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Functional characterization and expression study of sugarcane MYB transcription factor gene *PEaMYBAS1* promoter from *Erianthus arundinaceus* that confers abiotic stress tolerance in tobacco†

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Sugarcane is a glycophyte which has to confront various biotic and abiotic stresses while standing in fields. These stresses ultimately affect the growth and sucrose contents, causing heavy losses to farmers. A genetic approach through transgenic technology offers promising avenues to counter stresses and overcome the losses in production. In this study, *PEaMYBAS1* promoter from *Erianthus arundinaceus*, a wild relative of sugarcane, was isolated to reveal its stress tolerance mechanism at the transcriptional level. A series of *PEaMYBAS1* promoter deletions constructed from the transcription start sites F1 (−161 bp), F2 (−282 bp), F3 (−554 bp), F4 (−598 bp), F5 (−714 bp), F6 (−841 bp), and F0 (−1032 bp) were fused to the *uid*A reporter gene (GUS) separately, and each construct was analyzed by agroinfiltration in tobacco leaves subjected independently to drought, cold, salinity and wounding. Deletion analysis of the *PEaMYBAS1* promoter revealed that the F3 (−554 bp) region was required for basal expression. Interestingly, full length deletion fragment F0 (−1032 bp) showed the highest GUS activity in drought (4.9 fold), among the other abiotic stresses such as cold (3.89 fold), salinity (3.87 fold) and wounding (3.06 fold). GUS induction characterization of the promoter revealed the enhanced stress tolerance capacity against abiotic stresses in the model plant *Nicotiana tabacum*. Thus, the full length deletion fragment F0 (−1032) of the inducible promoter *PEaMYBAS1* can be advocated as an important genetic engineering tool to develop stress tolerant plants.

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1. Introduction

Sugarcane is an important cash crop cultivated in more than a hundred countries in tropical and subtropical zones. Sugarcane productivity is profoundly influenced by fluctuating climate conditions and ultimately the plants have to counter a variety of abiotic stresses. It is often subjected to several harsh environmental stresses that adversely affect growth, metabolism and yield. The yield difference can largely be explained by unfavorable environmental conditions and these conditions are capable of creating potentially damaging physiological changes within plants.[1] Abiotic stress factors such as drought, salinity, cold and water deficiency put a huge impact on the productivity of world agriculture and it has been suggested that they reduce average yields by more than 50% for a majority of the crop plants.[2] Among these environmental factors, water deficiency and salinity are the major abiotic factors limiting sugarcane production.[3] This has brought the scientific community to look at the urgent need to develop stress-tolerant and high yielding crop varieties.[4,5]

Multiple signaling pathways are known to regulate the stress response in plants.[6] Transcription factors (TFs) play a crucial role in the activation of different stress responsive gene expression pathways.[7,8] These TFs interact with *cis*-acting elements present in the promoter region of different stress-responsive genes and thus activate the cascade of genes that act synergistically in enhancing tolerance towards multiple stresses. This property of TFs makes them an effective category of candidate genes for the manipulation of abiotic stress

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tolerance. Most of the stress-related TFs are grouped into several large families, such as AP2/ERF, bZIP, NAC, MYB, MYC, Cys2, His2, zinc fingers and WRKY.[9] Among them, the MYB family is the most viable and durable target as well as an ideal genetic engineering tool for the development of abiotic stress tolerant plants.[10] Stress inducible promoters have already been studied to a large extent in plant transgenic technology. Such factors can be effectively used to transform sugarcane as well as other crops.[11] Recently, the sugarcane *SoMYB18* gene and *PScMYBAS1* promoter were successfully isolated from the sugarcane cultivar Co740 and were functionally validated by observing the overexpression of the stress responsive MYB transcription factor under various abiotic stress conditions.[12,13] Use of stress inducible and tissue specific promoters is becoming vital and imperative for the development of transgenic plants. *Erianthus arundinaceus*, a wild relative species of sugarcane has a strong potential to contribute valuable traits to sugarcane, including adaptation to biotic and abiotic stresses.[14] Isolation of a stress responsive promoter from *E. arundinaceus* may hence provide an insight to possibly sturdier abiotic stress responsive motifs. It should therefore be tried to make use of these motifs for further development of transgenic sugarcane equipped with notable capacity to counter abiotic stresses.

The current study deals with the isolation and functional characterization of the stress inducible *PEaMYBAS1* promoter of *E. arundinaceus* upon exposure to different abiotic stresses. The study was carried out using tobacco (*Nicotiana tabacum*) as the model plant system. A series of deletion constructs of the 5′-upstream region of the *PEaMYBAS1* promoter was fused to the GUS reporter gene in the pKGWFS7 vector to identify critical regions and motifs required for the stress-inducible gene activity.

2. Materials and methods

2.1. Plant materials, growth conditions and bacterial strains

Leaf samples of *E. arundinaceus* were collected from fields at Vasantdada Sugar Institute, Manjari (Bk), Pune, India. Tobacco plants were grown on a sterile half-strength Murashige and Skoog (MS) medium at 22 ± 2°C with 16/8 h photoperiod cycle in a growth chamber. Tobacco plants at a six leaf stage were used for the infiltration study. *Escherichia coli* strain DH5α was used for cloning and preparation of all recombinant plasmid vectors. *Agrobacterium tumefaciens* strain LBA4404 was subjected to tobacco leaf agroinfiltration.[15] Plasmid pKGWFS7 (Invitrogen) was used to create promoter fragment constructs.

2.2. Isolation of 5′ *PEaMYBAS1* promoter region

PEaMYBAS1 promoter primers were designed from the sequence of the *PScMYBAS1* promoter.[13] Genomic DNA was extracted from leaf samples of sugarcane cultivar *E. arundinaceus* using Plant DNeasy mini kit (QIAGEN). The promoter (*PEaMYBAS1*) of the *EaMYBAS1* gene was amplified by a PCR reaction containing 2.0 μL of *Taq* buffer, 2 mM $MgCl_2$, 0.8 mM dNTP, 400 nM each primer, FP:5′-GGCACCCTCAGTGGAAGAAT-3′ and RP:-5′GTGCTGAATTGCTGTCTTTAGC-3′, 1 U of *Pfu* polymerase (Sigma), 50 ng of genomic DNA and sterile H_2O under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 45 seconds and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were analyzed on 0.8% agarose gel which was further purified using the QIAquick Gel extraction kit (QIAGEN) and subsequently cloned into the pGEM-T cloning vector (Promega) and then transformed into DH5α. The positive clones obtained were further sequenced using an automated DNA sequencer (Set lab India Pvt. Ltd) and designated as pGEMT:*PEaMYBAS1*.

2.3. Promoter sequence analysis

The *PEaMYBAS1* promoter's *cis*-acting regulatory elements were analyzed using Plant CARE and PLACE bioinformatics analysis tools.[16,17]

2.4. Construction of the *PEaMYBAS1* promoter deletion fragments

The entire *PEaMYBAS1* region from −1032 to +1 designated as a full-length deletion fragment F0 (−1032 bp) and its six deletion fragments designated as F1 (−161 bp), F2 (−282 bp), F3 (−554 bp), F4 (−598 bp), F5 (−714 bp), and F6 (−841 bp) were generated by PCR amplification (Fig. 1). The full length cloned fragment F0 (−1032 bp) was amplified using P0 and R0 primers with an attb site. The obtained PCR product was purified and further used as a template to construct the deletion fragments. Forward primers such as P1, P2, P3, P4, P5, and P6 and the common reverse primer R0 with an attb site were used to construct F1, F2, F3, F4, F5 and F6 deletion fragments, respectively (Table 1). The PCR reaction was carried out as mentioned under the above conditions. The amplified PCR products flanked by attb recombination sites were directionally incorporated into the pENTR-207 entry vector (Invitrogen) using a BP clonase reaction mix. Subsequently, entry clone PCR products flanked by attL sites were incorporated into the desired destination vector pKGWFS7 (Invitrogen) with attR sites using an LR clonase reaction mix and the deletion fragment clones were obtained.[18] The recombinant positive colonies were selected using the antibiotic kanamycin (50 μg mL^{-1}) resistance marker gene, which ensured that the resulting colonies contained plasmids that have undergone recombination. A series of deletion constructs of the 5′-upstream region of the *PEaMYBAS1* promoter were fused with the GUS reporter gene in the pKGWFS7 vector (Fig. 2). Promoter fragment insertion was confirmed by PCR and sequencing in all plasmid constructs and later transformed into the *A. tumefaciens* strain LBA4404 using the freeze–thaw method.[15]

2.5. Transient expression assay of tobacco leaves

Agrobacterium-mediated transient expression assay of *PEaMYBAS1*:GUS constructs was carried out using tobacco leaves.[19] Each of the deletion constructs of the *PEaMYBAS1* promoter was further independently put in *A. tumefaciens* strain LBA4404 grown on a yeast extract peptone medium containing rifampicin (10 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) at 28 °C for 48

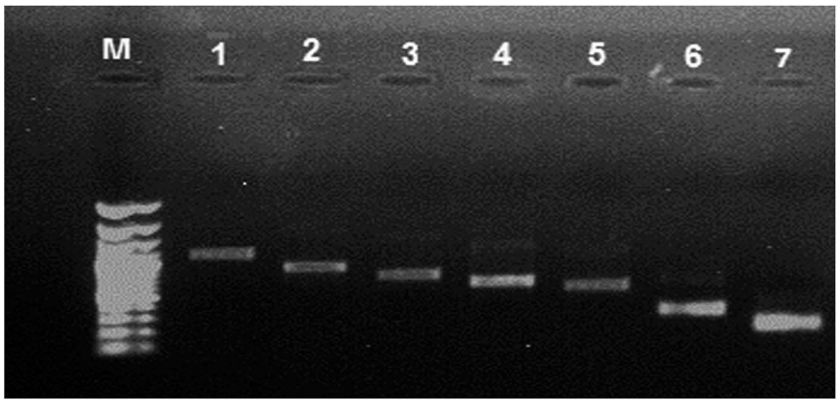

Fig. 1 PCR amplification of deletion fragments of *PEaMYBAS1* promotr. Lane M −100 bp marker, lane-1. F0 (−1032 bp), lane-2. F6 (−841 bp), lane-3. F5 (−714 bp), lane-4. F4 (−598 bp), lane-5. F3 (−554 bp), lane-6. F2 (−282 bp), lane-7. F1 (−161 bp).

h. The broths were centrifuged for 15 min at 6000*g* to obtain independent deletion constructs. The obtained constructs were resuspended later in 10 mM MES buffer (pH 5.5) and 10 mM $MgSO_4$ solution in MS basal medium. The bacterial culture was further activated with 200 μM acetosyringone. To perform the agroinfiltration of tobacco leaves, the bacterial suspension with a final absorbance of 0.8 measured at 600 nm was used. A needleless sterilized syringe was used for the agroinfiltration on the abaxial surfaces of tobacco leaves. After 48 h of agroinfiltration, the leaves were subjected to abiotic stress treatments and maintained in a moist chamber at 26 °C for 48 h.[19]

2.6. Abiotic stress treatment

The transgenic tobacco leaves were subjected to different abiotic stresses such as drought, cold, salinity and wounding for the characterization of promoter induction activity. For the dehydration and high salinity treatments, the tobacco leaves were soaked in 300 mM mannitol and 200 mM NaCl, respectively. To induce cold stress, the plants were kept at 4 °C while wounding stress was mechanically induced by pricking with needles. The treated leaves were then incubated at 22 ± 2 °C with 16/8 h photoperiod cycles in a growth chamber. The mock (control) tobacco leaves were kept in half strength MS medium.

2.7. Spectrophotometric measurement of GUS activity

Transient expression of GUS activity in the treated tobacco leaves (Test) was measured spectrophotometrically at 48 h after stress treatments as described previously.[20] Tobacco leaf tissue was homogenized in 1 mL of extraction buffer (50 mM NaH_2PO_4, pH 7.0, containing 10 mM EDTA, 0.1% Triton X-100, 0.1% (w/v) sodium lauroylsarcosine, and 10 mM β-mercaptoethanol) and centrifuged at 12 000*g* for 15 min at 4 °C. A 100 μL aliquot of the supernatant was mixed with 900 μL of GUS assay solution containing 1 mM PNPG (*p*-nitro phenyl-β-D-glucuronide) in extraction buffer. The mixtures were incubated at 37 °C for 2 h and 400 μL of stop buffer (2.5 M 2-amino-2-methylpropanediol) was added to terminate the reaction. This mixture was used for calibration and standardization. PNPG (*p*-nitro phenyl-β-D-glucuronide) is a chromogenic β-glucuronidase substrate. The *GusA* enzyme cleaves PNPG, yielding β-D-glucuronic acid and *p*-nitro phenol (PNP). When cleaved by GUS, *p*-nitro phenol (PNP) forms a yellow color showing a maximum absorbance at 405 nm. This method is highly sensitive and more accurate than the existing discontinuous methods.[21] The protein concentration was determined using bovine serum albumin (BSA) as a standard in the Bradford's method.[22] The absorbance of the mock and test samples was measured using 100 μL of the supernatant of a leaf sample after 48 h of stress treatments at a wavelength 405 nm to estimate the GUS activity. The fold change in GUS activities was calculated using eqn (1).

$$\text{Fold change in GUS activity} = \frac{\text{test} - \text{mock}}{\text{mock}} \quad (1)$$

Table 1 Sequence of the oligonucleotides used for the construction of the *PEaMYBAS1* deletion plasmids

Oligo name	Sequence (5′–3′)	Features
P0	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGCACCCTCAGTGGAAGAAT	−1032 to −1012 attb underlined
P6	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGACAGTTCCTAAAAGG	−841 to −823 attb underlined
P5	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGGGTAAAAGGTTCAGAT	−714 to −696 attb underlined
P4	GGGGACAAGTTTGTACAAAAAAGCAGGCTGATTGGACATTGTTGACG	−598 to −580 attb underlined
P3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTCGTTATGGGTTACC	−554 to −536 attb underlined
P2	GGGGACAAGTTTGTACAAAAAAGCAGGCTGAAGAGATAGGCGTTACATG	−282 to −262 attb underlined
P1	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGCACACACAGCCCCAGT	−161 to −143 attb underlined
R0	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTGCTGAATTGCTGTCTTT	−22 to −1 attb underlined

where test represents the GUS activity value in stressed leaves and control was the GUS activity value of the leaves without stress.

2.8. Data analysis

All GUS activity measurements were performed in triplicates. The results were expressed as the mean values ± SD. The error bars shown in the figures are the standard deviation (SD) of the experimental data.

3. Results and discussion

3.1. Analysis of *PEaMYBAS1* promoter

At first, the 5′*PEaMYBAS1* promoter region was isolated by the PCR.[13] The upstream region of the *PEaMYBAS1* promoter was analyzed using PLACE and PlantCARE databases to find putative motifs homologous to the *cis*-acting elements involved in the activation of abiotic stress-induced genes in tobacco. After PlantCARE analysis, it was observed that a number of potential *cis*-acting elements present in the *PEaMYBAS1* promoter respond to the induction of abiotic stress expression. In comparison with the earlier reported abiotic stress tolerant *PScMYBAS1* promoter from sugarcane cultivar Co740, the *PEaMYBAS1* promoter sequence from *E. arundinaceus* showed common motifs such as MBS (−87 and −731 bp), MYB (−941 bp), TCA (−618 bp), TGACG (−585 bp), box E (−632 bp), W box (−232 bp), WRKY (−95, −886, −966 bp), circadian (−775 bp), Skn-1 (−101, −726 bp), TCCC (−405 bp) and an anaerobic responsive element, *i.e.* ARE, located at −805 bp. The *PEaMYBAS1* promoter as well as the *PScMYBAS1* promoter also possess common motifs such as the CAAT-box and TATA-box located near many transcription start sites. The CAAT-box is well known to control transcription initiation, while the TATA-box is crucial for the initiation of transcription (Fig. 2, Table 2, ESI Fig. I†).

Some common motifs such as MBS (−731 bp), MYB (−941 bp) and ARE (−805 bp) from *PEaMYBAS1* have almost the same base pair position in the promoter *PScMYBAS1*, namely −732, −942, and −806 bp, respectively.[13] However, the *PEaMYBAS1* promoter possesses four new motifs compared to the *PScMYBAS1* promoter sequence, such as the GATA motif (−140 bp), 3-AF1 binding site (−340 bp), box III (−872 bp) and O2 site at −933 bp (Fig. 2, Table 2).

3.2. GUS expression analysis study

The GUS expression analysis study endorsed that the *PEaMYBAS1* promoter was a stress-inducing promoter and not constitutively expressed. A constitutive promoter such as CaMV35S is continuously expressed at the molecular level in all stages of plant growth and cannot be regulated by abiotic stresses. This makes transgenic plants grow relatively slow in the absence of stress than those plants with inducible promoters.[23–25] Inducible promoters are significantly used to regulate gene expression in plants as they are stimulated either by physical or chemical factors. These inducible promoters thus are preferred as powerful genetic engineering tools to develop stress tolerant transgenic plants.[25]

Compared to CaMV35S promoter mediated GUS expression, tobacco leaf agroinfiltrated with F1 (−161) and F2 (−282) showed minimal expression, while F3 (−554) showed basal GUS expression compared to the other fragments (F4, F5, F6, F0). *PEaMYBAS1* transient assays revealed an increased GUS induction of the promoter region from F3 (−554) to F0 (−1032 bp) under drought, cold, salt and wounding conditions (Fig. 3).

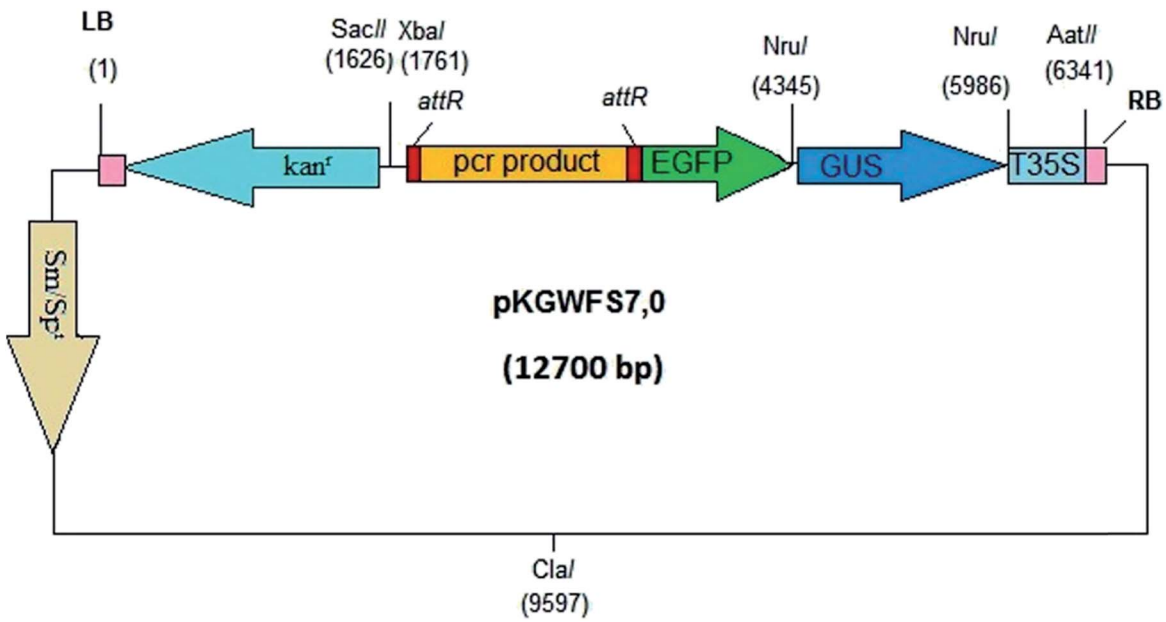

Fig. 2 Schematic representation of plant expression destination pKGWFS7.0 vector map. This vector contains LB: left border; kanamycin resitance gene; Egfp: green fluorescent protein gene; GUS: blue-coloring β-glucuronidase gene; T35S: cauliflower mosaic virus 35S terminator; and RB: right border.

Table 2 Positions and functions of the putative *cis*-acting elements in the *PEaMYBAS1* promoter

cis-element	Sequence	Position	Function	References
CAP site	CAC	+1	Transcription start site	Joshi (1987)
CAAT-box	CAAT, CAATT	−11, −198, −899, −735, −682, −669	Common *cis*-acting element in promoter and enhancer regions	Joshi (1987)
TATA-box	TATA, TATAA	−33, −131, −217, −248, −349, −565, −638, −669	Core promoter element around −30 of transcription starting point	Joshi (1987)
MBS	CAACTG	−87, −731	MYB binding site involved in drought-inducibility	Urao *et al.* (1993)
WRKY	TGAC	−95, −886, −966	WRKY factor-binding motif	Cormack *et al.* (2002)
Skn-1_motif	GTCAT	−101, −726	*cis*-Acting regulatory element required for endosperm expression	Washida *et al.* (1999)
GATA-motif	GATAGGA	−140	Part of a light responsive element	Reyes *et al.* (2004)
Box-W1	TTGACC	−232	Fungal elicitor responsive element	Eulgem *et al.* (1999), Kirsch *et al.* (2001)
3-AF1 binding site	AAGAGATATTT	−340	Light responsive element	Lam and Chua (1990)
TCCC-motif	TCTCCCT	−405	Part of a light responsive element	Bolle *et al.* (1996)
TGACG-motif	TGACG	−585	*cis*-Acting regulatory element involved in the MeJA-responsiveness	Reinbothe *et al.* (1994), Wang *et al.* (2011)
TCA-element	CAGAAAAGGA	−618	*cis*-Acting element involved in salicylic acid responsiveness	Reinbothe *et al.* (1994), Sobajima *et al.* (2007)
Box E	ACCCATCAAG	−632	Fungal elicitor-responsive element	Despres *et al.* (1995)
Circadian	CAANNNNATC	−775	*cis*-Acting regulatory element involved in circadian control	Jacobo-Velazque *et al.* (2015)
ARE	TGGTTT	−805	*cis*-Acting regulatory element essential for the anaerobic induction	Olive *et al.* (1991)
Box III	atCATTTTCACt	−872	Protein binding site	Weisshaar *et al.* (1991)
O2-site	GATGACATGA	−933	*cis*-Acting regulatory element involved in zein metabolism regulation	Vincentz *et al.* (1997)
MYB	GGATA	−941	MYB transcription factor-binding motif	Hua *et al.* (2006)

Such elevated expression of the GUS reporter gene might have occurred due to the regulation of the *cis*-acting elements present within the promoter region (Fig. 4 and 5).

3.3. Drought stress expression analysis

Plants require abundant quantities of water for growth. Transpiration is the most important factor driving water movement in plants, while photosynthesis and osmoregulation are other water dependent processes. The scarcity of water in drought conditions dramatically affects plant growth, reduces leaf size, stem extension, and root proliferation, and ultimately disturbs the plant water relationship. To face critical situations like drought, numerous drought-responsive genes, transcription factors and *cis*-acting motifs in plants are expressed at the molecular level to prevent drought-induced loss of crop yield.[26]

In this study, the full length *PEaMYBAS1* promoter region F0 (−1032 bp) showed the maximum GUS induction activity (4.9 fold) in agroinfiltrated tobacco leaves under drought stress conditions after mannitol treatment, while other deletion fragments like F6, F5, F4, and F3 exhibited GUS activity up to 3.35, 2.5, 2.35 and 1.51 fold, respectively (ESI Table I†). The F3 (−554) fragment showed marginal GUS induction activity and non significant GUS induction was observed in F2 (−282) and F1 (−161) deletion fragments (Fig. 4 and 5a). Prabu *et al.* have monitored the increased GUS activity from 2 to 4 fold in the *PScMYBAS1* promoter deletion fragment region from F6 (−777 bp) or longer up to F0 (−1033 bp).[13]

Drought stress enhanced the GUS activity of the full length F0 (−1032) deletion fragment of the *PEaMYBAS1* promoter, which can be endorsed due to the presence of *cis*-acting elements such as MBS (−731 bp), the MYB core sequence (−941 bp), circadian clock element (−775 bp), opaque-2, *i.e.* O2 site (−933 bp), BOX III (−872 bp) and WRKY (−886 and −996 bp) (Fig. 3). Interaction between these elements might have helped to boost the overall GUS expression in the *PEaMYBAS1* promoter with increased synthesis of drought stress regulatory proteins. While, comparatively, the *PScMYBAS1* promoter with MBS

Fig. 3 Nucleotide sequence of the EaMYBAS1 gene promoter (*PEaMYBAS1*). Numbering starts from the predicted transcription start site (+1, the letter A), which is labeled with arrow head. The putative core promoter consensus sequences and the *cis*-acting elements mentioned are boxed. The positions of the primers used in this study are indicated by an arrow.

(−732 bp) and MYB core sequences (−942 bp) has shown less GUS expression than the *PEaMYBAS1* promoter, which might be due to the lack of O2 sites and BOX III motifs.[13]

The MBS element provides a binding site for ABA dependent MYB, while the MYB-core sequence functions as a binding motif for the plant MYB proteins involved in the drought stress-induced gene expression.[27–30] The *OsMYB3R-2* gene, AtMYB2 gene and GmMYB177 gene from *Oryza sativa*, *Arabidopsis thaliana* and *Glycine max* have already been documented for drought stress response.[31–33] The *PScMYBAS1* promoter has been recommended for drought stress tolerance in *S. officinarum* due to the presence of MBS *cis*-acting element.[13] The circadian clock elements in *A. thaliana* and poplar have also been well known in response to drought stress during the daytime.[34]

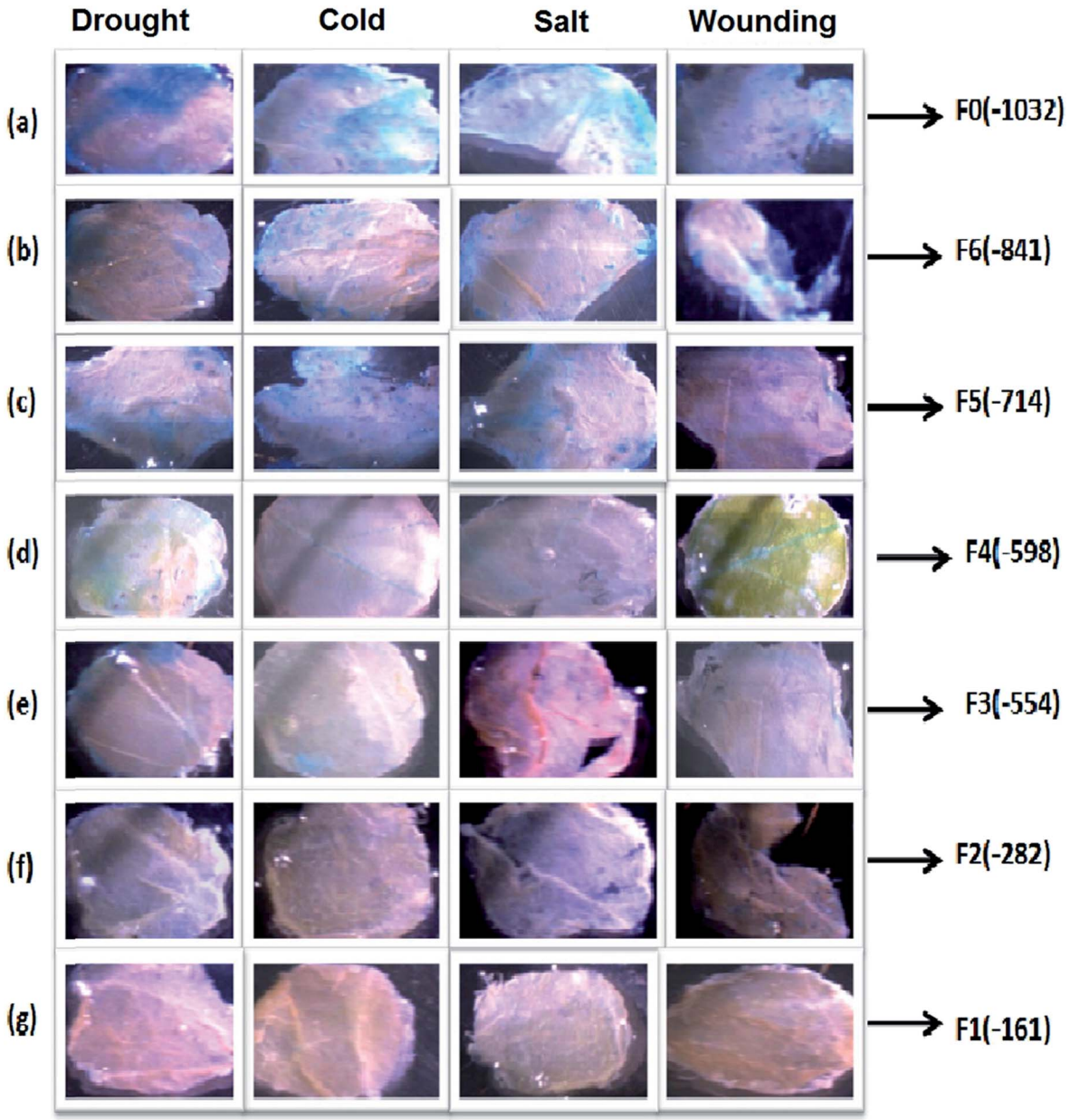

Fig. 4 GUS profile expression of *PEaMYBAS1* deletion fragments agroinfiltrated tobacco leaves. GUS was detected in X-Gluc solution followed by stress treatment.

Vincentz *et al.* have indicated that the O2 site is a regulatory locus that encodes a DNA-binding protein which activates the transcription of the b-32 gene and regulates the seed storage protein synthesis in maize.[35] The O2 site motif modulates endosperm-specific expression and encodes a bZIP (basic leucine zipper) transcriptional activator.[36] Ying *et al.* and Sun *et al.* have reported earlier that bZIP transcription factors in *A. thaliana* act as positive regulators of diverse functions such as plant development and drought stress response.[37,38] These results are also in agreement with studies on the OsbZIP23 transcription factor and GmbZIP44 gene from *O. sativa* and *G. max*, respectively.[39,40] These results clearly revealed that the *PEaMYBAS1* promoter is ideal for drought stress management.

3.4. Cold stress expression analysis

Cold temperature is necessary to break seed dormancy and vernalization to induce flowering, but a prolonged cold stress environment affects the physiological processes of plants. The *PEaMYBAS1* promoter region manifested increasing GUS expression from F4 (−598) to F0 (−1032 bp), *viz.* from 2.6 to 3.9 fold, under cold stress in the transiently expressed tobacco leaf tissues, compared to the respective mock samples (Fig. 4 and 5b, ESI Table I†). This enhanced GUS activity might be the result of the interaction of the WRKY transcription factor (−886 and −996 bp) with the TGACG (−585 bp), MBS (−731 bp), TCA (−618 bp) and MeJA-responsive *cis*-acting elements.[41–43] Prabu *et al.* have documented the GUS induction in the *PScMYBAS1*

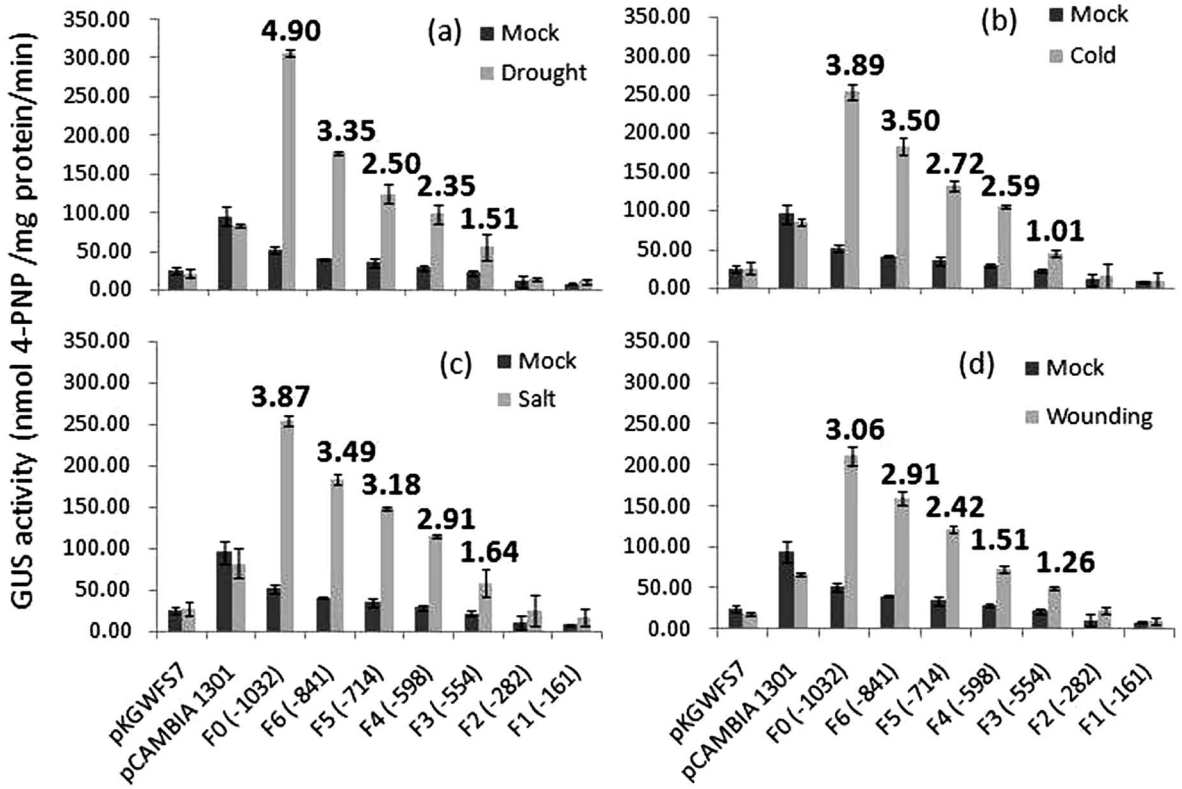

Fig. 5 Graphical representation of GUS activities fold change in deletion fragments F0, F6, F5, F4, F3, F2, F1 of promoter *PEaMYBAS1* in response to (a) drought, (b) cold, (c) salt and (d) wounding applied to transient tobacco leaf discs. Negative control (pKGWFS7), positive vector control (pCAMBIA1301). Data are means ± standard deviations from three independent assays of tobacco leaf extracts.

promoter region from F6 (−777) to F7 (−843), which was devoid of the WRKY transcription factor and circadian clock by 2.03 and 2.8 fold, respectively, under cold stress. However, the lack of interaction between the WRKY transcription factor, *cis*-acting elements and circadian clock can be considered to affect the GUS expression rate.[13] WRKY transcription factors, which are considered to be unique in plants, act as transcription activators as well as transcription repressors.[44,45] These WRKY transcription factors are sufficient for regulating the expression of the GUS reporter gene induced by cold stress. Kirsch *et al.* have demonstrated the preferential arrangement of *cis*-acting elements by the WRKY transcription factor enabling them to bind with the relevant target promoters.[46] The W1 box (−232 bp) *cis*-element provides a binding site for WRKY transcription factors, which plays an important role in plants during cold stress regulation. The GmWRKY21 gene from *A. thaliana* has been represented earlier by Zhou *et al.* for freezing condition management.[47] *A. thaliana* has indicated the expression of WRKY, ABRE-related, GT-1, and AT-rich motifs in response to regulation of cold stress.[27] WRKY transcription factors are additionally involved in the regulation of SA treatment, auxin elicitor responsive elements and light.[48–52]

Besides this, the plant circadian clock element is located at −775 bp in the *PEaMYBAS1* promoter. The circadian clock, important for regulation of growth, flowering time and metabolic activities also play a vital role in cold stress management.[53] The circadian clocks of *A. thaliana* have earlier been studied under cold stress and the expression of the stress responsive gene C-repeat Binding Factor (CBF) was observed.[53] These results clearly suggest that the *PEaMYBAS1* promoter containing the WRKY transcription factor and circadian clock play an important role in cold stress management.

3.5. Salt stress expression analysis

Salinity is one of the common environmental stresses which imbalances irrigated land, hampers the normal growth of plants by promoting early leaf senescence, and dramatically increases the photoprotective demand in plants. Excess salts and water in the soil affect plant growth by reducing the water uptake ability of the vasculature. This is also known as the osmotic or water-deficit effect of salinity. While in the salt-specific or ion-excess effect of salinity, excessive amounts of salts enter the plant in the transpiration stream and cause injury to the cells of transpiring leaves indirectly inhibiting the photosynthesis.[54,55] Salt tolerance is a complex phenomenon which involves the coordinated action of many gene families that perform cumulatively to launch antioxidative defence in plants.[56] Salt induced oxidative stress could be a protecting mechanism for plants from moderate doses of salt rather than causing damage to them. Plants have evolved to respond to this stress by several mechanisms such as physical adaptation and interactive molecular and cellular changes that commence after the onset of stress.

In this study, the deletion fragment region from F6 (−841) to F0 (−1032) bp of the *PEaMYBAS1* promoter showed enhanced GUS induction activity from 3.49 to 3.87 fold under salt stress in transient tobacco leaves than the respective mock samples (Fig. 4 and 5c, ESI Table 1†). This deletion fragment possesses the MBS core sequence (−731 bp), O2 site (−933 bp) and Skn-1 motif (−726 bp) (Fig. 3). While in comparison with the *PScMYBAS1* promoter, the deletion fragments F5 (−613) to F6 (−777) bp have exhibited GUS induction from 1.07 up to 2.68 fold.[13] This indicates comparatively less expression of the *PScMYBAS1* promoter under salt stress compared to the *PEaMYBAS1* promoter, because of the presence of the MBS motif and the absence of the O2 site. In a salt stress response, the MBS core sequence helps in the modulation of the MYB motif and plays a dual role in controlling drought and salt stress induction. MYB protein performs a key role in the transcriptional activation of the ABA-inducible gene under regulation in higher salt concentrations.[57] GmMYB76 from *G. Max*, and AtMYB2 and AtMYB7 genes from *A. thaliana* are popular to manage salt stress.[32,33,58] The O2 site encoding the bZIP transcription factor imparts a significant role in salt stress regulation in *A. thaliana via* the ABF3 gene.[59]

The Skn-1 motif, which is well known for the development of transcription factors, controlling the seed specific endosperm expression also functions in salt induced oxidative stress.[60] It has been published earlier that Skn-1, which is distantly related to the bZIP motif binds to DNA through a unique mechanism and orchestrates the oxidative stress response in *Caenorhabditis elegans.*[61] Salinity-stress tolerant tobacco plants were already raised by overexpressing a helicase gene, which suggests a new pathway to engineer plant stress tolerance.[62]

3.6. Mechanical wounding expression analysis

In plants, mechanical wounding by physical or biological agents lead to the stimulation of certain defense genes. When plants are continuously exposed to mechanical wounding, signaling molecules such as jasmonic acid (JA) and salicyclic acid (SA) which prevent pathogen attack are continuously synthesized at the injured site.[38] Expression of *cis*-acting elements like TGACG and TCA have earlier been verified for JA and SA production, respectively, under wound stress conditions.[63,64]

In the current study, the *PEaMYBAS1* promoter region from F4 (−598) to F0 (−1032 bp) containing TGACG (−585 bp), TCA (−618 bp), box E (−632 bp), ARE (−805 bp) and WRKY motifs (−886 and −996 bp) showed enhanced GUS induction from 1.51 to 3.06 fold, compared to the respective mock sample after mechanical wounding (Fig. 3, 4 and 5d, ESI Table 1†). ARE has an important role in the response to a variety of stresses including wounding, drought, cold and salinity, while box E is known to regulate the pathogen stress response genes during plant–pathogen interactions and to produce wound responsive proteins.[57] The interaction between the WRKY transcription factor and W box have been studied in the activation of pathogen or hormone responsive (SA, MeJA) genes.[65] The deletion fragment region of the *PScMYBAS1* promoter from F3 (−303) to F0 (−1033) have also been reported in response to wound stress.[13]

The promoter *PEaMYBAS1* region from F3 (−554) to F0 (−1032) containing other deletion fragments such as F4, F5 and F6 has consistently showed increasing GUS expression in transient tobacco leaves under various abiotic stresses such as drought, cold, salt and wounding. These results can be endorsed as the cumulative expression of different *cis*-acting elements and motifs in the promoter *PEaMYBAS1*. This helped to enhance the overall GUS activity in transient tobacco plants under various stress circumstances. Therefore, the *PEaMYBAS1* promoter can be utilized as a new and powerful tool for the study of tissue specific and stress responsive transgene expression in different crop plants.

4. Conclusion

PEaMYBAS1, the sugarcane *MYB* transcription factor gene promoter expressed in tobacco conferred and enhanced tolerance to drought, cold, salt and wounding stress. Implying from these results, *PEaMYBAS1* with novel *cis*-acting elements have an important role in countering abiotic stresses. A transient assay and GUS spectrophotometric assay together showed that the deletion fragment F0 (−1032 bp) upstream from the transcription start site of the *PEaMYBAS1* promoter triggers high levels of GUS expression in transgene tobacco leaves under abiotic stress. This work provides a thorough understanding about the function of *cis*-acting elements regarding drought, salt, cold and wounding stress. Further investigations are desirable to explicate the regulatory mechanism of *PEaMYBAS1* at the molecular level.

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