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Genetic Transformation of Sugarcane Variety HSF-240 with Marker Gene *GUS*

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Abstract

In the current research an efficient transformation system for sugarcane was established. Shoot tip of variety HSF-240, excised from a six months old field grown plants were used as explant. For transformation, *Agrobacterium tumefaciens* strain *EHA101* with vector *pIG121 Hm*, harboring *GUS*, *HPTII* and *NPTII* genes were used. *HPTII* is a hygromycin resistant while *NPTII* is a kanamycin resistant gene. Effects of Acetosyringone, duration of co-cultivation and pre-selection, concentration of cefotaxime and hygromycin in medium on transformation efficiency were studied. High transformation efficiency and 60% *GUS* expression was observed when 50 µM acetosyringone was added in the co-cultivation medium. Among different durations of co-cultivation, 48 h produced high (40%) transient *GUS* positives with an absolute control of bacterial growth. For pre-selection, seven days gave a high transformation efficiency of 10%. Cefotaxime concentration of 1000 mg/L proved optimal for pre-selection of the explants with efficient control of bacterial growth. A high regeneration (31%; $P < 0.01$) of the transformants was observed at 50 mg/L hygromycin. Presence of *GUS* gene was confirmed by PCR analysis and only the transgenic plants contained the 430 bp fragment of *GUS* gene. The new protocol developed in this study could be used for the efficient transformation of sugarcane with desired gene to produce insect/pest resistant, drought tolerant and high yielding sugarcane varieties in future. © 2013 Friends Science Publishers

Abbreviations: AA: Amino acid medium; As: Acetosyringone; BAP: 6-Benzyl amino purine; FAO: United Nations Organization for Food and Agriculture; GA_3: Gibberellic acid; *GUS*: *β-glucuronidase*; Hr: Hour(s); IBA: Indole-3-Butyric acid; min: Minute(s); Sec: second(s); µM: Micromolar

Keywords: *A. tumefaciens*; Hygromycin; Shoot tip; Sugarcane; Transgenic plants; *GUS* gene; PCR

Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the most important cash crops of Pakistan. Establishment of high yielding sugarcane varieties mainly depends on genetic variability and assemblage of potential genes. Genetic improvement of sugarcane in the country is hampered by the intricate flowering behavior under the prevailing climatic conditions. Currently, the breeding program in the country involves only the import of fuzz and selection of exotic lines. Micro-propagation is an important technique, which can help in rapid and safe production of sugarcane on commercial scale (Khan *et al*., 2008, 2009; Rashid *et al*., 2009; Uzma *et al*., 2012). However, the importance of sugarcane transformation as a mean to introduce traits of commercial interest into many cultivars is increasing rapidly. These traits include resistance to viruses, insects, fungi, resistance to herbicide, drought and improvement of the fiber quality. For these reasons, *Agrobacterium* mediated transformation must be an optimal system in order to set up the industrialization of sugarcane transformation technology.

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Agrobacterium mediated transformation has been the choice method of transformation in many monocotyledonous species including rice, wheat, barley and maize. Transgenic sugarcane plants were however, produced by other methods. For example, Taparia *et al*. (2012a) successfully transformed sugarcane with marker gene using biolistic direct gene transfer method. Transgenic sugarcane plants resistant to stem borer were produced by cell electroporation (Arencibia *et al.,* 1999). Arencibia *et al*. (1997) reported the generation of the first transgenic sugarcane lines resistant to stem-borer attack using particle bombardment method. Recently, Singh *et al*. (2011) reported successfully the transformation of sugarcane against the larvae of stalk borer, a serious sugarcane pest. However, *Agrobacterium* mediated transformation has remarkable advantages over direct transformation methods, including preferential integration of defined T-DNA into transcriptionally active regions of the chromosome (Olhoft *et al*., 2004), exclusion of vector DNA (Fang *et al*., 2002) and removal of unlinked integration of co-transformed T-DNA (Olhoft *et al*., 2004).

The present study was designed to establish a protocol for efficient transformation of sugarcane with marker gene (*GUS*) using shoot tip as explant. Several studies showed that sugarcane could be transformed with marker genes (Taparia *et al*., 2012a, b) using callus as explant. However, no report is available for developing *Agrobacterium* mediated transformation protocol in sugarcane using shoot tip as explant. The new protocol developed in this study could be used for the efficient transformation of sugarcane with desired gene. The transgenic sugarcane varieties developed by this system having desired genes will improve important traits like resistance to herbicide, insects, sugarcane mosaic virus and sugarcane yellow leaf virus in future.

Materials and Methods

Sugarcane Material and Explant Preparation

The plant materials were kindly provided by the Sugar Crops Program at Crop Sciences Institute, National Agricultural Research Centre (NARC), Islamabad, Pakistan. All the experimental work was carried out at Agricultural Biotechnology Program (ABP) in NARC, from March to November, 2005.

Fresh shoot tips (5-10 mm) from young plants holding active growing points *i.e.,* apical meristematic portions were used as explants throughout the transformation experiments. Explant materials were taken from six months old sugarcane plants. Due to excessive phenolic compounds the materials were treated with antioxidants solution containing (100 mg/L ascorbic acid + 150 mg/L citric acid) for 1 h to excrete these compounds. After treating with antioxidants, clorox (commercial bleach containing 5.25% v/v sodium hypochlorite) was used for surface sterilization of these explants. The explants were then washed three times with autoclaved distilled water each for 10 min and transferred to shoot initiation medium. Antibiotic cefotaxime @ 500 mg/L was added both in washing step and later in shoot initiation medium.

Transformation Media

The explants were cultured on solid MS (Murashige and Skoog, 1962) medium, supplemented with various concentrations of gibberellic acid (GA_3) and kinetin (Kin), for initiation of cultures. Same MS medium with 6-benzyl amino purine (BAP) + GA_3 and BAP +Kin in different concentrations was used as liquid medium for the multiplication of cultures. Co-cultivation medium (MS+ AA (amino acid) + 50 µM As), pre-selection medium (MS + 1000 mg/L cefotaxime), selection medium (MS + 1000 mg/L cefotaxime + 50 mg/L hygromycin) and regeneration medium (MS + 1000 mg/L cefotaxime + 50 mg/L hygromycin + 0.1 mg/L GA_3 + 1.0 mg/L BAP + 0.5 mg/L IBA) were used during the transformation experiments.

Bacterial Strain and Plasmid

Agrobacterium strain *EHA 101* with a binary vector *pIG121Hm* (Hiei *et al*., 1994) was used in all the transformation experiments. *pIG121Hm* is a binary vector that contains genes for hygromycin resistance and *GUS* in the T-DNA region, as well as for kanamycin resistance (Fig. l). The gene for *GUS* has an intron in the 5' end of the coding sequence and is connected to the 35S promoter of cauliflower mosaic virus. This intron-GUS reporter gene expresses *GUS* activity in plant cells but not in the cells of *A. tumefaciens.* The total size of the construct was 10 kb.

Transformation Procedure

Bacterial inoculum (10 µL) was taken from glycerol stock and cultured in 50 mL YEP medium (An *et al*., 1988) containing 50 mg/L each of kanamycin and hygromycin and incubated at 28ºC in a shaker for two days for growth. After this, 25 mL from the bacterial culture was centrifuged at 3000 rpm for 15 min and the pellet was re-suspended in equal amount of amino acid medium. The explants were drenched in this medium, blotted dry and placed on co-cultivation medium for two days. After two days of co-cultivation the explants were washed with 1000 mg/L cefotaxime solution and placed on pre-selection medium for seven days and then transferred to selection medium. Selection of the transformants was performed for 15 days. The selected explants were shifted to regeneration medium.

GUS Assay

The transformed explants were histochemically assayed for *GUS* expression as described by Jefferson *et al*. (1987). The plant parts (leaves) were put in the *GUS* solution overnight at 37ºC and then blue spots were observed under the light microscope. The presence of blue color confirmed the *GUS*

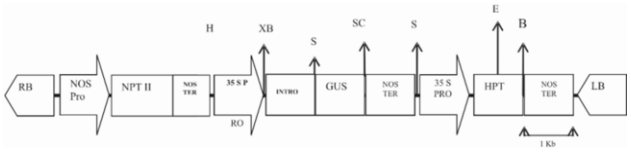

Fig. 1: T-DNA regions of construct *plG121Hm*. Abbreviations: RB: right border; LB: left border; *NPTII*: neomycin phosphotransferase; *GUS*: β-glucuronidase; *HPTII*: hygromycin phosphotransferase; NOS Pro: nopaline synthase promoter; 35S P: 35S promoter; NOS TER,:3' signal of nopaline synthase; 35S: 3' signal of 35S RNA; B: BamHI; E: EcoRI; H: Hindlll; S: Sail; SC: Sacl; XB: Xbal

gene activity.

Confirmation of Transgenic Plantlets by PCR Analysis

PCR analysis was performed for the amplification of the *GUS* gene. Leaf tissues from transgenic plantlets were taken and DNA was extracted according to CTAB method (Asif *et al.*, 2000). Forward primer 5'-ACACCGATACCATCAGCGAT-3' and reverse primer 5' TCACCGAAGTTCATGCCAGT-3' were used to amplify the 430 bp fragment of *GUS* gene. The PCR reaction was as: 94°C for 5 min, 55°C-72°C-94°C for 30 sec with a total of 25 cycles and finally 72°C for 7 min. The PCR products were then run on 1% agarose gel to detect the 430 bp fragment of *GUS* gene.

Experimental Design and Statistical Analysis

The research design used was Completely Randomized Design (CRD) with three replications of each treatment. Data for each experiment were collected visually where possible and with microscope where applicable. For each experiment 100 explants were used as starting material. MSTAT-C (1991) package was used for the statistical analysis of data. One-way ANOVA (Analysis of Variance) was used to analyze the treatments' effects. In case the results were significant, LSD test was performed to separate the means.

Results

Effects of Co-cultivation Conditions on Transformation Efficiency

Shoot tips infected for 5 min with *Agrobacterium* were tested for the effects of different concentrations of acetosyringone i.e., 25, 50, 75 and 100 µM in co-cultivation medium. Transformation efficiency was observed in all treatments of acetosyringone, while much less (5%) transformation efficiency was achieved in control (no acetosyringone). The highest *GUS* gene expression (60%) was obtained with 50 µM acetosyringone in the medium, followed by 35, 30 and 20% emergence of *GUS* positive shoot tips with 75, 25 and 100 µM of acetosyringone (Fig. 2).

In another experiment, infection of explants for 1, 2, 3, 4, 5 and 10 min, only 5 min showed a maximum survival rate of explants (25%), least bacterial growth (10%) and no browning. In our study overgrowth of bacteria (48.33%) occurred when explants were infected for 10 min. Percent browning was maximum (31.66%) for 10 min infection and minimum (0%) for both 1 min infection and control (Fig. 3). Significantly less survival (5 to 10%) was observed with 2,

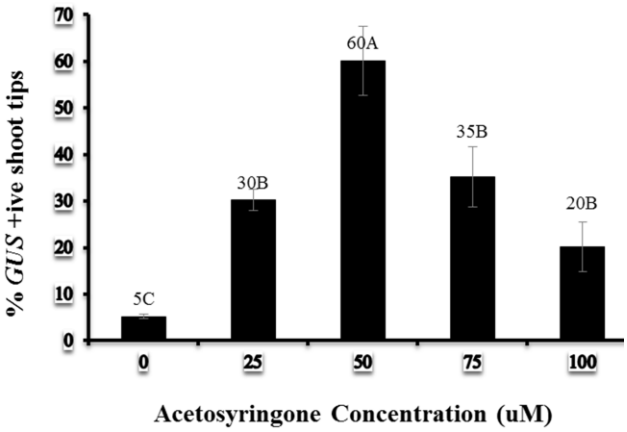

Fig. 2: Different acetosyringone concentrations effects on transformation efficiency. Each value represents the mean of three independent replicates. Error bars indicate ± standard deviation of three replicates. Letters on bars show whether the values are significant or not. Means having the same letter are not statistically significant ($p < 0.01$) according to least significant difference test

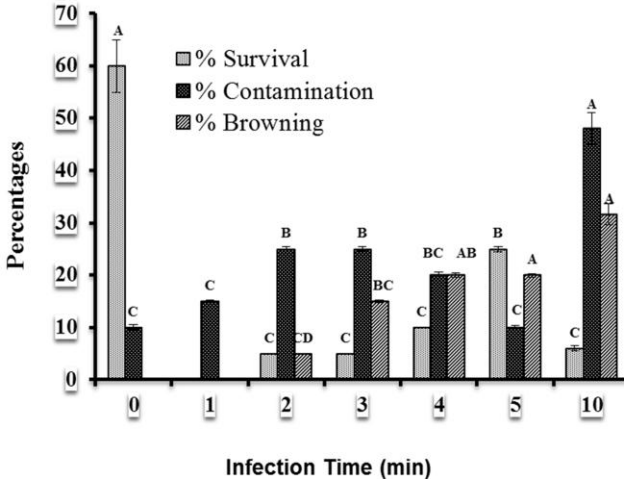

Fig. 3: Effects of different infection times on survival of shoot tips. Each value represents the mean of three independent replicates. Error bars indicate ± standard deviation of three replicates. Letters on bars show whether the values are significant or not. Means having the same letter are not statistically significant ($p < 0.01$) according to least significant difference test

3 and 4 min infection. However the browning percentages ranged from 5 to 20% and contamination observed was 20 to 25% (Fig. 3).

A very low transformation (10%) was obtained for a co-cultivation period of 4 h. Similarly 24 h incubation period was also not very effective and transformed only 20% of the explants. The 72 h duration of bacterial culture caused excessive growth and negatively affected the transformation process and only 10% transformation of shoot tips was observed. Co-cultivation for 48 h supported the highest transformation efficiency (40%) with a maximum control of bacterium in pre-selection step (Fig. 4). Both short (24 h) and a long incubation period (72 h) decreased transformation efficiency due to insufficient and over growth of bacterium, respectively.

Factors Affecting Pre-selection of Co-cultivated Shoot Tips

In this study, infected explants were placed on pre-selection medium for a period of 7 days. Cefotaxime concentration of 1000 mg/L during pre-selection was found optimal where 60% survival of the shoot tips was observed (Fig. 5). At a lower concentration of cefotaxime, more contamination was observed which greatly reduced the number of shoot tips. The lowest concentration of 250 mg/L cefotaxime was not effective because it failed to control the over growth of *Agrobacterium* and as a result 80% of the explants were contaminated (Fig. 5). Only 20% contamination and 30% growth of the explants were observed when cefatoxime @ 500 mg/L was used. Although at a high concentration of cefotaxime (1000 mg/L), no contamination of explants was observed however, it caused 15% browning of the shoot tips, while in control all the explants become contaminated (Fig. 4).

Time interval during pre-selection significantly affects the pre-selection process. A period of 7 days in pre-selection of shoot tips gave maximum (10%) transformation with 10% contamination and 10% browning while 30% contamination and the lowest transformation efficiency (5%) was observed for 3 days period of pre-selection (Fig. 6). Fig. 6 also describes that with increased time interval more browning was observed for example, 20% for 10 days duration. The percentage transformation increased with increase in exposure time up to 7 days, however, further increase continuously decreased the transformation efficiency and only 5% efficiency was observed after 10 days of pre-selection period (Fig. 6).

Selection of Transformed Shoot Tips and Transgenic Plantlets Production

Transformation efficiency (30%) was observed at 50 mg/L hygromycin concentration in combination with 1000 mg/L cefotaxime (Table 1). An increased up to 75 mg/L hygromycin caused the death of shoot tips. A lower concentration of 10 and 25 mg/L of hygromycin showed less browning and necrosis and more shoot growth. At 25 mg/L, 21.66% transformation was observed. After two weeks of selection, shoot tips were transferred to regeneration medium. The highest regeneration 31.66% was observed for 50 mg/L hygromycin followed by 25 mg/L hygromycin with 25% regeneration.

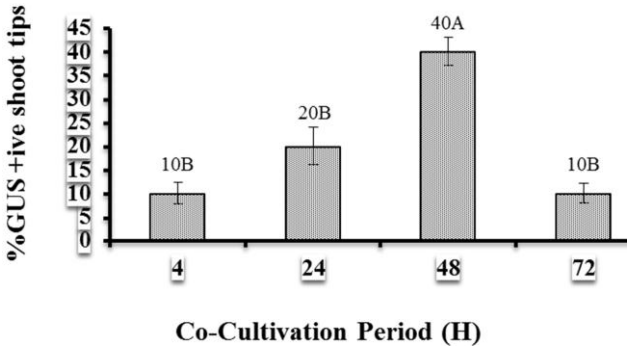

Fig. 4: Co-cultivation durations effects on transformation efficiency. Each value represents the mean of three independent replicates. Error bars indicate ± standard deviation of three replicates. Letters on bars show whether the values are significant or not. Means having the same letter are not statistically significant ($p < 0.01$) according to least significant difference test

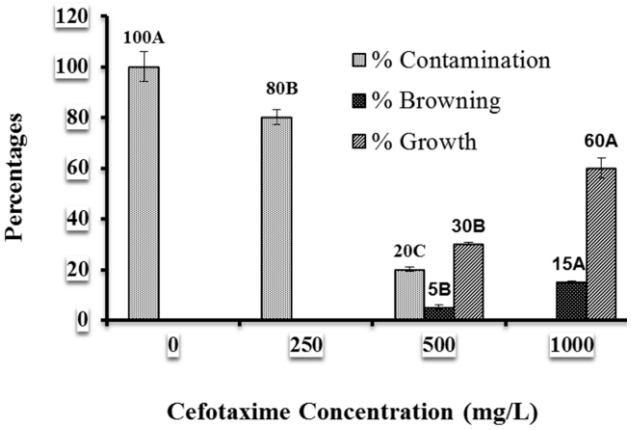

Fig. 5: Different cefotaxime concentrations in pre-selection of shoot tips. Each value represents the mean of three independent replicates. Error bars indicate ± standard deviation of three replicates. Letters on bars show whether the values are significant or not. Means having the same letter are not statistically significant ($p < 0.01$) according to least significant difference test

Histochemical Localization of *GUS* Expression

After regeneration, different parts of transgenic plants were histochemically tested for *GUS* activity. Different parts e.g. roots and leaves showed *GUS* positive results but *GUS* expression was clearer in leaves portions (Figs. 7 and 8).

Table 1: Transformation efficiency of sugarcane variety HSF-240 using hygromycin as a selective agent

Hygromycin (mg/L)	% Growth	% Proliferation	% Regeneration	% *GUS* positive	% Transformation
10	85 ± 4.5 A	68.3 ± 5.0 A	15.0 ± 0.5 BC	21.6 ± 2.0 B	10.0 ± 0.5 B
25	78 ± 5.0 A	65.0 ± 5.5 A	25.0 ± 2.0 AB	35.0 ± 2.0 B	21.6 ± 1.5 A
50	90 ± 6.5 A	80 .0 ± 6.5 A	31.6 ± 2.0 A	80.0 ± 4.5 A	30.0 ± 2.0 A
75	25 ± 4.0 B	15.0 ± 5.0 B	0.0 C	80.0 ± 5 A	0.0 B

*Each value represents the mean of three independent replicates ± standard deviation of three replicates. Means within a column having the same letter are not statistically significant ($p < 0.01$) according to least significant difference test

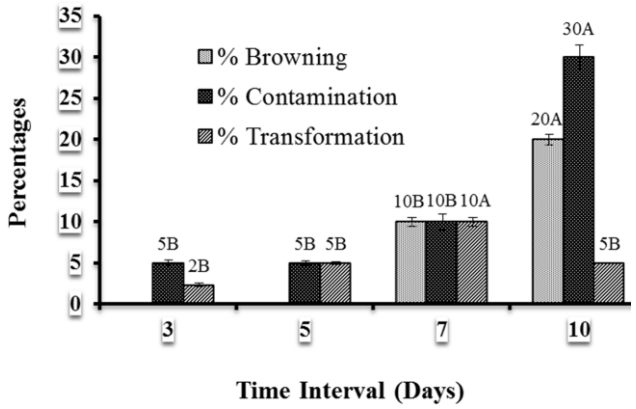

Fig. 6: Effects of different time intervals on pre-selection of shoot tips. Each value represents the mean of three independent replicates. Error bars indicate ± standard deviation of three replicates. Letters on bars show whether the values are significant or not. Means having the same letter are not statistically significant ($p < 0.01$) according to least significant difference test

Under the microscope different parts of transgenic var. HSF-240 showed blue color. A maximum of 80% *GUS* expression was observed (Table 1). The absence of blue color showed that either gene was not transferred or it was nonfunctional and not expressed or silenced.

Confirmation of Transgenic Plantlets by PCR

Genomic DNA from four transgenic plantlets, obtained from independent events, which were *GUS* positive and from an untransformed control plantlet were subjected to PCR for detection of the existence of transgene. Fig. 9 shows that all of the samples of transgenic plantlets (lane 1-4) gave the predicted DNA fragment of 430 bp for a part of *GUS* gene. No band was detected in the sample from an untransformed control plantlet (lane 6).

Discussion

Regeneration of transgenic sugarcane plantlets from shoot tips may become a very easy technique, if the conditions for efficient transformation are standardized. Shoot tip explants can be easily transformed and could be used in conducting functional studies of promoters and transgenic plants production for various agronomic traits, which are

Fig. 7: *GUS* expression in leaves of sugarcane variety HSF-240

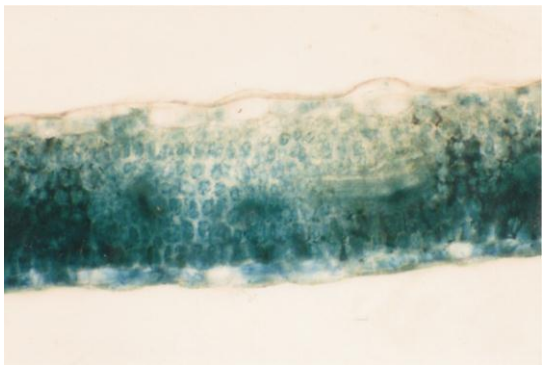

Fig. 8: *GUS* expression in leaves of sugarcane variety HSF-240

required for improvement of sugarcane productivity (Manickavasagam *et al.*, 2004; Mahmood *et al.*, 2007; Weng *et al.*, 2011). The lack of a reproducible methodology for stable transformation of sugarcane was a major obstacle for its genetic manipulation for many years in the past. However, recent developments in molecular biology and genetic transformation have made it possible to identify, isolate and transfer desirable genes into sugarcane such as for resistance against herbicide (Leibbrandt and Snyman, 2003), sugarcane yellow leaf virus (Gilbert *et al.*, 2005, 2009) and for other traits (Arruda, 2012). Recently, Mustafa and Khan (2012) reported on chloroplast transformation in sugarcane, which is an important target for plant scientists being involved in the photosynthesis. Transgenic sugarcane with modified *cry1Ac* gene, resistant to stem borers has been reported using micro projectile bombardment (Weng

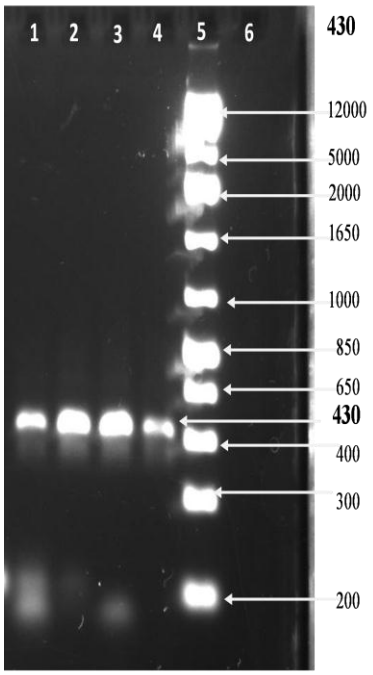

Fig. 9: PCR analysis to detect the presence of *GUS* gene in transgenic sugarcane plantlets (lane 1-4), 1 Kb plus DNA ladder (lane 5) and an untransformed control plantlet (lane 6). Ladder values are in base pair (bp). The bold 430 bp indicates the fragment of *GUS* gene

et al., 2011). Optimization of *Agrobacterium* mediated DNA transfer to sugarcane for various traits has also recently been reported (Molina *et al*., 2011; Singh *et al*., 2011). Manickavasagam *et al.* (2004) earlier reported an efficient protocol for *Agrobacterium* mediated transformation of two Indian sugarcane cultivars using auxiliary bud as the target tissue and production of transgenic sugarcane plants resistant to BASTA (herbicide).

We found that acetosyringone enhance the transformation in sugarcane. Matsuoka *et al.* (2001) used a high concentration of acetosyringone in co-cultivation medium for getting better transformation results in sugarcane. This supports our results strongly for using acetosyringone as transformation enhancer in sugarcane. Manickavasagam *et al*. (2004) also used 50 µM acetosyringone and obtained 7.2% transformation efficiency as compared to 3.2% transformation without acetosyringone. In the same study 5.2 and 3.8% transformation was recorded with 25 and 75 µM acetosyringone, respectively. These results are contrary to Enriquez-Obregon *et al.* (1998) who suggested that acetosyringone was not necessary for genetic transformation of sugarcane meristem. We found higher transformation efficiency with the inclusion of acetosyringone in co-cultivation medium. Infection time is another important factor that contributed in the successful transformation. We found 5 min as optimum infection time. Matsuoka *et al.* (2001) gave 10 min infection to the sugarcane calli. A possible explanation for this is that bacterium required enough time to transfer the gene to its host species while at higher infection time (10 min), it was very difficult to control the bacterium in subsequent pre-selection and selection steps. Matsuoka *et al.* (2001) found 72 h as the best co-cultivation time for sugarcane with 6% transformation, while in our studies we found 48 h as optimum co-cultivation period. Manickavasagam *et al.* (2004) also used 72 h incubation period using auxiliary buds and observed maximum of 11.6% and 23% transformation with two different strains of *Agrobacterium LBA 4404* and *EHA 105*, respectively.

Antibiotics such as cefotaxime, carbenecillin, and timentin have been used regularly in *Agrobacterium*-mediated transformation of crops following co-culture to suppress or eliminate *Agrobacterium* (Bottinger *et al*., 2001; Prabu and Prasad, 2012). Zhu *et al*. (2011) used 500 mg/L cefatoxime in pre-selection process to control bacterial contamination, while our results suggested 1000 mg/L cefotaxime as the optimum dose to kill the bacteria after co-cultivation process. It may be because of the different environmental conditions of different laboratories where the research was performed or also perhaps because of the origin of plant materials used. Matsuoka *et al*. (2001) used 40 mg/L hygromycin for selection of the transformants and observed 6% transformation. Zhu *et al*. (2011) reported 25 mg/L hygromycin as a best selective agent for the selection of transformed sugarcane tissues, while Kim *et al*. (2007) reported 20 mg/L hygromycin for optimum selection of the explants of a monocotyledon plant, alstromeria. We used relatively higher concentration of hygromycin i.e. 50 mg/L because at a lower concentration, selection of transformed was not very efficient. After two weeks of selection, shoot tips were transferred to regeneration medium. Highest regeneration 31.66% was observed for 50 mg/L hygromycin followed by 25 mg/L hygromycin with 25% regeneration. To our knowledge this is a highest regeneration rate, which is ever achieved for sugarcane transformation. After regeneration, different parts of transgenic plants were histochemically tested for *GUS* activity. This is a first step towards the confirmation of transformants. Such histochemical analysis of *GUS* expression was also observed by Prabu and Prasad, (2012) and Singh *et al*. (2011) who observed similar trend of analysis in their experiment while using sugarcane crop. In the next step the putative transformants were confirmed by the PCR. These sugarcane plantlets can then further be tested in the field for a stable genetic inheritance of the target gene.

In this study, acetosyringone inclusion in co-cultivation medium, duration of co-cultivation and pre-selection, concentration of antibiotics cefotaxime and hygromycin in a pre-selection and selection media, showed

significant effects on the transformation efficiency in sugarcane. For optimum and efficient transformation of sugarcane, optimization of these factors is very important. In this research, we established an easy protocol for efficient transformation of sugarcane by optimizing the conditions affecting transformation process. This optimized protocol can be used for sugarcane transformation to insert target genes controlling different important agronomic and biochemical traits in sugarcane. This can further help researchers to produce commercially a more healthy sugarcane crop for the benefit of the society.

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