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ESTABLISHMENT OF REGENERATION AND TRANSFORMATION SYSTEMS OF THE CULTIVAR 'M9681' OF SUGAR BEET (*BETA VULGARIS* L.)

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ABSTRACT

Sugar beet is one of the most important arable crops. In Egypt, sugar beet plant is a second main source of sugar after sugarcane plant. In this study, the regeneration and *Agrobacterium*-transformation systems of the M9681 sugar beet cultivar were established. The effect of the combinations of 6-bezylaminopurine (BAP) as cytokinin and indole-3-acetic acid (IAA) as an auxin on adventitious shoot regeneration from petiole explants excised from the seedlings, and the effect of NAA and IAA on root formation with the aim of improving the procedures for regeneration. *gus*-intron and *bar* as a reporter and selectable marker genes, respectively, were used for the establishment of *Agrobactirum*-transformation system. The integration of transgenes into sugar beet genome was detected by PCR. Two fragments of about 2070 and 540 bp were amplified regarding the two genes, respectively. Furthermore, the expression of *gus*-intron gene was also tested by GUS histochemical assay.

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) as a biennial plant species belongs to the *Chenopodiaceae* family, is one of the most economically important species in this family as 35-40% of world's sugar output is produced from this crop (Winner, 1993). The first experiments on tissue culture of sugar beet were done about 37 years ago (Butenko *et al*., 1972). Sugar beet is an allogamous and heterozygous crop plant; therefore, the micropropagation allows maintaining interesting geno types. Direct shoot formation from different plant tissues and/ or organs is widely used to achieve such aim, while indirect regeneration needs to be developed to obtain variants/ mutants. Adventitious shoot regeneration from several cultured explants has been often employed for the propagation of elite genotypes (Zhong *et al*., 1993 and Grieve *et al*., 1997) but there was a high degree of variability in the regeneration frequencies from various explants of different genotypes (Saunders and Tsai, 1999). Low-light and room-temperature conditions were favorable to sugar beet regeneration without callus when cytokinin had been added to the tissue

culture medium. Using this procedure adven-titious shoots from leaf pieces were obtained in a simple, one-step regene-ration procedure (Kuykendall *et al*., 2003). Breeding of sugar beet is mainly carried out conventionally but in the past couple of decades, the use of molecular techniques, more specifically genetic transformation technologies, has drastically increased (Mannerlof *et al*., 1997; Ivic *et al*., 2001). The develop-ment of an efficient protocol for plant regeneration from cultured explants is a prerequisite for the genetic manipu-lation and improve-ment studies. Sugar beet is known to be a recalcitrant species with respect to *in vitro* culture and genetic trans-formation (Krens *et al*., 1996). Lindsey and Gallois (1990) first reported the production of trans-formed sugar beet plants via *Agro-bacterium tumefaciens* infection and regeneration from shoot bases, which enable relatively rapid and frequent regeneration, as compared with petioles or leaf tissue.

Transgenic sugar beet plants carrying the reporter β-*glucuronidase* gene have been selected for their resistance to glufosinate ammonium herbicide. Integration of transgenes into sugar beet genome was cornered with GUS assay and polymerase chain reaction (PCR) (Kishchenko *et al*., 2005). In this study, the regeneration and *Agrobacterium* transformation systems of the sugar beet cultivar 'M9681' were established followed by detection of the presence and/or expression of the introduce genes.

MATERIALS AND METHODS

Plant materials: Seeds of M9681 sugar beet cultivar were used for regeneration and transformation studies.

Bacterial strain: *A. tumefaciens* LBA 4404 strain kindly provided by Plant Gene Transfer laboratory (PGTL), AGERI, ARC, Giza, Egypt was used for sugar beet transformation.

Plasmid: pISV2678 (Unpublished data) harboring *gus-intron* under the control of CaMV 35S promoter and nos termi-nator as well as *bar* gene under the control of *nos* promoter, AMV leader and pAg7 terminator, modified from plasmid pGPTV-BAR **(**Becker *et al*., 1992), cloned in pPin19 plasmid was used. This plasmid was kindly provided by Dr. P. Ratet, Institut des Sciences Vegetables (ISV), Centre National de la Recherche Scientifique (CNRS), Gif-Sur-Yvette, France.

Primers used: The following primers were synthesized by using the DNA Synthesizer 392, Applied Biosystems at AGERI, ARC, Giza, Egypt.

***gus* gene primers**:

P1 5'CCA GAT CTA ACA ATG CGC GGT GGT CAG TCC C'3

P2 5'CCA GAT CTA TTC ATT GTT TGC CTC CCT GCT GC'3

***bar* gene primers:**

P3 5'AAA AGC TTC CAC CAT GAG CCC AGA ACG ACG'3

P4 5'AAG GAT CCT CAG ATC TCG GTG ACG G'3

Sugar beet regeneration: Seeds of M 9681 sugar beet cultivar were treated with 70% (v/v) alcohol for 1 min, and sterilized with 40 % (v/v) sodium hypochlorite for 45 min in the presence of 0.5 ml /l Tween-20. Seeds were then rinsed several times with sterile distilled water and left in for 16-20 hours at room temperature. After sterilization, seeds were cultured in jars containing wet and sterilized cotton. The seeds were incubated in an incubator at 25°C, 16h light/ 8h dark regime for five

weeks. To initiate the petiole leaves, the seedlings were transferred on SB medium containing MS medium supplemented with 3% (w/v) sucrose, 1 mg/l BAP and 0.8% (w/v) agar, and the pH was adjusted to 5.8 with 1M KOH before adding agar.

Petiole explants were excised from seedlings and cultured on five different regeneration media contain combinations of different concentrations of BAP as cytokinin and IAA as an auxin to study their effects on adventitious shoot regeneration, i) control medium has no phytohormones, ii) SB15 medium (SB medium+ 0.25mg/L IAA), iii) the SB16 (SB medium + 0.5mg/L IAA), iv) SB17 medium (SB medium + 0.75mg/L IAA) and v) SB18 (SB medium+1mg/L IAA). Incubation conditions were the same as the germination medium. Experiments were repeated three times each using 15 explants per treatment. After three to four weeks the responded explants (which formed shoots) were sub cultured on the same medium for elongation. When the shoots reached 3-4cm height, they were transferred to three types of different rooting media, (RI 1) supplemented with 3mg/l IAA, containing of MS solid medium free phytohormones (used as a control),(RI 2) containing of MS solid medium supplemented with 3mg/l NAA and (RI 3) containing of MS solid medium supplemented with 3mg/l IAA.

Incubation conditions were the same as mentioned before. Three to four weeks later, the plantlets which have good roots were transferred to pots containing sterile peatmoss for adaptation under a controlled green house conditions.

Sugar beet transformation

Survival curve: Petiole leaves were excised from seedlings and cultured on SB17 (direct shoot formation medium) supplemented with different concentrations of bialaphos (0.5, 0.75. 1.0 and 1.25mg/L). Incubation conditions were the same as mentioned before. After three weeks, the concentration of bialaphos, which kill all petiole explants, was used in selection medium.

Agrobacterium transformatrion: Agro*bacterium* containing plasmid pISV was cultured for two days at 28ºC on a rotary shaker at 150rpm in liquid medium containing 50mg/l kanamycin. The petiole explants were immersed in the *Agrobacterium* culture for 5 min, and excess liquid was removed by placing the explants on a sterilized filter paper. Petiole explants were transferred to a solid medium and cultured for two days. The explants were rinsed with sterilized distilled water to remove *Agrobacterium* from the surface and then transferred to selection medium [SB17 medium+1mg/l bialaphos+200 mg/l cefotaxime]

Evaluation of transformed plant materials

GUS assay: GUS assays were done according to Jefferson *et al*., (1987). Two days post co-cultivation, the *gus* assay was performed for detection of the expression of *gus* gene. Samples were immersed in 1ml GUS assay buffer and rapped with aluminum foil, to prevent light effect. The samples were then incubated for 12 hours on a rotary shaker (150rpm) at 37°C for color development. To inhibit plant endogenous GUS activity the buffer was removed and 70% ethanol was added.

DNA isolation: About 200mg of putative transgene material was collected and the DNA was extracted according to the CTAB method of Lassner *et al*., (1989). DNA concentration was estimated with a spectrophotometer and by gel analysis.

PCR analysis: The PCR was conducted in a volume of 50 µl as described by El-Desoky *et al*., (2006) using two specific oligonucleotides as primers for each gene and the Gene Amp PCR System 2400 Perkin-Elmer. The PCR product was fractionated through gel electrophoresis and photographed under UV transilluminator using a Polaroid camera (Sambrook *et al.,* 1989).

RESULTS AND DISCUSSION

Sugar beet regeneration: Shoot regeneration through direct organo-genesis is the most effective way to produce true-to type regenerates in sugar beet (Toldi *et al*., 1996). Direct organogenesis is less genotype dependent and regenerates are genetically stable (Detrez *et al*., 1989; Jacq *et al*., 1992). Effect of different concentrations and combinations of cytokinin BAP and auxin IAA on direct shoot regeneration was studied. When petiole explants were cultured on SB15, SB16 and SB17 media, after two weeks, petiole explants formed shoots along the main vein and the highest number of petiole explants produced shoots has been observed on the petiole explants which cultured on SB17 as shown in Table-1 and Figures-1 and 2.

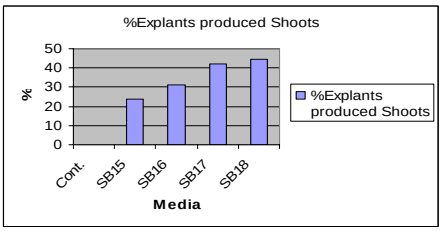

Fig.-1: Effects of different media (SB15, SB16, SB17 and SB18) on shoot formation from petiole explants.

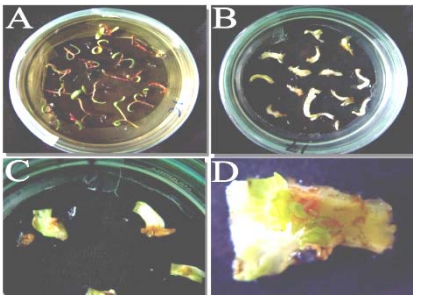

Fig. -2: (A). Germination of seedlings. (B) & (C): Petiole explants on SB17 regeneration medium, (D): Elongation stage on the same SB17 medium.

In contrast to our findings, Gürel *et al*., (2001) obtained indirect shoot regeneration from callus medium containing 1mg/l BAP and 0.3mg/l IAA. Gürel *et al*., (2003) demonstrated that the combination of lower concentrations of BAP and NAA produced nearly 50% more shoots from petiole explants than higher concentrations of the same combination which suppressed shoot development. Adventitious shoots which reached 3-4cm long were transferred to three types of different rooting media RI-1, RI-2 and RI-3 as illustrated in Table-2. RI-2 {MS solid medium supplemented with (3mg/l) NAA} was the pest rooting medium as it developed root after 3-4 weeks, earlier than the other types of rooting media with 80% rooted shoots. Results are illustrated in Figures -3, 4 and 5. Gürel and Wren (1995) previously pointed to the significance of NAA for adventitious root development from petiole explants of sugar beet cv. Primo. The plantlets which have good rooted were

transferred after 3-4 weeks from rooting stage to pots containing sterile peat-moss for adaptation under green house conditions for two months. Gürel *et al*., (2003) reported that the medium contains 3mg/l NAA was more effective for root induction. The main principle of acclimatization of sugar beet from *in vitro* culture is putting it under conditions where, air humidity can be gradually lowered (Hazarika, 2003).

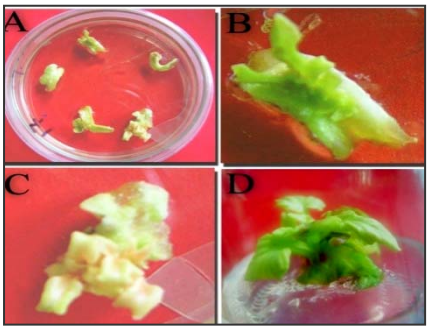

Fig. -3: Elongation stage of petiole explants of M 9681 sugar beet cultivar on SB17 medium.

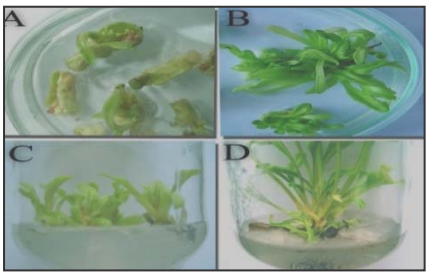

Fig.-4A, B: Direct shoot regeneration from petiole explants of M 9681 sugar beet cultivar on SB17 medium. C) Shoot elongation on the same medium and D) root formation on RI-2 medium.

Fig. -5: Acclimatization stage of the regenerated M 9681 sugar beet cultivar under a controlled Biocontainment conditions.

Sugar beet transformation

Survival curve: Petiol leaves explants were used to define optimal selection concentration. Different concentrations of biolaphos were tested 0.25, 0.5, 0.75, 1.0 and 1.25mg/L. 1mg/L of biolaphos was chosen as the selection concentration, since no escapes were observed under such condition.

***Agrobacterium* transformatrion:** Routine transformation system of plants requires cell cultures competent for efficient plant regeneration as well as an effective method of gene delivery. *A. tumefaciens* remains the preferred tool in genetic transformation of plants (Paul *et al*., 1987, Krens *et al*., 1988; Lindsey and Gallois, 1990; Hall *et al.,* 1996; El-Desoky *et al*., 2006). The advantageous features of *Agrobacterium* mediated transformation include transfer of relatively large segments of DNA with defined ends and with minimal rearrangement integration of small numbers of gene copies into plant chromosomes, high quality and fertility of resultant transgenic plants endogenous GUS activity was detected in transformed explants. All tested plant materials developed blue color, indicating a correlation between GUS enzymatic activities (Figure 6). The presence of the *gus* and *bar* genes was confirmed by

PCR using specific primers for each of the transgene. Fragments of 2070bp (Figure-7) and 540bp (Figure-8), respectively, were amplified with from the DNA of the transgenic tissues. These bands were not amplified with non-transformed plants.

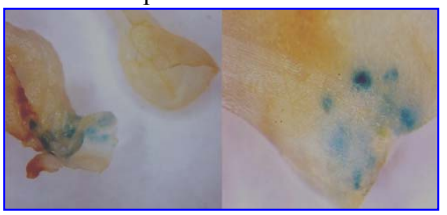

Fig. -6: GUS assay of petiole leaf explants of M 9681 sugar beet cultivar with *A. tumefaciens* LBA4404, contain plasmid (pISV).

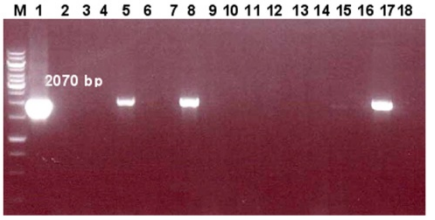

Fig.-7: PCR detection of *gus* gene in transformed plant materials.

Fig.-8: PCR detection of *bar* gene in transformed plant materials.

Table- 2: Effects of different concentrations of growth regulators on root formation.

Treatments	Growth Regulators (mg/l)	No. of Shoots	No. of Plantlets	%
RI (1)	Control	10	5	50
RI (2)	3 NAA	10	8	80
RI (3)	3 IAA	10	4	40

Table-1: Effects of different concentrations and combinations of cytokinin (BAP) and auxin (IAA) on direct shoot regeneration from petiole explants.

Media	Growth Regulators (mg/l)		No. of Petiole explants	Explants produced Shoots		No. of Shoots per explant
	BAP	IAA		No.	%	
Cont.	0.00	0.00	45	0.0	0	0.0
SB15	1.00	0.25	45	11	24	1.64
SB16	1.00	0.50	45	14	31	1.43
SB17	1.00	0.75	45	19	42	2.0
SB18	1.00	1.00	45	20	44.4	2.3

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