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

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Abstract

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 deletion construct from the transcription start site F1 (-161bp), F2 (-282bp), F3 (-554bp), F4 (-598bp), F5 (-714bp), F6 (-841bp), F0 (-1032bp) 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 *PEaMYBAS1* promoter revealed that F3 (-554 bp) region was required for basal expression. Interestingly, full length deletion fragment F0 (-1032 bp) showed 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, full length deletion fragment F0 (-1032) of inducible promoter *PEaMYBAS1* can be advocated as an important genetic engineering tool to develop stress tolerant plants.

Key Words: Abiotic stress; Agroinfiltration; GUS transient expression assay; Promoter *PEaMYBAS1; Erianthus arundinaceus*

1. Introduction

Sugarcane is an important cash crop cultivated in more than hundred countries under tropical and subtropical zones. Sugarcane productivity is profoundly influenced by fluctuating climatic 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; these conditions are capable for creating potentially damaging physiological changes within plants.[1] Abiotic stress factors such as drought, salinity, cold and water deficiency put a huge impact on world agriculture productivity and it has been suggested that they reduce average yields by more than 50% for 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 fraternity to look at the urgent need to develop stress-tolerant and high yielding crop varieties.[4, 5]

Multiple signaling pathways are known to regulate stress response in plants.[6] Transcription factors (TFs) play a crucial role in the activation of different stress responsive gene expression.[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 manipulation of abiotic stress tolerance. Most of the stress-related TFs are grouped into several large families, such as AP2/ERF, bZIP, NAC, MYB, MYC, Cys2, His2, zinc finger and WRKY.[9] Among them, the MYB family is most viable and durable target as well as an ideal genetic engineering tool for development of abiotic stress tolerant plants.[10] Stress inducible promoters have already been studied at 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 sugarcane cultivar Co740 and functionally validated by observing over expression 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 development of transgenic plants. *Erianthus arundinaceus,* a wild relative species of sugarcane has strong potential to contribute valuable traits to sugarcane including adaptation to biotic and abiotic stresses.[14] Isolation of stress responsive promoter from *E. arundinaceus hence* may 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 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 5′-upstream region of *PEaMYBAS1* promoter was fused to GUS reporter gene in pKGWFS7 vector to identify critical regions and motifs required for the stress-inducible gene activity.

2. Materials and methods

2.1. Plant materials, growth condition 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 sterile half-strength Murashige and Skoog (MS) medium at 22±2°c with 16/8 h photoperiod cycle in a growth chamber. Tobacco plants of six leaf stage were used for 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) were used to create promoter fragment constructs.

2.2. Isolation of 5´ *PEaMYBAS1* Promoter region

PEaMYBAS1 promoter primers were designed from sequence of *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 *EaMYBAS1* gene was amplified by PCR reaction containing 2.0 µL *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 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. PCR products were analyzed on 0.8% agarose gel which was further purified using QIAquick Gel extraction kit (QIAGEN) and subsequently cloned into pGEM-T cloning vector (Promega) and then transformed into DH5α. The positive clones obtained were further sequenced using automated DNA sequencer (Set lab India Pvt. Ltd) and designated as pGEMT::*PEaMYBAS1*.

2.3. Promoter sequence analysis

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

2.4. Construction of the *PEaMYBAS1* promoter deletion fragments

The entire *PEaMYBAS1* region from -1032 to +1 designated as full-length deletion fragment F0 (-1032bp) and its six deletion fragments designated as F1 (-161bp), F2 (-282bp), F3 (-554bp), F4 (-598bp), F5 (-714bp) and F6 (-841bp) were generated by PCR amplification. Full length cloned fragment F0 (-1032bp) was amplified using P0 and R0 primers having attb site. The obtained PCR product was purified and further used as a template to construct deletion fragments. Forward primers such as P1, P2, P3, P4, P5 and P6 and common reverse primer R0 with 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 in the above condition. The amplified PCR products flanked by attb recombination sites directionally incorporated into pENTR-207 entry vector (Invitrogen) using BP clonase reaction mix. Subsequently, entry clone PCR products flanked by attL sites were incorporated into desired destination vector pKGWFS7(Invitrogen) having attR sites using LR clonase reaction mix and deletion fragments clones were obtained.[18] The recombinant positive colonies were selected using antibiotic kanamycin (50µg/mL) resistance marker gene ensured that the resulting colonies contained plasmids that have undergone recombination. A series of deletion constructs of 5´-upstream region of the *PEaMYBAS1* promoter were fused with GUS reporter gene in pKGWFS7 vector (Fig. 2). Promoter fragment insertion was confirmed by PCR and sequencing in all plasmid constructs and later transformed into *A. tumefaciens* strain LBA4404 by 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 *PEaMYBAS1* promoter were further independently put in *A. tumefaciens* strain LBA4404 grown on yeast extract peptone medium containing rifampicin (10 µg/mL) and kanamycin (50 µg/mL) at 28°C for 48 h. The broths were centrifuged for 15 min at 6000 *g* to obtain independent deletion constructs. 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 agroinfilteration of tobacco leaves; bacterial suspension with final absorbance of 0.8 measured at 600 nm was used. Needleless sterilized syringe was used for agroinfiltration on abaxial surfaces of tobacco leaves. After 48 h of agroinfiltration, 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 characterization of promoter induction activity. For 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 cycle in a growth chamber. The mock (control) tobacco leaves were kept on 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 extraction buffer (50 mM NaH_2PO_4, pH 7.0, containing 10 mM EDTA, 0.1% Triton X-100, 0.1% (w/v) sodium laurylsarcosine, 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 yellow color showing maximum absorbance at 405 nm. This method is highly sensitive and more accurate than the existing discontinuous methods.[21] Protein concentration was determined using bovine serum albumin (BSA) as a standard by Bradford's method.[22] The absorbance of mock and test samples were measured by using 100 µL of supernatant of leaf sample after 48 h of stress treatments at wavelength 405 nm to estimate GUS activity. The fold change in GUS activities was calculated using equation (1).

$$\text{Fold change in GUS activity} = \frac{\text{Test} - \text{Mock}}{\text{Mock}}$$

……..Equation (1)

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 mean values with ±SD. Error bars shown in figures are standard deviation (SD) of the experimental data.

3. Results and discussion

3.1. Analysis of *PEaMYBAS1* promoter

In the beginning, 5´*PEaMYBAS1* promoter region was isolated by PCR.[13] Upstream region of *PEaMYBAS1* promoter was analyzed using PLACE and PlantCARE databases to find putative motifs homologous to *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 *PEaMYBAS1* promoter respond to induction of abiotic stress expression. In comparison with earlier reported abiotic stress tolerant *PScMYBAS1* promoter from sugarcane cultivar Co740; *PEaMYBAS1* promoter sequence from *E. arundinaceus* showed common motifs such as MBS (-87 and -731bp), MYB (-941bp), TCA (-618bp), TGACG (-585bp), Box E (-632bp), W box (-232bp), WRKY (-95, -886, -966bp), Circadian (-775bp), Skn-1 (-101, -726bp), TCCC (-405bp) and an anaerobic responsive element *i. e.* ARE located at -805bp. The *PEaMYBAS1* promoter as well as *PScMYBAS1* also possesses common motifs such as CAAT-box and TATA-box located near many transcription start site. CAAT-box is well known to control transcription initiation, while the TATA-box is crucial for initiation of transcription. (Fig. 3, Table 2, Supplementary Fig. I).

Some common motifs such as MBS (-731bp), MYB (-941bp) and ARE (-805bp) from *PEaMYBAS1* have almost same base pair position in promoter *PScMYBAS1* such as -732, -942 and -806 bp, respectively.[13] However, *PEaMYBAS1* promoter possesses four new

motifs than those of *PScMYBAS1* promoter sequence such as GATA motif (-140bp), 3-AF1 binding site (-340bp), Box III (-872bp) and O2 site at -933bp (Fig.3, Table 2).

3.2. GUS expression analysis study

The GUS expression analysis study endorsed that *PEaMYBAS1* promoter was a stress-inducing promoter and not constitutively expressed. A constitutive promoter such as CaMV35S is continuously expressed at molecular level in all stages of plant growth and cannot be regulated by abiotic stresses. This makes the transgenic plants grow relatively slow in the absence of stress than those plants with inducible promoters.[23, 24, 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 a powerful genetic engineering tools to develop stress tolerant transgenic plants. [25]

As compared to CaMV35S promoter mediated GUS expression, tobacco leaf agro-infiltrated with F1 (-161) and F2 (-282) showed minimal while F3 (-554) showed basal GUS expression than the other fragments (F4, F5, F6, F0). *PEaMYBAS1* transient assays revealed increased GUS induction of the promoter region from F3 (-554) to F0 (-1032 bp) under drought, cold, salt and wounding. Such elevated expression of the GUS reporter gene might have occurred due to regulation of *cis*-acting elements present within the promoter region (Fig. 4, Fig. 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, osmoregulation are other water dependent processes. Scarcity of water in drought condition dramatically

affects the plant growth, reduces leaf size, stems extension, root proliferation and ultimately disturbs the plant water relation. To face the critical situation like drought, numerous drought-responsive genes, transcription factors and *cis*-acting motifs in plants are expressed at molecular levels to prevent drought-induced loss of crop yield.[26]

In this study, the full length *PEaMYBAS1* promoter region F0 (-1032 bp) showed maximum GUS induction activity (4.9 fold) in agro-infiltrated tobacco leaves under drought stress condition 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 (supplementary Table I). Whereas F3 (-554) fragment showed marginal GUS induction activity and non significant GUS induction was observed in F2 (-282) and F1 (-161) deletion fragment (Fig. 4, Fig. 5a). Prabu et al. have monitored increased GUS activity from 2 to 4 fold in *PScMYBAS1* promoter deletion fragment region from F6 (-777 bp) or longer up to F0 (-1033 bp).[13]

Drought stress enhanced the GUS activity of full length F0 (-1032) deletion fragment *PEaMYBAS1* promoter can be endorsed due to presence of *cis*-acting elements such as MBS (-731bp), MYB core sequence (-941bp), circadian clock element (-775bp), Opaque-2 *i.e.* O2 site (-933bp), BOX III (-872bp) and WRKY (-886 and -996bp) (Fig. 3). Interaction between these elements might have helped to boost overall GUS expression in *PEaMYBAS1* promoter with increased synthesis of drought stress regulatory proteins. While comparatively *PScMYBAS1* promoter with MBS (-732bp) and MYB core sequence (-942bp) have shown less GUS expression than that of *PEaMYBAS1* promoter might be due to lack of O2 site, BOX III motifs.[13]

MBS element provides a binding site for ABA dependant MYB while MYB-core sequence function as a binding motif for plant MYB proteins involved in drought stress-

induced gene expression.[27,28,29,30]. *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, 32, 33] *PScMYBAS1* promoter has been recommended for drought stress tolerance in *S. officinarum* due to 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 at day time.[34]

Vincentaz et al. have indicated that O2 site is a regulatory locus that encodes a DNA-binding protein which activates the transcription of the b-32 gene and regulates 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 earlier reported that bZIP transcription factors in *A. thaliana* act as a positive regulator of diverse functions such as plant development and drought stress response.[37, 38] These results are also in agreement with studies on 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 prolonged cold stress environment affects the physiological process of plants. *PEaMYBAS1* promoter region manifested increasing GUS expression from F4 (-598) longer up to F0 (-1032 bp) viz from 2.6 to 3.9 fold, respectively under cold stress in the transiently expressed tobacco leaf tissues while compared with respective mock (Fig. 4, Fig. 5b, Supplementary Table I). This enhanced GUS activity might be the result of interaction of WRKY transcription factor (-886 and -996bp) with TGACG (-585bp), MBS (-731bp), TCA (-

618bp) and MeJA-responsive *cis*-acting element.[41, 42, 43,] Prabu et al. have documented the GUS induction in *PScMYBAS1* promoter region from F6 (-777) to F7 (-843) which was devoid of WRKY transcription factor and circadian clock as 2.03 to 2.8 fold, respectively under cold stress. However, lack of interaction between 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 WRKY transcription factor enables them to bind with the relevant target promoters.[46] *Cis*-elements W1 box (-232bp) provides a binding site for WRKY transcription factors which plays an important role in plants during cold stress regulation. 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 regulation of SA treatment, auxin elicitor responsive element and light.[48, 49, 50, 51, 52]

Besides this, plant circadian clock element is located at -775bp in *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] Circadian clock 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 *PEaMYBAS1* promoter containing 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 stress which imbalances the irrigated land, hampers normal growth of plants by promoting early leaf senescence as well as dramatically increases photoprotective demand in plants. Excess salts and water in the soil affect plant growth 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 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 performs 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, interactive molecular and cellular changes that commence after onset of stress.

In this study, deletion fragment region from F6 (-841) to F0 (-1032) bp of *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 (Fig. 4, Fig. 5c, Supplementary Table 1). This deletion fragment possesses MBS core sequence (-731bp), O2 site (-933bp) and Skn-1 motif (-726bp) (Fig. 3). While in comparison with *PScMYBAS1* promoter, deletion fragment 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 *PScMYBAS1* promoter under salt stress than that of *PEaMYBAS1* promoter because of the presence of MBS motif and absence of O2 site. In salt stress response, MBS core sequence helps in modulation of MYB motif and plays a dual role in controlling drought and salt stress

induction. MYB protein performs a key role in transcriptional activation of ABA-inducible gene under regulation in higher salt concentrations.[57] GmMYB76 from *G. Max*, AtMYB2 and AtMYB7 gene from *A. thaliana* are popular to manage salt stress.[32, 58, 33] O2 site encoding bZIP transcription factor imparts significant role in salt stress regulation in *A. thaliana* via ABF3 gene.[59]

The Skn-1 motif which is well known for development of transcription factor, controlling the seed specific endosperm expression also functions in a salt induced oxidative stress.[60] It has been published earlier that Skn-1 which is distantly related to bZIP motif binds to DNA through a unique mechanism and orchestrates oxidative stress response in *Caenorhabditis elegans*.[61] Salinity-stress tolerant tobacco plants were already raised by over expressing 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 drive certain defense genes. When plants are continuously exposed to mechanical wounding; signaling molecules such as jasmonic acid (JA) and salicyclic acid (SA) which prevent pathogens attack are continuously synthesized at the injured site.[38] Expressions of cis-acting elements like TGACG and TCA have earlier been verified for JA and SA production, respectively under wound stress condition.[63, 64]

In current study, *PEaMYBAS1* promoter region from F4 (-598) to F0 (-1032 bp) containing TGACG (-585bp), TCA (-618bp), Box E (-632bp), ARE (-805bp) and WRKY motifs (-886 and -996bp) showed enhanced GUS induction from 1.51 to 3.06 fold than the respective mock after mechanical wounding (Fig. 3, Fig. 4, Fig. 5d, Supplementary Table 1).

ARE has an important role in 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] Interaction between WRKY transcription factor and W Box have been studied in the activation of pathogen or hormone responsive (SA, MeJA) genes.[65] Deletion fragment region of *PScMYBAS1* promoter from F3 (-303) to F0 (-1033) have also been reported in response to wound stress.[13]

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 cumulative expression of different *cis*-acting elements and motifs in promoter *PEaMYBAS1.* This helped to enhance overall GUS activity in transient tobacco plant under various stress circumstances. Therefore, *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

The *PEaMYBAS1*, sugarcane *MYB* transcription factor gene promoter expressed in tobacco conferred and enhanced tolerance to drought, moderate to cold, salt and wounding stress. Implying on these results, *PEaMYBAS1* with novel *cis*-acting elements have an important role in countering abiotic stresses. 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. Although this work provides thoughtful 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 molecular level.

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Figure legends:

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

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.

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.

Fig . 4 GUS profile expression of PEaMYBAS1deletion fragments agroinfiltered tobacco leavs. GUS was detected in X-Gluc solution followed by stress treatment.

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.

526 **Table1.** Sequence of the oligonucleotides used for the *PEaMYBAS1* deletion plasmids construction

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

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543 **Table 2**. Positions and functions of 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 start	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	
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)

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Fig. 1 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.

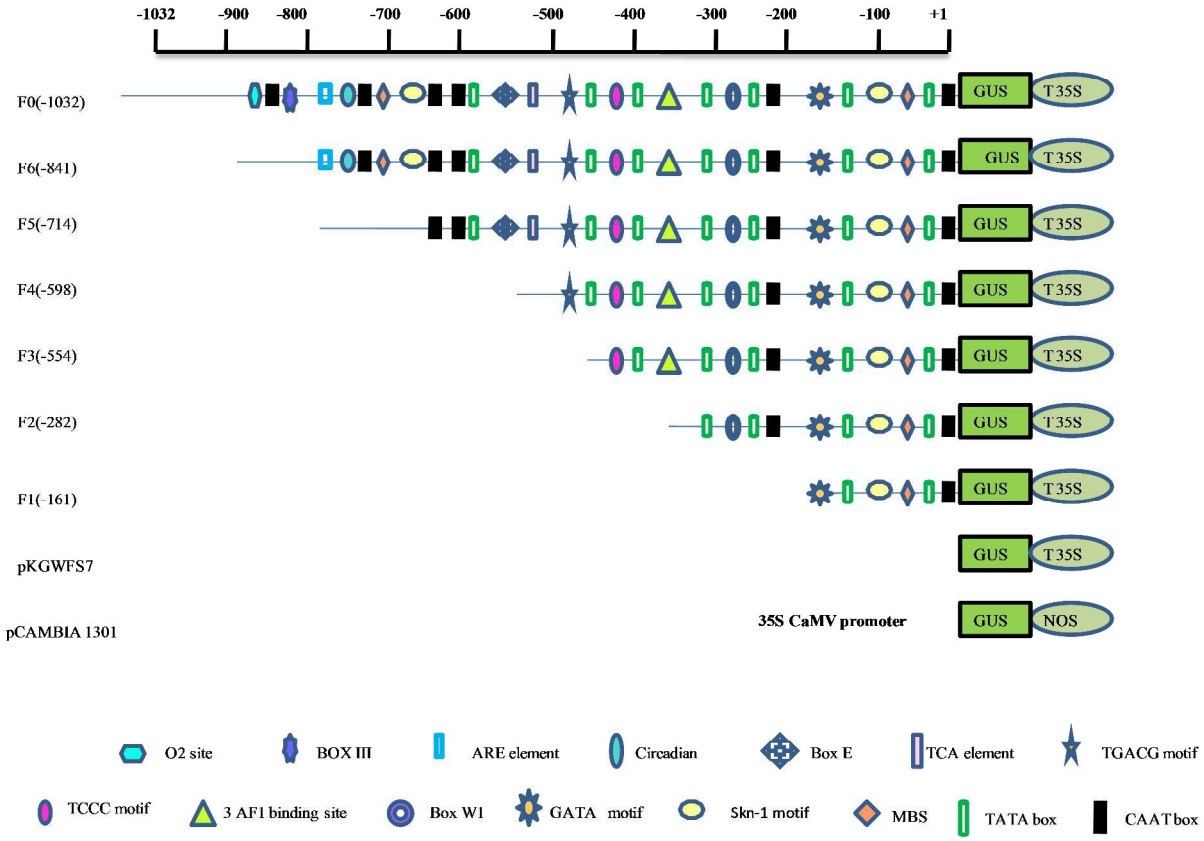

Fig. 2 Schematic representation of *PEaMYBAS1* promoter constructs for assaying GUS expression in tobacco leaves. The serially 5′-deleted promoter constructs of the *PEaMYBAS1* were fused to the GUS reporter gene in the vector pKGWFS7.

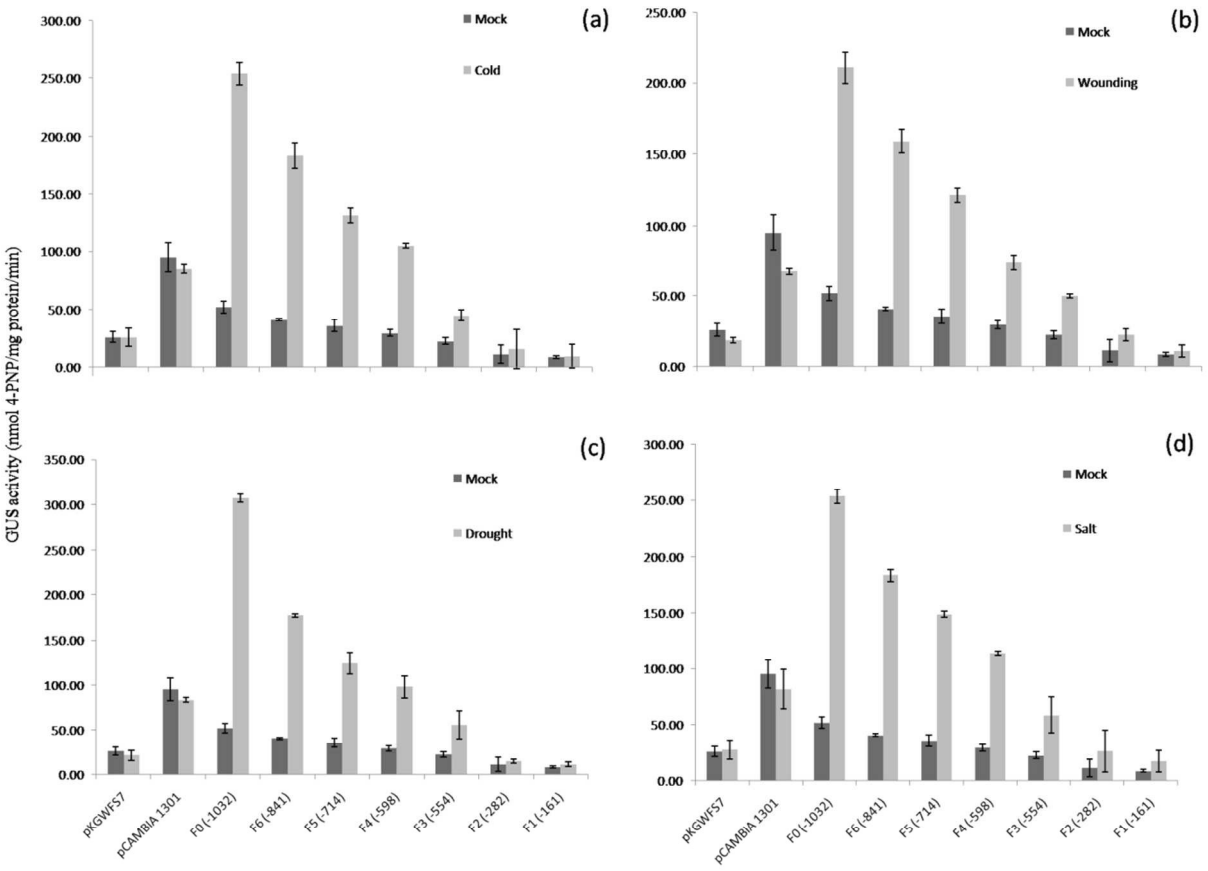

Fig. 3 *PEaMYBAS1* activation in response to a. cold, b. wounding, c. drought and d. salt applied to tobacco leaf tissues transiently transformed with *PEaMYBAS1*::GUS constructs. Transient transformation was conducted by agroinfiltration of negative control (pKGWFS7), positive vector control (pCAMBIA1301). GUS activity was analyzed spectrophotometrically and is displayed quantitatively in terms of nmol 4-PNP/mg protein/ min. The numbers over the bars indicate the fold increase in induction of GUS activity after stress treatment versus mock treatment. Data are means ± standard deviations from three independent assays of tobacco leaf extracts.