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Full Length Article

Enhanced Transformation Efficiency of *Saccharum officinarum* by Vacuum Infiltration Assisted *Agrobacterium*-mediated Transformation

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Abstract

Transgenic plants have been extensively produced for years with improved agronomic characteristics, enhanced disease and insect pest resistance. However, improved and reproducible system of gene transformation remains the main focal point in plant genetic engineering. In the present study, an efficient *Agrobacterium*-mediated stable transformation protocol for sugarcane (*Saccharum officinarum* L.) was optimized using embryogenic calli and green fluorescent protein (*gfp*) as explants and reporter gene, respectively. Parameters studied were optical density of bacterial culture and agro-infiltration under different pressure regimes. Results indicated that out of four different treatments, inoculation of calli with bacterial culture of OD_{600}0.4 through vacuum infiltration at -50 kPa produced maximum number of transgenic plants. This newly developed protocol of *Agrobacterium*-mediated transformation of sugarcane has shown significant improvement over conventional procedure in terms of its transformation efficiency. This protocol can be employed to develop transgenic sugarcane plants, having tolerance/resistance against various biotic and abiotic stresses. © 2014 Friends Science Publishers

Keywords: *Agrobacterium tumefaciens*; Inoculation density; GFP; *Saccharum officinarum*; Vacuum infiltration

Introduction

Sugarcane (*Saccharum officinarum*) is a cash crop that is mainly grown in warm temperate to tropical regions of Asia; moreover, it contributes 75% of world sugar production followed by sugar beet. Despite its economic importance and share in world market, sugarcane production suffers with many problems that are mainly caused by both biotic and abiotic factors. Susceptibility to various diseases, insect pest attack and drought are some of the major yield limiting factors that mainly contribute towards low sucrose contents and recovery in cultivated varieties (Raza *et al.,* 2010). Improvement of currently grown sugarcane cultivars through conventional breeding is difficult due to its complex genome and low fertility while genetic engineering has emerged as a potential tool to introduce desirable traits directly into commercially cultivated elite varieties from any endogenous and exogenous sources (Arruda, 2011).

Agrobacterium tumefaciens and biolistic are two methods that are mainly used for the introduction of genes encoding for desirable traits in plant's genome (Tingay *et al.,* 1997; Dai *et al.,* 2001; Li *et al.,* 2013). However, *Agrobacterium*-mediated transformation is preferred over biolistic because of its more stable expression, less chances of transgene silencing, precise insertion, introduction of few copies in genome and higher chances of transgene to segregate in Mendelian manner (Alimohammadi and Bagherieh-Najjar, 2009). Besides, only few studies have reported successful *Agrobacterium*-mediated transformation and plant regeneration in sugarcane (Zhangsun *et al.,* 2007; Joyce *et al.,* 2010).

All critical factors affecting the transformation efficiency need to be properly defined for the development of a reproducible plant transformation method. Co-cultivation conditions, selection system and cultivars are some of the important factors, which considerably affect the *Agrobacterium*-mediated transformation efficiency (Joyce *et al.,* 2010). Present study was conducted to develop a reproducible *Agrobacterium*-mediated transformation system for local sugarcane cultivar (CPF-246). A combination of various inoculation methods and co-cultivation conditions along with *gfp* as reporter gene were used for the transformation of sugarcane embryogenic calli.

Materials and Methods

Plant Material

The sugarcane (*Saccharum officinarum*) plants of commercial cultivar CPF-246 were obtained from the Sugarcane Research Institute (SRI), Ayub Agriculture Research Institute, Faisalabad, Pakistan.

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Embryogenic Callus Cultures

Embryogenic calli were raised from apical meristem of sugarcane variety CPF-246 following the procedure described by Raza *et al.* (2010). Apical portions of healthy shoots were stripped to the terminal bud and sterilized with 70% ethanol. Leaf rolls were peeled, under sterile conditions inside a laminar air flow hood, to cylindrical pieces of approximately 3–5 mm diameter in size. Leaf roll slices were cultured in three replicates on an optimized callus induction medium (CIM) containing MS medium (4.43 g/L); casein hydrolysate (0.5 g/L); myo-inositol (1 g/L); 2,4-D (4 mg/L); thiamine HCl (4 mg/L); sucrose (2%); phytagel (0.38%) and pH adjusted to 5.7 (prior to autoclave). Culture plates were left in the dark at 26 ±1°C for 8–10 weeks to induce embryogenic calli with sub culturing onto fresh medium after every 2–3 weeks. Three days before the inoculation compact, cream colored nodular embryogenic callus was subcultured onto fresh CIM plates.

Vector Construction

A variant of shuttle vector (pART 7) named pART-N'gfp used by Saeed *et al.* (2007) was restricted with *Not1* restriction site to lift the gene cassette comprising CaMV35S promoter, *gfp* gene and CaMV35S terminator. This gene cassette was then sub-cloned in pGreen0029 at the same *Not*1 restriction site and resulting vector was named as pGFP35S (Fig. 1).The pGFP35S expression vector was then electroporated into *A. tumefaciens* strain LBA4404 for plant transformation.

Optimization of Inoculation Method

A single colony of *Agrobacterium* harboring pGFP35S was picked using sterile tooth pick to inoculate 50 mL luria broth (LB) medium and incubated at 28°C for 48 h. After incubation culture was confirmed using PCR specific primers and for each treatment OD_{600} was adjusted accordingly by spectrophotometer (Spectro 22 serial number 220275, USA). Prior to inoculation the calli were air dried by placing on sterile filter paper sheets in laminar hood. The slightly dried compact granular calli were then co-cultivated with bacterial culture under various conditions. Four different treatments (T_1, T_2, T_3 and T_4) with varying inoculation conditions were tested along with controls. In first treatment (T_1), embgryogenic callus was inoculated with *A. tumefaciens* culture OD_{600} of 1.0 through vacuum infiltration at -50 kPa for 5 min. In second treatment (T_2) bacterial culture was pelleted by centrifugation at low speed and resuspended in Murashige and Skoog (1962) basal medium/vitamins (MS medium) [Phytotechnology Laboratories M 519] with an adjusted OD_{600} of 0.4; afterwards calli was inoculated through vacuum infiltration at -50 kPa for 10 min. In third treatment

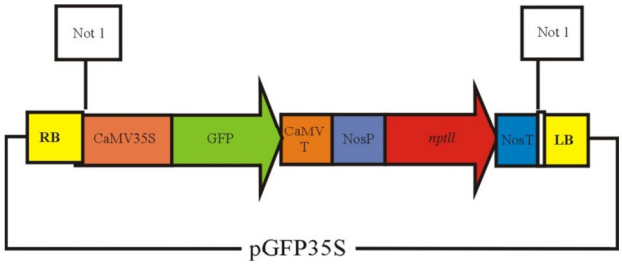

Fig. 1: Map of expression construct pGFP35S used for harboring *gfp* as a reporter gene

(T_3) calli was inoculated with bacterial culture with OD_{600} of 1.0 at room temperature (100 kPa) for 25 min. In fourth treatment (T_4) calli was inoculated with bacterial culture (same as described in T_2) at room temperature (100 kPa) for 45 min. Two control treatments were also included in experimental design; in first control treatment (C_1), calli was inoculated by immersing in liquid LB media and then placed on CIMS (Callus Induction Medium with Selection) containing MS medium (4.43 g/L);casein hydrolysate (0.5 g/L); myo-inositol (1 g/L); 2,4-D (4 mg/L); thiamine HCl (4 mg/L); sucrose (2%); phytagel (0.38 %); pH adjusted to 5.7 (prior to autoclave); cefotaxime (500 mg/L) and geneticin (40 mg/L). In second control treatment (C_2), callus was placed on CIM (Callus Induction Medium without selection) without any inoculation, which served the purpose of non-transgenic control in further experiments. Acetosyringone (100 µM) was added to each culture sixty min prior to the inoculation of explants. Excessive bacterial culture was blotted following the transfer of co-cultivated callus on filter paper sheets placed on CIM plates and incubated in dark for three days in controlled temperature (25 ± 1°C). After co-cultivation, explants were washed three to four times with sterile double distilled water followed by cefotaxime (500mg/L) and shifted to CIMS plates. Callus was allowed to grow for four weeks before transferred to regeneration medium.

Regeneration of Sugarcane

Four week old geneticin resistant calli were transferred to ReM (Regeneration Media) containing MS medium (4.43 g/L); myo-inositol (1g/L); 2-4-D (1 mg/L); sucrose (3%); 6-benzylaminopurine (2 mg/L); 1-naphthaleneacetic acid (1 mg/L); kinetin (0.5 mg/L); phytagel (0.38%); pH adjusted to 5.7 (prior to autoclave); cefotaxime (250 mg/L) and geneticin (40 mg/L). After 10-14 weeks of subculturing on ReM, regenerated plantlets were transferred to rooting media [MS medium (4.43 g/L); thiamine HCl (1 mg/L); sucrose (4%); 1-naphthaleneacetic acid (1 mg/L); kinetin (0.5 mg/L); indole-3-butyric acid (3.0 mg/L); phytagel (0.38%), pH adjusted to 5.7, geneticin (40 mg/L)]. After 8-10 weeks, plants with well-developed root system were

transferred to containment in earthen pots containing sterilized sand.

PCR and Microscopic Studies of Putative Transgenic Plants

DNA of putative transgenic plants was isolated by CTAB method (Iqbal *et al.,* 1997). Presence of the transgene in putative transgenic plants was confirmed by PCR, using *gfp* sequence specific primers (GFP forward primer: AAGGTGATGCTACTTACGG and GFP reverse primer: AATGGTTGTCTGGTAACAAG). The GFP expression of all PCR positive plants was compared with non-transgenic plants (C_2) under UV lamp. In addition, small leaf sections of 5-8 mm_2 size were also analyzed for GFP expression using Olympus SZX-ILLD$_2$-200 UV Fluorescent microscope.

Statistical Analysis

Data obtained from all transformation experiments were recorded, pooled and subjected to Chi square statistical analysis to check the significant difference among treatments. Further Z-test was applied for pair wise comparison of each treatment with other in all possible combinations.

Results

Regeneration Response of Sugarcane

Agrobacterium culture with two different OD_{600} levels was used to inoculate the calli at two different atmospheric pressures with various time intervals, which resulted in 35 independent transgenic events (Table 1). In T_1 and T_3, *Agrobacterium* culture was used for inoculation with varying exposure time under both vacuum (-50 kPa) and room (100 kPa) conditions. In both treatments (T_1 and T_3) explants were directly inoculated with *Agrobacterium* culture but inoculation time of explants used in T_3 was longer as compared to T_1. Due to this difference in inoculation time, during co-cultivation over growth of *Agrobacterium* was observed in T_3 resulting in its lower transformation efficiency as compared to T_1. However, this difference between transformation efficiencies of T_1 and T_3 was not statistically significant (Table 1) but the efficiency of both treatments was significantly different from control C_1. Transformation efficiency in T_2 was significantly higher as compared to T_4, where culture OD was same but with varying infiltration conditions. Overall both treatments with vacuum infiltration (T_1 and T_2) gave better regeneration response with subsequent higher transformation efficiency when compared with the treatments involving the explant inoculation at ambient pressure (T_3 and T_4). Vacuum infiltration with 10 min of co-cultivation duration was found to be the most effective treatment with transformation efficiency of 9.42% and it was significantly different from rest of the treatments (T_1, T_3 and T_4) and C_1 control (Table 1). Different steps involved in *in vitro* culturing of sugarcane are shown in Fig. 2.

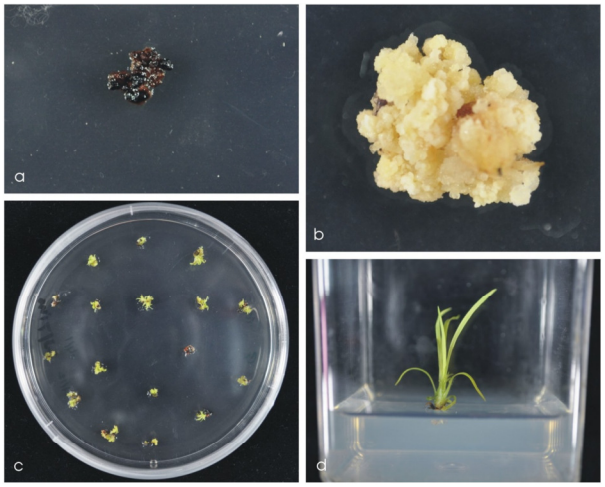

Fig. 2: Different steps involved in in vitro culturing of *Agrobacterium* mediated transformation of sugarcane; a Non-transgenic control (C_1), callus inoculated with LB media and then placed on callus induction medium with selection; b Embryogenic callus placed in callus induction medium; c Callus regeneration after inoculation on regeneration medium; d Putative transgenic plant on rooting medium with selection

Table 1: Detail of inoculation treatments and *in vitro* culturing of sugarcane explants

Treatments	No. of calli plates	No. of inoculated calli parts	No. of regenerated plants	No. of transgenic plants positive for GFP	*Transformation efficiency %
T_1	15	216	20	8	3.70^b
T_2	15	223	34	21	9.42^a
T_3	15	217	5	2	0.92^b
T_4	15	232	7	4	1.72^b
C_1	15	220	0	0	0^c

Where T_1: inoculation of embryogenic calli with *A. tumefaciens* culture (OD_{600} 1.0) through vacuum infiltration for 5 min at -50 kPa, T_2: inoculation of calli with *A. tumefaciens* culture (OD_{600} 0.4) through vacuum infiltration for 10 min at -50 kPa, T_3: inoculation of calli with *A. tumefaciens* culture (OD_{600} 1.0) at room temperature (100 kPa) for 25 min,T_4: inoculation of calli with *A. tumefaciens* culture (OD_{600} 0.4) at room temperature (100 kPa) for 45 min, C_1: inoculation of calli with liquid LB media. Values with different alphabets indicate that they are significantly different. *Transformation efficiency = (no. of transforments / no. of inoculated calli parts)×100. Values within a column with different superscript letters indicates that those are significantly different from each other

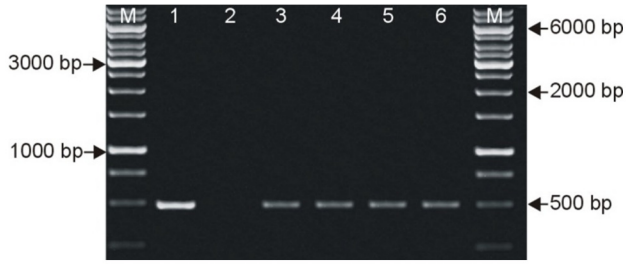

Fig. 3: PCR confirmation of putative transgenic plants by using sequence specific primers for *gfp*; Lane M, 1kbp DNA ladder; lane 1, positive control; lane 2, negative control; lanes 3-6, amplified PCR products (500 bp) from genomic DNA of putative transgenic plants.

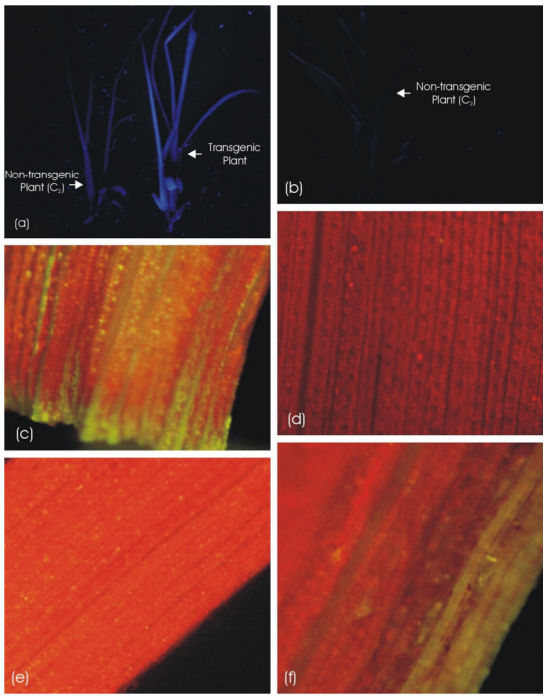

Fig. 4: Expression of GFP in PCR positive transgenic sugarcane plants under UV light with and without microscope; a Comparison of *gfp* expression in both transgenic (right) and non-transgenic (left C_2) plants under direct UV light; b Non-transgenic sugarcane plant (C_2) under UV light; c, f Microscopic view of transgenic sugarcane plants expressing GFP under UV light; d, e Non-transgenic sugarcane plants

Bacterial Cultural Concentration

Transformation efficiency varied significantly at different OD_{600} levels of *Agrobacterium* inoculation culture. Culture with lower OD_{600} values showed good results as compare to bacterial culture with higher OD_{600}. Although among some treatments this difference was not significant between higher and lower OD_{600} cultures (i.e., T_1 and T_3) but transformation efficiency of T_2 was significantly higher from rest of the treatments (Table 1). Bacterial culture of higher OD_{600} showed over growth on inoculated calli and reduced its proliferation, consequently changing the calli texture from granular light creamy to dark brown and ultimately cause the loss of regeneration potential.

Molecular Analysis and Microscopy of Putative Transgenic Plants

Putative transgenic plants were subjected to PCR to determine the presence of transgenes. Gene specific primers were used to amplify the *gfp* internal sequence of 500 bp (Fig. 3). PCR positive sugarcane transgenic plants were further visualized under UV light for the confirmation of GFP expression. Both transgenic (right) and non-transgenic (left) plants are shown in Fig. 4a. After confirmation of GFP expression under direct UV light, leaf sections of both transgenic and non-transgenic plants were analyzed under microscope with GFP fluorescence. The microscopic visualization of leaf sections from transgenic plants confirmed the florescent protein expression (Fig. 4c and f), which was completely absent in leaf sections of negative control plants (Fig. 4d and e).

Discussion

Protocols describing successful *Agrobacterium*-mediated transformation of sugarcane callus and axillary buds are available but their practical application on large scale is hindered due to genotype specific response and low transformation efficiency (Manickavasagam *et al.,* 2004; Joyce *et al.,* 2010; Khan *et al.,* 2013a, b). In present study, factors influencing the transformation efficiency such as the concentration of bacterial culture and method of inoculation were optimized to establish the *Agrobacterium*-mediated gene transformation protocol for *S. officinarum* (CPF-246). Among different tested inoculation methods, vacuum infiltration was found to be the most effective with maximum number of regenerated plants (Table 1). Duration of vacuum infiltration in all tested treatments is very critical factor as it considerably affects the regeneration potential of explants (Table 1). Increased exposure under vacuum results into preponderant bacterium attachment with tissue, a condition optimum for T-DNA delivery but might not be the best for explants survival and subsequent regeneration (Joyce *et al.,* 2010).

Among two tested vacuum infiltration treatments, ten min of vacuum infiltration was found to be the most effective (Table 1) which correlates with the findings of de Oliveira *et al.* where ten min of vacuum infiltration along with two sec of sonication resulted into highest transformation efficiency of citrus epicotyls segments. Moreover, in different studies vacuum infiltration has been used in combination with other techniques like sonication and needle piercing to accelerate the traditional

Agrobacterium-mediated transformation process (Lin *et al.,* 2009). Subramanyam *et al.* (2011) reported an efficient *Agrobacterium* mediated transformation method for transformation of banana where they used both sonication and vacuum infiltration alone as well as together. They recorded highest value of percentage of GUS positive shoots in treatment where a combination of six min of sonication followed by six min of vacuum infiltration was used. However this percentage was not statistically different from other two treatments where six min of sonication and six min of vacuum infiltration were used alone, indicating that results of all these three treatments are statistically equal. In another study Akbar *et al.* (2012) developed a new vacuum infiltration assisted *Agrobacterium* mediated transformation system for pear cultivars. The transformation efficiency of vacuum assisted *Agrobacterium* mediated transformation was significantly higher than the traditional system. The results of both these studies are in agreement with the use of vacuum infiltration in this study and obtained higher transformation efficiencies.

Culture density of inoculum is another important factor, which needs careful consideration for optimizing *Agrobacterium*-mediated plant transformation. In current study transformation efficiency of cultures with high and low inoculum density (OD_{600} 0.4 and 1) was compared and culture with low bacterial density showed significantly higher transformation efficiency (Table 1). This lower transformation efficiency at higher OD level could be due to the excessive washings of explants that are required to control the overgrowth of bacteria. Studies on different plant species indicated that inoculum density of OD_{600} 0.4-0.6 is an optimum range for obtaining high transformation efficiencies (Kumar *et al.,* 2010; Mishra *et al.,* 2012), which further supports the findings of current study. However, Reyes *et al.* (2010) reported that the change in OD of inoculums have no effect on transformation efficiency in maize. Other factors like nature of explants and plant species might be responsible for these contradictory results with current study.

Concentration of selection agent is also very crucial for successful differentiation of transgenic from non-transgenic plants. Optimum concentration of selective agent varied with respect to the cultivar to be transformed (Basnayake *et al.,* 2011). Sugarcane transformants of different cultivars were obtained through particle bombardment (Raza *et al.,* 2010). Low concentration of geneticin at rooting stage was found effective, whereas the higher concentration may be among one of the factors delaying the root initiation (data not shown).

In nut shell, efficiency of *Agrobacterium*-mediated transformation system can be significantly enhanced by adopting the vacuum infiltration. Moreover, density of bacterial culture is another important factor that significantly contributes in the efficiency of *Agrobacterium*-mediated transformation. *Agrobacterium* culture density (OD_{600} 0.4) and inoculation through vacuum infiltration are two critical factors that have been optimized in current study for stable transformation of sugarcane. This reproducible system of sugarcane transformation can be utilized for the production of transgenic sugarcane plants having resistance/tolerance against major biotic and abiotic stress factors.

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