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ESTABLISHMENT OF REGENERATION AND TRANSFORMATION SYSTEMS OF F144 SUGARCANE CULTIVAR

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ABSTRACT:

Sugarcane is one of the most important (two sugar) crops for sugar production in Egypt. In this study, an experiment was conducted to establishment the regeneration system for sugarcane., F144 using leaf bases as a explants through somatic embryogenesis. The effect of 2,4-D, NAA, IAA and kinetin hormones on the sugarcane regeneration was studied. The microprojectile-mediated method was used to transform plasmid pAB6 carrying the *gus* and *bar* genes as reporter and selectable markers respectively. The presence of the trans-genes was confirmed by PCR analysis through which amplified fragments with sizes of about 1800(*gus*) and 540 bp (bar) were detected in transgenics. GUS assay and leaf painting detect the expression of both *gus* and *bar* genes.

INTRODUCTION:

Sugarcane is cultivated on a large scale in tropical and subtropical regions as a raw material for sugar and industrial products (Enríquez-Obregón *et al*., 1998). The proportion of sugar obtained from sugarcane represents 65% of the world sugar production (Agra–Europe, 1995). The improvement of sugar crop was one of the main objectives of the Egyptian Agricultural Policy. Sugarcane yield has increased to 51 tons/ Feddan besides increasing the area to cultivated cane in Upper Egypt now to 323400 feddans, which represents 73% of the sugar productivity in Egypt (Anonymous, 2003).

The sugar contents decreased after few cycles because of aging, accumulation of pests, systemic bacterial, viral diseases such as ratoon stunting disease, leaf scald disease, SCMD and SSD (EL-Kholi and Esh, 1999). Sugarcane tissue culture was first initiated in Hawaii in 1961 (Liu, 1984), while its regeneration system through somatic embryogenesis and organogenesis have been developed by using callus induction (Chengalrayan and Gallo-Meagher,2001), from various explants, i.e., somatic embryogenesis from young leaves (Brisibe *et al.*, 1994; Gallo-Meagher *et al.*, 2000), immature inflorescences (Blanco *et al*., 1997), apical meristems (Ahloowalia and Maretzki, 1983) and organogenesis from young leaves (Fitch and Moore, 1990). Microprojectile mediated plant transformation can serve an important tool to introduce an agronomically useful genes into sugarcane which is best especially for monocots than other standard procedures (Liu *et al*., 2003; Bower and Birch, 1992). This work

was designed to study the following items: 1) Establishment of the regeneration and transformation systems in a sugarcane variety F144 and 2) Evaluation of the transformed tissues and/or plants for the detection of the transgenes. This established protocol might be useful for the future improvements in other sugarcane cultivars.

MATERIALS AND METHODS:

Plant materials: F144, sugarcane (sacetranum officinarum'L) variety was kindly provided by Dept. of Genetics, Cairo University, and used for establishing its regeneration and transformation systems.

Plasmid: Plasmid pAB6 (9.45 Kbp, Ahmed Bahieldin, unpublished data) was obtained from ESL, AGERI, ARC, Giza, Egypt. The plasmid is harboring the *gus* gene under the *Act*1 promoter and *bar* gene, which directed by CaMV 35S promoter. The *nos* sequence was used as a terminator for both genes**.**

Primers: The gene specific primers for both *gus* and *bar* genes (Table 1) were used for detection of the transgenes in the transformed tissues and/or plant materials. The used primers were synthesized by using the DNA Synthesizer 392, Applied Biosystems at AGERI, ARC, Giza, Egypt.

Sugarcane regeneration:

Preparation of explants: The apical regions (stem) of the sugarcane variety F144 were collected from 6 months old-field grown plants. All of the outer mature leaves were removed. The remaining apical part of the shoot, which consists of several pale yellow leaves, was trimmed to 10 cm in length, and surface sterilized by 20% solution of Clorox (commercial of sodium hypochlorite) for 20 min and washed 3 times with sterilized and distilled water (dH_2O) under aseptic conditions. Transverse segment (3mm) thick were excised from the leaf bases up to 5cm towards the tip, used as explants.

Callus induction stage: The explants were cultured on MS medium (MS salts of vitamins) supplemented with and without 2,4–D (2,3,4mg/L separately). The cultures were incubated at 28°C for 8 weeks under dark conditions. The explants were subcultured to the fresh medium after every 3 weeks, on the same media. The selected embryogenic calli were cultured on MS medium supplemented with kinetin (1.0, 1.5 and 2.0 mg/L) and (5.0 mg/L NAA) for 6 weeks. The regenerated shoots, 3-4 cm in heights were excised from shoot clusters and cultured individually for root formation in magentas containing MS medium with different concentrations of IAA (0.25,0.50, 0.75 and 1.0 mg/L). All the cultures were incubated at 28^{o}C under diffuse cool-white fluorescent lamps (4 k 1X) with a 16h photoperiod.

Table-1: Nucleotide sequences of oligonucleotides as primers used in this investigation.

Primers	Sizes (nt)	Sequences (5'----------3')
gus (P3)	31	CCA GAT CTA ACA ATG CGC GGT GGT CAG TCC C
gus (P4)	32	CCA GAT CTA TTC ATT GTT TGC CTC CCT GCT GC
bar (P5)	30	AAA AGC TTC CAC CAT GAG CCC AGA ACG ACG
bar (P6)	25	AAG GAT CCT CAG ATC TCG GTG ACG G

Bold & underlined: *Bam*H 1 site.

Acclimatization stage: The plantlets were rinsed and soaked in a fungicide solution (0.1%) for 2 min and transferred to pots (10 cm in diameter) containing sterilized soil: peatmoss, clay and sand (1:1:1, v/v/v), potted plants were covered with transparent bags to maintain high humidity for two weeks and left for another 6 weeks under a controlled greenhouse (Bioconta-inment).

Sugarcane transformation *via* micro projectile bombardment:

DNA plasmid miniprep: A single colony of *E. coli* strain DH5α carrying the plasmid pAB6 containing *gus* and *bar* genes was inoculated in a 5 ml liquid LB medium containing ampicillin (100 µg/ml). The method of Sambrook *et al.* (1989) was then carried out for extraction, purification and determination of DNA concentration.

Preparation of microcarriers for bombardment: 30mg of gold particles (1.0 µm) were put in a microtube, and 0.5 ml absolute ethanol was added. The tube was vortexed at high speed for 1-2 min, and spun down at 10,000 rpm for 10 sec (This step was repeated 3 times). After spinning down for 1 min, the supernatant was removed and 0.5 ml of sterile dH_2O was added.

Coating of gold particles: To 50µl aliquot of gold particles and the followings under hood conditions were added: 50µl of DNA (1µg/µl), 50µl $CaCl_2$ (2.5 M) and 20µl spermidine (0.1 M). After vortexing for 3 min, spinning down for 10 sec, and discard supernatant and a volume of 250µl of 100% ethanol was added. On vortexing and spinning down the supernatant was removed. After washing, the DNA coated-gold particles (gold/DNA complex) were resuspended in 70µl of 100% ethanol and vortexed at low speed.

Bombardment of explants: Embryo-genic calli were bombarded by the Biolistic Particle delivery System (PDS/1000/He) under the following conditions: microcarrier (gold), 1.1 and 1.6µM (60 mg/ml d H_2O); rupture disk macrocarrier (6 mm), microcarrier travel distance (9cm); chamber vacuum (25 in Hg); Helium pressure (650 kg/cm^2 and 1100 kg/cm^2). 10µl of the gold/DNA complex were placed on a macrocrrier, which was placed into the acceleration tubes, and the acceleration was loaded. The sample chamber was evacuated till the chamber pressure was at vacuum (25 inches Hg), then the PDS- 1000 was fired.

Evaluation of transformed plant materials:

GUS histochemical assay: After two days of bombardment, the *gus* assay was performed for the detection of the expression of *gus* gene. The callus samples were immersed in 1 ml GUS assay buffer and rapped with aluminum foil, to prevent light effect (Jefferson *et al*., 1987). The callus samples were then incubated for 24 h on a rotary shaker (160 rpm) at 37°C for colour development. To inhibit plant endogenous GUS activity the buffer was removed and 70% ethanol was added.

Leaf painting: The leaves of the transgenic plants, which were developed from the embryogenic calli that gave positive reaction in GUS assay. They were painted with BASTA herbicide (2.0g/L) to detect the expression of *bar* gene. The leaves that gave dark brown color (necrosis) were considered as negative while, others which reained with their normal green colour were considered as positive.

DNA extraction: DNA was extracted from transformed and non-transformed plants, according to the method of Dellaporta *et al*. (1985). The genomic DNA was resuspended in 40µl-deionized d H_2O. 5µl were added to 95µl dH_2O and the nucleic acid concentration was determined by spectrophotometer at 563nm and adjusted to100ng/µl.

PCR detection: The PCR was conducted (Sadik *et al*., 1999) with two specific oligonucleotides as primers (Table 1) for each transgene with Perkin-Elmer (Gene Amp PCR System 2400). The PCR product was fractionated through gel electrophoresis **(**Sambrook *et al*., 1989) and photographed under UV trans-illuminator using a Polaroid camera.

RESULTS AND DISCUSSION:

Sugarcane regeneration *via* embryogenesis: The regeneration (*Sacchamus pfficinarum L*.) system of the sugarcane cultivar F144 was established *via* embryogenesis. Leaf bases (apical parts of the shoot, Liu, 1984) of sugarcane cultivar F144 were used as explants, sterilized, sectioned into small pieces and cultured on callus induction media (MS medium with different concentrations of 2,4-D) as reported by Chengalrayan and Gallo-Meagher, (2001). After 8 weeks, the number of explants in which embryogenic calli induction occurs was recorded (Table 2). 2,4-D at concentration of 4 mg/L (18.1 µM) was the most effective one, with the frequency (68.8%) of responding explants (number of explants produced calli) and development of embryogenic calli (48.8%). While, when 2,4-D at a concentration of 2 mg/L (9.06 µM) was added to the MS medium, 24 explants (53.3%) gave response for producing calli and 11 calli out of the 24 represent 24.4% (Figure 1) were developed to embryogenic calli (Ahloowalia and Maretzki, 1983; Ho and Vasil, 1983; Chen *et al*., 1988;Brisibe *et al*., 1994; Chengalrayan and Gallo-Meagher, 2001).

Table (2): Effect of different concentrations of 2,4-D on callus induction from young leaves explants in sugarcane.

2,4–D (mg/L)	Replicates	EPC		Embryogenic Calli	
		No.	**%**	**No.**	**%**
2	R1	10	66.6	05	33.3
	R2	06	40.0	02	13.3
	R3	08	53.3	04	26.6
	Total	24	53.3	11	24.4
3	R1	09	60.0	06	40.0
	R2	08	53.3	05	33.3
	R3	10	66.6	06	40.0
	Total	27	60.0	17	37.7
4	R1	10	66.6	05	33.3
	R2	13	86.6	10	66.6
	R3	08	53.3	07	46.6
	Total	31	68.8	22	48.8
***Cont.**	**Total**	**0**	**0**	**0**	**0**

Note: 15 explants for each replicate were used. **EPC**= Explants producing calli. ***** = without 2, 4-D.

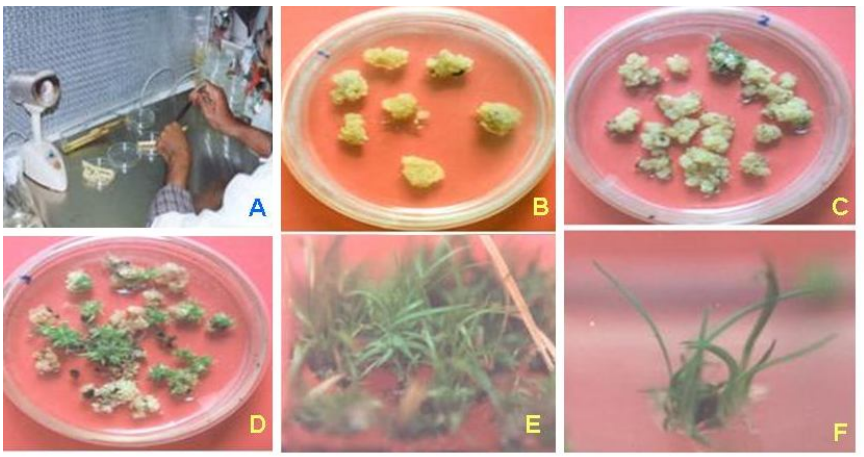

Figure 1. Different Regeneration steps for sugarcane cv. F144. (A) explant preparation; (B) callus induction; (C) callus differentiation; (D-F) shoot formation; shoot multiplication & root induction in the regenerated plants.

Highly proliferating embry-ogenic calli were transferred to MS medium supplemented with different concen-trations of kinetin (1.0, 1.5 and 2.0 mg/L 4.65, 6.98 and 9.3 µM) in combination with NAA (5 mg/L

26.85 µM) for shoot induction. When kinetin at a concentration of 2mg/L was combined with NAA (5mg/L or 26.85 µM), the embryogenic calli gave 77 % response for shoots induction (Table 3). Irvine and Benda (1987); Irvine *et al*. (1991) and Gallo-Meagher *et al*. (2000) have also reported similar results. On the other hand, a few numbers of plantlets were also developed from the embryogenic calli, which were cultured on simple based medium (Aftab and Iqbal, 1999; Aftab Alam *et al*., 1995).

Several methods for encouraging root production, such as culturing the shoots in water, storing them at 15°C, trimming the leaves and adding 5 mg/L NAA to the medium were used (Liu, 1981). In this study the effect of IAA on root formation was determined Table (4). The isolated shoots were cultured on A3 medium-supplemented with 0.75 mg/L IAA give highest number of plantlets (100%) followed by 0.5 mg/L. No difference between 0.25 mg/L IAA and the IAA-free A3 medium was found. The rooted shoots (Figure 2) were transferred into a beaker containing a fungicide solution (0.1%) for 2 min and then transferred to pots containing peat moss, clay and sand (1:1:1, v/v/v) for 2 months (Figure 3).

Sugarcane transformation: Genetic transformation has been identified as a useful tool for sugarcane improvement while breeding advances by conven-tional crossing programs is slow and laborious work (Falco *et al*., 2000). Sugarcane transformation approaches rely upon its efficient *in vitro* regeneration system.

Number transgenic events in the sugarcane plants have been developed through particle bombard-ment method by using embryogenic callus as an explant (Bower and Birch, 1992; Bower *et al*., 1996; Arencibia *et al*., 1998; Elliott *et al*., 1998). Until now sugarcane transgenic are developed by inserting genes for developing herbicide resistance in sugarcane (Chowdhury and Vasil, 1992; Gallo-Meagher and Irvine, 1993; Enríquez-Obregón *et al*., 1998; Falco *et al*., 2000).

In this experiment, 4 weeks old embryogenic calli were used as explants, each callli were divided into small sections and placed in the center of a 10-15 mm disposable Petri dish containing osmotic medium (A1 medium, 0.4 M sorbitol and 0.4 M mannitol) (Figure 4) and incubated at 28°C in a dark incubator for 4 h before their bombardment.

The bombarded calli were placed on a fresh A1 medium and incubated for 2 weeks. Few of putative transformed calli were subjected to GUS assay, while others were regenerated in to plantlets. The transformed, rooted shoots (plantlets) were acclimatized and subjected to leaf painting (Franks and Birch, 1991; Chowdhury and Vasil, 1992; Bower and Birch, 1992; Liu *et al*., 2003).

Table - 3: Effect of Kinetin concentrations on shoot formation in the presence of 5 mg/L NAA.

Kinetin (mg/L)	Replicates	ECPS		ANSPC	ES	
		No.	%		No.	%
1	R1	05	50	11	05	45.4
	R2	06	60	06	02	33.3
	R3	05	50	10	03	30
	Total	**16**	**53.3**	**144/9***	**10**	**37**
1.5	R1	09	90	17	11	46.7
	R2	05	50	12	04	33.3
	R3	06	60	14	06	42.8
	Total	**20**	**66.6**	**286/14.3***	**21**	**48.8**
2	R1	06	60	24	19	79
	R2	10	100	21	11	52.3
	R3	07	70	18	08	38.8
	Total	**23**	**77**	**483/23***	**38**	**60**
****Cont.**	R1	04	40	7	03	42.8
	R2	03	30	9	02	22.2
	R3	03	30	5	01	20
	Total	**10**	**33.3**	**70/7***	**06**	**28.5**

ECPS = Embryogenic calli producing shoots. **ANSPC =** Average number of shoots per embryogenic callus. **ES =** Elongated shoots. **Note**: 10 embryogenic calli for each replicate were used. *****: Total number of produced shoots/ ANSPC. **: Control, i.e., medium hormone-free.

Table - 4: Effect of different concentrations of IAA on root formation.

IAA (mg/L)	Total shoots	Produced plantlets	
		No.	%
0.25	45	40	88.8
0.50	45	44	97.7
0.75	45	45	100
1.00	45	37	82.2
Cont.	45	40	88.8

Note: A number of 45 shoots were used for each treatment in three replicates.

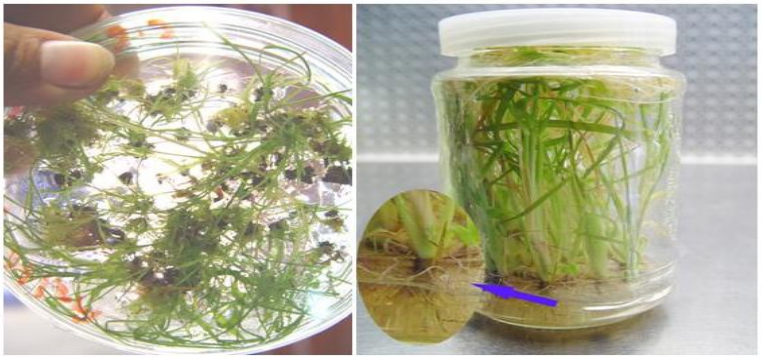

Figure 2. *In vitro* rooting stage of the cv. F144 of sugarcane.

Figure 3. Successful acclimatization of the *in vitro* regenerated sugarcane plantlets under biocontainment conditions at AGERI.

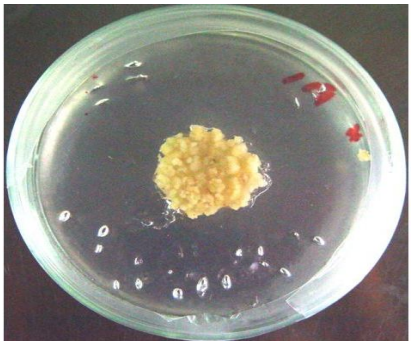

Figure 4. Preparation of explants for Micro-projectile mediated transformation with plasmid pAB6.

Evaluation of putative transgenics was carried out to prove the expression of the introduced genes. Gus assay exhibit (Table 5) that when 1100 psi was used for introducing the plasmid DNA in sugarcane cells, transformation frequency increased from 24% there in case of 650 psi (40%) Figure 5. The transgenic sugarcane plants were successfully regenerated and acclimatized under a control green house conditions in the bio containment as shown in Figure 6.

Figure 5. GUS assay of embryogenic calli derived from leaf bases of sugarcane cv. F144 as explants bombarded with plasmid pAB6.

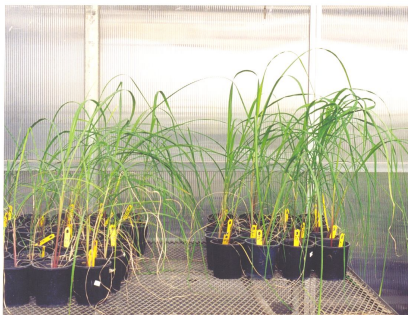

Figure 6. Transgenic plants acclimatized under a control biocontainment conditions at AGERI, containing *gus* and *bar* genes.

Leaf painting with the herbicide BASTA at one-half the recommended dose was used to detect the expression of *bar* gene in the transgenic sugarcane plants. The herbicide resistance of putative transgenics was tested by painting the middle green parts of the plant leaves from both sides with 2 g/L BASTA (Enríquez-Obregón *et al*., 1998). The transgenic plant leaves were resistant to the herbicide (stay green), while non transgenic as well as control plant leaves turned yellow and ultimately leads to their deaths within two days (Figure 7).

Table-5: GUS assay of calli of bombarded embryogenic sugarcane cv. F144 at different pressures forces.

Pressures (psi)	Replicates	Positive GUS assay	
		No.	%
650	R1	05	25
	R2	05	35
	R3	04	20
	Total	14	23.3
1100	R1	10	50
	R2	06	30
	R3	08	40
	Total	24	40

Note: 20 bombarded calli for each replicate were used.

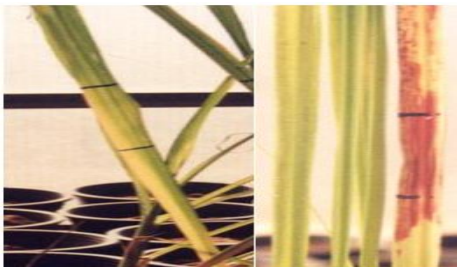

Figure-7. Leaf painting of putative transgenic sugarcane plants regenerated from embryogenic calli (GUS+^{ve}) while necrotic leaf of non-transgenic is presented (Right).

PCR was conducted to confirm the presence of the two genes, i.e., *bar* and *gus* in the genomes of the transformed

sugarcane plants. The positive plants showed the expected band sizes (1800 bp 540 bp amplified) for *gus* gene (Figure-8 & 9), while fragment confirmed the presence of *bar* gene. These amplified bands were also present in the positive controls **(**pAB6 plasmid), while they were absent in the negative control (non transgenic plant), which confirm the presence of the foreign genes in sequence genome (Gambley *et al*. 1993).

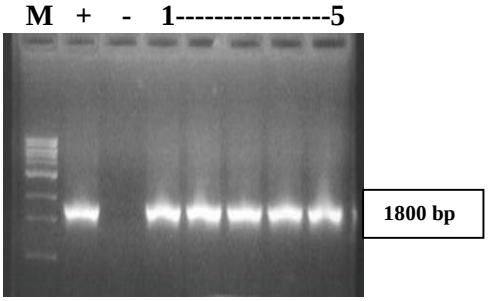

Figure 8. PCR analysis for *gus* gene in the putative transgenic plants. (1-5). M: DNA marker. +: Positive control (pAB6 plasmid). -: Negative control (non-transgenic plant)

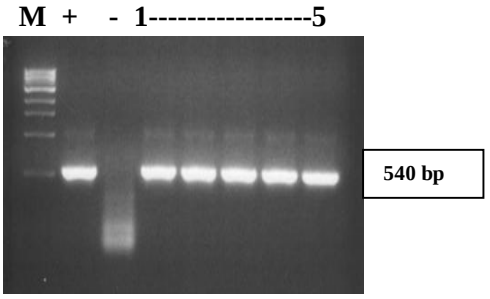

Figure 9. PCR analysis for *bar* gene in putative transgenic plants (1-5), M: DNA marker. +: Positive control (pAB6 plasmid). -: Negative control (non transgenic plant).

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