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TRANSFORMATION OF THE *OSC$_3$H$_{52}$* GENE PROMOTER IN TISSUE CULTURE OF SUGARCANE

M. S. Khan[1*], W. Ahmad[1], M. Z. Ahmad[2], M. Ullah[1] and A. Jan[1]

[1]Genomics and Bioinformatics Division, Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture, Peshawar, Khyber Pakhtunkhwa, Pakistan.
[2]Dr. M. Ajmal Khan Institute of Sustainable Halophyte Utilization (ISHU), University of Karachi, Karachi, Pakistan
[*]Corresponding author's email: sayyarkhankazi@aup.edu.pk

ABSTRACT

Sugarcane growth is negatively affected by abiotic stresses. Genetic engineering of sugarcane with abiotic stress-responsive genes has been the method of choice to confer abiotic stress tolerance. In the present work, we optimized callus induction and transformation of the sugarcane variety CP-77-400 with the promoter region of the *OsC$_3$H$_{52}$* gene to analyze its regulatory function under drought and salt stress. Calli were induced using different callus induction media (CIM). The promoter region of the *OsC$_3$H$_{52}$* gene that belongs to the zinc finger protein family was cloned into two different expression vectors i.e. pBI221 and pGreenII0129, which were subsequently transformed to sugarcane calli through agrobacterium and biolistic transformation methods. Among various callus induction media, CIM3 (5 mg L^{-1} 2,4-D + 10% coconut water) showed high callus induction (93%). Biolistic transformation using recombinant pGreenII0129 at 8.5µg/µl concentration resulted in higher efficiency of transformation (28%). While the agrobacterium mediated transformation using recombinant pBI221 plasmid gave 13% transformation efficiency. Amongst the various concentrations of acetosyringone used in agrobacterium mediated transformation, 100 µM showed higher efficiencies (13%) of transformation. Selection of the transformed calli was performed at 25 mg L^{-1} Hygromycin and 25 mg L^{-1} Hygromycin + 300 mg L^{-1} cefotaxime for 15 days, both for biolistic and agrobacterium mediated transformation, respectively. Transformed calli were then successfully confirmed through PCR and GUS expression. The regulatory role of the *OsC3H52* promoter in transgenic calli was further evaluated under drought and salt stress. Quantitative real-time PCR analysis of the *GUS* reporter gene showed high expression in transgenic calli under drought and salt stress conditions.

Key words: Abiotic stress, Callus induction, Gene Transformation, GUS Analysis, *Saccharum officinarum.*

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INTRODUCTION

Sugarcane (*Saccharum officinarum*) is an octoploid (2n = 80~270) and belongs to family Poaceae (Dillon *et al*., 2007). It is an important crop for sugar and biofuel production throughout the world (Chaudhary and Naseer, 2008). Sugarcane cultivation and production are hampered by several biotic and abiotic stresses. Among abiotic stresses, drought and salt are the major constraints on its production (Nasir *et al.,* 2000; Begam *et al*. 2011; Ferreira *et al.,* 2017). With rapid progress on plant transformation technologies, genetic engineering of sugarcane for drought and salt tolerance has recently been the preferred choice of most breeders and biotechnologists. However, optimization of callus induction, regeneration, and gene transformation is challenging in sugarcane. Several factors like the type of variety, explants source, conditions of tissue culture, the concentration of acetosyringone and antibiotics and choice of the transformation methods, agrobacterium and biolistic approaches affect the transformation of sugarcane (Briza *et al*., 2008; Cho *et al.,* 2008; Nerkar *et al*., 2018). Both agrobacterium mediated gene transformation and biolistic methods have been reported in sugarcane with variable transformation efficiencies (Mayavan *et al*. 2015). In comparison to direct gene transfer through the biolistic method, the success of agrobacterium mediated transformation is limited (Mayavan *et al*. 2013). The transformation methods may produce variable transformation efficiencies depending upon the sugarcane variety. Therefore, both transformation methods should be tested on any target variety to investigate their efficiencies.

Members of the zinc finger protein family are transcription factors which help plant adaptation under stress condition (Wang *et al.,* 2008). Classification of the zinc finger proteins depends on the difference in number and order of Cysteine and Histidine amino acids. These residues bind to the zinc ion (Yilmaz and Mittler, 2007). The *OsC$_3$H$_{52}$* gene (zinc finger protein) was identified along with other 66 members of this family in rice, and 68 family members in Arabidopsis (Wang *et al*., 2008).

These families were further divided into 8 and 11 subfamilies, respectively. Expression profile of these genes revealed that many of these are regulated by some biotic and abiotic stresses. Many of the rice zinc finger genes and their promoter regions are not functionally characterized. Due to their potential role in abiotic stress tolerance, functional characterization may lead to the identification of suitable genes and promoters for the transformation of crop plants with abiotic stress tolerance. Several studies reported the functional characterization of zinc finger family genes of Arabidopsis and rice under multiple abiotic stresses (Sun *et al.,* 2007). Liu *et al.* (2015) reported the up-regulation of C2H2-ZF genes in popular under salt, drought, and heat stress. Wang *et al.* (2015) reported the expression of the *C3H47* gene in rice under salinity and drought stress. Similarly, salt tolerance was enhanced by overexpression of AtZFP1 in Arabidopsis (Huang *et al.,* 2014). The rice zinc finger OsTZF1 was found induced under high salt stress, hydrogen peroxide, and drought stress (Jan *et al.,* 2013).

In the present study, the sugarcane variety CP-77/400 was transformed with the promoter region of the *OsC$_3$H$_{52}$* gene to investigate its potential role as a stress-inducible promoter under drought and salt stress conditions. Besides, the sugarcane transformation was also optimized.

MATERIALS AND METHODS

Plant materials: Sugarcane variety (CP 77/400) was used in this study which was grown in the field at the Institute of Biotechnology and Genetic Engineering (IBGE), Peshawar. The immature leaf whorls were used for callus induction.

Callus induction media preparation: Murashige and Skoog (MS) medium was used for callus induction. MS medium was supplemented with Gamborg's vitamins and 30 g L^{-1} sucrose. MS media was prepared with varying concentrations of 2,4-D and coconut water: CIM1 (2 mg L^{-1} 2,4-D + 0% coconut water v/v), CIM2 (3 mg L^{-1} 2,4-D + 5% coconut water) and CIM3 (5 mg L^{-1} 2,4-D + 10% coconut water) (Fig. 1). The media was solidified with 0.8% agar and was then maintained at pH 5.8 with 1N NaOH. The media was autoclaved for 15-20 minutes at 121 ºC and 15 psi and then poured into Petri plates. All the petri plates were stored at 4 ºC for explant inoculation.

Sterilization and inoculation of explants: The immature leaf whorls were collected from the field-grown sugarcane plants and were sterilized according to the procedure previously reported (Ullah *et al*., 2016).

Construct preparation: The Construct pBI221 containing P*OsC$_3$H$_{52}$*:GUS, and pGreenII0129 containing P*OsC$_3$H$_{52}$*:GUS were previously prepared at our laboratory at IBGE (Khan *et al*., 2015). The size of the pBI221 and pGreenII0129 constructs were 12200 and 2945 bp, respectively.

Preparation of E. coli (DH5α) competent cells: The *DH5α* culture was grown overnight in 5 mL LB media and after that 1 mL was inoculated in 100 mL LB media within 500 mL flask until the OD_{600} reaches to 0.2-0.3. The preparation of competent cells and transformation was performed according to the previously reported method by Khan *et al*. (2015). The cells were spread on antibiotic LB plates. After incubation for 12 hrs at 37 ºC, the plates were checked for bacterial colonies.

Preparation of Agrobacterium (EHA 105) competent cells: The *EHA 105* culture was grown overnight and after that 1 mL was added to 10 mL fresh LB medium containing Rifampicin (20 mg L^{-1}). The cells were grown to log phase (OD_{550} 0.5-0.8). The preparation of competent cells was performed according to the previously reported method by Tu *et al.* (2005). Recombinant pBI221G plasmid was used for the transformation of competent cells and then spread on LB agar plates containing 100 mg L^{-1} Kanamycin (100 mg L^{-1}) and 20 mg L^{-1} Rifampicin (20 mg L^{-1}). Cells were incubated at 28ºC for 2 days to observed colonies.

Infection; co-cultivation and washing of the calli: Transformed agrobacterium colonies were selected and grown overnight in LB medium containing Kanamycin (100 mg L^{1}) at 28 ºC and 200 rpm. Six weeks old calli were used for transformation. In the laminar flow hood, 300 µl of the overnight culture and different concentrations of Acetosyringone (55 µM, 100 µM and 150 µM) were added to 35 mL liquid MS medium and mixed thoroughly. The calli were placed in sterile mesh in a sterilized petri plate. The mesh was then placed on the sterile tissue paper to dry the calli plate. The calli were then co-cultivated on CIM3 media supplemented with different concentrations of acetosyringone (55 µM, 100 µM and 150 µM). Approximately, 10 calli were inoculated on each plate. Petri plates were kept at 28 ºC under the dark condition for 3 days for co-cultivation. The infected calli were then washed 3-4 times with 3% sucrose solution + 300 mg L^{-1} cefotaxime to avoid the overgrowth of agrobacterium.

Selection and regeneration of the putative transgenic calli: Putative transgenic calli were placed on selection media (MS + cefotaxime- 250 mg L^{-1} + Hygromycin- 25 mg L^{-1}) for 20 days. After selection, calli were moved to regeneration media containing BAP (2 mg L^{-1}); 1 mg L^{-1} NAA (1 mg L^{-1}), and 25 mg L^{-1} Hygromycin (25 mg L^{-1}).

Biolistic transformation

Gold particle preparation: Gold particles (30 mg) were mixed with 1 mL of Ethanol (70%) and then vortexed for

15 minutes. The gold particles were incubated at 25 ºC for 5 minutes before centrifugation at 10,000 rpm for 5 seconds. The supernatant was discarded and 1 mL ddH_2O was added to the pellet and vortexed for 1 minute. After centrifugation at 10000 rpm for 2 minutes, the pellet was taken and the supernatant was discarded. The above steps were repeated 3 times and finally, the pellet was dissolved in 1 mL 50% glycerol.

Binding DNA to gold particles: Gold particles (40 µl) were transferred into an Eppendorf tube before adding 10 µl of DNA and then vortexed. Then 40 µl $CaCl_2$ and 16 µl spermidine were added to the gold particles followed by vortexing for 5 min. The mixture was centrifuged at 10000 rpm for 2 min and then the supernatant was discarded while the pellet was further washed with 100% ethanol. The pellet was finally dissolved in 100% ethanol (40 µl) and vortexed for 1 min.

Bombardment of calli: The osmoticum medium was prepared using CIM3 supplemented with sorbitol and Mannitol (0.2 M for both). The calli were transferred to the osmoticum medium 3 hours before bombardment. The induced calli of sugarcane were bombarded with gold particles coated with the desired construct (pGreenII0129), at concentrations of 2.8, 5.6 and 8.5µg/µl, 1100 psi and vacuum pressure 25 in Hg using biolistic particle delivery system (Bio-Rad). Calli were then incubated at 28 ºC for 48 hours.

GUS analysis of the putatively transformed calli: For the confirmation of the putatively transformed calli with *OsC_3H_{52}* promoter, GUS analysis was performed after co-cultivation. GUS solution was prepared by mixing Triton X-100 (0.1%), X-Gluc A (0.5 mg L^{-1}), methanol (5%), and 50 mM sodium phosphate buffer having pH 7.0 and. Autoclaved distilled water was used to adjust volume up to 10 mL. The putatively transformed calli (1.5 mL) were mixed with the GUS solution (250 µL) in an Eppendorf tube and was incubated at 37 ºC for 2 days to validate the expression and presence of the *GUS* gene.

Extraction of genomic DNA and PCR confirmation of transgenic calli: Sucrose method was used to extract DNA from the putative transgenic calli as previously reported by Wilcox *et al.,* 2009. PCR was performed using the expression vector and the transgene specific primers as given below.

Primers for pBIG containing the putative promoter region of the *OsC_3H_{52}* gene are given below.

pBI221-F 5′-
TTCTGCGGACTGGCTTTCTACGTGT-3′
OsC_3H_{52}-Pr-R1505′-
TATTACTGCAACATGGCATTCGTGCAT-3′

Primers for pGreenII0129 containing the putative promoter region of the *OsC_3H_{52}* gene are given below.

M13-F 5′-
CAGGAAACAGCTATGAC-3′
OsC_3H_{52}-Pr-R1505′-
TATTACTGCAACATGGCATTCGTGCAT-3′

Calli treatment: Transgenic sugarcane calli harboring the GUS gene under the control of the stress-inducible promoter were used to investigate the effects of drought and salt stress on the expression of the *GUS* reporter gene. For drought stress treatment, calli were treated with 20% polyethylene glycol (PEG). All PEG treated calli samples were harvested at 1, 4, 6, 10 and 24h. Calli were subjected to salt stress by culturing on media containing 0 mM, 50 mM and 100 mM NaCl stress. In both treatments, non-transformed calli, and calli, transformed with an empty vector were used as negative controls. After each treatment, calli samples were frozen in liquid nitrogen followed by storage at -80˚C for RNA isolation.

Quantitative Real Time-PCR Analysis: Quantitative Real Time-PCR (qRT–PCR) was performed to determine the relative expression of the GUS gene in control and transgenic sugarcane calli. Total RNA was isolated from stressed and non-stressed sugarcane calli using RNeasy Plant Mini Kit (Thermoscientific, Germany). Around 5 µg total RNA was reverse transcribed into cDNA using superscript II (Invitrogen, USA). qRT–PCR analysis was carried out using the ABI3700 system (Applied Biosystem, USA) as described by Nakashima *et al.* (2007). The sugarcane Actin gene mRNA was used as an internal control. The GUS and Actin gene primers used were as follows: Actin forward, 5′-CTCAACCCCAAGGCTAACAG-3′, and reverse, 5′-GGCATGAGGAAGGGCATA-3′, GUS forward 5′-CGGGATCCTGCCGTGATACCGACTTGA -3′ GUS reverse 5′- CCCATGGTTCAGCTTGCTTGTTGCTTG-3′.

Data analysis: All data were examined in at least five replicates (n = 5). Data were statistically analyzed using the statistical package "Statistix 8.1" and the values represented as means with standard deviations. Student's t-test was used to compare individual means.

RESULTS

Callus induction: Among different callus induction mediums, significantly high ($P \leq 0.05$) number of calli (93% ± S.D) were induced on CIM3 media followed by CIM2 (76.7% ± S.D) and CIM1 (60% ± S.D) (Figs. 1 and 2).

Agrobacterium-mediated transformation of sugarcane calli: As the pBI221G expression vector is generally used in agrobacterium mediated gene transformation; therefore, the agrobacterium *EHA105* cells were transformed with pBI221G $POsC_3H_{52}$:GUS binary vector and was cultured on 100 mg L^{-1} Kanamycin containing LB media plates. The calli were successfully

infected and transformed with agrobacterium. The infected calli were maintained on different acetosyringone concentrations on the plates. Browning was observed in the non-transformed region of the calli. However, the transformed cells continued growth on the selection media (Fig. 3A). Three different doses of acetosyringone were used to analyze its effect on transformation efficiency. Amongst the different acetosyringone concentrations, 100 µM concentration showed significantly higher ($P \leq 0.05$) efficiency (13%) of agrobacterium to infect calli as compared to those of 150 µM with 8% and 55 µM with 4.8% efficiencies, respectively (Fig. 3B).

Regeneration of the transformed calli: After selection, the putative transgenic calli were placed on regeneration media added with BAP (2 mg L^{-1}) and NAA (1 mg L^{-1}). For the selection of the transgenic calli, the regeneration medium was also supplemented with 5 mg L^{-1} Hygromycin. After 20 days on regeneration media, greening was observed in some calli lines. After every two weeks, calli transfer on to a fresh regeneration media. All the transformed calli started regeneration after the second sub-culture on regeneration media (Fig. 4A).

PCR confirmation of the transgenic calli: The putatively transformed plants were successfully confirmed through PCR using PBI221-F primer and OsC_3H_{52}-Pr-R150 reverse primer to confirm the amplified fragment and the amplified fragment using these primers was approximately 300 bp (Fig. 4B).

Biolistic transformation of sugarcane calli: Sugarcane calli were successfully survived and regenerated on the selection media after bombarded with gold particles coated with recombinant pGreenII0129 plasmid. The bombarded calli showed browning in some portions while some portions of the calli were whitish-yellow in appearance (Fig. 5A). Among three different plasmid concentrations (8.5, 2.8 and 5.6 µg), the highest efficiency of transformation i.e. 28% was achieved in 8.5 µg (Fig. 5B).

PCR confirmation of the bombarded calli: The transformed calli subjected to DNA extraction were confirmed with M13-F Primer and OsC_3H_{52}-Pr-R150 reverse primer. The amplified fragment of PCR was approximately 300 bp (Fig. 6C).

GUS analysis: Transient GUS expression was observed in both agrobacterium mediated and biolistic transformed calli after two days incubation in GUS solution. The non-transformed calli showed no GUS expression. The transgenic calli were stained blue while the non-transformed calli remained colorless (Fig. 6A,B).

GUS gene expression under drought and salt stress: The regulatory role of the *OsC3H52* gene promoter was further investigated under drought and salt stress conditions. Transgenic calli were subjected to 20% PEG for drought stress application. Relative expression of the GUS reporter gene was checked at different time intervals after stress application (Fig. 7). Transgenic calli showed GUS expression that was significantly higher after 4 h of stress application. Similar induction of the expression of the *GUS* gene was observed under salt stress (Fig. 8). Transgenic calli showed a relative expression of the *GUS* gene under 0 mM NaCl stress. However, the *GUS* gene expression enhanced with increasing NaCl stress from 0 mM to 50 and 100 mM.

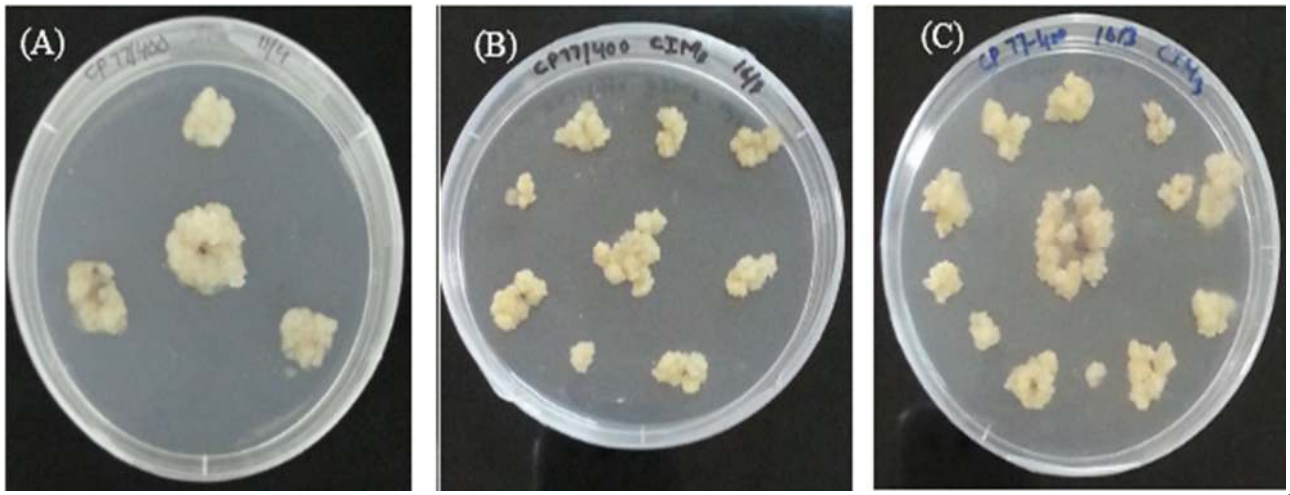

Figure 1. Calli induced on callus induction media added with 2,4-D and coconut water in various doses after 3rd sub-culture (A) calli induced on CIM1 (2 mg L^{-1} 2,4-D + 0% coconut water), (B) calli induced on CIM2 (3 mg L^{-1} 2,4-D + 5% coconut water), and (C) calli induced on CIM3 (5 mg L^{-1} 2,4-D + 10% coconut water).

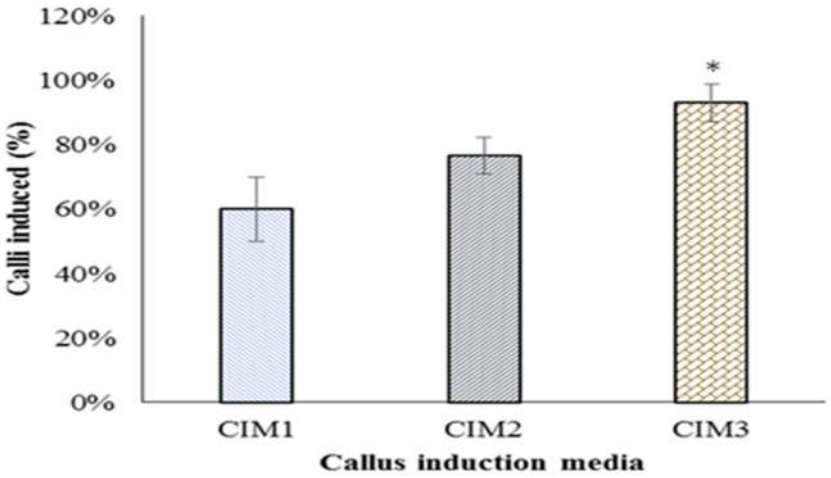

Figure 2. The effect of various doses of 2,4-D and coconut water on callus induction of sugarcane. Data are the percentage of three replicates ± SD. CIM1 (2mgL^{-1} 2,4-D), CIM2 (3 mg L^{-1} 2,4-D + 5% coconut water) and CIM3 (5 mg L^{-1} 2,4-D + 10% coconut water). The asterisk represents a significant difference ($P \leq 0.05$) revealed by the student's t-test.

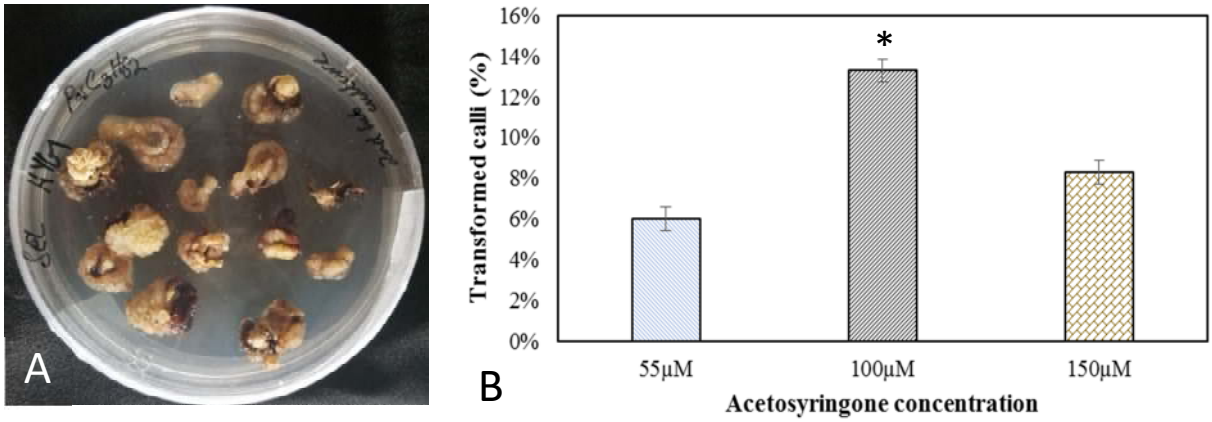

Figure 3. Selection of the infected calli and the effect of acetosyringone concentration on the transformation efficiency of agrobacterium method (A) Selection of the putatively transformed calli on media added with 300 mg L^{-1}cefetoxime and 25 mg L^{-1} Hygromycin,(B) The effect of different concentrations of acetosyringone (55 µM, 100 µM, and 150 µM) on the transformation efficiency. The asterisk represents a significant difference ($P \leq 0.05$) revealed by the student's t-test.

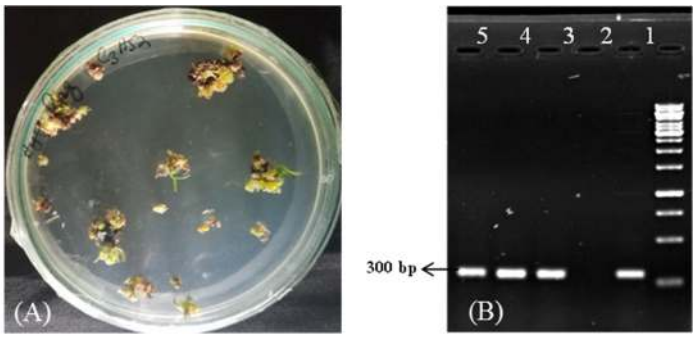

Figure 4. Regeneration and PCR analysis of the Transformed Calli. (A) pGreen transformed calli on media having BAP (2 mg L^{-1}) and NAA (1 mg L^{-1}) augmented with Hygromycin (25 mg L^{-1}). (B) PCR analysis of pBI221 PosC_3H_{52}:GUS transgenic plant on 1% agarose gel. L is ladder and lane 1-5 shows the amplified fragment.

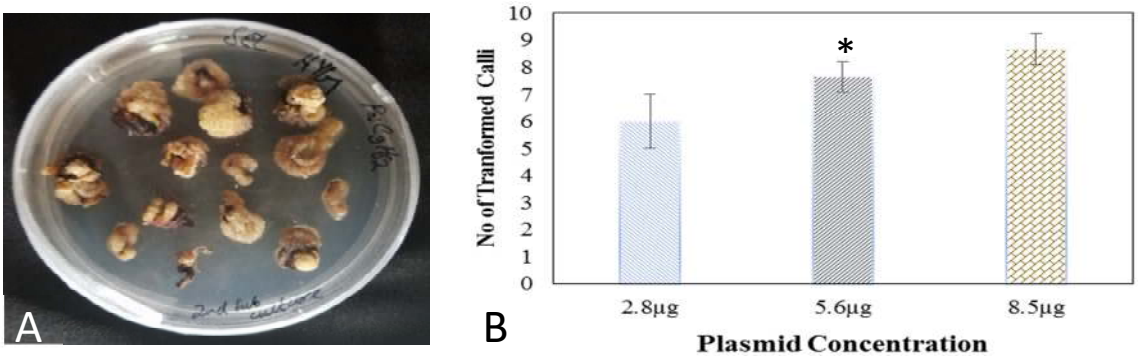

Figure 5. Selection of the bombard calli and the effect of plasmid concentration on the efficiency of biolistic transformation. (A) Selection of putatively transformed calli on media containing Hygromycin. (B) Effect of different plasmid concentrations (2.8, 5.6, and 8.5µg/µl) on transformation efficiency of sugarcane calli. Values are averages ± SD. The asterisk represents a significant difference ($P \leq 0.05$) revealed by the student's t-test.

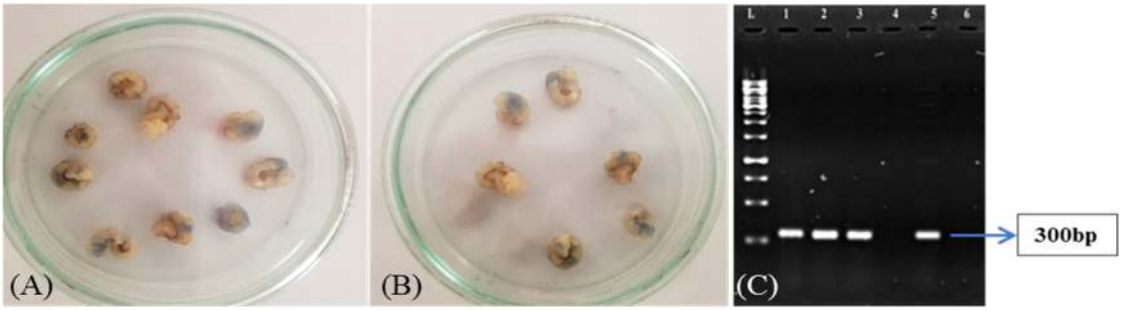

Figure 6. The histochemical activity of the *GUS* gene and PCR confirmation of putative transgenic calli. (A) GUS activity of putative transgenic calli using pBI221 plasmid containing the promoter region of the OsC_3H_{52} gene: GUS. (B) GUS activity of putative transgenic calli using pGreenII0129 harboring the promoter region of the OsC_3H_{52} gene: GUS. (C) PCR confirmation using M13 forward and C_3H_{52}-Pr-R150 reverse primer, Where L = 1kb ladder and lane # 1-5 shows transformed PCR amplified fragments.

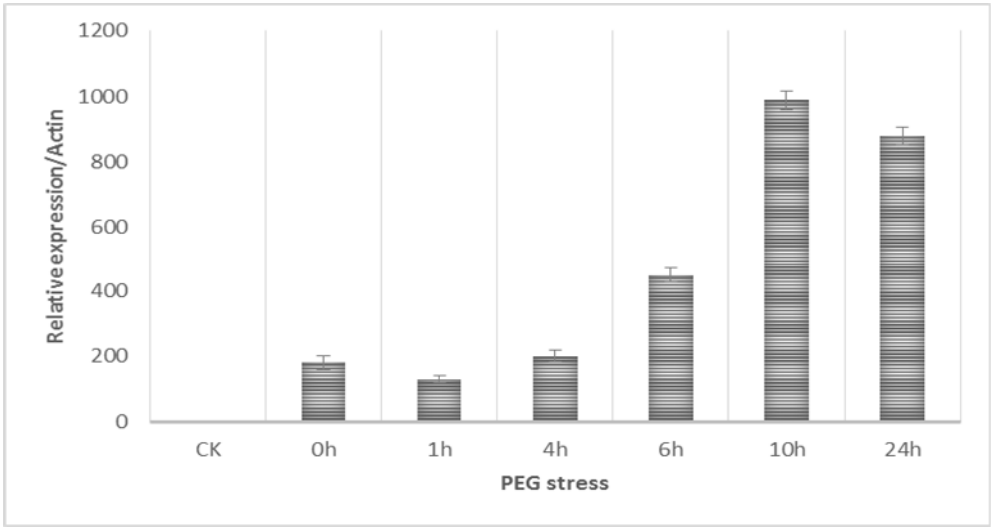

Figure 7. Time course of GUS transcript levels in sugarcane calli after treatment with polyethylene glycol (PEG). CK (control) and 0 h-treated calli were left untreated as controls. The experiment was repeated thrice for each sample. The sugarcane *Actin* gene mRNA was used as an internal control. Data are averages ± SD values.

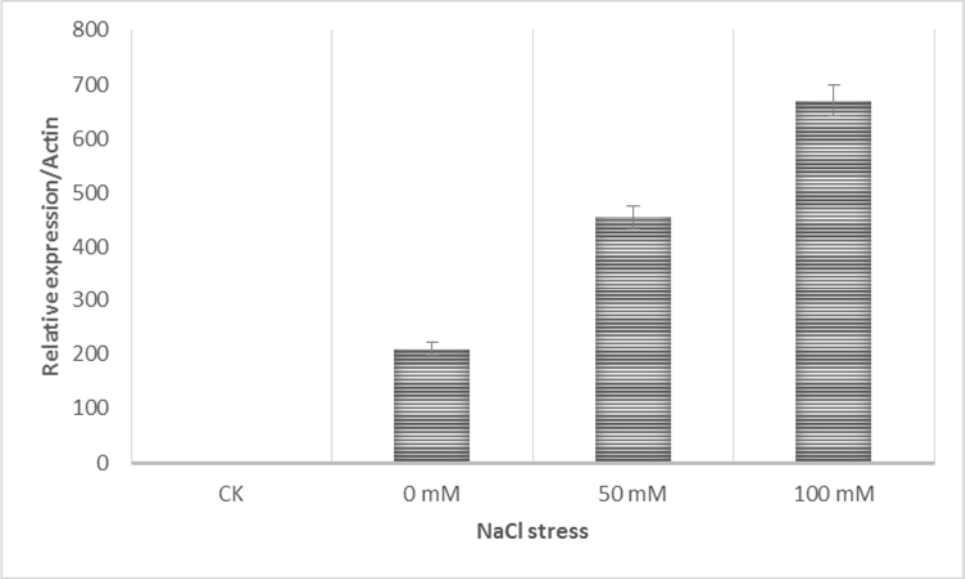

Figure 8. Relative expression of the *GUS* gene in transgenic calli at various NaCl stress conditions. CK represents non-treated, non-transgenic control calli. Data are averages ± SD values.

DISCUSSION

Zinc finger protein family members are responsible for biotic and abiotic stress tolerance (Wang *et al*., 2008). Several genes and promoter regions of the zinc finger family of Arabidopsis and rice have been functionally characterized under various harsh environmental conditions (Sun *et al*., 2007). Previously, Liu *et al.* (2015) mentioned that C2H2-ZF genes in popular are involved in heat, salt, and drought responses. Similarly, rice tolerance to salinity and drought stress was linked with the *C3H47* gene expression (Wang *et al*., 2015). Huang *et al*. (2014) reported that salt tolerance was enhanced by overexpression of *AtZFP1* in Arabidopsis. Rice zinc finger OsTZF1 is induced under high salt stress, hydrogen peroxide, and drought stress (Jan *et al*., 2013). After characterization, these genes need to be transformed into crop plants to analyze their potential contribution in abiotic stress tolerance. Promoter sites have regions, which tend to up- or down-regulate gene expression in response to changes in environmental conditions (Butler and Kadonga 2002). In this connection, the promoter regions of these zinc finger genes also need functional characterization in crop plants. Sugarcane is one of the target plants for transformation and subsequent functional analysis of the zinc finger genes under abiotic stress conditions. The present study was designed to transform sugarcane calli with the promoter region of zinc finger OsC_3H_{52} and to check its activity using PCR and GUS assay. This promoter was previously cloned and transformed into a local rice variety through GUS gene expression (Khan *et al*., 2015). GUS assays were previously used for the characterization of promoters and gene activities (Kajita *et al*., 1994; Vidal *et al*. 2003).

For transformation, the sugarcane calli were first induced on various callus inducing media such as CIM1, CIM2, and CIM3. Out of these, the CIM3 produced comparatively enhanced callus induction. The composition of the CIM3 was previously found highly responsive to callus induction (Ullah *et al*., 2016). In our experiment, the callus response under CIM3 media was comparable with the outcome of Nawaz *et al.* (2013). However, Nawaz *et al.* (2013) used calli induction media without coconut water. Our results are in contrast with Karim *et al.* (2002), in which callus induction was maximum when media contained 3 mg L^{-1} 2,4-D + coconut water. Huang *et al.* (2014) also used a 3 mg L^{-1} concentration of 2,4-D for callus induction during the transformation experiment. Similar studies were also performed by Islam *et al.* (2016).

Two different transformation techniques such as agrobacterium mediated transformation and biolistic were used and their efficiencies were compared. The present study showed biolistic as an efficient approach for transformation (i.e. 28%) as compared to agrobacterium mediated transformation (i.e. 13%). Basnayake *et al*. (2011) reported a 15% transformation efficiency using the biolistic technique in sugarcane calli. Enriquez-Obregon *et al*. (1998) reported a maximum (35%) efficiency of promoter transformation by agrobacterium method. Kalunke *et al*. (2009) reported 28% transformation efficiency using agrobacterium mediated transformation in sugarcane. In contrast, Wu *et al.* (2015) mentioned no substantial difference between biolistic and agrobacterium methods for transformation efficiencies in rice calli. Arvinth *et al*. (2010) also reported higher

transformation efficiency of sugarcane using biolistic which confirms the findings of the present research. Duan *et al*. (2012) reported high transformation efficiency using agrobacterium mediated transformation in rice.

Besides, plasmid type and its concentration must also be considered to improve the biolistic transformation efficiency. In our study, we obtained the maximum transformation efficiency of sugarcane calli with 8.5 µg of recombinant pGreenII0129 plasmid. Matroodi *et al*. (2013) reported a higher concentration of plasmid as vital for enhanced transformation efficiencies; however, they used $pUBI_{121}$ plasmid instead of pGreenII0129. Kim *et al*. (2012) reported the impact of plasmid concentration on the biolistic transformation of sugarcane calli. Tee and Maziah (2005) reported that different concentrations of plasmid had a significant impact on transformation efficiency.

Although agrobacterium mediated transformation of monocots such as sugarcane has been achieved with the addition of acetosyringone, still several variables need to be further optimized to achieve high transformation efficiency (Huang *et al*., 2014). In this study, we optimized the acetosyringone concentration to improve the transformation efficiency of sugarcane calli. Increasing acetosyringone concentration from 50-100 µM enhanced transformation efficiency. However, bacterial overgrowth and subsequent death of the infected calli were observed with acetosyringone concentrations beyond 100 µM. Kumar *et al*. (2014) also suggested an optimal concentration of acetosyringone as a pre-requisite for efficient agrobacterium mediated transformation. The present findings regarding the optimized level of acetosyringone are supported by those reported by Fu *et al*. (2015). Mayavan *et al*. (2015) reported 32.6% transformation efficiency while using the same concentration of acetosyringone for agrobacterium mediated transformation in sugarcane. However, Khan *et al*. (2013) reported lower concentration (50 µM) sufficient for transformation. Manickavasagam *et al.* (2004) reported higher agrobacterium mediated transformation while using 50 µM acetosyringone. Whereas, Martins *et al*. (2015) reported 200 µM acetosyringone is adequate for the agrobacterium technique that contradicts with our findings.

To confirm that the promoter was transformed successfully and was induced under stress condition, the putatively transformed sugarcane calli were analyzed using GUS analysis. Transgenic calli showed mild *GUS* gene expression even under the non-stressed condition as the media contained osmoticum, Mannitol, and Sorbitol, which triggers stress in the growing calli. However, the *GUS* gene expression was induced when calli were subjected to drought and salt stress conditions. These results confirmed that the *OsC3H52* gene promoter is induced under both drought and salt stress and maybe a better choice for expression induction of stress-inducible genes. Previous studies revealed the potential role of zinc finger family genes in multiple stress tolerance of several plants (Huang *et al*., 2014; Liu *et al*., 2015; Wang *et al*., 2015). Moreover, the *GUS* expression analysis showed that this promoter can serve as a regulatory element for high levels of transgene expression in sugarcane under drought and salt stress conditions.

Conclusion: Sugarcane calli were successfully transformed with the promoter of the OsC_3H_{52} gene fused with the *GUS* gene. For optimization, the biolistic and agrobacterium methods were used and compared for transformation efficiencies. Biolistic transformation showed maximum transformation efficiency as compared to agrobacterium mediated transformation. The optimal concentration of plasmid for biolistic transformation and acetosyringone concentration for agrobacterium mediated transformation enhanced the transformation efficiencies. Finally, the *GUS* gene expression was checked in the transgenic calli subjected to drought and salt stress. Induction of the *GUS* gene activity under these stresses confirmed the stress-inducible nature of the *OsC3H52* gene.

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