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ORIGINAL ARTICLE

A novel PR10 promoter from *Erianthus arundinaceus* directs high constitutive transgene expression and is enhanced upon wounding in heterologous plant systems

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Abstract In genetic engineering, inducible promoters play an important role as the expression of genes driven by them can be turned on or off under situations like biotic or abiotic factors. There are few reports on inducible promoters that can be employed in the development of transgenic plants, particularly in sugarcane. In the present study, four wound inducible genes (Chitinase, PR1A, PR10 and HRGP) were selected and were amplified from *Erianthus arundinaceus,* a distant relative of sugarcane. In order to determine the gene that is highly induced upon wounding, RT-qPCR was performed, which showed that PR10 gene expression was instantaneous and higher upon wounding when compared to the other three genes. Using the random amplification of genomic ends technique, a 592 bp promoter sequence was obtained and in silico analysis of the upstream regulatory region revealed a 469 bp promoter and 123 bp of 5′ untranslated region (UTR). Functional analyses of the promoter sequence (with and without 5′ UTR) in tobacco, rice and sugarcane using β-glucuronidase (GUS) as the reporter gene revealed the constitutive and inducible nature of the PR10 promoter. Our studies have demonstrated that the PR10 promoter, though highly constitutive, was quickly induced upon wounding as well as on treatment with abscisic acid and methyl jasmonate hormones. This is the first report on the isolation and characterization of a PR10 promoter from a wild grass and is expected to have application for development of transgenic plants.

Keywords Inducible promoter · PR10 · Wounding · *Erianthus* · 5′ regulatory sequence

Introduction

Plants are sessile organisms that are continuously exposed to several biotic and abiotic stresses such as heat, cold, high salinity, drought or pathogen [1]. Plants initiate many inducible defense responses following wounding/pathogen attack. These responses comprise (a) phytoalexin synthesis (b) deposition of lignin, callose, hydroxy proline rich glycoproteins (HRGPs) (c) production of lytic enzymes like chitinases and glucanases and (d) accumulation of defense related proteins such as the pathogen related (PR) proteins. PR proteins are induced both due to pathogen attack and also in response to different abiotic stresses like wounding, drought and high salinity. They have been grouped into 17 classes based on their amino acid composition, small molecular masses and acidic pIs [2]. Many PR proteins possess anti-microbial activities like b-1, 3-glucanase (PR2), chitinases (PR3, PR4, PR8 and PR11) and RNase (PR10). Abscisic acid (ABA), methyl jasmonate (MeJA), salicylic acid (SA) and ethylene (ET) have been described as the chief signaling molecules in plant stresses, and their signaling pathways have been studied extensively [3–5]. Genes that are induced by stress and/or defense have led to the identification of a number of inducible promoters. Many of these valuable promoters help expression of the target genes at the site of wounding or infection making them potentially useful for genetic engineering of plants for disease/insect resistance that are turned on at the wound

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site. However, limited inducible promoters are known to be used in plant transformation studies. In this study, four wound inducible genes namely PR1A, Chitinase, PR10 and HRGP were chosen and their expressions upon wounding were studied with a view to isolate and characterize the promoter of the gene that is highly induced. Such an inducible promoter would be of great value in genetic engineering of plants particularly for biotic stress resistance.

Materials and methods

Isolation of partial gene sequences

Based on literature, four wound inducible genes (PR1A, Chitinase, PR10 and HRGP) of monocot plants were chosen and to amplify the partial gene sequences primers were designed from the conserved regions, as listed in Table 1. Using these gene specific primers the genes were amplified from *Erianthus arundinaceus* (Ac. No IK 76-81) genomic DNA, cloned in pTZ57R/T vector following manufacturer's instructions and sequenced.

Table 1 Primer sequences used in the study

Primer	Sequence 5′–3′
PR1A F	AGTACGGCGAGAACATCTTC
PR1A R	AGTTGCAGGTGATGAAGACG
CHI F	CTGCTACATCAGCGAGATC
CHI R	SGTGCACGTTGTTCGTCCAG
PR10 F	ACAGCTGGACCCWCGAGAT
PR10 R	GTARGCGTCSGGGTTGGCGA
HRGP F	AGTGGCAGGGTGAGCGTCTC
HRGP R	CGGCTGGCTAGGACACAAC
ACT F	GTGACAATGGCACTGGAATC
ACT R	GACCTGACCATCAGGCATCT
RT PR1A F	TGGGTCTCCGAGAAGCAGTA
RT PR1A R	AGTTGCAGGTGATGAAGACG
RT CHI F	AACAACGCCTACTGCGACTC
RT CHI R	TTGTTCGTCCAGAACCAGAG
RT PR10 F	CGTCAGGCAGTTCAACTTCACCTCA
RT PR10 R	ATGCACGTCATGCGCTAGCTGT
RT HRGP F	AAACCCACTCCCACTCCTCCGA
RT HRGP R	CTTAGGGCTTGGAGTGTACG
RT ACT F	AAGTACCCGATTGAGCATGG
RT ACT R	TACATTGCTGGGCATTCAAA
RT GUS F	AGTCCATCTCCGCCAGGAATTT
RT GUS R	GATCAATGTCGTGAAAGCCCGCAA
WPF	GCATGAATTCCGACCCGGGATCCGA
GUS R1	GATCAATGTCGTGAAAGCCCGCA

Wounding treatment

Erianthus arundinaceus plants were raised from single buds in replicates in green house and were covered with polystyrene cages to prevent any insect attack. For wounding experiments, a modified method of Delessert [6] was followed. In order to study the gene expression upon wounding, the middle of the third leaf was wounded using a serrated forceps and samples were collected from three different locations i.e.—local (wound site), adjacent (adjacent to wound site) and systemic (unwounded next lower leaf) at an interval of 0, 3, 6, 12 and 24 h after wounding. The samples were immediately frozen in liquid nitrogen for RNA extraction. A diagrammatic representation of sampling is given in Fig. 1.

Expression analysis through RT-qPCR

Total RNA was extracted with TRIzol Reagent (Sigma) following manufacturer's instructions and after treating with DNase, from 1 µg of total RNA, cDNA was synthesized using revertaid first strand cDNA synthesis kit (Thermo Scientific). The real-time quantitative polymerase chain reaction (RT-qPCR) was carried out using a Step-One plus Real-Time PCR system (Applied Biosystems, Canada) and each reaction was carried out in triplicates. The primers used for RT-qPCR are given in Table 1. For qPCR experiments cDNA concentration was standardized for each sample and melt curve analysis was performed to

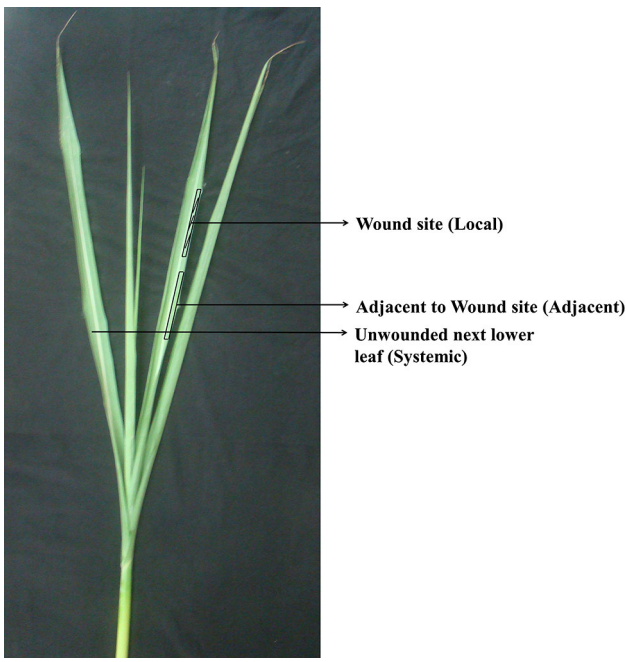

Fig. 1 A pictorial representation of sampling for wounding in *E. arundinaceus*

check primer specificity. The cocktail for RT-qPCR reaction contained 200 ng cDNA, 3 pmol each of forward and reverse primers, 12.5 µl of 2X MESA GREEN RT qPCR master mix plus (Eurogentec, Belgium) and with nuclease free water the total reaction mixture was made up to 25 µl. The reaction was performed for 40 cycles (first cycle for 10 min at 95 °C and followed by 40 cycles and each cycle alternating with 95 °C for 15 s and 1 min for 60 °C). To quantify the relative transcript levels, actin transcript was used as endogenous control. For calculating the relative expression of target gene $2^{-\Delta\Delta Ct}$ method was used [7], where $\Delta\Delta Ct = \Delta Ct$ sample $- \Delta Ct$ actin. $\Delta\Delta Ct$ values reflect the relative expression of the target gene.

Isolation of 5′ regulatory region of wound inducible gene

The random amplification of genomic ends protocol [8] was followed for isolating the 5′ regulatory region. The genomic DNA (gDNA) was digested with three different blunt end cutting restriction enzymes (*Dra*I, *Hinc*II and *Ssp*I) at 37 °C for 30 min. The adapter with annealed long (5′-CTAATACGACTCACTATAGGGCTCGAGCGGCC GCCCGGGCAGGT-3′) and short (5′-ACCTGCCC-NH2-3′) arms was ligated to the restricted gDNA [8, 9].

The RAGE primary PCR was performed using the adapter specific primer I (ASPI-5′-GGATCCTAATA CGACTCACTATAGGGC-3′) and PR10 gene-specific primer I (GSPI-5′-AAGTTGAACTGCCTGACGCT-3′) as forward and reverse primers, respectively. The PCR reaction was performed with 1.5 mM of dNTPs, 0.4 µM of forward and reverse primers and 0.9 unit of the XT-5 polymerase enzyme (Merck, India) along with the appropriate buffer. For RAGE nested PCR the primary PCR product was used as a template and the reaction cocktail contained primary PCR product, dNTP mix (1.5 mM), forward and reverse primers (0.25 µM each) and XT-5 polymerase enzyme (0.9 units) along with the specific buffer. The primers used for the nested PCR were adapter specific forward primer II (ASPII-5′-AATAGGGCTCGA GCGGC-3′) and gene-specific reverse primer II (GSPII-5′-TGGGCGCTGGCGACGAC -3′) and the PCR product was separated in 1 % agarose gel through electrophoresis, eluted, cloned, and sequenced.

In silico sequence analysis

The sequence obtained through RAGE was analyzed through GenScan (http://genes.mit.edu/GENSCAN.html) to identify putative introns and exons. Putative core promoter elements and transcription factor-binding sites (*cis*-elements) were predicted through PLACE database search [10]. Based on the position of the predicted TATA box, the Transcription Start Site (TSS) (+1 site) was assigned. The distribution of the *cis*-elements was plotted using Cytoscape software [11]. The regulatory sequence was named Eri PR10.

Plasmid constructs

The Eri PR10 PCR fragment was ligated into the pCAMBIA1305.1 vector after digesting the vector with *Eco*RI and *Bgl*II enzymes so as to release the CaMV 35S promoter driving the GUS reporter gene and the PCR fragment to insert the PR10 5′ regulatory sequence to form pEri PR10.1-GUS. Similarly PR10 promoter without 5′ UTR was cloned instead of CaMV 35S promoter to form pEri PR10.2-GUS.

Plant transformation

Agrobacterium-mediated transformation was carried out in rice [12], tobacco [13] and sugarcane [14], with pEriPR10.1-GUS and pEriPR10.2-GUS. Transgenics were produced with pCAMBIA1305.1 (for tobacco and rice) and pMubi1-GUS (for rice and sugarcane) and were used as controls. The transgenics obtained were maintained in transgenic glasshouse.

PCR confirmation of transgenics

Genomic DNA was extracted from putative transgenics using DNeasy plant mini kit (Qiagen). The integration of the promoter-GUS in putative transgenics was confirmed by PCR with WPF forward primer and GUS-R1 reverse primer given in Table 1. The reactions were carried out using 25 ng of template DNA with 1.5 mM dNTPs, 0.25 µM each of the primers, and one unit of Taq polymerase enzyme along with the specific buffer. PCR products were separated through electrophoresis in 1 % agarose gel and the molecular weight of the products was determined by comparing with 1 kb DNA marker.

Wounding treatment in transgenic plants

Wounding was performed in transgenic plants as explained earlier and the samples were collected from three different locations i.e., local, adjacent and systemic at 0 and 3 h after wounding for GUS expression analysis and RNA isolation for qRT-PCR. GUS expression analysis was carried out in transgenic tobacco, rice and sugarcane and qRT-PCR was carried out in transgenic tobacco (T_1) and transgenic sugarcane (T_0).

In situ histochemical localization of GUS expression

GUS staining was performed following Jefferson [15]. Tissues were excised aseptically and were washed in 50 mM sodium phosphate buffer prior to incubation in phosphate buffer containing 1 % Triton X-100 at 37 °C for an hour. Further, the explants were transferred to 1 mM X-Gluc staining solution, vacuum infiltrated for a short period and incubated for 16 h at 37 °C. After de-staining in 70 % ethanol, the tissues were observed under a stereo light microscope.

GUS fluorometric assay in transgenic plants

Fluorometric GUS assay was performed following Jefferson [15] in transgenic plants. Two hundred and fifty milligrams of the leaf tissue was ground in GUS extraction buffer [50 mM $NaPO_4$ (pH 7.0), 10 mM dithiothreitol, 1 mM Na_2EDTA, 0.1 % sodium lauryl sarcosine, and 0.1 % triton X-100]. Fifty microlitres of the extract was added to 0.5 ml of pre-warmed (37 °C) assay buffer (1 mM 4-methyl umbelliferyl b-D-glucuronide in extraction buffer). From this, 100 µl was transferred to a microfuge tube containing 0.9 ml stop buffer (0.2 M Na_2CO_3) at room temperature. The liberation of 4-methyl umbelliferone (4-MU) was followed by quantification of the fluorescence with excitation at 365 nm and emission at 455 nm in a fluorometer (Promega). The assays were performed in triplicates for 5–10 independent transgenic events and GUS activity was determined as nanomoles of 4-MU hydrolyzed/minute/milligram of total protein.

Analysis of GUS expression on exogenous application of hormones involved in wound signaling in T_1 transgenic tobacco

Two month old transgenic tobacco plants (T_1) were assayed for GUS expression on application of SA, MeJA and ABA hormones which are involved in wound signaling. For the hormone treatments, the procedure described by Curtis [16] was followed. Plants were sprayed with a solution of 0.01 % ethanol, 0.1 % Triton X-100 in water containing any one of the following: 5 mM SA (pH 6.5), 100 µM ABA (pH 6.8) or 100 µM MeJA (pH 6.8). Control plants were treated with 0.01 % ethanol and 0.1 % Triton X-100 in water. Leaf samples were collected in triplicates after 6, 12 and 24 h of hormone treatments and assayed for GUS activity.

GUS expression analysis using RT-qPCR in transgenic tobacco and sugarcane

For transgenic tobacco (T_1), three independent transgenic events, with pCAMBIA1305.1, pEriPR10.1-GUS and pEriPR10.2-GUS that showed better GUS expression were selected and T_1 tobacco plants were raised in a transgenic glasshouse. In the case of transgenic sugarcane, three independent events with pMubi1-GUS, pEriPR10.1-GUS and pEriPR10.2-GUS that showed higher GUS expression were selected for RT-qPCR analysis. Leaf samples were harvested from three locations (local, adjacent and systemic) at 0 and 3 h after wounding and were immediately frozen in liquid nitrogen. Total RNA was extracted from the samples and after treating with DNase, first strand cDNA was synthesized and RT-qPCR was performed as explained earlier.

Statistical analysis

GUS assays were performed in triplicates and the data was subjected to statistical analysis using a one tailed paired Student's *t* test. Significance of treatments was evaluated using one-way ANOVA. A probability (P) value of ≤ 0.05 was considered to be statistically significant.

Results

Isolation of partial sequences of inducible genes

The genomic DNA was isolated from *Erianthus* and used for the amplification of wound inducible genes—PR1A, Chitinase, PR10 and HRGP. The actin gene was also amplified (for use as endogenous reference in RT-qPCR). The amplicons obtained were cloned and sequenced using M13 specific primers. The sequences were deposited in GenBank with the following accessions: KP284555–KP284559. Based on these sequences, the primers were designed for RT-qPCR.

Gene expression upon wounding

The relative expression of the four wound inducible genes was studied at 0, 3, 6, 12 and 24 h after wounding from three different locations. RT-qPCR results showed that PR10 gene expression was higher and faster upon wounding compared with the other three genes. At the wound site, after 3 h of wounding, the PR10 gene expression was 33-fold, 460-fold and 27-fold higher compared to PR1A, Chitinase and HRGP respectively (Fig. 2a). The relative expression of PR10 adjacent to the wound site (at 0 h) was 163-fold, 84-fold and 389-fold higher compared to PR1A, Chitinase and HRGP respectively (Fig. 2b). Systemic gene expression of PR10 after 3 h of wounding was 84-fold, 41-fold and 15-fold higher compared to PR1A, Chitinase and HRGP respectively (Fig. 2c). The expression of PR10 gene was significantly

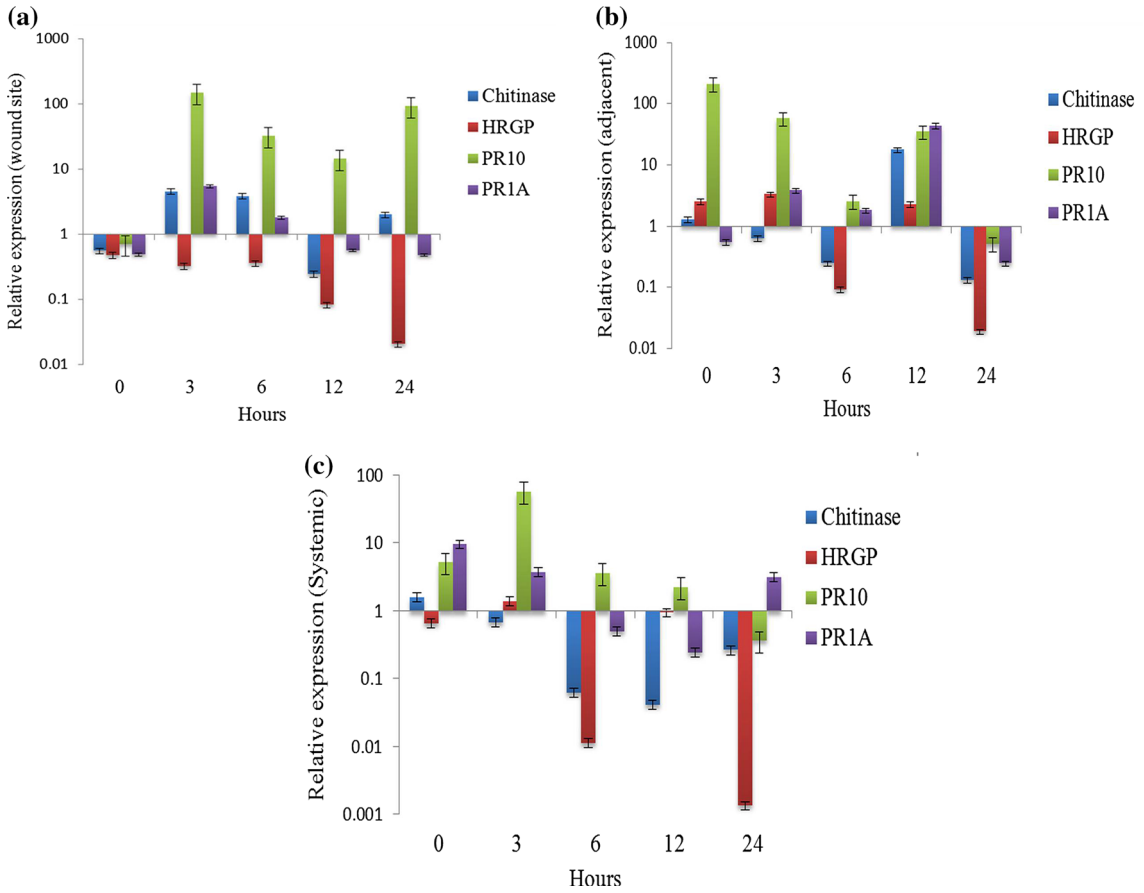

Fig. 2 Relative expression of the four genes **a** at wound site, **b** adjacent to wound site, **c** systemic gene expressions in unwounded next lower leaf; each *bar* represents average of three replicates and error bars indicate standard deviation (SD)

higher at the wound site 3 h after wounding and gradually reduced after 6 and 12 h, though the expression level increased after 24 h (90 fold). Based on the expression patterns to wounding, PR10 gene was selected for the isolation and characterization of the regulatory sequence.

Isolation of PR10 5′ regulatory sequence

PCR amplification with ASPII and GSPII primers yielded an amplicon containing the upstream region of PR10 gene. Out of three restriction enzymes used, *Hinc*II gave an amplicon size of 800 bp in the nested PCR and this fragment was further cloned and sequenced.

In silico analysis

In silico analysis revealed that the PR10 5′ regulatory sequence contained 592 nucleotides, with a promoter sequence of 469 nucleotides upstream of the transcription start site (+1). The sequence also contained an untranslated region (5′ UTR) of 123 bp (Fig. 3a). The TATA box was predicted at −30 position and CAAT box at −260 position in addition to another CAAT box (Fig. 3b). The isolated PR10 5′ regulatory region of 592 bp was compared with the earlier reported PR10 promoters from other species. When the promoter alone (−469 position from TSS) was compared with the corresponding regions of sorghum, rice, *Asparagus*, chilly and pine PR10 promoters, a sequence similarity ranging from 27.1 to 37.2 % was observed. When the PR10 regulatory sequence with 5′ UTR (592 bp) was compared with the corresponding regions of sorghum, rice, *Asparagus*, chilly and pine PR10 promoters, a sequence similarity ranging from 31.4 to 37.4 % was observed.

With a view to identify the potential *cis* acting elements, the regulatory sequence was analyzed using PLACE database. The Fig. 6 depicts the nucleotide sequence of EriPR10 promoter. Besides TATA and 2 CAAT boxes, several plant conserved motifs were observed. Some selected *cis*- acting elements are given in Table 2. The *cis* acting elements are illustrated schematically using Cytoscape (Fig. 4). Several pathogen/defense responsive *cis* elements were present throughout the regulatory sequence which include four WRKY boxes, an ethylene responsive

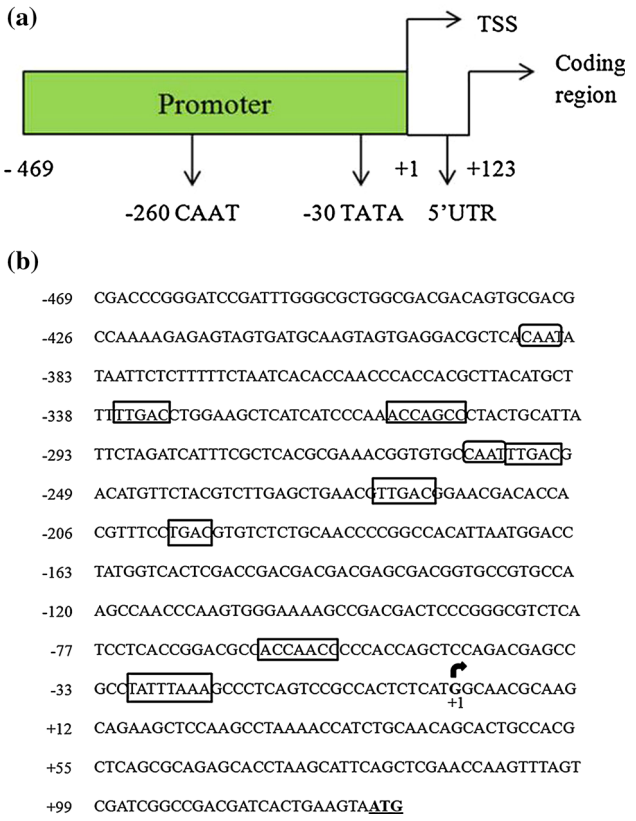

Fig. 3 *Erianthus* PR10 regulatory sequence **a** Schematic representation of 5′ regulatory region of *Erianthus* PR10 gene, **b** Nucleotide sequence of PR10 5′ regulatory region; the translation initiation codon ATG is *bold* and *underlined*; major motifs like TATA, CAAT and pathogen/defense responsive elements are boxed; the *black arrow* indicates transcription start site (TSS)

element, two ABA responsive elements, three MeJA responsive elements, two elicitor responsive elements and three SA inducible *cis* acting elements. Tissue specific *cis* acting elements like mesophyll, seed, pollen and nodule specific elements were present in varying numbers; however mesophyll elements were the highest (five). The abiotic *cis* elements include four light responsive, three low temperature responsive and two dehydration responsive elements. In addition, *cis* elements that are involved in flavonoid biosynthesis and those which are induced on etiolation were also identified.

Functional validation of PR10 5′ regulatory sequence

The plasmid constructs pEriPR10.1 and pEriPR10.2 (Fig. 5) were further mobilized individually in *Agrobacterium* strain LBA4404 and transformed in rice, tobacco and sugarcane through *Agrobacterium* mediated transformation. The transgenic plants obtained were assayed for GUS expression after PCR confirmation. In transgenic tobacco (T_0), five independent transgenic events with PR10.1 and PR10.2 promoters were assayed for GUS expression after wounding. Histochemical analysis was also performed with X-gluc substrate and profound GUS activity could be observed in the leaf tissues (Fig. 6). The GUS assay results showed that in transgenic tobacco at 0 h, both the promoters drove significantly higher expression in the area adjacent to the wound site, whereas 3 h after wounding, the wound site showed higher expression compared to the other two locations (adjacent and systemic) which corresponded to the data obtained from qRT PCR for PR10 gene in *E. arundinaceus* plants. In all the three locations PR10.1 and PR10.2 promoters drove significantly higher expression when compared to CaMV 35S promoter (Fig. 7a). In transgenic rice (T_0), upon wounding at 0 h, both the promoter sequences conferred higher GUS expression compared to the CaMV 35S and maize ubiquitin promoters (Fig. 7b). In the case of CaMV 35S and maize ubiquitin transgenics there was no change in the expression pattern of the GUS gene upon wounding. However, 3 h after wounding there was a significant reduction in the GUS expression level, which was much lower than that of CaMV 35S and maize ubiquitin promoters, with an exception in the case of PR10.1 promoter at the wound site.

The transgenic sugarcane plants (V_0) expressing GUS driven by PR10.1, PR10.2 and maize ubi-1 promoters were subjected to GUS fluorometric assay at 0 ^{h} and 3 h after wounding. The GUS fluorometric assay results showed that there was a significant increase in GUS activity upon wounding (0 h) in the leaves of transgenic sugarcane driven by both PR10.1 and PR10.2 promoters at the wound site (Fig. 7c). However after 3 h of wounding there was a reduction in GUS expression in both promoter sequences. The GUS expression levels in both PR10.1 and PR10.2 transgenics were significantly higher than that of maize ubiquitin transgenics. There was no significant change in the GUS expression levels in the case of maize ubiquitin transgenics with wounding (Fig. 7c).

The transgenic tobacco plants (T_1) were assayed for GUS activity at 0 h and 3 h after wounding. The GUS fluorometric assays showed that at 0 h in the wound site, there was a distinct increase in GUS activity in the transgenics driven by both PR10.1 and PR10.2 promoters (Fig. 8) .When compared with the CaMV 35S promoter, the GUS activity was 23 times higher in the wound site at 0 h. However, there was negligible GUS activity after 3 h in all the locations.

Table 2 List of *cis*-acting elements identified using PLACE program in Eri PR10 regulatory region

	Cis element	Consensus	Position	No. (+, −)	Function	References
Pathogen/defense responsive elements	WRKY71OS	TGAC	−335, −254, −222, −199	4 (4, 0)	Repressor of gibberellin signaling	[17]
	WBOXATNPR1	TTGAC	−336, −255, −223	3 (3, 0)	Salicylic acid responsive	[18]
	WBOXNTERF3	TGACY	−335, −159	2 (2, 0)	Wound inducible	[19]
	GCC CORE	GCCGCC	−36	1 (1, 0)	Ethylene responsive	[20]
	BOXLCOREDCPAL	ACCWWCC	−362, −61	2 (2, 0)	Elicitor	[21]
	ASF1MOTIFCAMV	TGACG	−243, −222, −199	3 (3, 0)	Auxin, MeJA responsive	[22]
Tissue specific elements	GTGANTG10	GTGA	−413, −401	2 (2, 0)	Pollen specific	[23]
	EBOXBNNAPA	CANNTG	−111, +33	2 (1, 1)	Seed specific	[24]
	CACTFTPPCA1	YACT	−303, −156, −9, +45, +114	5 (3, 2)	Mesophyll specific	[25]
	NODCON2GM	CTCTT	−378	1 (1, 0)	Nodule specific	[26]
Abiotic elements	LTRECOREATCOR15	CCGAC	−149, −98, +106	3 (2, 1)	Low temp responsive	[27]
	SORLIP1AT	GCCAC	−178, −64, −11, +49	4 (3, 1)	Light regulated	[28]
	MYBPZM	CCWACC	−361, −117, −60	3 (3, 0)	Flavonoid biosynthesis	[29]
	MYBPLANT	MACCWAMC	−363, −62	2 (2, 0)	Myb binding site	[30]
	MYB1AT	WAACCA	−313, +29	2 (1, 1)	Dehydration responsive	[31]
	ACGTATERD1	ACGT	−240, −226, −207, −197	4 (4, 0)	Etiolation induced	[32]
Quantitative elements	CAAT BOX	CAAT	−388, −260	2 (2, 0)	CAAT consensus	[33]
	DOF CORE	AAAG	−423, −102, −25	3 (3, 0)	Carbon metabolism	[34]

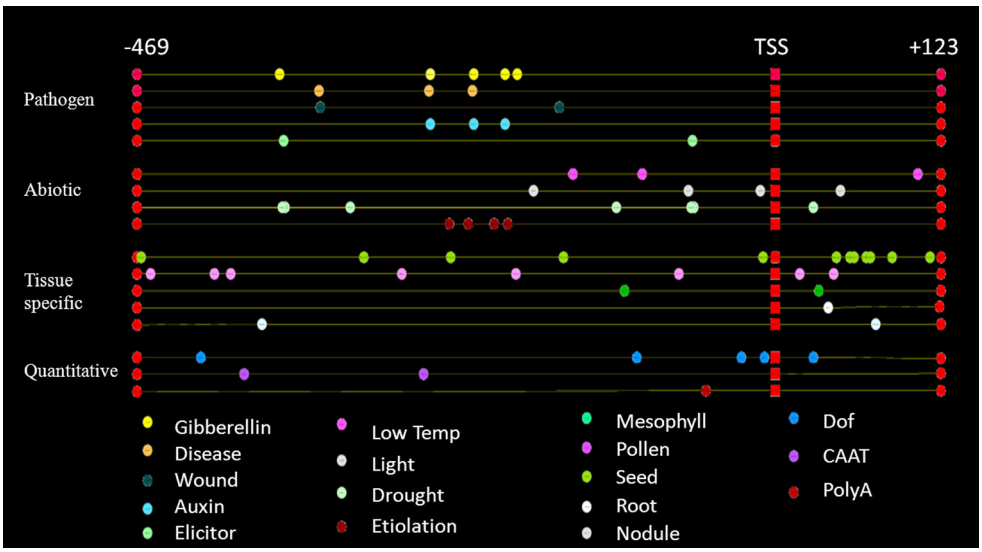

Fig. 4 Graphical representation of the major *cis* elements in the *Erianthus* PR10 regulatory sequence

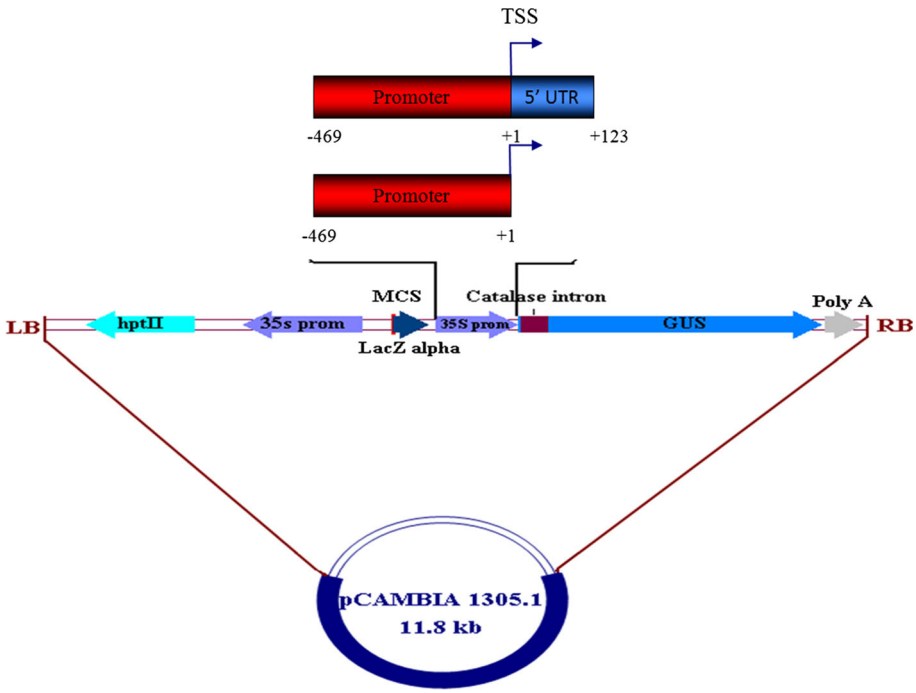

Fig. 5 Pictorial representation of binary vectors generated with PR10 promoter-GUS fusion

GUS expression on exogenous application of hormones involved in wound signaling in transgenic tobacco (T_1)

GUS expression was assayed in 2 month old transgenic tobacco plants (T_1) driven by CaMV 35S, PR10.1 and PR10.2 promoters after applying SA, MeJA and ABA. Three independent events were assayed for GUS activity with three plants per event at 6, 12 and 24 h after hormone treatment. The results demonstrated that SA was negatively regulated in all the treatments, whereas, there was a significant increase in GUS activity both in PR10.1 and PR10.2 transgenics 12 h after treatment with MeJA (2.5-fold and 1.6-fold) and ABA (4.5-fold and threefold) (Fig. 9), which decreased 24 h after both the treatments.

Relative GUS expression

The transgenic tobacco plants expressing GUS driven by CaMV 35S, PR10.1 and PR10.2 promoters were further validated using RT-qPCR. The RT-qPCR results showed that immediately after wounding (0 h), GUS expression driven by PR10.1 and PR10.2 promoters increased 180-fold and 150-fold compared to CaMV 35S promoter

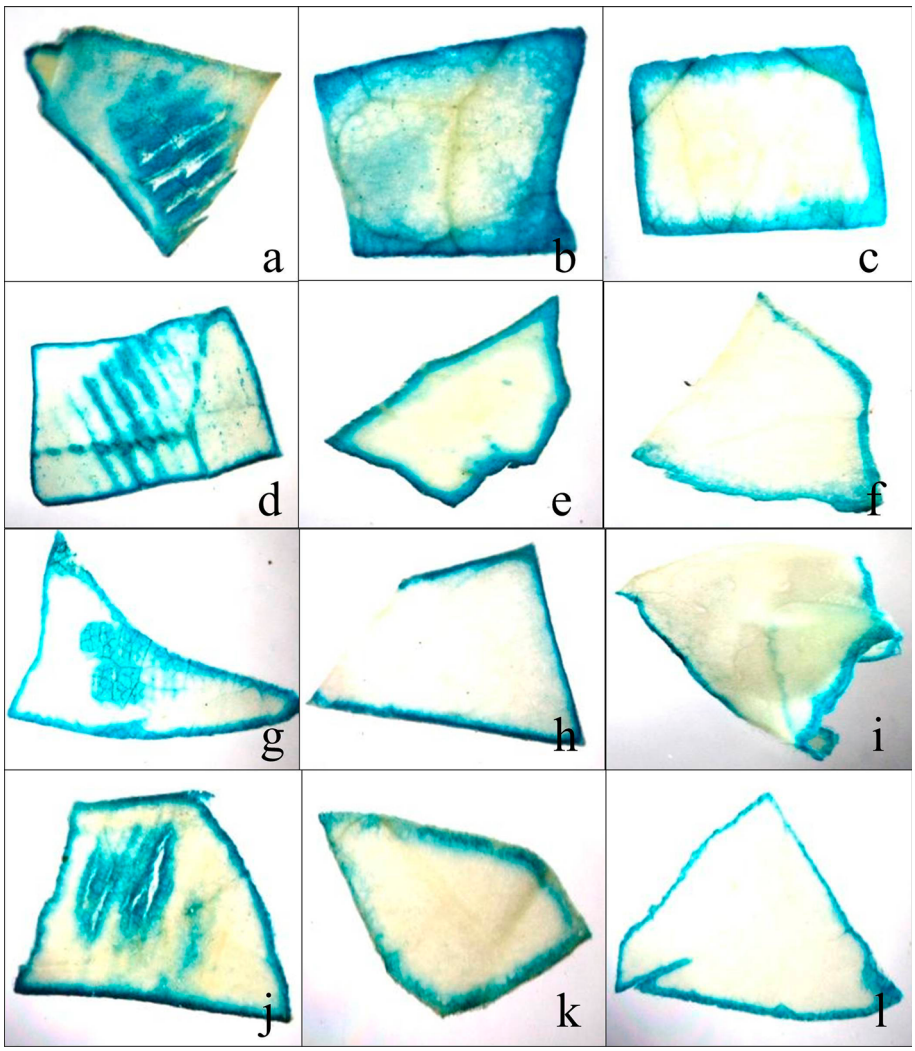

Fig. 6 Histochemical GUS staining of transgenic tobacco leaves driven by PR10.1 and PR10.2 promoters **a** GUS expression driven by PR10.1 promoter in wound site immediately after wounding, **b** GUS expression driven by PR10.1 promoter in the adjacent site immediately after wounding, **c** Systemic GUS expression driven by PR10.1 promoter immediately after wounding, **d** GUS expression driven by PR10.1 promoter in wound site after 3 h of wounding, **e** GUS expression driven by PR10.1 promoter in the adjacent site after 3 h of wounding, **f** Systemic GUS expression driven by PR10.1 promoter after 3 h of wounding, **g** GUS expression driven by PR10.2 promoter in wound site immediately after wounding, **h** GUS expression driven by PR10.2 promoter in the adjacent site immediately after wounding, **i** Systemic GUS expression driven by PR10.2 promoter immediately after wounding, **j** GUS expression driven by PR10.2 promoter in wound site after 3 h of wounding, **k** GUS expression driven by PR10.2 promoter in the adjacent site after 3 h of wounding and **l** Systemic GUS expression driven by PR10.2 promoter after 3 h of wounding

(Fig. 10a). However, 3 h after wounding there was a reduction in GUS expression.

The transgenic sugarcane plants expressing GUS driven by maize ubi1, PR10.1 and PR10.2 promoters were also validated using RT-qPCR and the results demonstrated sevenfold and 8.5-fold higher GUS expression driven by PR10.1 and PR10.2 promoters at the wound site immediately after wounding (Fig. 10b). The expression was retained even after 3 h of wounding, but in adjacent and systemic sites there was negligible expression.

Discussion

Initial gene expression studies had revealed that PR10 gene expression was instantaneous and higher upon wounding when compared with the other three genes (PR1A, Chitinase and HRGP). In the current study, an attempt was made to isolate the regulatory sequence of PR10 gene from *E. arundinaceus*. Using the RAGE technique, an 800 bp amplicon was obtained in the secondary PCR using *Hinc*II digest and the PCR product was cloned in pTZ57R/T

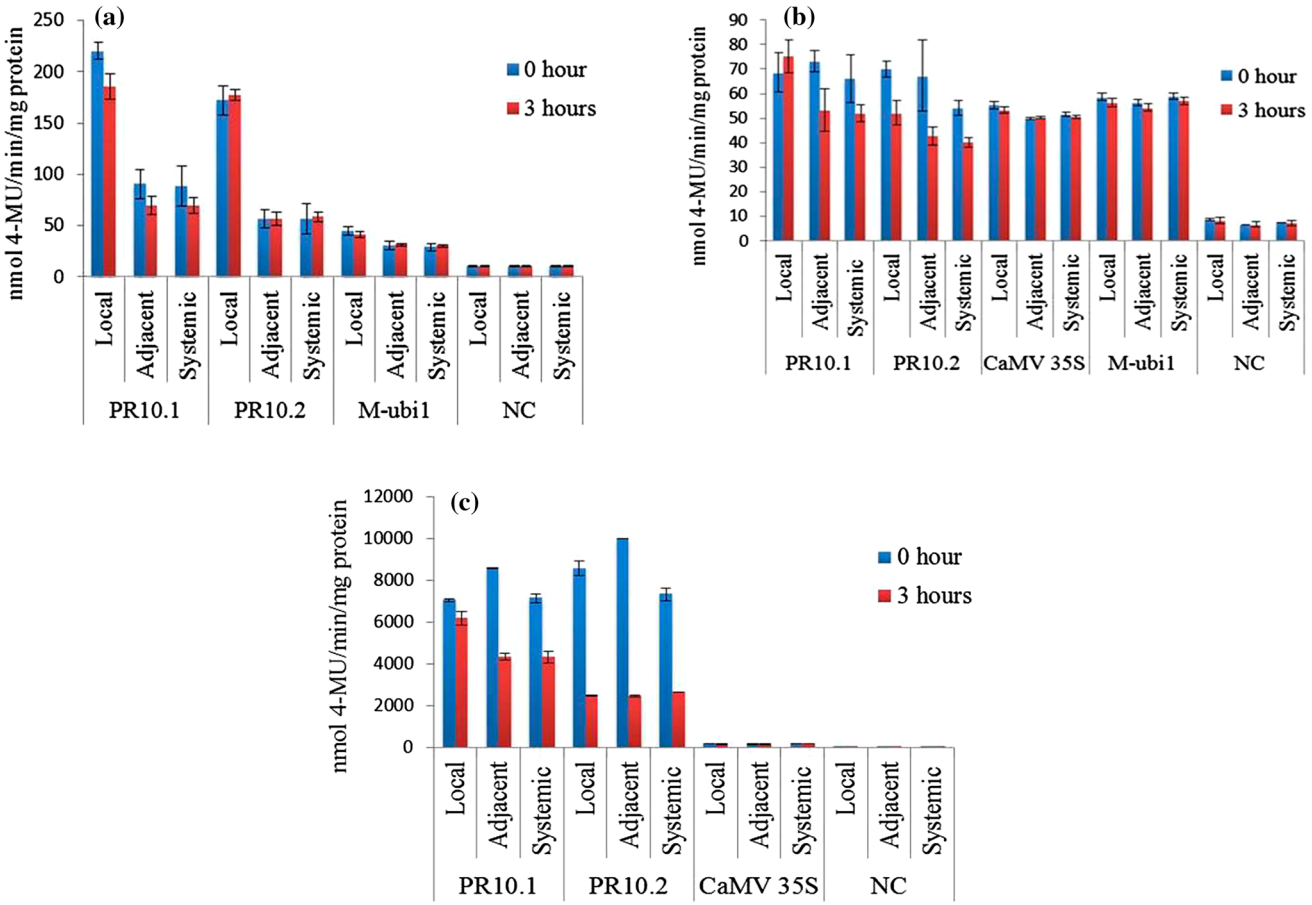

Fig. 7 Fluorometric GUS analysis in transgenic plants **a** Transgenic tobacco with PR10.1, PR10.2 and CaMV 35S promoters at 0 and 3 h after wounding, **b** Transgenic rice with PR10.1, PR10.2, CaMV 35S and Maize ubiquitin promoters at 0 and 3 h after wounding, **c** Transgenic sugarcane with PR10.1, PR10.2 and M-ubi1 promoters at 0 and 3 h after wounding. *NC* negative control; each value denotes the average of five to ten independent transgenic events; the *error bars* indicate SD; $p \leq 0.05$ was considered statistically significant as determined by Student's *t* test

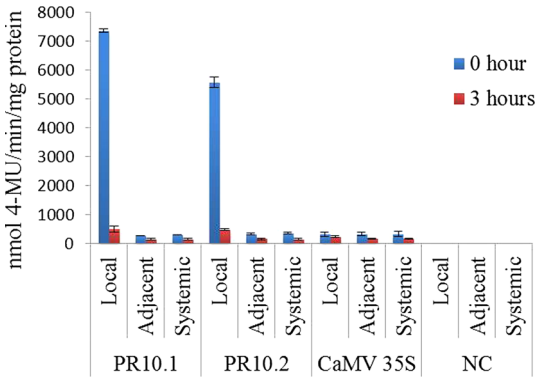

Fig. 8 Fluorometric GUS analysis in transgenic tobacco (T_1) plants with PR10.1, PR10.2 and CaMV 35S promoters at 0 and 3 h after wounding. *NC* negative control; each value represents the average of three independent transgenic events; the *error bars* indicate SD; $p \leq 0.05$ was considered statistically significant as determined by Student's *t* test

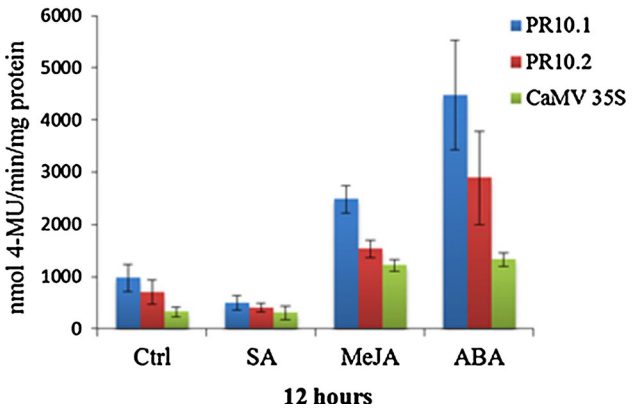

Fig. 9 GUS fluorometric analysis in transgenic tobacco T_1 plants with PR10.1, PR10.2 and CaMV 35S promoters after 12 h of hormone treatment; each value denotes the average of three independent transgenic events; the *error bars* indicate SD; $p \leq 0.05$ was considered statistically significant as determined by Student's *t* test

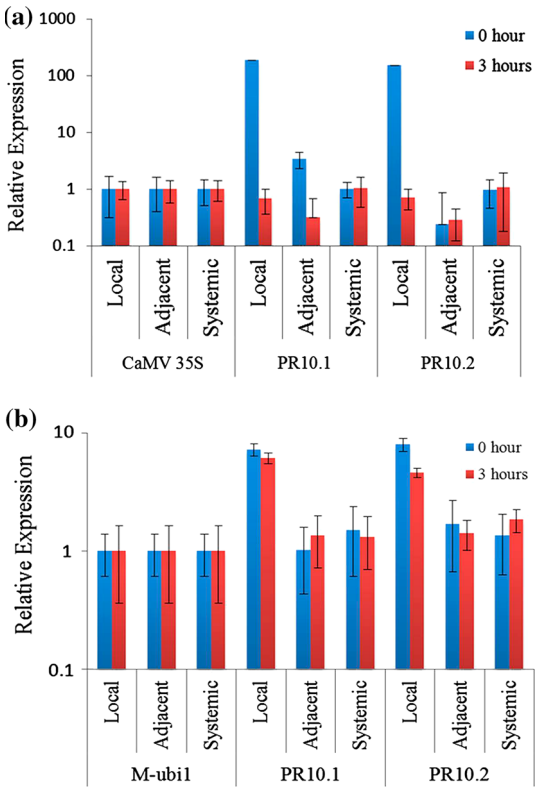

Fig. 10 Relative GUS expression of transgenic plants **a** Transgenic tobacco (T_1) with PR10.1, PR10.2 and CaMV 35S promoters at 0 and 3 h after wounding. **b** Transgenic sugarcane with PR10.1, PR10.2 and maize ubiquitin promoters at 0 and 3 h after wounding; each value denotes the mean of three independent transgenic events; the *error bars* indicate SD

vector and sequenced. In silico analysis of the isolated sequence revealed that the *Erianthus* PR10 5′ regulatory sequence contained 592 nucleotides, with a promoter sequence of 469 nucleotides upstream to TSS.

Several pathogen/defense responsive *cis* elements were present throughout the regulatory sequence which included four W boxes (binding sites for WRKY transcription factors containing TGAC motif), an ethylene responsive element (ACCGCC), three SA responsive elements (TTGAC), two ABA responsive elements (ACGTG) and three methyl jasmonate responsive elements (TGACG). TGAC motifs (W box) are binding sites of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway. WRKY proteins of parsley bind specifically to TGAC elements present in PR10 genes [17]. Ohme-Takagi and Shinshi [35] have shown that the GCC box is conserved in the 5′ upstream region of ethylene-inducible PR protein genes in *Nicotiana* spp. and in a few other plants. Several proteins that bind to GCC boxes have been isolated which were found to be members of the ethylene-responsive element binding proteins (EREBP). These transcription factors also bind to the dehydration-responsive element (DRE) present in the promoters of drought related genes. The regulatory sequence contained three TGACG motifs which are the ASF-1 binding sites present in CaMV 35S promoter. TGACG motifs are found in many promoters and are essential for jasmonate inducibility and are the binding sites for bZIP transcription factors [36].

Two *cis* elements that are known to be responsible for ABA responsiveness [37] were deduced in the PR10 regulatory sequence. This element might be involved in the induction of PR10 by ABA. In addition, various tissue specific *cis* acting elements were found in the promoter which included mesophyll, seed, pollen and nodule specific elements of which mesophyll elements were the highest in number (five). The abiotic *cis* elements include four light responsive, three low temperature responsive and two dehydration responsive elements. Few *cis* elements that are involved in flavonoid biosynthesis and which are induced on etiolation were also identified.

Transgenic tobacco (T_0) plants (five independent events each) expressing GUS driven by both PR10.1 and PR10.2 were assayed for GUS activity and compared with that of CaMV 35S promoter. At 0 h, both the promoters drove higher GUS expression at the area adjacent to the wound site when compared with local and systemic locations. This result correlates with those of earlier reports in which the accumulation of PR10 transcripts has always been localized in the regions of wounding or fungal inoculation and adjacent areas [38–41]. After 3 h of wounding, GUS activity driven by PR10.1 promoter was significantly higher at the site of wounding. There was no appreciable change in the GUS activity in all the locations in the transgenics driven by the PR10.2 promoter after 3 h of wounding.

When compared with the CaMV 35S promoter, the constitutive expression of GUS driven by PR10 promoters showed significantly higher activity indicating that the PR10 promoter is a better candidate for constitutive expression of transgenes. Some PR10 proteins display constitutive expression patterns independent of the pathogenic response. For instance, the yellow lupine PR10 promoter conferred constitutive expression but its level of expression varied with developmental stages and was also affected by wounding, oxidative stress and SA treatment [42].

When transgenic tobacco (T_1) plants with PR10.1 and PR10.2 promoters were subjected to fluorometric assay, significantly higher GUS activity was observed in the wound site immediately after wounding (0 h) when compared with CaMV 35S transgenics. This result was further confirmed through RT-qPCR analysis which clearly indicated that the PR10 promoter is quickly induced upon wounding. The relative GUS expression in PR10.1 transgenics was higher (180-fold) than PR10.2 transgenics (150-

fold) suggesting the role of 5′ UTR in enhancing transgene expression. The 5′ UTR is necessary for translation initiation and plays a significant role in determining the translational efficiency. The role of eukaryotic cellular 5′ UTRs or leader sequences for regulation of translation initiation is well understood and several factors involved in this process have been characterized. Rice ubi3 promoter along with its 5′ UTR enhanced the expression of GUS both at transcriptional and translational levels suggesting the important role of 5′ UTR in gene expression [43, 44].

In transgenic rice (T_0), upon wounding at 0 h, both promoter sequences conferred higher GUS expression compared to the CaMV 35S and maize ubiquitin promoters. In the case of CaMV 35S and maize ubiquitin transgenics there was no change in the expression pattern of the GUS gene upon wounding. However, 3 h after wounding there was a significant reduction in the GUS expression level, which was much lower than that of CaMV 35S and maize ubiquitin promoters, with the exception in the case of PR10.1 promoter at the wound site. In contrast to transgenic tobacco, there was no appreciable increase in GUS activity in rice transgenics when compared with the CaMV 35S and maize ubiquitin promoters. This difference in GUS activity between transgenic tobacco and rice is contrary to the expectation that monocot promoters would confer higher expression in monocots than in dicots.

Transgenic sugarcane plants (V_0) expressing GUS driven by PR10.1, PR10.2 and maize ubi-1 promoters were subjected to GUS fluorometric assay at 0 h and 3 h after wounding. The GUS fluorometric assay results showed that there was a significant increase in GUS activity upon wounding (0 h) in the leaves of transgenic sugarcane driven by both PR10.1 and PR10.2 promoters at the wound site. However after 3 h of wounding there was a reduction in GUS expression in both transgenics. The GUS expression levels in both PR10.1 and PR10.2 transgenics were significantly higher than that of maize ubiquitin transgenics with wounding. There was no significant change in the GUS expression levels in the case of maize ubiquitin transgenics with wounding. Transgenic sugarcane plants (three events each) were also analyzed for GUS expression at 0 h and 3 h after wounding through RT-qPCR. The qRT results correlated with the fluorometric assay results thereby proving the wound inducible nature of the *Erianthus* PR10 promoter.

PR10 gene expression is also controlled by defense-related signaling molecules and plant hormones, including MeJA [45], ABA [46], and SA [47]. These data suggest that defense-related signaling chemicals implicate in the signal transduction pathway leading to activation of PR10 gene. Based on investigation using lily anthers, Wang [45] have proposed that two distinct signal transduction pathways take part in the induction of PR10 genes by ABA and MeJA, respectively.

An attempt was made to study the role of these signaling molecules (SA, ABA and MeJA) for their inducibility of the *Erianthus* PR10 promoter. Transgenic tobacco plants with PR10.1, PR10.2 and CaMV 35S promoters were sprayed with these signaling hormones and GUS activity was determined at 6, 12 and 24 h after hormone spray. It may be noted that multiple numbers of *cis* elements responsive to SA, ABA and MeJA hormones were present in the *Erianthus* PR10 regulatory sequence. The fluorometric assay results showed that GUS activity was greatly reduced when the transgenic plants were sprayed with SA compared with control plants and the activity was lowered in all the three time intervals. SA treatment did not induce GUS expression indicating that PR10 promoter activity is unaffected by SA treatment. Significant increase in GUS activity was observed 12 h after hormone treatment with MeJA and ABA in both PR10.1 and PR10.2 transgenics. However, GUS activity was found to decrease 24 h after the treatments. This clearly shows that *Erianthus* PR10 promoter activity is induced by MeJA and ABA treatments which can be explained as due to the presence of multiple *cis* acting regulatory elements involved in ABA and MeJA responsiveness. Induction of PR10 gene expression upon treatment with MeJA [48], SA [49] and ABA [50] has been reported. Hwang [51] reported that the rice PR10 promoter was induced on application of SA, JA and ABA. MsPR10.1A gene of alfalfa was induced by ABA and ethylene [52].

To summarize, we have identified a novel PR10 promoter of 592 bp from *E. arundinaceus*. Functional characterization of the PR10 promoter in tobacco, rice and sugarcane revealed that though this sequence could drive constitutive expression, there was an enhanced expression of GUS gene upon wounding demonstrating the wound inducibility of this promoter sequence. In addition, the PR10 promoter was also induced upon MeJA and ABA hormone treatments.

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