In vitro production of transgenic tomatoes expressing defensin gene using newly developed regeneration and transformation system

(Received: 06.03.2007; Accepted: 15.03.2007)

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ABSTRACT

Tomato is considered one of the most important vegetable crops grown in Egypt. Fungal diseases are the most dangerous diseases of tomato on the basis of yield losses. Most previously published procedures for tomato transformation are genotype dependent and still far from routine and universal methods. This study was conducted to enhance regeneration and transformation efficiency of the local Egyptian tomato cultivar "Edkawy". The developed regeneration system involves the culturing of decapitated seedlings (one cotyledon and a merstimatic shoot tip were removed and the rest of explants include radicals, hypocotyls and one cotyledonary leaf only) on basal MS medium. Multiple shoots per explant were developed after three weeks of cultivation on basal MS medium. The developed system was employed to transfer defensin gene, which renders plants resistance against fungal diseases to the Egyptian cultivar "Edkawy". Pluronic acid and Agrobacterium concentration were found to be key factors for efficient Agrobacterium-mediated transformation of cultivar "Edkawy". Transformation was carried out using disarmed A. tumefaciens strain LBA4404 harboring a binary vector pITB-AFP. The plasmid contains defensin gene (AFP) under the control of a CaMV35S promoter and nopaline synthase (NOS) terminator, hygromycin phosphotransferase gene (hpt) and β-glucuronidase. The modfied developed regeneration/transformation system herein, which orignally rely on flmaingo bill-like explant and floral-dip transformation method, enabled us to produce transgenic tomato shoots without the necessity of a complicated tissue culture system. GUS expression was observed in transformed tomato shoots but never in non-transformed (control). The possibility of false GUS positives was ruled out because the GUS gene was interrupted by intron. GUS positive explants reacted positively to polymerase chain reaction (PCR) and gave the expected amplicon (300bp) corresponding to AFP gene. The obtained results indicated that the gene of interest was introduced in tomato genome. The results of the present study can be seen as a step towards production of transgenic Egyptan tomtoes resistance to fungal diseases.

Key words: *In vitro, transgenic tomato, defensin gene.*

INTRODUCTION

Tomato (*Lycopersicon esculentum L.*) ranks foremost among the important vegetable crops in the world. Egyptian farmers in Edko region, El-Beherah Governorate, Egypt, using traditional selection, succeeded to develop a tomato cultivar named Edkawy. This cultivar showed superiority in salt tolerantce and is the most successful candidate for expansion of tomato-cultivated area in Egypt, especially in new reclaimed lands; where irrigation water is relatively saline underground or drainage water (Saker and Rady, 1999). The productivity and value of this cultivar could be greatly increased by the introduction of stably inherited traits such as natural auto-resistance against fungal diseases.

According to FAO data, tomato cultivated area and productivity in Egypt is estimated by 180,000 hectares and 35.5 t/ha, respectively. The current tomato productivity in Egypt is about half of USA productivity (66.57 t/ha). Losses of tomato yield due to diseases have been estimated by 40%. Late and early blights caused by *Phytophthora infestans* and *Alternaria solani* are devastating diseases of tomato worldwide (Rotem, 1998). Early blight of tomato, caused by the fungus *Alternaria solani*, is perhaps the most common foliar disease of tomatoes overall the world. This disease causes direct losses by the infection of fruits and indirect losses by reducing plant vigor. Fruits from defoliated plants are also subject to sunscald. Moreover, *Alternaria* toxins have been reported in various fruits, including tomatoes, olives, mandarins, melons, peppers, apples, raspberries and also in processed fruit products (Scott, 2001).

Application of chemical fungicides to control fungal diseases is not a welcomed strategy due to associated health risks and environmental concerns. Genetic engineering brings new hope for the effective control of plant diseases. However, losses due to diseases have recorded dramatic increase in spite of the release of new resistant cultivars and more effective chemical fungicides. Therefore, effective and sustained control of fungal pathogens is one of the most important issues in modern agriculture. Accordingly, a significant effort has been directed toward the identification of more effective antifungal proteins (Shah, 1997). Recently, different classes of natural genes of plant origin were successfully used to develop transgenic crops with enhanced resistance against fungal diseases through the overexpression of chitinases. Chitinases are the largest group among pathogen related proteins (Boller *et al.* 1983 and Lin *et al.*, 1995).

More recently, different classes of genes encoding antifungal peptides, defenisin, proved to be effective against fungal diseases. Plant defensins are a family of small (45 to 54 amino acids), usually basic peptides occurring in various plant species. Many plant defensins can inhibit the growth of a broad range of fungi at micromolar concentrations, but are nontoxic to both mammalian and plant cells. In some plant tissues, the expression of defensin genes is induced in response to fungal infection (Broekaert *et al.*, 1995 & 1997). One of the most promising reports in this context is the obtainment of fungal pathogen protection in potato by expression of a plant defensin peptide (Gao *et al.*, 2000). They demonstrated that the alfalfa antifungal peptide (alfAFP) displays strong activity against the agronomically important fungal pathogen *Verticillum dahliae*. They reported that the expression of alfAFP peptide in transgenic potato plants provides robust resistance in the green house. El-Awady *et al.*, (2008)

developed enhanced resistant canola plants through *A. tumefaciens*-mediated transformation. They succeed in transforming the gene *kate* causing defense against the fungi *Peronospora parasitica* and *Erysiphe polygoni* responsible for downy mildew and powdery mildew.

Unfortunately, the application of aforementioned breakthrough in cloning of safe plant genes rendering plants resistant against pathogens, is still far from routine applications, even for tomato, whereas tomato transformation using *Agrobacterium* was reported in mid-eighty's (Horsch *et al.*, 1985). The reason beyond this delay is partially due to regenerative capacity of local genotypes and the absence of a universal procedure suitable to transform different cultivars within each species. During the last two decades, *in vitro* regeneration and transformation of tomato is well established worldwide (for example, Velcheva *et al.*, 2005; Roy *et al.*, 2006; Shahriari *et al.*, 2006; Koc *et al.*, 2007 and Qui *et al.*, 2007). Also several trails on regeneration and transformation of Egyptian tomato cultivars were done (El Bahr *et al.*, 1993; Hamoud *et al.*, 1993; Abo-Shady *et al.*, 1993; Saker and Rady, 1999 and Moghaieb *et al*, 2000). However, there is a real need for more efficient genotype independent, rapid and universal method for production of true-to-type transgenic tomatoes (Frary and Earle, 1996). All previously published regeneration/transformation methods of tomato are in general tedious, time consuming and involve the use of growth regulators, sometime expensive one such zeatin (Hamza and Chupeau, 1993).

The emergence of a new class of safe and effective genes of plant origin such as defensin, encouraged us to develop genotype independent enhanced regeneration and transformation system for the most valuable salt tolerance Egyptian tomato cultivar Edkawy and to produce transgenic tomatoes expressing defensin gene which renders recipient plants resistant against fungal and bacterial diseases.

MATERIALS AND METHODS

The bacterial strain

The *A. tumefaciens* strain LBA4404, harboring the plasmid pIBT-AFP which contains the target gene, defensin under to control of 35S promoter and NOS terminator, intron interrupted GUS, as a reporter gene, kanamycin and hygromycin resistance genes for bacterial and plant selection, respectively, was used in this study. Liquid cultures were prepared by suspending one colony of bacterium grown on solid LB medium containing 50 µg/ml kanamycin and 30 µg/ml sterptomycin and incubated at 28°C with shaking (200 rpm) for 24 hr. Bacterial concentration was diluted to optical density of 0.8 at 600 nm. Cultures were then centrifuged for 15 min at 3000 rpm and the pellet was suspended in 10 ml of liquid MS medium containing 4% sucrose.

Agroinfection

Seeds were surface sterilized and germinated under aseptic conditions as described by Saker and Rady (1999). Seven day-old seedlings were used as a source of explants. Explants were prepared by removing one cotyledonary leaf and the merstimatic shoot tips were removed. The rest of the seedling, i.e. the radical, hypocotyl and one cotyledonary leaf represents the target explant. The resulting explants (likes flamingo bill), as described by Pozueta-Romero *et al.* (2001). Explants were immersed in bacterial culture suspension for 10 min with gentle agitation, blotted to sterilized whatman paper and cultured on solid basal MS medium for two days of co-cultivation. Explants were then transferred to the same basal medium

supplemented with 25 µg /ml hygromycin and 300 mg/l carbenicillin and incubated in a growth room at 22 °C under 16 hr/day photoperiod of 2000 lux (Fig. 1). Explants were subsequently transferred to fresh medium every three weeks. Proliferated shoots developed in hygromycin containing medium were micropropagated and used for subsequent biochemical and molecular analysis.

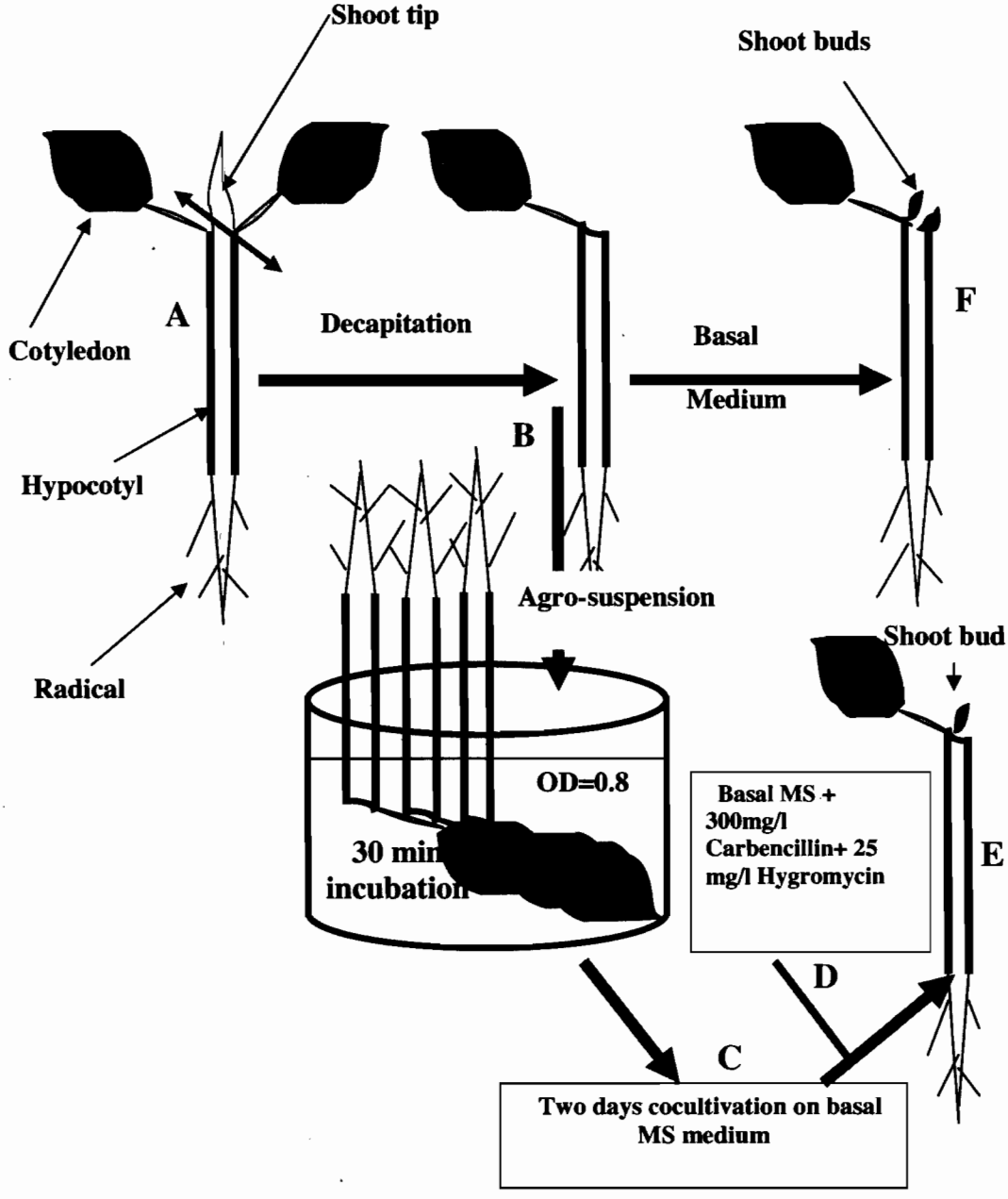

Fig.(1): Schematic diagram showing the different steps of the developed regeneration and transformation system for the Egyptian tomato cultivar "Edkawy". A): One week old seedling, B-E): Agro infection of flamingo bill-like explants, co cultivation and recovery of putative transformed shoot buds, F): Multiple shoot bud proliferation (control-non transformed).

Histochemical detection of GUS expression in putative transformants

Transient expression level was assayed for expression of the *gus-intron* gene in the infected explants 3 days after co-cultivation. Transformation frequency was evaluated as the total number of blue spots observed under binuclear. Spots were estimated approximately ≤1 mm in diameter, representing one or few *gus*-expressing cells (De Clercq *et al,* 2002). GUS expression was assayed in putative transformed calli and regenerated shoots. β-glucuronidase activity was histochemically detected as described previously (Jefferson, 1987) with some modification. Briefly, Calli were dropped with 4 ml Gus reaction buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, 10 mM EDTA, 0.5 mM K-ferricyanide, 0.5 mM ferrocyanide, 1 µl/ml tween-20, 1 mg/ml x-gluc and pH 6.8) per 10 ml Petri dish and incubated in dark at 37°C overnight.

DNA isolation and PCR

DNA isolation was done using a kit of Purgene Company (USA). DNA concentration was determined by electrophoresing 10 µl of the purified DNA along with serial dilutions of Lambda DNA in 0.8 % agarose. PCR amplification of target gene, i.e. defensin was performed in a 20 µl reaction mixture containing 2 µl 10 X amplification buffer, 200 µM dNTPs mix., 10 pmole each of upper and reverse primers, 40 ng template DNA, 1 unit Taq polymerase (Promega) and volume was completed to 20 µl using sterilized distilled water. The reaction mixture was assembled on ice, overlaid with a drop of mineral oil and amplification was conducted for 35 cycles using preheated thermal cycler of Biometra (Germany). The following temperature profile was used: denaturation at 94 °C for 1.5 min, annealing at 65 °C for 1.5 min and extension at 72 °C for 2 min and finally incubated at 72 °C for 5 min. Amplification products were electrophoresed in 2 % agarose using TAE buffer, for 2 hr at 70 volt, visualized under UV light after staining in 0.2 µg/ml ethidium bromide and photographed using Polaroid film. Primers used for amplification of *AFP* gene are 5\` CGC GGA TCC ATG GCG AGG TGT GAG AAT TTG GCT 3\` (forward) and 5\` TGC TCT AGA ATG GCG AGG TGT GAG AAT TTG GCT 3\` (reverse). The expected size of the amplified band is about 300 bp.

RESULTS AND DISCUSSION

Effect of *Agrobacterium* concentration

In preliminary experiments, we used the same transformation conditions described for transformation of the local Egyptian cultivar Edkawy, as described by Saker and Rady (1999), for the agroinfection of flamingo bill-like explants. A notable less browning was observed following co-cultivation with *Agrobacterium*, as noticed in other transformation methods. This less browning can be attributed to indirect contact between the explants (wounded edges) and the medium. This observation, to some extent, indicates that browning phenomenon observed following *Agrobacterium* infection can be minimized using the regeneration/transformation protocol described herein. For further minimizing the hypersensitivity-like reaction and browning caused by *Agrobacterium* infection, different concentrations of *Agrobacterium* were tested. Data summarized in Table (1) indicate that notable increase in transient GUS expression (97.2%), expressed as number of explants showed positive GUS expression, was recorded on higher bacterial concentrations (1.1 OD) and prolonged duration of infection (40 min). Data of Table (1) also indicate that the recorded increment in transient GUS expression was at the expense of explants

viability (survival) and the lowest survival (20 %) was observed following infection with the highest concentration of *Agrobacterium* (1.1 OD) and prolonged infection (40 min). It is concluded that the best conditions to achieve the highest GUS expression (60.5%) and survival rate (70%) of explants include the infection of explants with *Agrbacterium* solution (0.8 OD) for 20 min (Table 1).

In this context, pervious studies dealt with the effect of *Agrobacterium* concentration (density) indicated that the highest density could increase transient GUS expression but not correlated with higher stable transformation (Cheng *et al.*, 1997). Therefore, the increment in transient GUS expression at the expense of explant survival reported herein can be attributed to the damage effect of higher concentrations on the plant cell, which leads to reduction in cell recovery and accordingly, explant survival and stable transformation. Similar conclusion was suggested by Opabode (2006).

Effect of Pluronic acid

For further enhancement of transformation rate, expressed as positive transient GUS expression and explant survival, optimized conditions of the pervious experiment, i.e. *Agrobacterium* concentration (0.8 OD) and infection period (20 min), were used in this experiment.

Table (1):Effect of Agrobacterium concentration on the rate of GUS expression and explants survival after cocultivation.

Agrobacterium cell density (OD_{600})	No. of GUS^+ explants[z]	Mean percentage of GUS explants (%)	No. survival explants
		After 10 min incubation	
0.5	40	50.0	80
0.8	43	57.3	75
1.1	36	60.0	60
		After 20 min incubation	
0.5	42	52.3	80
0.8	42	60.0	70
1.1	35	70.0	50
		After 40 min incubation	
0.5	29	73.0	40
0.8	26	87.0	30
1.1	19	95.2	20

[z]No. of explants per item = 100.

Pluronic acid was included during infection process. The effect of pluronic acid on transient GUS expression and explant survival was demonstrated in Table (2). A glance on data summarized in this table indicates very clearly that all explants treated with pluronic acid showed positive GUS expression and the survival rate was 85% (Table 2). It could be concluded that inclusion of pluronic acid enhances the transient GUS expression by 40% and expalnt survival by about 15%. Including surfactant such as pluronic acid in inoculation medium, greatly enhanced T-DNA transfer in tomato explants. Similar observations were also reported by Bechtold *et al.* (1993), Cheng *et al.* (1997), and Desfeux *et al.* (2000) on wheat and *Arabidopsis*, respectively. Pluronic acid, as other surfactants, may enhance Agroinfection by aiding *Agrobacterium* attachment to the tissue or by elimination of certain substances

that inhibit *Agrobacterium*, as proposed by Opabode (2006).

Table (2): Effect of pluronic acid on the rate of GUS expression and explant survival after cocultivation with A.tumefaciens.

Pluronic acid	No. of GUS$^+$ explants	Mean percentage of GUS$^+$ explants (%)	No. of survival explants
Control explants	43	60	72
Treated explants	85	100	85

Regeneration and transformation

The aforementioned optimized conditions were followed to transform the Egyptian tomato genotype Edkawy. The previously described regeneration protocols of this local genotype rely on zeatin and the regenerative capacity was adversely affected by *Agrobcaterium* infection. To improve the yield of valuable local genotypes of tomato quantitatively and qualitatively, genotype independent regeneration system is of great importance. Also precautions have to be taken into consideration to eliminate, or reduce somaclonal variations which hinder gene transfer studies. Therefore, the best regeneration system is the system which uses minimal concentrations of growth regulators (Larkin and Scowcroft, 1981). Herein, we described a new enhanced system for regeneration and transformation of the Egyptian tomato cultivar Edkawy. This system is summarized in Figure (1) and involves the decapitation of one week-old aseptically growing seedlings, i.e. removing of shoot tip and one cotyledonary leaf. The rest of the seedling represents the explant and looks like flamingo bill. This type of explant, i.e. flamingo bill like explant, was described by Pozueta-Romero *et al.* (2001). In our case, multiple shoot buds were developed on the wounded edge after three weeks of cultivation on basal MS medium (MS free of plant growth regulators). The proliferated shoots from multiple shoot buds can be easily removed from flamingo bill explants, elongated and rooted on basal MS medium. Figure (2-A) shows the proliferation of multiple shoot buds from flamingo explants on basal MS medium.

The developed regeneration system described herein relies on the endogenous plant growth regulators in the seedling root and cotyledon (Hicks, 1994). Pozueta-Romero *et al.* (2001) hypothesized that the balance of plant growth regulators produced by cotyledon and root might induce *de novo* regeneration of meristems after decapitation of apical and axillary meristems. Based on the aforementioned hypothesis, we succeeded to induce *de novo* regeneration for the local Egyptian tomato cultivar Edkawy on plant growth regulators-free MS medium. The number of shoots per explant ranged from 2 to 6 bud shoots per expalnt (Table 3). The produced number of shoots per explant reported herein was similar or greater than the number of shoots per explant reported in other regeneration protocols, which relies on expensive growth regulators such as zeatin or complex combinations of plant growth regulators (Hamza and Chapeau, 1993; Velcheva *et al.*, 2005; Roy *et al.*, 2006; Shahriari *et al.*, 2006; Koc *et al.*, 2007 and Qui *et al.*; 2007).

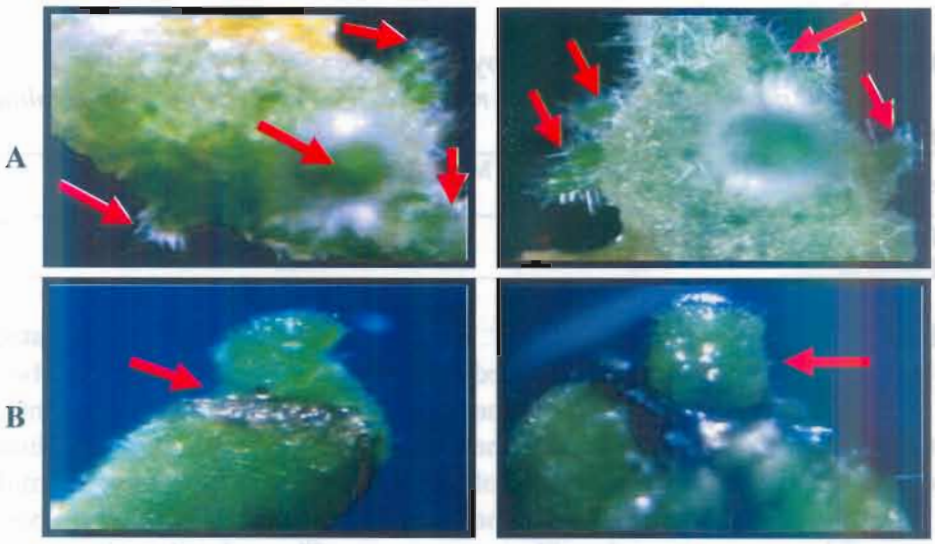

Fig.(2):Regeneration via direct organogenesis. A): Emergence of multiple shoot buds from flamingo bill-like explants of the Egyptian tomato cultivar, Edkawy, cultured for three weeks on basal MS medium. B): Emergence of shoot buds from flamingo bill-like explants infected with A. tumfaciens strain LBA4404 harboring the plasmid pITB-AFP.

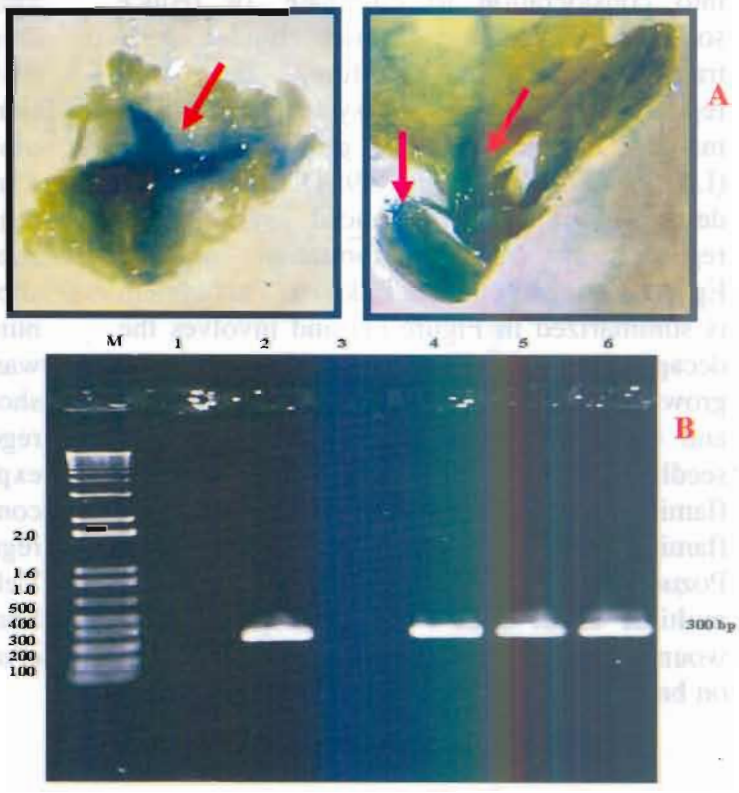

Fig.(3):Biochemical and molecular evaluation of putative transformants. A): Histochemical GUS assays of shoot bud primordia (left) and shoot bud (right). B): PCR analysis of different putative GUS positive transformants for the presence of AFP (defensin) gene. Lane 1 (non-transformed, control), Lane 2 (positive control), Lane 3 (putative transformant, -ve GUS), lanes 4-6 (putative transformants, +ve GUS), and M (1 kb Ladder DNA marker).

Table (3): Effect of Agrobacterium infection on the regenerative capacity of flamingo explants of Edkawy cultivar.

Explant	*De novo regeneration frequency*[z]	*No. of proliferated shoot buds per explant*
Control	95	4.0±1.0
Infected with *Agrobacterium*	70	1.5±0.5

Agro-infection of flamingo bill-like explants with the *Agrobcaterium* strain LB4404 harboring the *AFP* gene using the aforementioned optimized conditions as in the diagram presented in Fig. (1), led to the recovery of putative transformed shoot buds (Fig. 2-B). However, the number of the proliferated shoot buds per explant is 25% of that of control explants (Table 3). All the proliferated shoots following *Agrobacterium* infection onto selective medium, i.e. basal MS medium containing 25 mg/l hygromycin were subjected to histochemical GUS assay. Data presented in Fig. (3-A) shows the positive GUS expression in the developed shoots. The shoots which exhibited positive GUS activity were subjected to PCR analysis using specifically designed primers for *AFP* (defensin) gene. PCR analysis confirmed the presence of *AFP* gene in putative transformants, which gave the expected amplicon (about 300bp) for *AFP* gene as in positive control Fig. (3-B). This is the first successful preliminary report describing production of transgenic tomato shoots from the local Egyptian tomato cultivar Edkawy using a protocol modified from floral dip method, firstly described by Clough and Bent, (1998) for *Arabidopsis* and flamingo bill-explants originally developed by Pozueta-Romero *et al.* (2001). The described system enabled us to produce putative transformants without the need of complicated tissue culture systems. Similar successful trails were reported by Curtis and Nam (2001). Also similar observations were reported by Bechtold *et al.* (1993); Cheng *et al.* (1997) and Desfeux *et al.* (2000) in Wheat and *Arabidopsis*, respectively, and by El-Awday *et al.* (2008) who could develop transgenic canola plants over-expressing bacterial catalase exhibiting enhanced resistance to fungi causing downy and powdery mildews in plants.

ACKNOWLEDGMENT

We thank Dr. S. V. Reddy, Head of Plant Transformation Group (PTG), ICGEB, Delhi, India for kindly providing the bacterial strain harboring the defensin gene used in this study.

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الملخص العربي

الأنتاج المعملي لطماطم معدلة وراثياً لجين الديفنسين باستخدام نظام استيلاد و تحول جديد

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*قسم البيوتكنولوجيا النباتية – المركز القومى للبحوث – الدقى – جيزة – مصر

**قسم الوراثة – كلية الزراعة – جامعة القاهرة – جيزة – مصر

تعتبر الطماطم من أهم محاصيل الخضر فى مصر و هى تصاب بالعديد من الأمراض والتى من أهمها الأمراض الفطرية و الفيروسية لذا تم الأهتمام فى هذه الدراسة بزيادة كفاءة عملية الاستيلاد و كذا التحول الوراثى للصنف المحلى إدكاوى، و أيضاً دون الحاجة للنظم المعقدة لزراعة الأنسجة. تمت عملية الإستيلاد عن طريق أستخدام البادرات كجزء نباتى بعد إزالة الجزء المرستيمى للقمة النامية مع أحدى الورقتين الفلقتين مع ترك الورقة الأخرى مع ساق البادرة (Flamingo bill-like explants) و زراعة هذه البادرات على بيئة MS بدون اى أضافات. ظهرت العديد من الأفرع للجزء النباتى الواحد بعد3 أسابيع و هذه الطريقة أستخدمت فى عملية التحول الوراثى لنقل جين الديفنسين *AFP* لأكساب النباتات المحوله المقاومة للأمراض الفطرية. و قد وجد أن حمض البلورونيك و تركيز الأجروباكتريم يؤثران فى التحول الوراثى. أستخدمت سلالة الأجروباكتريم LBA 4404 و الحامله للبلازميد PITB-AFP المحتوى على الجينات *AFP* ، و *hpt* المسئولة عن المقاومة للمضاد الحيوى الهيجروميسين، و الـ *GUS* المعبر عن التحول الوراثى. و قد شوهد تعبير جين الـ *GUS* فى النباتات المحولة فقط دون النباتات المقارنة و تم التأكد من التحول الوراثى عن طريق الـ PCR. اتضح من النتائج دخول الجين *AFP* فى جينوم الطماطم و هذا يعتبر خطوة هامة فى انتاج نباتات طماطم مصرية مقاومة للأمراض الفطرية.