector were transformed into *Agrobacterium tumefaciens* strain EHA105 using electroporation. Positive colonies were used for tobacco transformation. *Nicotiana tabacum* (L.) var. Xanthi was used to develop transgenic tobacco plants according to Divya et al., (3). The putatively transformed plantlets were transplanted into soil and transferred to a greenhouse. Genomic DNA was isolated from leaf tissue using the DNeasy Plant Mini Kit (Qiagen). Transgene integration of these plants was confirmed by PCR using the promoter-specific F - *uid*A gene R as well as Hyg F - Hyg R, and maintained in a containment glasshouse for selfing and seed set. While the T_1 transgenic tobacco events were analyzed for Mendelian segregation, the T_2 generation plants of *PgHsp10PC*, *PgHsp10AS*, *PgHsp10D1* and *PgHsp10D2* were used for functional analysis. Abiotic stresses, including salt, drought, heat and cold stresses, were imposed on 30-day-old plants. These plants were incubated for 4 h in heat and cold conditions at 45 °C and 4 °C for the respective stresses. For dehydration stress, water supply was withheld for 5 days and for salt stress 250 mM NaCl solution was applied for 48 h to the plants. Detailed methodology of the abiotic stress treatments were described in one of our earlier publications (3). Plants with regular water supply were used as controls. Seedling, leaf, stem and root tissues of control and treated plants were used for GUS histochemical studies, and respective samples were snap frozen in liquid nitrogen and stored at −80 °C for RNA extraction. All experiments were performed in triplicate.

2.3. GUS Histochemical assay

Control and abiotic stress-treated transgenic tobacco tissues were subjected to histochemical GUS studies according to [19]. Tobacco tissues (Seedling, leaf, stem and root) were immersed in GUS staining solution (50 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, 0.1% Triton X-100 and 1 mM X-Gluc), and incubated at 37 °C for 24 h. The tissues were bleached with 70% ethanol, and the results documented.

2.4. qRT-PCR analysis

Total RNA was extracted from the seedling, leaf, stem and root tissues of transgenic tobacco plants using the RNeasy Mini Kit (Qiagen). The quantity and quality of RNA were analyzed by a NanoVue plus spectrophotometer (GE Health Care, USA) in addition to the ratio of the absorbance at 260 and 280 nm (260/280). qRT-PCR reactions were performed in a total volume of 10 μl containing 1 μl of RNA (30 ng), 400 nM of each primer, 5 μl of 2× one step SYBR RT-PCR buffer 4 (Takara, Japan) and 0.5 μl of Prime Script One Step Enzyme Mix 2 (Takara, Japan) made to a volume of 10 μl with RNase-free H_2O. The qRT-PCR reactions of all samples were performed using the following standard thermal profile: 42 °C for 5 min and 95 °C for 10 s (reverse transcription), followed by 40 cycles of 15 s at 95 °C, 15 s at 62 °C with fluorescent signal recording and 15 s at 72 °C. The amplicon dissociation curves were measured after 40 cycles by heating from 58 °C to 95 °C with fluorescence measured within 20 min. Experiments were performed in triplicate and repeated three times, and the data from three experiments were averaged. The relative expression levels of the *uid*A gene in response to different abiotic stress treatments were estimated using qBase software [20] by normalizing with corresponding control samples and with *L25* and *EF-1α* as the internal control genes [21].

2.5. Statistical analysis

The transgenic tobacco seeds in T_1 generation were germinated on MS medium [22] containing hygromycin (50 mg/l) were analyzed for the Mendelian inheritance pattern by a chi square test ($\chi 2$) [23]. The $\chi 2$ values were calculated with 0.5 significance level used to test for statistical significance. The results were expressed as the mean values ± SD (standard deviation). The relative expression of the *uid*A gene is expressed as the mean ± SE from at least three experiments. For all the stress experiments (heat, cold, salt and drought), relative expression data were analyzed with CoStat version 6.204 (Cohort Software, Monterey, CA, USA). One-way ANOVA was performed to check for expression differences among the transgenic events and wild types. The means were compared using the Tukey-Kramer test as well as LSD (at $p = 5\%$ significance).

3. Results

3.1. Cloning and sequence analysis of the PgHsp10pro region

The *Hsp10* promoter region (*Hsp10pro*) was isolated from pearl millet genomic DNA according to Nitnavare et al., [15]. The full-length (PC), anti-sense (AS) and truncated promoters (D1 and D2) were PCR amplified using specifically designed primers with sizes of 896 bp, 896 bp, 561 bp and 319 bp corresponding to the *Hsp10PC*, *PgHsp10AS*, *Hsp10D1* and *PgHsp10D2* promoters, respectively. All promoter sequences were analyzed *in silico* using the PlantCARE database (Fig. 1) which showed that *PgHsp10pro* harbors *cis*-acting elements, including CAAT-box, G-box, and two copies of LTRE present in all four promoter fragments. CCGTCC-box (meristem tissue specific) and MBS (drought-inducibility) were present in the *PgHsp10PC*, *PgHsp10D1* and *PgHsp10D2* promoters. MeJA-responsive TGACG-motif, AAGAA-motif and CGTCA-motifs, endosperm Skn-1_motifs were present in two truncated promoters. ARE (anaerobic induction), Box4 (light responsiveness), CAT-box (meristem expression), CCAAT-box (MYBHv1 binding site), CE3 (ABA and VP1 responsiveness), and the O2-site (zein metabolism

Table 2
Cis-elements present in the promoter region of *PgHsp10*, *PgHsp10 AS*, *PgHsp10 D1* and *PgHsp10D2*.

Motif	No.of elements in				Function
	Hsp10PC	Hsp10 AS	Hsp10D1	Hsp10D2	
ACE	1	–	–	–	Light responsiveness
ARE	1	–	–	–	Anaerobic induction
Box 4	1	–	–	–	Light responsiveness
CAT-box	1	–	–	–	Meristem expression
CCAAT-box	1	–	–	–	MYBHv1 binding site
CCGTCC-box	1	1	1	–	Meristem specific activation
CE3	1	–	–	–	ABA and VP1 responsiveness
CGTCA-motif	1	1	–	–	MeJA-responsiveness
GAG-motif	1	1	–	–	Light responsive element
GARE-motif	1	1	1	–	Gibberellin-responsive element
GC-motif	–	1	–	–	Anoxic specific inducibility
GCN4_motif	–	1	–	1	Endosperm expression
LTR	1	1	1	1	Low-temperature responsiveness
MBS	1	1	1	–	Drought-inducibility
O2-site	1	–	–	–	Zein metabolism regulation
Skn-1_motif	1	1	–	–	Endosperm expression
TCA-element	–	1	–	–	Salicylic acid responsiveness
TGACG-motif	1	1	–	–	MeJA-responsiveness
TGG-motif	1		–	–	Part of a light responsive element
chs-CMA2a	1	1	1	1	Part of a light responsive element
circadian	1	–	1	–	Circadian control

Table 3
Segregation analysis and seed germination efficiency of transgenic tobacco plants carrying the *PgHsp10*, *PgHsp10AS*, *PgHsp10D1* and *PgHsp10D2* constructs: * $\chi 2$ value at 0.05% probability at 1 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 $\chi 2$ test) was not significant among any of the progeny.

Construct	Event No.	No. of seeds plated	No. of seeds germinated	Total no. of seeds non-germinated	Segregation ratio	$\chi 2$ value as to expected ratio of 3:1	Likelihood (P) according to $\chi 2$ test
PgHsp10	18	343	246	97	2.5:1	1.96	<0.05
PgHsp10	29	386	290	96	3.0:1	0.0034	<0.05
PgHsp10	34	368	287	81	3.5:1	1.75	<0.05
PgHsp10AS	2	335	256	79	3.2:1	0.35	<0.05
PgHsp10AS	17	378	298	80	3.7:1	2.96	<0.05
PgHsp10D1	2	394	308	86	3.5:1	2.11	<0.05
PgHsp10D2	2	390	288	102	2.8:1	0.27	<0.05

regulation) were present in the *PgHsp10PCpro*. The presence or absence of motifs in these promoters showed their involvement under different abiotic stress conditions and suggest their vital role in promoter regulation in response to different abiotic stress conditions (Fig. 1 and Table 2).

3.2. Development of the transgenic tobacco plants

All four promoters were separately cloned along with the *uid*A gene into the pMDC164 vector through gateway cloning. Recombinant plasmids with the pMDC164 backbone containing *PgHsp10PC*, *PgHsp10AS*, *PgHsp10D1* and *PgHsp10D2* promoters were transformed into *A. tumefaciens strain* EHA105, and positive colonies were transformed into tobacco using the leaf-disc method [3]. Aseptically maintained leaf explants were infected with *A. tumefaciens* containing the gene of interest and then regenerated into a whole plant on basal MS media supplemented with hormones and 50 mg/l hygromycin [3]. A total of 39, 39, 4 and 3 hygromycin-resistant putative T_0 transgenic tobacco plants were generated with *PgHsp10PC*, *PgHsp10AS*, *PgHsp10D1* and *PgHsp10D2* promoters, respectively. Transgenic tobacco events that showed a segregation pattern according to the Mendelian segregation ratio of 3:1 in the T_1 generation were selected for advancement T_2 generation. These positively confirmed T_2 plants of all four constructs were used for *uid*A expression studies.

3.3. Statistical analysis

Based on the chi-square ($\chi 2$) test and the goodness of fit, all progenies of the confirmed transgenic plants followed the segregation ratio, consistent with the presence of a T-DNA insertion locus. Any deviation from monogenic segregation (as shown by the $\chi 2$ test) was not significant in any of the progenies tested (Table 3). The relative expression data for abiotic stresses showed significant differences between the expression levels of *PgHsp10PC* and the other three promoters (*PgHsp10AS*, *PgHsp10D1* and *PgHsp10D2*) when compared to their respective controls.

3.4. Tissue-specific expression of PgHsp10 promoter regions

Seedling, leaf, stem and root tissues of the T_2 transgenic tobacco plants were used to study the spatiotemporal *uid*A gene expression under control conditions. While the *PgHsp10PC* was active in seedlings and stems, *PgHsp10AS* was less active in roots, *PgHsp10D1* was proactive completely in seedlings and *PgHsp10D2* in roots. In comparison, the wild-type seedlings did not show any expression. Lower or no expression was observed in the leaves and roots of *PgHsp10PC* and in the leaves, stems and roots of *PgHsp10AS*, and no expression was observed in the leaf, stem and root tissues of *PgHsp10D1* and *PgHsp10D2*

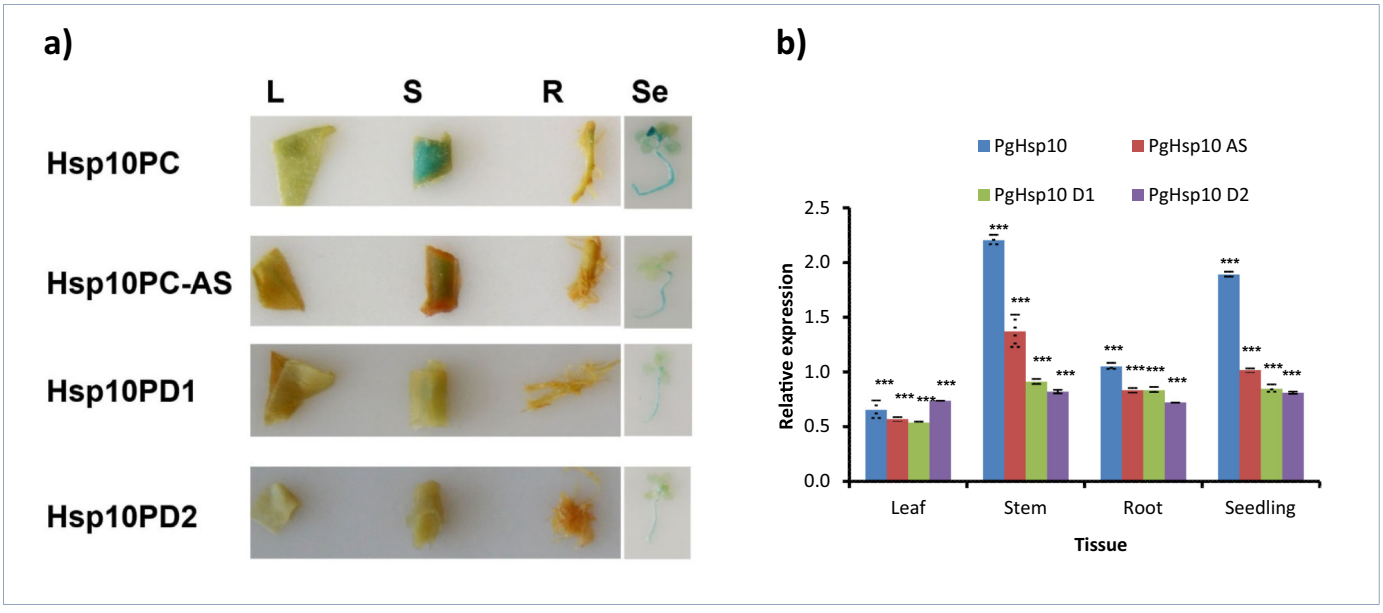

Fig. 2. Tissue-specific expression of the *PgHsp10*, *PgHsp10AS*, *PgHsp10D1* and *PgHsp10D2* promoters in transgenic tobacco plants under control conditions in leaf (L), stem (S), root (R) and seedling (Se) tissues. a) GUS histochemical assay b) Expression analysis by qRT-PCR analysis. Values represent the normalized expression values after normalizing with the reference genes. All samples were analyzed in triplicate in three independent experiments. Different tissues are represented on the X-axis, whereas normalized expression of the *uid*A gene expression is represented on the Y-axis. Standard error bars are shown.

promoters. These results indicate that *PgHsp10* promoters are not only stress-inducible but are also tissue-specific.

3.5. PgHsp10pro regions expression under abiotic stress conditions based on histochemical assay

To unravel the functional region of the *PgHsp10pro* region involved in conferring abiotic stress tolerance, we analyzed the complete, deletions and antisense region of the *PgHsp10pro* under different abiotic stress conditions. To check the regulation of these four promoter fragments, expression of *uid*A in T_2 transgenic tobacco plants under different abiotic stress conditions were imposed. Under heat stress, *PgHsp10PCpro* showed the highest expression in the leaf and stem tissues, with lower expression in the roots. Mild expression of the *uid*A gene was observed in the stem of *PgHsp10ASpro* (Figs. 3 and 4). However, no expression was observed in *PgHsp10D1pro* and *PgHsp10D2pro* transgenic plants (Figs. 3 and 4). Under cold stress, all four promoters showed the lowest expression in all the tested tissues. Mild expression was observed in the leaf and stem of the *PgHsp10PCpro* plants. In comparison to all the tested stresses, cold stress had no influence on the *Hsp10pro* activity (Figs. 3 and 4). The expression pattern of salt stress was similar to that of cold stress. Very low expression was observed in the stems of the *PgHsp10PCpro* transgenic plants. No expression or lower expression was observed in *PgHsp10AS*, *PgHsp10D1* and *PgHsp10D2*. Among all the stresses, salt and cold stresses showed lowest expression (Figs. 3 and 4). Under drought stress, *uid*A gene activity was highest in *PgHsp10PCpro* transgenic plants in the stem and leaf and lower in the root. In contrast, *PgHsp10ASpro* and *PgHsp10D1pro* showed mild expression in the stem compared to *PgHsp10PCpro*. The *uid*A gene expression was negligible in the tissues of *PgHsp10D2* plants (Figs. 3 and 4).

3.6. qRT-PCR analysis

Quantitative real-time PCR (qRT-PCR) was performed to quantify *uid*A gene expression in seedlings, leaf, stem and root tissues under control and different abiotic stress conditions. In response to either salinity or low temperature, there was similarity in the expression levels in all four promoters in different tissues, indicating the presence of similar elements in all (Fig. 4). The loss of the segment in *PgHsp10D1pro* and *PgHsp10D2pro* resulted in a decrease in *uid*A activity in both stem and roots, thereby suggesting the absence of their drought and heat inducible *cis*-element(s) in the deleted regions. However, *PgHsp10AS pro* expressed only in the stem under heat stress. *PgHsp10PCpro* that was solely responsible for its expression under heat and drought stresses because of the presence of the respective *cis*-acting elements, viz. CCAAT-box, and ARE, which were absent in the *PgHsp10AS*, *PgHsp10D1* and *PgHsp10D2* promoters. The level of *uid*A activity under salt and low temperature stress was observed to be high in leaves and even higher in stems and roots of *PgHsp10PCpro*, thereby indicating the presence of

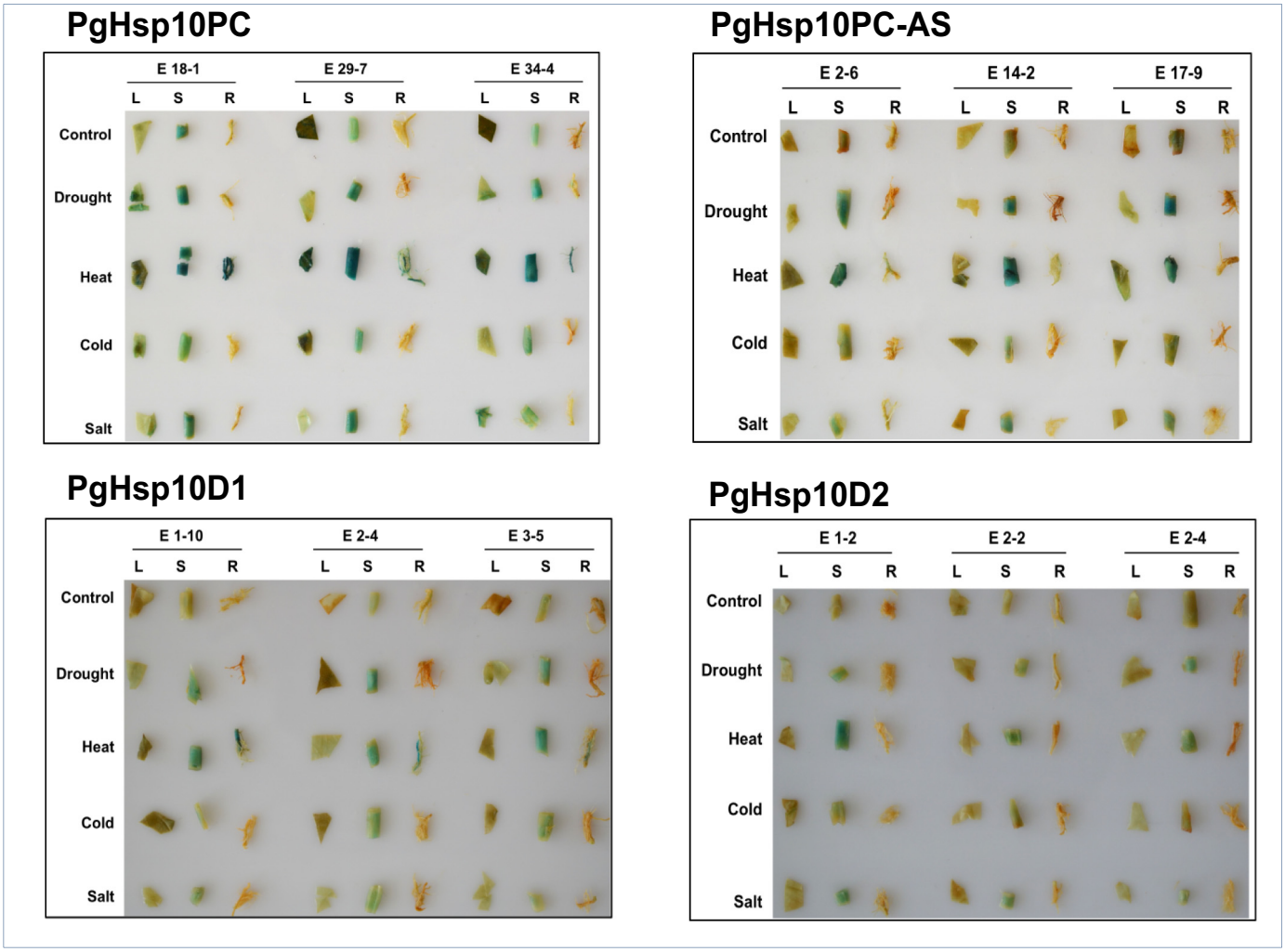

Fig. 3. Expression analysis of the *uid*A gene controlled by the *PgHsp10*, *PgHsp10AS*, *PgHsp10D1* and *PgHsp10D2* promoters under different experimental conditions. Transgenic tobacco leaves, stems and roots were treated with different abiotic stress conditions and stained with GUS staining solution to study the localization of their activity and intensity under non-treated (control) conditions and upon treatment with salt (250 mM NaCl for 48 h), heat (4 h at 45 °C), cold (4 h at 4 °C) and drought (withholding water for 4 days).

corresponding regulatory element(s) in this region. The results obtained from GUS histochemical assay and qRT-PCR expression data of stress-imposed plants in all four promoters were in congruence to a large extent.

4. Discussion

Earlier studies showed that constitutive promoters overexpressing genes and/or TFs, resulted in abnormal phenotypes that are undesirable [24]. Hence, transgenic plants should be generated such a way that they can accumulate transgene products exclusively under unfavorable or stress conditions and in specific tissues. An alternative option that can perhaps be employed is the stress-inducible/tissue/temporal expression of promoters regulating the candidate gene expression for tolerance to abiotic stress conditions [24–27]. For the induction of promoters under different abiotic stresses, *cis*-acting elements, such as DRE, ABRE, LTRE, and HSE are regulated [28,29]. The necessity of such a system for transgene expression may be vital for precise and efficient temporal regulation of transgene expression. This is especially true under conditions where unwanted gene products may reach high or lethal threshold, undesirable especially if this happens during plant development and in absence of any stress [30]. In our earlier studies, several stress-inducible genes were identified and characterized [5,15,31,32]. *PgHsp10* is a promising candidate gene that is highly regulated under heat stress and mildly active under salt and drought stresses. Hence, characterization of the promoter of this gene (*PgHsp10pro*) could play a potential role in abiotic stress tolerance. One effective way to study the regulation of promoters in genetic engineering is through the *uid*A reporter gene system [33]. Hence, in the present study, we evaluated the heat, cold, salt and drought stress reflexes of *PgHsp10pro* in the leaves, stems and roots of transgenic tobacco plants.

The function of the *PgHsp10pro* was determined through a promoter deletion approach (Fig. 1). Histochemical GUS assays for the *uid*A gene showed that under control conditions, the *PgHsp10PCpro* was active in the stem and whole seedling, *PgHsp10AS* was mildly active in roots and seedlings, *PgHsp10D1* was mildly active in seedlings and stems and *PgHsp10D2* was only active in seedlings, whereas the wild-type seedlings did not show any GUS staining due to its expression in the presence of a CCGTCC box in their promoter regions, except for *PgHsp10D2* (Fig. 2a and b). These results indicate that these promoters are not only stress-inducible but also regulate tissue-specific expression. The histochemical activity of *uid*A gene was not detected in untransformed wild-type plants. Tobacco transgenic plants containing either of the *PgHsp10PC*, *PgHsp10AS*, *PgHsp10D1* and *PgHsp10D2* constructs were subjected to cold and salt stresses. However, in response to either salinity or low temperature treatment, there was some similarity in the intensity of *uid*A expression in the latter three constructs in the leaf, stem and root, respectively, thereby indicating the absence of specific elements. While *PgHsp10PCpro* showed very mild expression in the stem tissue, no upregulation of the *uid*A gene was observed in *PgHsp10AS*-, *PgHsp10D1*- and *PgHsp10D2*-containing tissues (Figs. 3 and 4). This could be because of the absence of cold- and salt-specific elements in these promoter regions. The level of *uid*A activity was observed to be high in the leaf and even higher in the stem and root when the *PgHsp10 pro*-containing plant tissue was subjected to heat stresses, indicating the presence of the corresponding regulatory

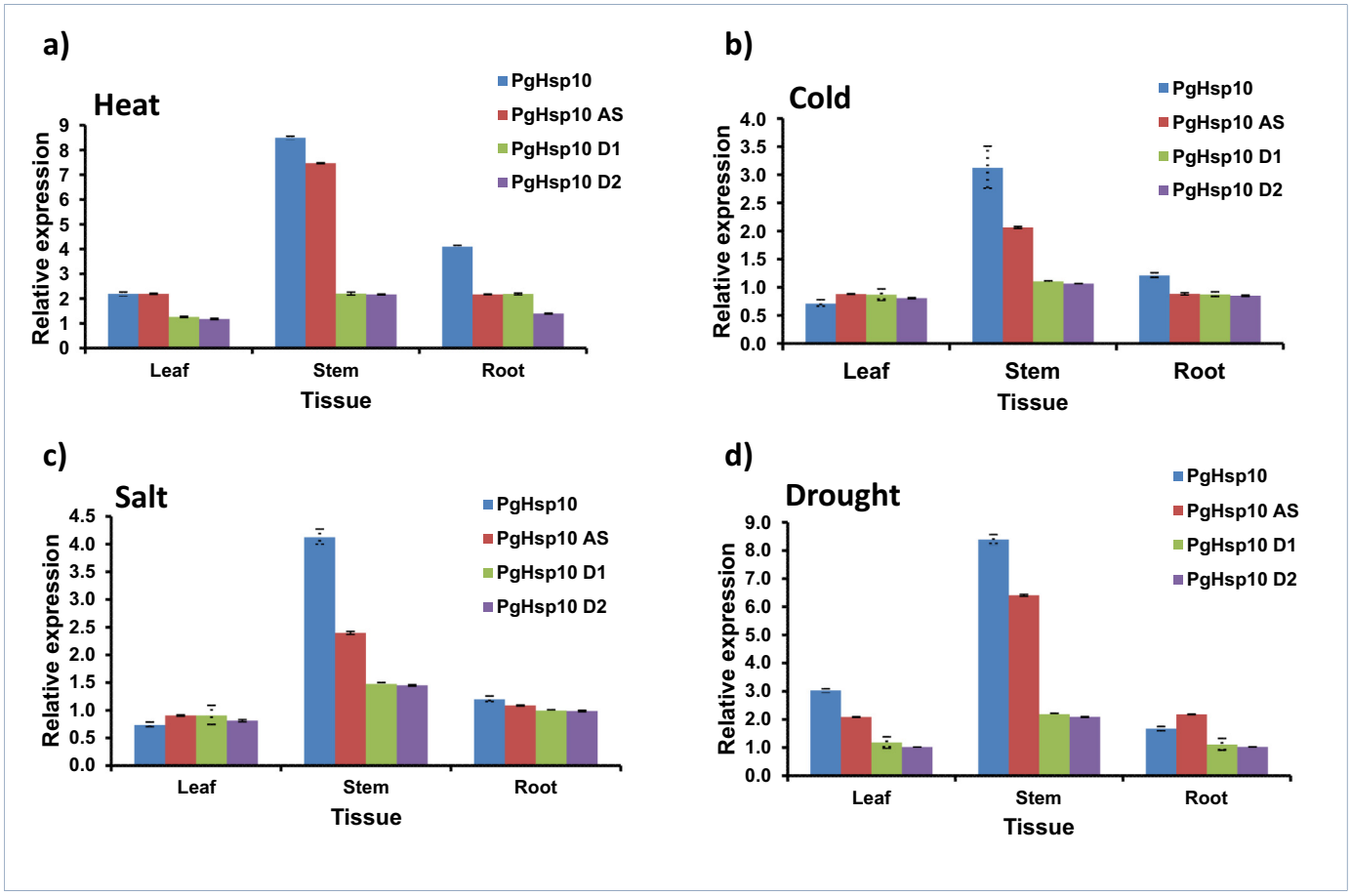

Fig. 4. Expression analysis of the *uid*A gene transcript in leaf, stem and root tissues of *PgHsp10*, *PgHsp10AS*, *PgHsp10D1* and *PgHsp10D2* promoter transgenic tobacco plants under different abiotic stress conditions. Values represent the expression fold obtained after normalizing against the reference genes. All samples were analyzed in triplicate in three independent experiments. Different tissues and abiotic stress treatments are represented on the X-axis, whereas normalized expression of *uid*A gene expression is represented on the Y-axis. Standard error bars are shown.

element HSE in this region. The loss of this segment in *PgHsp10D1* and *PgHsp10D2* resulted in a decrease in *uid*A activity in both the stem and roots, thereby suggesting the absence of their heat-inducible positive regulatory element(s) in the deleted regions. A study by [34] showed that *Hsp18.2pro* when fused with the *uid*A gene in transgenic *Arabidopsis* plants showed that heat stress induced *uid*A gene activity in almost all organs of the plant. Likewise, heat-shock-induced *uid*A activity was observed in transgenic *Arabidopsis* when the promoter of *Hsp81* was used [35]. *GmHsp17.5Epro* was differentially expressed in all the tested organs and floral tissues under heat stress [36]. These studies show the possible reasons for *PgHsp10pro* being protective and up-regulated under heat stress conditions. Under drought stress, the stem of *PgHsp10pro* was highly expressed due to the presence of CCAAT-box and MBS in its promoter regions (Figs. 3 and 4). However, *PgHsp10AS* and *PgHsp10D1* contain MBS in their promoter region, and *PgHsp10AS*, *PgHsp10D1* and *PgHsp10D2* lack the presence of a CCAAT-box specific for drought inducibility. Hsp10 proteins alone are widely involved in protecting prokaryotic or eukaryotic cells from stresses caused by infection, inflammation and other abiotic stresses [37–39]. Based on our studies, it could be concluded that *PgHsp10pro* is drought-inducible and could be employed in the development of abiotic stress-tolerant crops.

5. Conclusion

With changing global temperatures, it is likely that heat stress imposed on sessile plants will increase, thereby resulting in a high decrease in yield and posing a serious concern for crop production. Stress-inducible promoters can be used to drive gene expression for increased abiotic stress tolerance without compromising on adverse effects that often occur with the constitutive promoters. In this study, the promoter region of *PgHsp10* gene was functionally characterized by using transgenic tobacco plants, where the histochemical and qRT-PCR assays confirmed its inducibility under heat and drought stresses specifically in leaf and root tissues. This study provides a significant contribution to the understanding of *PgHsp10pro* and broadens the toolbox for stress-inducible promoters and promoter elements for its use in basic and/or applied research. Hence, the promoter of *PgHsp10* gene could be used as a heat- and/or drought-inducible promoter to drive gene expression under different abiotic stresses in genetically engineered crops.

Author contributions

PSR, VV and KKS conceived and designed the experiments, PSR, PBM and KD executed the study, PSR, VV, KD and KKS analyzed data. KD, PSR and KKS wrote the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2020.04.069.

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