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Characterization of a drought inducible *Dehydrin* promoter from sugarcane (*Saccharum officinarum* L.) in tobacco (*Nicotiana tabacum* L.)

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Abstract

Dehydrin (DHN) is known to play an important role in plant response and adaptation to abiotic stresses (drought, high salinity, cold, heat, etc.). Previous research reported the increased expression of DHN in sugarcane stems exposed to drought stress for 15 days which may be controlled by its corresponding stress inducible promoter. The *DHN* promoter was succesfully isolated from sugarcane variety PSJT 941 (Pr-*1DHNSo*) and was cloned to pBI121 expression vector fused to a β-glucuronidase (GUS) reporter gene. The aim of this research was the functional testing of the Pr-*1DHNSo* promoter through transformation into tobacco plant treated with *in vitro* drought stress. Genetic transformation of Pr-1DHNSo construct was conducted by *Agrobacterium tumefaciens.* The transformed tobacco was then subjected to drought stress treatment using 40% PEG 6000 for five sequential incubations (0, 12, 24, 48 and 72 hours). The GUS assay reveal that the transformed tobacco treated with drought stress showed a blue color denoting GUS activity in leaf, stem and root tissues and this expression increased along with the length of the drought treatment. The analysis of *gusA* gene using real time-qPCR normalized to the L25 reference gene also showed that the expression increased in line with the length of time of drought stress. The results presented in this study indicated that the *Pr-1DHNSo* promoter from sugarcane was expressed and induced by drought stress treatment in tobacco.

Keywords: drought stress, dehydrin, DHN, promoter study, sugarcane, tobacco

Introduction

Increasing sugarcane productivity to achieve national sugar self-sufficiency can be conducted by growing sugarcane on marginal land. However, this process is likely to require the use of varieties with a high yield and tolerance to drought stress. Therefore, sugarcane breeding programme have been undertaken to generate new varieties elevated tolerance to drought conditions. To date, numerous plant genes or transcripts that are known to be induced by osmotic stress have been identified and characterized (Chavez et al., 2003). The function of some of these genes in sugarcane has been examined by expression analysis and *via* transgenic plant to elucidate their role in tolerance mechanisms against stresses (Ferreira et al., 2017). The gene families that encode for proteins and play a role in responding to osmotic stress includes genes from chaperone proteins such as *HSP* (*Heat Shock Protein*) (Kelley, 1999), *Dehydrin* (*DHN*), and *LEA* (*Late Embryogenic Abundant*) (Ingram & Bartels, 1996; Bray, 1997). Those genes encode proteins that commonly found in cytoplasm and play an important role in the protection of the protein from degradation and from the effects of proteinase as well as folding process in protein synthesis. *DHN* is a member of the group 2 LEA proteins that play a fundamental role in plant response and adaptation to abiotic stresses (drought, high salinity, cold, heat, etc.) in vegetative and generative tissues (Bray, 1997; Hanin et al., 2011).

Previous research reported that there was an increase expression of *DHN* in sugarcane stems treated with drought stress for 15 days (Iskandar et al., 2011). The DHN gene promoter of wheat (*Triticum* sp) namely Pr*DHN*-5 has been reported to be induced by abiotic stress (Ben Amar et al., 2013). In tobacco, overexpression of DHN gene was demonstrated to increase the tolerance to osmotic stress including high salinity (Hanin et al., 2011). DHN gene families were also reported have a role in increasing tolerance to biotic stress such as DHN5 that affect the signalling process of Jasmonic acid (JA) and have an ability to activate PR (pathogenesis related) protein (Hanin et al., 2011).

Prior research leads to the isolation of full length coding sequence of *DHN* from the sugarcane variety PSJT 941 with increasing expression under drought stress (Minarsih et al., 2018). Moreover, the *DHN* promoter from sugarcane were isolated contained *cis*-regulatory elements motifs that were identified to play roles in adaptation to abiotic stress (Minarsih et al., 2020).

The DHN promoter Pr-*1DHNSo* was cloned into the pBI121 vector to characterize the expression in sugarcane and other plants. This research was performed to study the function and expression profile of sugarcane *DHN* promoter fused to a reporter gene, *gusA,* in tobacco plant cultivar Besuki under normal and drought simulated environment. The results of this research could become a basis of a genetic engineering program of sugarcane tolerant to drought stress in order to ultimately improve sugarcane productivity.

Materials and Methods

Plant Materials

Seeds of “Besuki” Tobacco variety were sterilised with 70% ethanol in 2 mL tube for 1 min and then washed with sterile distilled water (dH_2O) once followed by soaking in Clorox 1.5% for 15 min and three times re-washed with dH_2O. The seeds were then dried on sterile tissue paper and cultured on basal MS medium containing 100 $mg.L^{-1}$ myo inositol, 0.5 mgL^{-1} nicotinic acid, 0.5 $mg.L^{-1}$ thiamine HCl, 0.1 pyridoxine HCl, sucrose 40 gL^{-1}, at pH 5.8. Induction of tobacco shoots were performed by adding BAP 0.5 $mg.L^{-1}$ hormone. Cultures were kept in the light for 2 months until ready for transformation. Leave pieces of tobacco were cultured on solid MS1 medium and incubated in the dark for 4-6 weeks until germinated. Shoots grown from leaf explants after 2-4 times subculturing steps were used as explants for genetic transformation of the DHN promoter construct *via Agrobacterium* strain LBA4404.

Transformation of pBI121- Pr1DHNSo into Agrobacterium LBA4404 Competent Cells

The plasmid pBI121-Pr-DHNSo was transformed into 200 µl of competent cells of *Agrobacterium* LBA4404 followed by incubation on ice for 15 min and liquid Nitrogen for 5 min and the sample was then placed in an incubator at 37°C for 5 min. About 800 µL YEP liquid medium was added and shaken for 3 hours at 28°C. The mixture was then centrifuged at 6000 rpm for 3 min. The supernatant was subsequently removed and 200 µl of it was kept and spread onto selection medium containing 50 ppm Kanamycin, and incubated for 2 days at 28°C. Colonies grown on the selection medium were isolated for colony PCR using the *DHN1* primer pair with the sequences of: Forward: AGCTTGTCCTTGCCATAAA; Reverse: GTCGTGCGGCTTGGTTTCT.

Agroinfiltration Transformation on Tobacco Model Plant

The transformation method was conducted according to Hasan *et al*. (2008) and Srinivas *et al*. (2008) with modifications. The colony of the *Agrobacterium* strain LBA4404 carrying the plasmid pBI121-Pr-1DHNSo was taken and put into a falcon tube with 10 mL YEP liquid medium containing 25 ppm rifampicin antibiotic and 50 ppm kanamycin. The culture was incubated at 25°C in the dark and agitated at 200 rpm. After 16 hours of incubation (overnight), about 10% of the culture was taken and mixed with liquid YEP medium containing rifampicin (25 ppm) and kanamycin (50 ppm) and incubated again at 25°C with agitation (200 rpm) in the dark until the OD600 reached 0.8. The culture was then centrifuged at 11.000 g for 1 min at 4°C and the pellet was collected and resolved in 4 mL of infiltration medium. The tobacco plantlet was placed into a 250 mL Erlenmeyer flask with infiltration medium containing the transformed *Agrobacterium*. Co-cultivation was conducted at 22°C in the dark for 48 hours without vacuum.

Transient Analysis with GUS Assay

The histochemical GUS assay was conducted according to the method of Jefferson (1987). Transformants were first sterilized using 500 ppm antibiotic cefotaxime. Each of transformant carried a construct of CaMV 35S in pBI121 and Pr-1DHNSo incubated with 4 mL X-gluc solution and incubated at 37°C in the dark for 18-24 hours. A positive result was indicated by the appearance of blue colour on the tissue of tobacco plantlets. The explant was

then soaked in 90% ethanol until the chlorophyll on the leaf and stem disappeared. Following this the tissues were examined under a microscope. A further test of the successful transformation of the tobacco was done by PCR using specific primer to *gusA* gene with the sequences: GUS Forward: CTAGTGCCTTGTCCAGTTGC; GUS Reverse: GAACAACGAACTGAACTGGC. Genomic DNA was isolated from samples of transgenic tobacco 2 months after transformation using Genomic DNA Mini Kit (Plant) (Geneaid) according to the protocol provided by the manufacture.

Drought Stress Treatment and Relative Water Content (RWC) Analysis

Tobacco plantlets transformed with the *Pr-1DHNSo* construct was treated with drought stress *in vitro* using 40% Polyethilene glycol (PEG) 6000 and incubated for 0, 12, 24, 48 and 72 hours. The measurement of relative water content (RWC) was conducted as described by Barrs and Wheatherley (1962). Leaf samples were weighed after osmotic treatment in three different conditions; fresh weight, after soaking in the water for 4 hours and after drying in the oven at 60°C for 18 hours. After drying for 18 hours, the samples were weighed every 18 hours until a steady dry weight was reached. The RWC value was calculated using the formula of Sade et al (2015):

$$\% \text{ RWC} = \frac{(FM-DM)}{(TM-DM)} \times 100$$

Total RNA Isolation and cDNA Synthesis

The RNA isolation was done as described in Chang et al. (1993). All of the reagents and devices were treated with DEPC (diethylpyrocarbonate) solution and sterilized by autoclaving at 121°C for 30 min. Treated tobacco samples were washed and dried using tissue paper and subsequently cut into pieces and kept in the freezer (-40°C) prior to the isolation. Samples were then ground to powder in a mortar with the addition of liquid Nitrogen and 0.1 gr of gram poly(1-ethenylpyrrolidin-2-one) (PVP). The RNA extraction was conducted using GenAll Ribospin™ Plant kit (GeneAll). The RNA was dissolved in 50 µL of NFW (Nuclease Free Water) (Invitrogen) and kept in a freezer.

The cDNA synthesis was conducted using RT-PCR Premix kit (Bioneer) using 1000 ng total RNA samples. The PCR program was as described by Iskandar et al. (2004) with some modifications; pre-denaturation 95 °C, 3 minute, 12 cycles with denaturation 95 °C, 5 minute, annealing 58 °C, 1 minute, extension 72 °C, 10 minute, and post extension 72 °C, 5 minute and finalized with a cooling phase at 10 °C. Qualitative analysis of cDNA was achieved by PCR using *ACTIN* primer pair.

Quantitative Real Time PCR

Real time-qPCR master mix was prepared by adding NFW 1.8 µL and 2.5 **µ**L Power Syber Green (PSG) (Bioline), GUS primer with the sequences described previously at a concentration of 10.0 pmolL^{-1} 0.1 µL forward and 0.1 µL reverse primer respectively as well as 0.5 **µ**l cDNA template. The amplification process was conducted in ABI Step One Plus real time-qPCR (Applied Biosystem). The PCR program followed the procedure of Iskandar et al. (2004) with slight modification; pre-denaturation 95 °C, 10 minutes, followed by 40 cycles of denaturation 95 °C, 15 second, annealing 60 °C, 1 minute. The dissociation (melt curve analysis) program was set up at 95 °C, 2 minute, 60 °C, 15 second and 95 °C, 15 second. The reference gene used in the qPCR was L25 ribosomal protein (Schmidt & Delaney, 2010).

Results

Confirmation of pBI121-Pr-1DHNSo in Agrobacterium tumefaciens

The plasmid construct that have been confirmed by sequencing (Minarsih et al., 2020) was transformed into *Agrobacterium* strain LBA 4404. Transformed colonies that grew were isolated using the alkaline lysis method (Sambrook et al.,1989) and confirmed using PCR. PCR results using *Pr-1DHNSo* specific primer showed 2000 bp of amplicon, indicated that the plasmid in *Agrobacterium* was the correct plasmid (Figure 1).

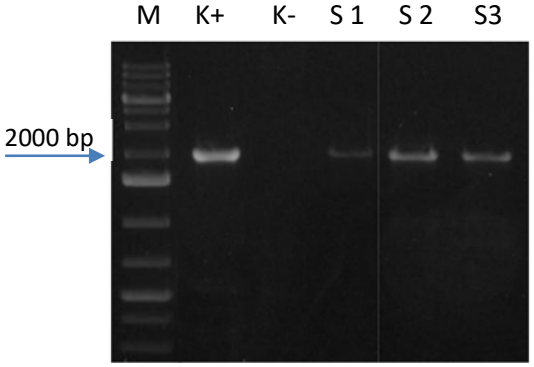

Figure 1. Detection of pBI121- Pr-1DHNSo integration in *Agrobacterium* strain LBA4404. M: 1000 bp DNA ladder as size marker Thermo Scientific); K-: negative control (dH_2O template); S1 – S3 (colonies 1, 2, and 3) and K+: positive control that produced 2000 bp DNA plasmid fragment

Genetic Transformation of Tobacco using Agrobacterium Carrying pBI121-Pr-1DHNSo and Its Transient Expression Analysis under Drought Stress Condition

Genetic transformation results showed that after three months the transformed tobacco grew normally as well as the wild type (WT) plants (Figure 2). Molecular analysis to confirm the presence of *GUS* (*gusA*) gene in the genome of transformant plants was performed on plantlets of transformant candidates that were grown on medium containing kanamycin. All transformant showed positive results for the presence of *GUS* in the genome. This was indicated by the detection of amplified DNA fragments in the size range of 300 bp of *gusA* gene in gel electrophoresis analysis (Figure 3 lane 2-6). The band did not appear on the sample non transformant plantlet (Figure 3 lane 1). This data suggest that the T-DNA constructs were successfully integrated into the genome of tobacco transformant candidates.

Further analysis of transformant plants was carried out by GUS screening. The expression of the *gusA* gene encoding the β-glucuronidase enzyme is marked by the formation of blue colour in the plants tissue, thus indicating the localization and patterns of promoter expression.

Relative water content (RWC) measurements performed by the turgidity method on plants treated with drought stress under 40% PEG 6000 s carried out *at* 0, 12, 24, 48 and 72 hours were shown in Table 1. The results indicated that with increasing time of drought stress treatment, the percentage of RWC decreased to circa 46-47% after 72 hours in transformant and wild type plants. Whereas in control plants without drought stress treatment, RWC values remain high above 90%.

GUS expression analysis showed that the CaMV 35S promoter controlled the *gusA* gene and the expression was observed in leaf, stem and roots

Figure 2. Regeneration of transformant tobacco plants on kanamycin selection medium. A: positive control (wild type tobacco cultured in a non-antibiotic medium); B: negative control (wild type tobacco cultured in a medium with antibiotic; C: two-months-old plants after transformation; D: three-months-old transformant

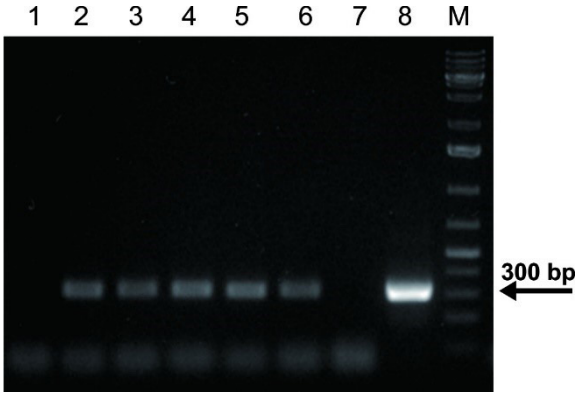

Figure 3. A band of the expected size (300 bp) was observed in transgenic tobacco plants (lanes 2-6) and pBI-*Pr-1DHNSo* plasmid (positive control; lane 8). Negative control (H_2O) (lane 7) and WT (wild type) plant (lane 1). M: 1 kb plus DNA marker (Thermo Scientific)

tissues at 0 hours of drought stress treatment (Figure 4, control +). Furthermore, the expression of *gusA* under the control of Pr-1DHNSo promoter in drought stressed transformant plants appeared to increase with the duration of stress treatment (0, 12, 24, 48 hours). After 48 hours treatment the blue colour became more intense compared to earlier timepoints in leaf, stem and root respectively. While non-transformant plants treated with drought stress formed no blue colour in any tissues. Hence, there was no indication of any expression of the *gusA* gene in non-transformed plants. This finding indicated that the Pr-1DHNSo promoter from sugarcane was induced by drought stress condition as shown by the increased expression of *GUS* gene.

To study the patterns of expression of the Pr-DHNSo promoter, *gusA* cDNa samples from transformant plants treated with drough stress and

Table 1. Relative Water Content (RWC) of transformant plants

Sample	Mean RWC	SD
A 0 hours	95.46%	0.007713
A 12 hours	77.67%	0.011537
A 24 hours	63.24%	0.007502
A 48 hours	57.17%	0.004629
A 72 hours	45.64%	0.009587
B 0 hours	94.35%	0.006381
B 12 hours	91.45%	0.030535
B 24 hours	95.03%	0.011219
B 48 hours	93.01%	0.004451
B 72 hours	90.89%	0.023394
C 0 hours	95.54%	0.007284
C 12 hours	75.27%	0.002941
C 24 hours	65.00%	0.013033
C 48 hours	56.35%	0.010589
C 72 hours	47.99%	0.008091

Note: (A) Drought treated transformant plants; (B) non-treated transformant plants; (C) treated wild type plants. SD = standard deviation

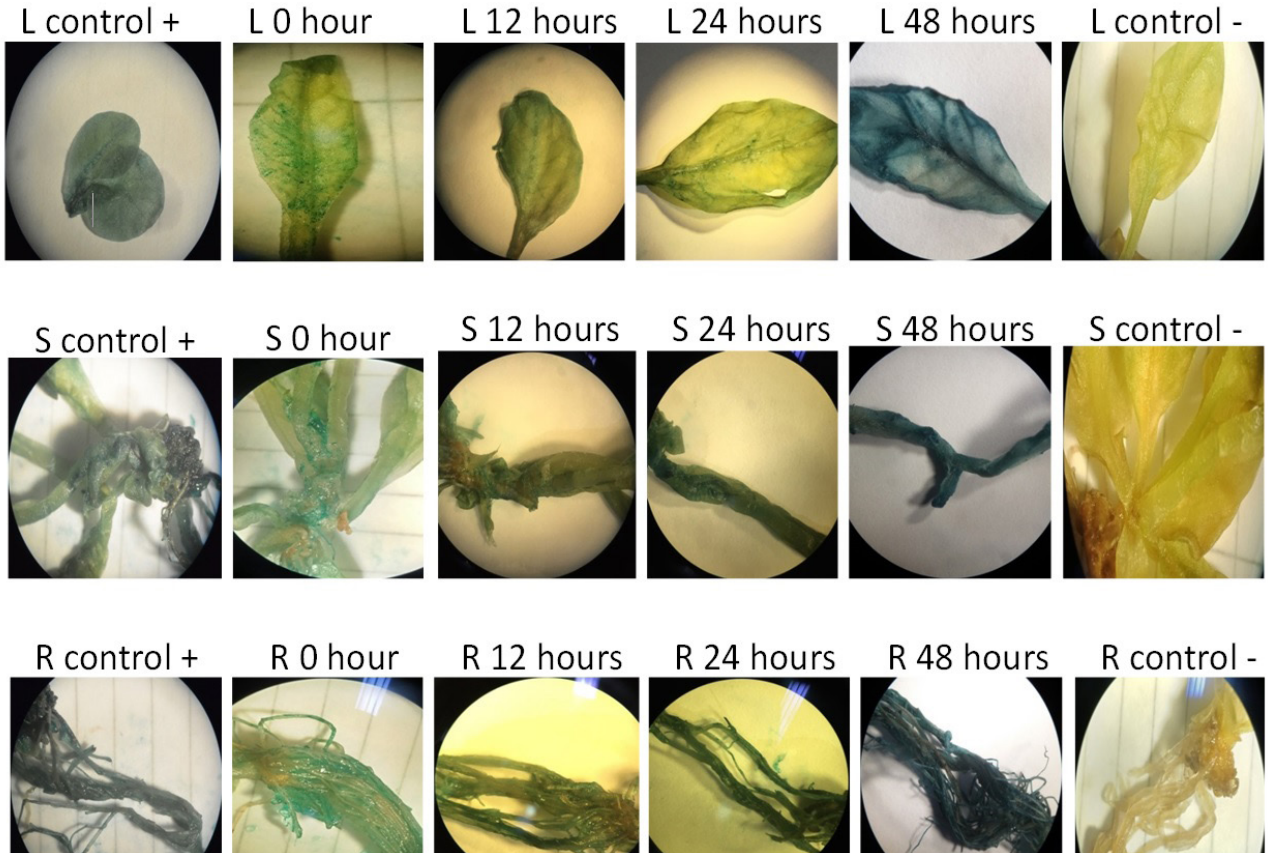

Figure 4. Histochemical staining of GUS activity in transgenic tobacco plant treated with 40% PEG 6000. Control (+): Tobacco transformed with the CAMV 35S-pBI121 construct; control (-): non-transformant plant (wild type); L: leaf; S: stem, R: root

WT as control were quantified. The value of *gusA* expression compared to the *L25* reference gene showed significantly increased expression through time. The highest expression value observed under 72 hour drought stress condition was almost 8 times compared to 0 hours (Table 2). Importantly, *gusA* gene expression was very low in treated but non-transformant plant.

gene to increase plant tolerance to drought using tobacco as model plant.

Relative water content (RWC) is one of the important characteristics influencing plant water relations. RWC is a measure of plant water status, reflecting the metabolic activity in tissues and is a frequently used as well as the most meaningful index for dehydration

Table 2. Relative value of *gusA* expression to the L25 regerence gene in transgenic tobacco plants

Time (hour)	Expression value		
	Transformant plants with drought stress treatment	Transformant plants without drought stress treatment	Non-transformant plants with drought stress treatment
0	4.8946±1.02^{f}	5.7591±0.93^{ef}	0.0961±0.01^{g}
12	8.2362±1.11^{d}	5.2598±1.77^{ef}	0.0891±0.02^{g}
24	17.6055±0.63^{b}	7.0857±0.70^{def}	0.1461±0.01^{g}
48	15.0523±2.38^{c}	6.1029±1.08^{def}	0.1313±0.03^{g}
72	30.9742±0.91^{a}	7.5306±0.45^{de}	0.1765±0.04^{g}

Discussion

Drought is one of the most deleterious abiotic stresses and negatively affects crop productivity worldwide (Rampino et al., 2006). Sugarcane is an important crop, as it is a major source of sugar yet its growth is greatly impacted by water deficit (Lakshmanan and Robinson, 2014). A voluminous body of research relevant to drought stress adaptation has enabled scientist to engineer sugarcane plants with improved tolerance to such stress (Ferreira et al., 2017; Nerkar et al., 2018; Marcos et al., 2018). Abbas et al. (2014) reported different levels of drought tolerant between 13 varieties of sugarcane. While, Kumar et al. (2014) successfully transformed the *Arabidopsis Vacuolar Pyrophosphatase* (*AVP1*) gene into sugarcane to increase tolerance against both drought and salinity. Further research from de Souza et al. (2018) showed that overexpression of the transcription factor *AtDREB2A CA* conferred drought tolerance in sugarcane subjected to water deficit under glass house conditions.

Notably, genetic transformation can serve as a useful and practical technology to develop sugarcane with distinct agronomically useful traits. In genetic transformation, transgene expression mainly depends on the use of potent promoters that regulate expression patterns of transgenes. The use of monocot promoters is preferable for the production of transgenic lines that have high transgene expression. Ubiquitin (*Ubi*) promoters such as maize *Ubi* (Christensen and Quail, 1996) have been the benchmark for transgene expression in sugarcane. Herein, we used endogenous DHN promoter (Pr-1DHNSo) to drive the expression of the *DHN* or other

tolerance (Allahverdiyev, 2015). In this study we evaluated RWC in tobacco transformant plants and found that drought stress reduced the water content in both transformant and non-transformant plants. The RWC status were also an indicator whether the plants were in stress condition or not. Therefore, we might related the expression level with the stress condition of the plants. Interestingly, RWC in transformant plants was relatively higher than non-transformant plants, indicating that transformant plants might be able to adapt better to drought stress conditions. Sugiharto (2017) also reported that transgenic drought-tolerant sugarcane expressing NXI-4T gene stay green after 8 days of drought stress, and maintain a higher RWC compared to non-transformant plants. Non-transformant plants were permanently wilting and dried out after 28 days without watering. Moreover, Ramiro et al. (2016) reported that leaves of *ScBI* transgenic sugarcane plants can maintain their internal water level above 80%, even after 21 consecutive days without irrigation.

In this study, we also examined the expression of the *gusA* gene under CaMV35S and Pr-1DHNSo promoters respectively related to drought stress by GUS activity analysis. The CaMV35S is a constitutive promoter that not affected by any stimuli or specific developmental phases, so it can regulate genes within the whole plant tissues. We found that in 0 hours of drought stress, the GUS activity under CaMV35S promoter was already detected in all tobacco tissues. Similarly, under Pr-1DHNSo promoter, the GUS activity was also occurred from 0 hours of drought treatments in all tissues and subsequently increased along with the time points. The expression of *gusA* under the sugarcane Pr-1DHNSo promoter revealed

that there was a compatibility between the promoter and the type of plant which in here tobacco and the activity of transcription factors to bind a specific subset or motif of a promoter to support the transcription process (Suhandono et al., 2014).

Conclusion

The Pr-1DHNSo promoter that was isolated from sugarcane and transformed into tobacco leaves showed that it can be expressed in tobacco as well. Expression analysis using *gusA* gene indicated that this promoter was also induced by drought stress where the expression increased along with the time of the drought treatments. Therefore, this study further corroborates that Pr-1DHNSo has the potential to be used in biotechnological applications to control the expression of other genes conferring tolerance to drought stress.

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