Inducibility of dehydration responsive element (DRE)-based promoter through gusA expression in transgenic tobacco

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Received 16 January 2013; revised 6 April 2013; accepted 11 May 2013

The current study focuses on the pattern of stress-inducibility of a synthetically designed promoter, viz., 4X DRE (four tandem repeats of dehydration-responsive element), in response to the various stress-inducers like NaCl-mediated salinity, polyethylene glycol (PEG)-mediated water deficit, cold and abscisic acid (ABA). The construct, containing the 4X DRE promoter linked to the reporter gene gusA (that encodes β-glucuronidase, GUS), was introduced in tobacco plants via Agrobacterium tumefaciens-mediated transformation. The T\(_2\) progenies showed the integration of gusA, as verified by polymerase chain reaction (PCR) and DNA Blot hybridization analysis. The gusA induction was noted upon treatment of T\(_2\) transgenics with 20% (w/v) PEG for 24 h, while remained undetected even in PEG-treated wild type (WT) plant. The maximum expression was observed in the transgenic plant T\(_2\)/3, which also showed induction in gusA expression with other stressors, viz., 200 mM NaCl and cold (4°C). However, PEG-mediated water stress seemed to be the most effective signal for promoter activation, followed by salinity stress and with lesser stimulation by cold. The promoter was activated in a time-dependent manner of stress application, i.e., greater gusA expression was detected after 24 h of stress as compared to 6 h. ABA application, even at a concentration of 100 µM for 24 h, failed to activate reporter gene expression, proving that the promoter is stress-inducible but ABA-independent. Our observation showed the potentiality of 4X DRE for use as a stress-inducible promoter in overexpressing transgene(s) for salinity, drought and cold tolerance.

Keywords: Cold stress, dehydration responsive element (DRE), β-glucuronidase (GUS), polyethylene glycol, promoter inducibility, salinity, transgenic tobacco, water deficit

Introduction

The water deficit or dehydration stress constitutes the heart of any form of abiotic stress, whether salinity (due to increased ion flux) or chilling (due to ice crystal formation in the apoplast or xylem). Plants have evolved different adaptive strategies to alleviate the adverse effects of these stresses at molecular, cellular and physiological level\(^{1-4}\). The phytohormone abscisic acid (ABA) is considered as the common mediator during plant stress response. The endogenous ABA level increases during stress to initiate protective responses. Several genes are up regulated by ABA in different plant species. A striking feature of the upstream regions of these genes is the presence of 8-10 bp conserved sequence, called the abscisic acid responsive element (ABRE), with the ACGT core\(^{5,6}\). An ABRE often functions with GC-rich second sequence element, called coupling element (CE), which together constitute the abscisic acid responsive complex (ABRC). The synthetically designed promoters containing tandem repeats of ABRE or ABRC were earlier found to induce gusA expression upon stress imposition in transgenic tobacco in an ABA-dependent manner\(^7\).

In addition to the ABREs, dehydration responsive elements (DREs), with the sequence TACCGACAT, and similar cis-acting sequences called C-repeat (CRT) and low temperature responsive element (LTRE), both containing a TGG/ACCGAC motif, were reported in the upstream regions of the genes responding to drought and low temperature, but not to ABA\(^8\). The DRE was first identified as an essential cis-element in the promoter of the gene responsive to dehydration 29A (rd29A), also known as cold-regulated 78 (cor78) and low temperature-induced 78 (lti78), which showed ABA-independent expression in response to dehydration and cold stresses\(^8\). The CRT/DRE regulatory elements thus have the conserved 5 bp core sequence of CCGAC, to which the cold/drought responsive transcriptional activators called C-repeat/DRE-binding factors (CBFs/DREBPs) bind\(^{10-13}\). The fact that the DRE-mediated transcription is

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ABA-independent has been supported by several reports. A truncated rd29A promoter containing DREs without an ABRE was found to exhibit a loss of induction in response to exogenous ABA application, but retains induction under osmotic stress treatments\(^8,14\). Moreover, the osmotic stress-responsive transcription of rd29A was maintained in ABA-deficient mutant plants\(^15\).

A targeted manipulation of transcriptional activity through synthetic promoter engineering holds great promise to further our understanding of regulatory complexity. The use of a synthetically designed promoter with inducible regulatory sequences is highly desirable for genetic engineering program\(^16\). This is because strong constitutive overexpression of transgene hampers plant growth, leading to reduced productivity. The multimerization of conserved sequences or combinations of different conserved motifs in tandems were earlier used to create synthetic promoters\(^17\). Despite several efforts to functionally characterize stress-inducible promoters, limited studies have focused on their responses to different inducers like NaCl, dehydration, cold and ABA. In our earlier communications, we studied the pattern of induction under osmotic stress treatments\(^8,14\). Moreover, the osmotic stress-responsive transcription of rd29A was characterized in transgenic tobacco by monitoring the expression of gusA reporter gene, after exposure of the plants to salt stress (200 mM NaCl), water deficit (20% PEG-mediated) and cold (4°C) treatment for varying durations of time.

### Materials and Methods

#### Preparation of Promoter Construct

The synthetic promoter containing four tandem copies of DRE (4X DRE, from that of Arabidopsis rd29A), viz., CAGTTACTACCGACATGAGTACATACCGAC ATGAGTACTACCGACATGAGTATGACGCG, fused with 3′ ends, providing the basal promoter up to -70 from cauliflower mosaic virus 35S (CaMV35S) promoter and supplying CAAT and TATA boxes (70 LS), were designed. Since the basal transcription factors are required to assist RNA polymerase II to initiate transcription from the start site, the CAAT and TATA boxes were included downstream of the synthetic promoter. The oligonucleotide-primer pairs, 4X DRE and 70 LS, which are complementary on their extreme 3′ ends were annealed, followed by filling in with Klenow enzyme to generate double-stranded, 176 bp-4X DRE promoter. The filled-in products were amplified through PCR by using two other primers designed from the two ends. Finally, the 4X DRE inducible promoter was subcloned in the binary vector pBI121 at HindIII and BamHI site by replacing CaMV35S constitutive promoter, following the method as described earlier\(^7,19\).

#### Plant Transformation

The recombinant plasmid was mobilized into LBA4404 strain of Agrobacterium tumefaciens by freeze thaw method\(^20\). The leaf discs (2.5-5 cm in length) of tobacco (Nicotiana tabacum var. SR1) were infected with LBA4404 strain containing pBI: 4X DRE: gusA\(^21,22\) by immersing the leaf discs in liquid Murashige and Skoog (MS) medium containing Agrobacterium culture (1:10 dilution) for 20 min. The leaf discs were blot dried and placed on solid MS media for 2 d in the dark at 25°C. After 2 d of co-cultivation, the leaf discs were transferred to the regeneration medium supplemented with benzyl amino purine (BAP, 0.5 mg L\(^{-1}\)), cefotaxim (250 mg L\(^{-1}\)) and kanamycin (200 mg L\(^{-1}\)). The cultures were maintained at 26°C under 16/8 h light-dark cycles. The shoot bud differentiation started after 14-16 d of culture, which elongated into shoots within 30-35 d. The shoots with a defined stem were transferred to the solid MS rooting medium containing naphthaleneacetic acid (NAA, 0.2 mg L\(^{-1}\)), kanamycin (200 mg L\(^{-1}\)) and cefotaxim (250 mg L\(^{-1}\)). After profuse rooting, the plantlets were placed on soil rite to allow hardening for 10-12 d. The plants were finally transferred to soil and grown under normal environmental conditions for seed production by preventing cross-pollination. All the T\(_0\) plants were fertile with normal phenotype.

They were confirmed for the presence of gusA (data not shown). The T\(_0\) seeds collected were germinated on medium containing kanamycin to raise T\(_1\) seedlings. Similarly, T\(_1\) seeds were also collected to raise transgenic plants of T\(_2\) generation. Three T\(_2\) transgenic plants were considered for further molecular analyses.

#### Polymerase Chain Reaction (PCR) and DNA Blot Hybridization

Genomic DNA was isolated from the young leaf tissues of WT (wild type) and T\(_2\) transgenic plants according to the modified CTAB method\(^23\). PCR
amplification of the integrated gusA transgene was performed with 0.2 µg of genomic DNA, using GUS5 (5′-GTTTGCGTCAAGGCGTACAG-3′) and GUS3 (5′-GGTGTCCTTTCTTCCGCA-3′) oligonucleotide-primer pairs. The PCR cycle was as follows: 94°C for 1 min, 53°C for 1 min and 72°C for 2 min. The amplification was done for 30 cycles. For DNA Blot hybridization analysis, about 12 µg of each of the genomic DNA samples was digested overnight with HindIII and SacI together and transferred onto OPTITRAN pure nitrocellulose membrane (Schleicher & Schuell BioScience, USA) using 10× SSPE as transfer buffer. The ~2.0 Kbp (4X DRE-gusA) DNA fragment was [32P]-labeled using the Prime-ItII random primer labeling kit (Stratagene, USA) and the probe was purified on Sephadex G50 columns. The hybridization was performed at 55°C for 24 h in aqueous buffer at high stringency conditions following the standard protocol.

Application of Salinity Stress, PEG-mediated Water Stress, ABA and Cold Stress to Transgenic Plants

The 25 d-old transgenic tobacco plants were used for promoter characterization. The healthy T2 seedlings were treated with ¼ th strength MS medium supplemented with 200 mM NaCl for 6 h and 24 h (for salinity stress); with ¼ th strength MS medium supplemented with 20% (w/v) PEG-6000 for 6 h and 24 h (for water stress); with ¼ th strength MS medium supplemented with 100 µM ABA for 6 h and 24 h; or were incubated at 4°C for 6 h and 24 h (for cold treatment). The healthy T2 seedlings treated with only ¼ th strength MS medium served as control.

Reverse Transcriptase (RT)-PCR

Total RNA was extracted from the leaves of untreated and stressed WT and T2 transgenic plants. RNA samples were treated with RNase free DNase I (Fermentas, India). About 5 µg of total RNA was used for first-strand cDNA synthesis using Sensiscript RT-PCR kit (Qiagen). PCR was then carried out with GUS5 and GUS3 primer pairs as described above.

Results and Discussion

Although the constitutively active promoters like CaMV35S and its derivatives can drive high levels of transgene expression in monocot and dicot plants, they are usually not desirable for the generation of transgenic plants. This is because constitutive overexpression of functional genes may compete for the building blocks required for plant growth under optimal conditions and hence the transgenic plants show undesirable phenotypes like growth retardation and reduction in seed production. For example, the use of CaMV35S to drive the expression of DREB1A cDNA resulted in severe growth retardation of the transgenics, while the use of rd29A promoter caused minimal negative effects on plant growth with better tolerance. Hence, it is necessary to generate transgenic plants accumulating gene products only under stress conditions. The stress-inducible promoters get activated only when triggered by appropriate stressors, but not at the background level. So they are ideal for driving the expression of candidate genes for abiotic stress tolerance. Despite the isolation and characterization of several stress-inducible promoters, there is dearth of reports regarding their pattern of inducibility upon stress application. The regulatory elements are important molecular switches involved in the transcriptional regulation of a dynamic network of gene activities controlling various biological processes. Two major cis-acting elements, ABRE and DRE, are involved in ABA-dependent and ABA-independent regulatory pathways respectively. Multiple copies of ABREs were earlier shown to confer ABA response to a stress-inducible promoter. Not only that, the synthetically designed 4X ABRE and 2X ABRC promoters showed gusA expression in the presence of NaCl, ABA and PEG, when the respective gene constructs were overexpressed in transgenic plants.

In our present study, we wanted to test whether another synthetic promoter that contains multiple copies of DREs in tandem repeats, along with minimal promoter sequences (-70 to +5) of CaMV35S, can similarly induce expression of the reporter gene gusA after stress application to the plants. The engineered construct 4X DRE:gusA was used and gusA expression was monitored in the transgenics against WT plants.

Confirmation of T2 Transgenics

The synthetic promoter construct, pBI: 4X DRE: gusA used in the present study is shown schematically (Fig. 1). The different intermediate stages of raising transgenic tobacco harbouring 4X DRE: gusA

![Fig. 1—Schematic representation of the construct pBI121: 4X DRE:gusA (-CaMV35S).](image-url)
construct is also shown (Fig. 2 A-E). PCR confirmation of the kanamycin-resistant, three T2 transgenic plants (Fig. 3A) showed the presence of ~1 Kbp band of gusA (Lanes 3-5), whereas the band was absent in the WT plant (Lane 2). DNA Blot hybridization analysis (Fig. 3B) also confirmed the presence of ~2 Kbp 4X DRE: gusA fragment in the above three transgenic plants (Lanes 2-4), while the band was undetected in the WT plant (Lane 1).

RT-PCR Analysis

Water stress condition can be mimicked by PEG treatment. The cells do not readily take up PEG, so that it reduces the external free water concentration without attaining the ionic composition of the cell. Thus, we initially screened for gusA expression in three T2 transgenic plants in response to 20% PEG treatment for 24 h. The ~1 Kbp gusA transcripts were detected in all the three transgenic plants, viz., T2/1, T2/3 and T2/7, when water stress was induced by 20% PEG treatment for 24 h, while the transcript was undetectable even in PEG-treated WT plant (Fig. 4A). Though the gusA expression, driven by 4X DRE, could be observed even at the background level (unstressed condition) in the three transgenic plants, the expression was appreciably induced with stress (20% PEG, 24 h). The maximum expression was found in PEG-treated, T2/3 transgenic plant (Fig. 4B). Further, the gusA expression was noted under several abiotic stress conditions in the transgenic plant T2/3 (Fig. 4C), viz., 200 mM NaCl treatment for 6 h and 24 h (Lanes 3 & 4), 20% PEG imposition for 6 h and 24 h (Lanes 6 & 7) and cold (4°C) treatment for 6 h and 24 h (Lanes 9 & 10). The untreated transgenic plant (control) also showed background gusA expression (Lanes 2, 5 & 8). However, there was a clear

Fig. 2 (A-E)—Stages of regeneration of transgenic tobacco plants after infection of tobacco leaf discs with A. tumefaciens containing pBI:4X DRE: gusA construct: Selection of infected leaf discs on kanamycin-containing medium (A); Shoot and root formation (B); Hardening of plantlets (C); Mature plant stage in soil-containing pot (D); and Flowering plants (E).

Fig. 3 (A & B)—Confirmation of T2 transgenic plants for the presence and integration of gusA by genomic DNA PCR and DNA Blot hybridization analysis: (A) PCR amplification of the 1 Kbp gusA from equal amount of genomic DNA (0.2 µg) in three transgenic tobacco plants (Lanes 3-5). No PCR product was visible in the WT plant (Lane 2). (B) DNA blot hybridization showing the introgression of ~2 Kbp 4X DRE: gusA fragment (0.176+1.8 Kbp) in the transgenic plants (Lanes 2-4), when equal amounts of genomic DNA (12 µg) from each were digested with HindIII and SacI and probed with [32P]-4X DRE: gusA. No hybridization signal was obtained in case of WT plant (Lane 1), the mol wt standards indicated on the left.
induction in gusA expression over control with the above stressors, viz., 2-3 folds with NaCl, 3-5 folds with PEG and 1.2-1.6 folds with cold (as observed after densitometric scanning of band intensity). Thus, the promoter activation was the most pronounced with PEG-mediated water stress. The gusA transcript was totally undetectable with ABA (100 µM) treatment for either 6 h or 24 h, clearly showing that the synthetic promoter is not ABA-inducible (Fig. 4D). The gusA gene was also not expressed in WT plants with any of the stress treatments for either 6 h or 24 h (data not shown).

Thus, the 4X DRE promoter used in our study was found to be highly recommendable for use in inducible expression of any transgene under salinity, drought or cold-mediated oxidative stress in an ABA-independent manner. Water stress appeared to be the better elicitor of promoter activation and gusA expression, followed by NaCl and cold stress. The promoter activation was also purely time-dependent, i.e., increased steadily with the duration of stress exposure. It is expected that 4X DRE promoter will be equally functional in monocot crops like rice, maize, barley and wheat under salinity, drought or cold stress. The present work thus paves the future scope for utilization of this promoter to overexpress gene(s) for abiotic stress tolerance, so as to generate transgenic crops with broad spectrum resistance to salinity, drought and cold stress simultaneously, thereby highlighting its importance in genetic engineering program for plant protection.

Acknowledgement

ARC gratefully acknowledges the Science and Engineering Research Board (SERB), Government of India, New Delhi for financial support (Grant No SR/FT/LS-65/2010).

References