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ORIGINAL ARTICLE

Isolation of 5′ regulatory region of *COLD1* gene and its functional characterization through transient expression analysis in tobacco and sugarcane

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Abstract

Chilling Tolerant Divergence 1 (*COLD1*) gene consists of Golgi pH Receptor (GPHR) as well as Abscisic Acid-linked G Protein-Coupled Receptor (ABA_GPCR), which are the major transmembrane proteins in plants. This gene expression has been found to be differentially regulated, under various stress conditions, in wild *Saccharum*-related genera, *Erianthus arundinaceus*, compared to commercial sugarcane variety. In this study, Rapid Amplification of Genomic Ends (RAGE) technique was employed to isolate the 5′ upstream region of COLD1 gene to gain knowledge about the underlying stress regulatory mechanism. The current study established the *cis*-acting elements, main promoter regions, and Transcriptional Start Site (TSS) present within the isolated 5′ upstream region (Cold1P) of COLD1, with the help of specific bioinformatics techniques. Phylogenetic analysis results revealed that the isolated Cold1P promoter is closely related to the species, *Sorghum bicolor*. Cold1P promoter-GUS gene construct was generated in pCAMBIA 1305.1 vector that displayed a constitutive expression of the GUS reporter gene in both monocot as well as dicot plants. The histochemical GUS assay outcomes confirmed that Cold1P can drive expression in both monocot as well as dicot plants. Cold1P's activities under several abiotic stresses such as cold, heat, salt, and drought, revealed its differential expression profile in commercial sugarcane variety. The highest activity of the *GUS* gene was found after 24 h of cold stress, driven by the isolated Cold1P promoter. The outcomes from *GUS* fluorimetric assay correlated with that of the *GUS* expression findings. This is the first report on Cold1P isolated from the species, *E. arundinaceus*.

Keywords *Erianthus* · GUS · Promoter · RAGE · Sugarcane · Transient expression

Introduction

The development of stress-tolerant plant varieties has become crucial in the recent years, due to the rapidly increasing human population and the scarcity for arable land and fresh water (Mohanan et al. 2020). Across the globe, sugarcane (*Saccharum* spp.) is the most important crop cultivated to produce sugar and ethanol (Mohanan et al. 2021). Several physiological aspects of the plants get affected by abiotic elements such as cold, heat, salt, and drought, resulting in a considerable loss of crop yield for the farmers. A sparse study was conducted earlier to explain the tolerance mechanisms in plants at molecular level.

Genetic engineering is an advantageous and a powerful technique to enhance the crop varieties and yield. This technique is applicable especially in case of perennial crops like sugarcane that have polyploid nature and consume long duration to develop a new variety (14 years) and impair the trait-specific traditional breeding (Singh and Rajam 2009; Gambino and Gribaudo 2012). Even though genetic transformation is the fastest method to achieve varietal improvement, it takes 4–6 weeks to form a single plantlet through

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direct regeneration, from the most responsive transformed cell or tissue (Takebe et al. 1971; Horsch et al. 1985). On the other hand, development of transgenic sugarcane plants requires almost one year. To counteract this lengthy time requirement, alternative genetic transformation strategies such as transient expression have been developed. Transient transformation method brings temporary alterations in the gene expression and is useful as a rapid analysis technique that can provide insights about the functionality of a gene or its regulatory elements (Gunadi et al. 2019). Particle bombardment is the most reliable technique that can rapidly analyze the gene regulatory components like promoters, introns and terminators (Hernandez-Garcia et al. 2010).

Promoter is a key *cis*-acting region that plays a crucial role in both gene construction as well as genetic engineering processes for the purposes of gene expression and transcription. Promoter sequences are located upstream of the coding area and are confirmed by the proteins, involved in the transcription process. Constitutive promoters correspond to active promoters that are present in every cell type and during every developmental stage (Porto et al. 2014; Potenza et al. 2004). Primarily, constitutive promoters are isolated from the upstream regions of highly-expressed house-keeping genes, such as one that encodes ubiquitin (Hernandez-Garcia et al. 2010; Li et al. 2012), actin (He et al. 2009; Beringer et al. 2017), elongation factors (Suhandono et al. 2014; Zhang et al. 2015), nutrient uptake genes (Murugan et al. 2022; 2023), etc. Only a few constitutive promoters, for instance the maize ubiquitin promoter (M-ubi), are frequently employed in monocots, particularly sugarcane, to the best of researcher's knowledge.

Ma et al. (2015) recognized a correlation between COLD1 and low temperature stress, which induces a downstream response. The transcriptome analysis, conducted upon wild sugarcane species (*Saccharum spontaneum* IND 00-1037) in terms of low temperature stress, have shown that COLD1 is highly upregulated and has a key role in stress regulatory mechanism (Dharshini et al. 2016; Selvarajan et al. 2018 Dharshini et al. 2020a; 2020b). Recently, the authors (Anunanthini et al. 2019) identified the COLD1 gene and reported its functional confirmation during abiotic stress in monocots. With this finding as a preliminary evidence, a novel COLD1 gene promoter was isolated from *Erianthus arundinaceus*, a highly abiotic stress-tolerant wild relative of the *Saccharum* family and analyzed under different abiotic stresses such as cold, heat, salt and drought under transient expression using real-time PCR technique.

Materials and methods

Plant materials and growth conditions

To isolate the COLD1 promoter region, single bud sets of *E. arundinaceus* Bethuadahri clone were planted and grown under greenhouse conditions (at a temperature of 25 ± 2 °C, 16/8 h of photoperiodic light and 75% Relative Humidity) for about 3 months. To conduct transient expression analysis, *Saccharum* hybrid Co 86032 single bud sets and *Nicotiana benthamiana* seeds were sown and grown under greenhouse conditions (at a temperature of 28 ± 2 °C, 16/8 h of photoperiodic light and 65–70% Relative Humidity, Narayan et al. 2021) until the tobacco reached three-leaf stage and the sugarcane reached internode formation stage.

Isolation of the upstream region of *COLD1* gene using RAGE

Genomic DNA was isolated from the leaf extract of *E. arundinaceus* Bethuadahri using CTAB method (Doyle and Doyle 1990). Genomic DNA (2 µg) was partially digested using *DraI*, *EcoRV* and *SmaI* blunt-end restriction enzymes (Thermo Fisher Scientific Company Ltd., USA) individually. Then, the restricted products were purified and ligated with flanking adapter sequences (Table 1) using T4 DNA ligase at 4 °C overnight. Then, primary PCR was executed by employing Adapter Specific forward Primer 1 (ASP1) and *COLD1* gene-specific reverse primer 1 (CSR1) under standard conditions as given herewith; 94 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 58 °C for 30 s and 72 °C for 2 min. The PCR reaction was terminated by a final extension of 72 °C for 10 min. Then, the primary PCR product was diluted to 50 ng/µL and used as a template for secondary PCR using adapter-specific forward primer 2 (ASP2) and *COLD1* gene-specific reverse primer 2 (CSR2) under the same primary PCR conditions. The amplified PCR product was then purified, cloned using InsTAclone PCR Cloning Kit (Thermo Fisher Scientific, USA), sequenced and named as Cold1P. ASP1, ASP2, CSR1 and CSR2 primers for which the details are listed in Table 2.

Table 1 Adapter sequences used for RAGE technique to isolate Cold1P

Long arm	5′ CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCC GGGCAGGT 3′
Short arm	5′ ACCTGCCC 3′

Table 2 Gene specific and adapter specific primers used for the isolation of Cold1P using RAGE

Name	Sequence
ASP1	GGATCCTAATACGACTCACTATAGGGC
ASP2	AATAGGGCTCGAGCGGC
CSR1	TCTGTTGGAAATTTAACTCGGTGTGG
CSR2	CTGCGGCCATGTTCTTTTTCC

Table 3 Primers used to confirm promoter of interest (Cold1P) and hygromycin in transformed *Agrobacterium* cells through colony PCR

Name	Sequence
HPT FP	ATGTCCTGCGGGTAAATAGCT
HPT RP	ATCGCGCATATGAAATCACGCC
C1P FP	ATGCGGATCCTAATAGGGGGAAAGCGGC
C1P RP	ATGCCCATGGCCTTTGCTGTATTCCTTGC

In silico analysis of 5′ regulatory region of *COLD1* gene

Homology of the isolated Cold1 promoter (Cold1P) was analyzed using BLASTN search that is available at NCBI (http://blast.ncbi.nlm.nih.gov). Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) and PLACE (https://sogo.dna.affrc.go.jp/cgibin/sogo.cgi?lang=en&pj=640&action=page&page=newplace; Suhandono et al. 2014) tools were used to predict the core and *cis*-acting elements, present in the 5′ regulatory region of *COLD1* gene sequence, obtained through RAGE technique. Transcriptional Start Site (TSS), located in the sequence, was predicted with the help of Neural Network Promoter Prediction (NNPP) (https://www.fruitfly.org/seq_tools/ promoter.html) tool (Basyuni et al. 2018). Secondary RNA structure of the isolated 5′ region was projected using Vienna suit (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi; Philip et al. 2013). Both Multiple Sequence Alignment (MSA) and phylogenetic tree were constructed using BLASTN search sequence results obtained from NCBI using MEGA6 software.

Plasmid construction

The isolated Cold1P was ligated into pCAMBIA 1305.1 vector with the help of *BamHI* and *NcoI* restriction enzymes by switching the CaMV35S. Finally, the obtained construct was named as pSBI C1P::GUS (Fig. S1A). In this study, the commercially available pCAMBIA 1305.1 (pCAMBIA 1305.1 CaMV35S::GUS) vector was used as a control.

Agrobacterium transformation

Agrobacterium tumefaciens LBA4404 strain, harbouring binary vectors such as pSBI C1P::GUS and pCAMBIA 1305.1 used for this study was prepared by freeze–thaw method, followed by 3 h of shaking at 28 °C in YEP medium. The grown culture was then pelleted and resuspended in 100 µL of fresh YEP medium to plate it over YEP plates, containing 10 mg/L rifampicin and 50 mg/L kanamycin. The positive colonies were screened with *hygromycin* and Cold1P through colony PCR using specific primers (Table 3).

Transient plant transformation

Sugarcane transformation

To conduct transient plant expression experiments, shoots were selected from 6 to 8 months grown sugarcane of genotype Co 86032. Young meristematic leaf bits were incubated in dark at 25 °C for seven days under basal Murashgee and Skoog (MS) medium, standardized as per Chakravarthi et al. (2015). The 1-week old meristematic leaf bits, of 1–2 cm^2 in length, were then transferred to Murashgee and Skoog (MS) osmotic media, containing 50 g/L Sorbitol and 50 g/L Mannitol, 4 h prior to particle bombardment so as to enhance the transformation efficiency. The leaf bits were arranged in a circular manner at the center and the petri dishes were placed in a particle inflow chamber at 4 and 8 cm distance. About 1 µg of plasmid DNAs such as pSBI C1P::GUS and pCAMBIA 1305.1 were bombarded separately in the plates containing meristematic leaf bits.

Tobacco transformation

Agrobacterium, containing pSBI C1P::GUS and pCAMBIA 1305.1 plasmid DNAs, were cultured in Luria Bertani (LB) broth overnight. Then, the cultures were centrifuged and dissolved in agroinfiltration medium (full strength MS with 0.5 M $MgCl_2$ and pH 5.7) with 100 µM acetosyringone. Afterwards, these *Agrobacterium* cells were infiltrated into intercellular spaces of the intact plant leaves (*N. benthamiana*) with a needleless syringe. About 100 µL of *Agrobacterium* suspension was infiltrated into 2–4 spots (about 2–4 cm in the infiltrated area) in a single *N. benthamiana* leaf as per the procedure recommended by Yang et al. (2000). The infiltrated *N. benthamiana* plants were then transferred to controlled transgenic glasshouse conditions.

Abiotic stress treatment

For heat stress, the bombarded leaf bits were incubated at 40 °C whereas for cold stress, the bombarded explants were incubated at 4 °C. To expose the plants to salinity stress, the explants were incubated in MS media (3 mg/L 2,4-D and 30 mg/L hygromycin) containing 150 mM NaCl, whereas for drought treatment, the explants were incubated in MS media (3 mg/L 2,4-D, 30 mg/L hygromycin) containing 200 mM Mannitol (Errabii et al. 2007). Expression analysis was conducted using the explants collected after treatment up to 12 and 24 h of stress induction, against a set of untreated controls.

Expression analysis using quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from treated leaf samples and untreated control samples by TRIzol method. Genomic DNA contaminant was removed by the addition of DNaseI (Invitrogen, USA). 1 µg of RNA samples was taken for cDNA synthesis using the kit as per manufacturer's instructions (Thermo Fisher Scientific Company Ltd., USA).

cDNA synthesized from treated and untreated control RNA samples was used to perform quantitative *GUS* expression analysis using real-time PCR (RT-PCR) technique. IDT software was used to design the primers for specific amplification of *GUS* and housekeeping gene i.e., glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Table 4). Quantitative Real-Time PCR (qRT-PCR) was performed in a final volume of 25 µL, consisting 12.5 µL of SYBR Green Master Mix (Thermo Fisher Scientific Company Ltd., USA), 2.0 µL of ROX reference dye, 1 µL of cDNA, 0.2 µL of forward and reverse primers and 9.1 µL of nuclease-free water. qRT-PCR was conducted for all the cDNA samples (retrieved from cold, heat, salt and the drought-exposed leaf samples) along with control in Applied Biosystems, USA, using the procedure recommended by Manoj et al. (2019) and Narayan et al. (2019). These experiments were repeated with three biological and three technical replications. $2^{-\Delta\Delta CT}$ method was used to estimate the relative expression as per the literature, Livak and Schmittgen (2001).

Table 4 qRT-PCR primers designed for expression analysis

Name	Sequence
Forward primer (GAPDH RT)	AAGGGTGGTGCCAAGAAGG
Reverse primer (GAPDH RT)	CAAGGGGAGCAAGGCAGTT
GUSPLUS RT	GGAATGGTGATTACCGACG
GUSPLUS RT	ATACCTGTTCACCGACGACG

Histochemical *GUS* staining

Both bombarded leaf bits and the agro-infiltrated leaf bits were incubated with 50 mM phosphate buffer (pH 7.2) for about 1 h after which leaf bits were transferred to 5-bromo-4 chloro-3-indoyl-β-D-glucuronide (X-gluc) and incubated at 37 °C overnight. The leaf bits were then decolorized using 70% ethanol. The modified procedure of Kapila et al. (1997) was followed in this study. The observations, in terms of formation of number of blue loci on leaf fragments, were recorded using a stereomicroscope. Transient GUS expression was quantified based on the average number of GUS foci per explant.

Protein extraction and GUS quantitative assay

Both treated as well as the control leaf samples were ground using protein extraction buffer (Na_2 EDTA, dithiothreitol (DTT), $NaPO_4$ (pH 7.0), triton X-100 and sodium lauryl sarcosine). Then, the extracted proteins were added to assay buffer (4-methyl umbelliferyl b-D-glucuronide), followed by the addition of stop buffer (Na_2CO_3). The liberated 4-methyl umbelliferone was measured for its fluorescence using spectrofluorometer with excitation at 365 nm and emission at 455 nm (Jefferson et al. 1987).

Results

Isolation of 5′ regulatory region of *COLD1* gene through RAGE and in silico analysis

COLD1 gene, isolated from the highly abiotic stress tolerant wild relative of sugarcane *i.e.*, *E. arundinaceus* IK 76-81, was studied earlier under abiotic stress conditions (Anunanthini et al. 2019). The current study attempted to isolate and characterize the 5′ regulatory region so as to extensively analyze its expression and signals that drive the expression of *COLD1* gene. The secondary PCR results arrived at only one 600-bp band in the template DNA that was digested with *DraI* restriction enzyme (Fig. S2). The 600-bp promoter contained a few parts of the gene sequence along with 5′ upstream promoter region (Cold1P). Therefore, the 484 bps of *COLD1* 5′ regulatory region was further processed for functional characterization.

PlantCARE database and PLACE tool were utilized to analyze the putative transcriptional binding site/*cis*-acting elements that revealed the presence of putative abiotic and biotic stress-inducing elements and sites (Rombauts et al. 1999; Koul et al. 2019) as listed in Table 5. MYB, AP2, Sp1, ERF, Dehydrin, LEA_5, bZIP, WRKY and MYB are some of the commonly known stress-inducible transcriptional binding sites that are present across the stress-inducible

Table 5 Putative *cis*-acting elements/motifs present in Cold1P analyzed using PlantCare and PLACE tool

S. no.	*Cis*-elements/motif	Hit sequence	Functions	Position	References
1	CAAT-box	CCAAT, CAATT, CAAAT	Common *cis*-acting element in promoter and enhancer regions	105, 378, 262, 263	Wenkel et al. (2006), Shirsat et al. (1989)
2	GARE-motif	TCTGTTG	Gibberellin-responsive element	135	Sazegari and Niazi (2012), Skriver et al. (1991)
3	TATA-box	ATATAT, TATA	Core promoter element around −30 of transcription start	189, 209	Hahn (2004), Shirsat et al. (1989)
4	Sp1	GGGCGG, CC(G/A)CCC	Light responsive element	17, 335, 450	Wang et al. (2002), Wen et al. (2014)
5	RY-element	CATGCATG	*Cis*-acting regulatory element involved in seed-specific regulation	84	Reidt et al. (2000)
6	TCA-element	CCATCTTTTT	*Cis*-acting element involved in salicylic acid responsiveness	98	Kim et al. (2008)
7	rbcS-CMA7a	GGCGATAAGG	Part of a light responsive element, ribulose-bisphosphate carboxylase element	216	Wen et al. (2014), Nomura et al. (2000)
8	I-box	AGATAAGG	Part of a light responsive element	218	Wen et al. (2014), Donald and Cashmore (1990)
9	MYB	cgaGATCCga	*Cis*-acting regulatory element involved in regulation of drought inducible gene expression	389	Berendzen et al. (2012)
10	AP2	actCCGACaa, tcGCCGGcca, caGCCGGctc, agCGGCCgcc, tgCGGCCggc, ggCCGGCc, ttcCAACAgaat	DNA binding domain which are necessary and sufficient to bind the GCC-box	398, 416, 133, 178, 181, 435	Mizoi et al. (2012), Okamuro et al. (1997)
11	Dof	caAAAAGtag, actTAAAGgcc, gaAAAAG, AAAGC, GCTTT	*Cis*-acting regulatory element involved in DNA binding with one finger	50, 161, 171, 241	Cominelli et al. (2011)
12	GATA	cgAGATCcga	*Cis*-acting regulatory element involved in chlorophyll a/b binding protein and for light-regulated and tissue-specific expression.	389	Reyes et al. (2004)
13	tify	AATCA, CATCA, CATCT, TATCA, AGATA, AGATC, GATCC	A putative transcription factor involved in inflorescence and flower development	79, 82, 100, 193, 219, 272, 309, 391	Zhang and Peterson (2005), Kaiser et al. (1995)
14	ZF-HD	ATTAG	Zinc finger homeobox family protein	266	Fujita et al. (2011)
15	SBP	AGTAC, GTACC	*Cis*-acting elements involved in flower and fruit development	234, 256, 235, 257	Wang et al. (2009), Unte et al. (2003)
16	TALE	TGACC		41	Liu et al. (2016)
17	TCP	GCCCG, CGGGC, CGGCC, GGCCC, TGGCC, GGCCG	Plant development and defense responses	20, 290, 23, 15, 154, 180, 34, 61, 455	Manassero et al. (2013), Li (2015)

Table 5 (continued)

S. no.	*Cis*-elements/motif	Hit sequence	Functions	Position	References
18	AP2; ERF	ATCTT, TAAAT, ATATA, TATAT, GAGAT	*Cis*-regulatory element known as the Ethylene-Responsive Element (ERE)	101, 128, 190, 191, 218, 390	Mizoi et al. (2012), Fujita et al. (2011)
19	B3	GCAGG, ACATG, CATGG, TCATG, CATGC, GCATG, GCACG	Auxin response factor	26, 66, 58, 59, 84, 175, 85, 176, 88, 151	Reidt et al. (2000), Suzuki et al. (2007)
20	NF-YB ;NF-YA ;NF-YC	ATAGG, CCACT, ATTGG, CCAAC, CCCAT, CCACT, CCAAT, ATTAG, CAAAT, AATGG, CCAAT	*Cis*-regulatory element specifically binds to the CCAAT box	3, 37, 106, 135, 187, 239, 263, 266, 300, 302, 379	Gusmaroli et al. (2001), Fujita et al. (2011)
21	Dehydrin	CCGCC, GTCTG, CCCAC, CTGAC, TTCGG, GCCGG, CCGGC, CCGAA, CCCAC, CCCAC, GTGGG	Response to biotic and abiotic stress	18, 30, 36, 40, 46, 182, 214, 183, 292, 201, 238, 296, 332	Hanin et al. (2011), Yu et al. (2018)
22	Trihelix	GTTCC, GTTAA, GTACC	Involved in plant development and stress responses	96, 325, 126, 235, 257	Nagano et al. (2001), Xie et al. (2009)
23	WRKY	TGACC	*Cis*-acting regulatory element involved in transcriptional repressor of the gibberellin signaling pathway	41	Fujita et al. (2011)
24	bZIP	TGACC, CATCA, TGCCG, TGGCG, CATCA, CGGCA, AGACG, CGCCA, CGACA	Transcription factors regulate processes including pathogen defence, light and stress signalling, seed maturation and flower development	41, 82, 272, 199, 304, 309, 317, 344, 347, 402	Berendzen et al. (2012), Fujita et al. (2011), Jakoby et al. (2002)
25	CG-1; CAMTA	CCGCGG, GCGCGC	*Cis*-acting regulatory element involved in calcium and calmodulin signaling	314, 462	Doherty et al. (2009), Mitsuda et al. (2003)
26	bHLH	CACGAG, CAAATG, AGCCGTAT	*Cis*-acting regulatory element involved in cell development and differentiation	387, 300, 205	Fujita et al. (2011)
27	SRS	GAAGAGT, ACTCTCC	Responsive to auxin signalling	355, 409	Lu et al. (1998)
28	ERF	AGCCGGC, GCCGGCT	Ethelene Responsive Element	436, 437	Xu et al. (2008)
29	LEA_5	CATGCATG, CATGCTGG	Responsive to abiotic stress	85, 89	Du et al. (2013)
30	E2F/DP	gggGGAAA	Responsive to G1/S transition in cell cycle	6	De Veylder et al. (2003)
31	Myb/SANT; trp; MYB; NF-YC	CTCAGCA	DNA-binding domain involved in regulation of drought inducible gene expression	442	Weirauch and Hughes (2011), Pandey et al. (2019)

promoter region. Figure S1B illustrates the *cis*-acting elements present in Cold1P. The TSS, predicted using Neural Network Predictor, helped in identifying its region (Table 6). RNA secondary prediction revealed that the promoter has a free energy of thermodynamic ensemble −170.70 kcal/mol with ensemble diversity being 149.36 (Fig. S1C). Phylogenetic tree constructed using MEGA6 software displays that the isolated promoter region has high similarities towards the closely-related cereal crops like *Sorghum bicolor*, *Setaria italica* and *Zea mays* (Fig. 1).

Histochemical GUS analysis

Bombarded sugarcane leaf bits and agro-infiltrated tobacco leaves were visualized for blue colour, attributed by the expression of *GUS* reporter gene (Shi et al. 2019; Yan et al. 2019), under a light microscope and the blue coloration proved the expression driven by Cold1P (Fig. 2A, B). This phenomenon initially proved that the 484 bps of the 5′ regulatory region have the ability to transcribe a gene sequence that results in its expression. The elevated GUS readings, from quantitative fluorometric assay, also proved that Cold1P can drive the GUS gene with precision during stress induction.

Table 6 Transcription start site prediction of Cold1P isolated from *E. arundinaceus* using neural network predictor

Start	End	Score	Promoter sequence
181	231	0.98	GGCCCATATATCACT **G**CCGAAGCCGTA TAGCCGGAGA**T**AA GGCATA
270	320	0.97	TCAGGCTTAAAGTCC **A**GCCCGGCCCAC AAATGGCGCA**T**CA CCGCGG

Bold value indicates the possible transcription start site

Relative transient expression profiling of GUS gene by qRT-PCR

Comparative C_T method was used to examine the transcript expression pattern of *GUS* gene under cold, heat, salt and drought conditions using real-time experiment. Overall, the expression patterns driven by CaMV35S and Cold1P under cold stress were comparatively higher than the rest of abiotic stresses. During cold stress, Cold1P-driven GUS gene got upregulated up to 1.6 folds whereas CaMV35s-driven GUS got upregulated up to 1.3 folds, in comparison with the control after 12 h of stress. Cold1P-driven *GUS* gene had a 3.1-fold upregulation after 24 h whereas CaMV35S-driven *GUS* gene exhibited an upregulation up to 2.1 folds than control.

The expression patterns of Cold1P-driven *GUS* gene, under heat, salt and drought conditions, got upregulated in terms of 1.4, 1.3 and 1.5 folds respectively. However, CaMV35S promoter-driven *GUS* gene got upregulated in terms of 1.1, 1.1 and 1.2 folds only after 12 h. *GUS* gene, driven by Cold1P, also got upregulated up to 1.9, 1.4 and 1.7 folds whereas *GUS* gene, driven by CaMV35S promoter, exhibited 1.6, 1.3 and 1.4 folds upregulation after getting exposed to 24 h of heat, salt and drought stress respectively (Fig. 2C).

Discussion

Although the application of transgenic technology has improved a number of crop species, the significance of transcriptional control and the underlying mechanisms that involve gene promoters still remain insignificant. Only a handful of monocot-specific ubiquitin promoters have been described to date (Philip et al. 2013; Moyle and Birch 2013).

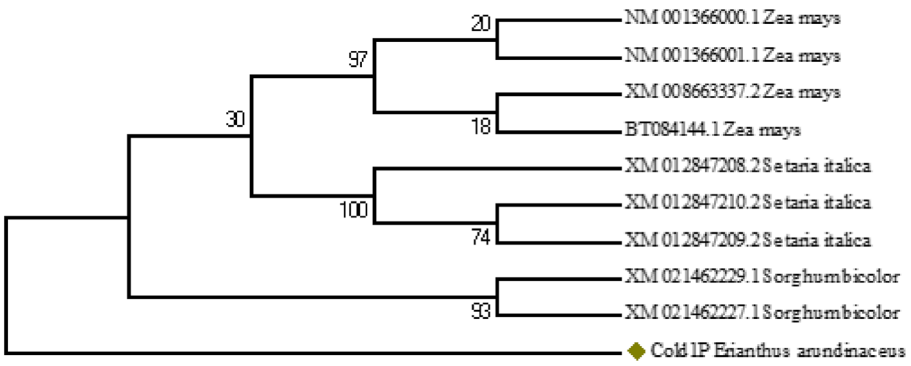

Fig. 1 Phylogenetic tree of Cold1P promoter from different related genera with *Erianthus arundinaceus* was constructed using Neighbour-Joining method with 1000 bootstrap replicates using MEGA6 software. The figures next to the branch demonstrate the result of 1000 bootstrap repeats expressed in percentage. Isolated Cold1P promoter has shown highly related to *Sorghum bicolor*

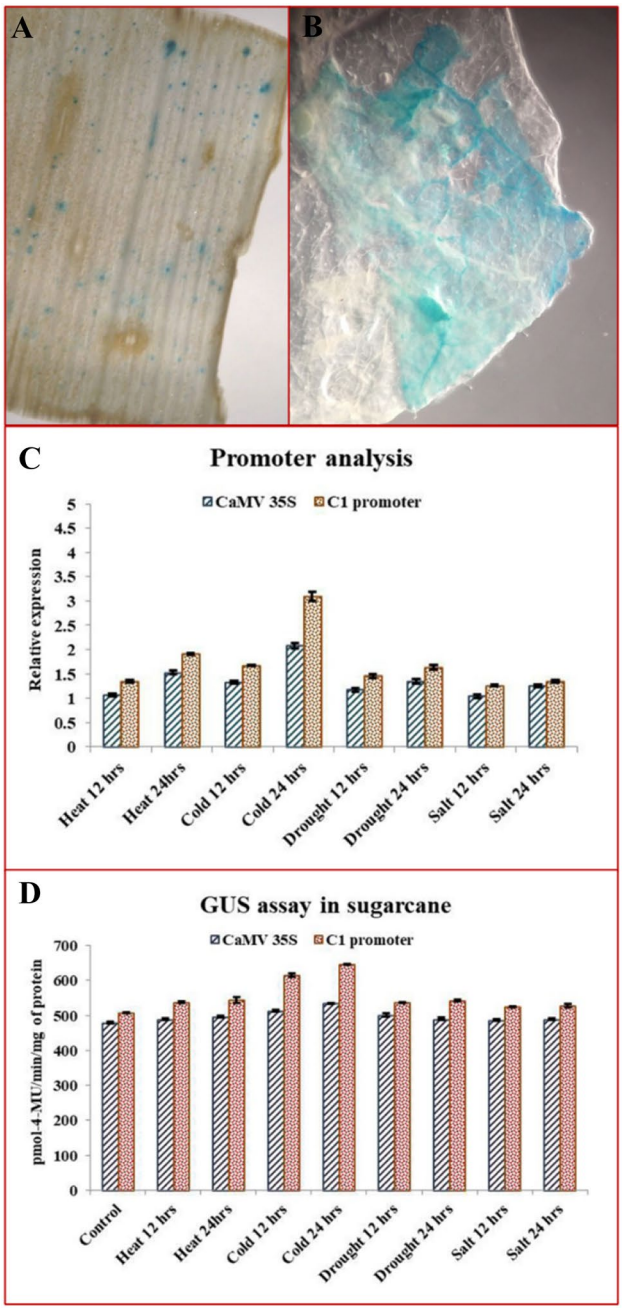

Fig. 2 GUS histochemical staining of bombarded sugarcane (**A**) and infiltrated tobacco (**B**) leaf segments visualized under a light microscope. The blue colour represents expression of *GUS* gene in the tissues. Expression profiling of *GUS* gene driven by Cold1P compared to CaMV35S constitutive promoter in bombarded sugarcane leaf bits upon various abiotic stresses (Heat, Cold, Drought, Salt) through qRT-PCR (**C**). Where, different stress treatments are given in *X*-axis and relative expression is given on *Y*-axis. GUS histochemical analysis in bombarded sugarcane leaf bits on exposure to different abiotic stresses (**D**). Where, different stress treatments are given in *X*-axis and GUS activity is given on *Y*-axis. Higher level of GUS activity was observed with Cold1P compared to CaMV35S in both expression profiling and GUS assay

Using RAGE approach, a new promoter (Cold1P) was isolated for the first time in this study, from wild *Saccharum* genus, *E. arundinaceus*. The primary objective of this work was to comprehend how plants perceive abiotic stress and transmit this information to induce gene expression changes that lead to tolerance. One of the strategies followed in this study to achieve the objective was to discover the *cis*-acting elements that are present upstream of the gene sequence that encodes the abiotic stress regulatory components (Doherty et al. 2009). The in silico characterization of Cold1P, using PlantCare and PLACE tools, detailed about the probable presence of *cis*-acting elements within the promoter region. Even though the isolated Cold1P was short (484 bp), it was found that a large number of potential biotic and abiotic stress-responsive components is dispersed across the region. The extremely compact distribution of stress-responsive elements that surround the TSS, demonstrates the adaptability of gene expression in eukaryotic systems (Zhang and Peterson 2005; Lin et al. 2010). Further, deletion studies should be conducted to confirm the function of each element present within the isolated regulatory region of COLD1.

The 5′UTR area of *E. arundinaceus* COLD1 was found to be highly homologous to the 5′UTR regions of *S. bicolor*, *S. italica*, and *Z. mays*, from Multiple Sequence Alignment (MSA) of Cold1P region and BLAST search results (Fig. S3). The phylogenetic tree demonstrates that Cold1P region is closely linked to the cereals. These connections have been investigated earlier and can be correlated with the current study findings on *COLD1* gene (Anunanthini et al. 2019).

Transient expression briefs the gene expression process and is often considered as a preliminary screening stage for the evaluation of the components, involved in gene expression (Gunadi et al. 2019). This method is useful since the gene expression can be evaluated with in a short period, without the need for regeneration of transformed cell or tissue (Porto et al. 2014). pSBI C1P::GUS, a binary vector, was constructed for plant expression study. Histochemical GUS assay results revealed that Cold1P can drive the expression in both monocot and dicot plants and this inference opens new opportunities for the development of stress-tolerant crops through overexpression of abiotic stress-related genes. Several studies have been conducted earlier globally, in which GUS gene was employed as a reporter gene on numerous plant species, including sugarcane (Nomura et al. 2000; Maghuly et al. 2008; Philip et al. 2013; Chakravarthi et al. 2015; Palaniswamy et al. 2016). Translation, splicing, and several key biological processes depend on the interaction between RNA and protein molecules. However, it is not possible to empirically evaluate their intensity and specificity (Kappel et al. 2019). RNA secondary structure prediction, by Vienna suit for the 5′UTR of COLD1, exhibited short secondary structural branches. The finding is supportive in

envisaging the RNA degradation and protein production processes.

Real-time measurement of the desired gene, in transiently-expressed explants, can shorten the time required for analysis than the stable transgenic plants. In this study, transient expression of the desired genes was quantified via RT-PCR expression analysis to reduce the long duration, taken for its validation (Zeinipour et al. 2018; Klay et al. 2018; Vera-Guzmán et al. 2019). The outcomes from the comparative expression analysis, conducted using qRT-PCR, infer that the expression of GUS gene, driven by Cold1P, is higher than that of the CaMV35S promoter-driven GUS gene expression among the meristematic leaf bits of sugarcane. Of all the abiotic stresses analyzed in this study, GUS gene had a significantly elevated rate of expression, when driven by Cold1P during cold stress after 24 h of cold induction. This indicates that the Cold1P region plays a prominent role in driving the GUS gene expression in sugarcane, a monocot plant. When this promoter was deployed in dicot plants such as tobacco, the expression rate was limited. The results achieved from the transient expression analysis of Cold1P in tobacco, clearly show the elevated levels of GUS in tobacco sample, a dicot plant. It proves its suitability to drive transgene in dicot plants as well.

The expression pattern of Cold1P was monitored after inducing stress, along with control explants, through GUS assay in sugarcane meristematic leaf bits at different time points. The analysis exhibited an increase in GUS gene activity (Fig. 2D) to further demonstrate the activity of the isolated promoter region, which correlates with the values of earlier studies (Agarwal et al. 2017; Yedahalli et al. 2018; Gallo-Meagher and Irvine 1993).

Conclusion

The binary vector construct, developed with *COLD1* gene regulatory region (Cold1P), has proved to drive the expression of transgene among different plant species viz., sugarcane and tobacco. Overall, the current study on Cold1P, isolated from *E. arundinaceus*, is built in an appropriate manner for transgene overexpression in monocot and dicot plants. The new promoter, isolated in this study, opens the possibility to replace existing promoters or to be used in addition to existing promoters in genetic engineering strategies for the improvement of both monocot and dicot crops.

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Author contributions MVM carried out major experiments and wrote the manuscript. AP helped in isolation of promoter, and GUS assay. ANJ helped in cloning and stress analysis. DS helped in in silico experiments. SR and HG corrected the manuscript and gave critical comments on the manuscript. AC conceptualized the work design, compiled the data and corrected the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials All data generated or analyzed during this study are included in this article (and its additional files). Materials are available with corresponding author.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Ethics approval Not applicable. This is to confirm that no specific permits were needed for the described experiments, and this study did not involve any endangered or protected species.

Originality of the work The authors state that this is an original research work carried out at ICAR-Sugarcane Breeding Institute, Coimbatore, Tamil Nadu, India.

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